Plant Physiology and Biochemistry 104 (2016) 81–91

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Phenolic compounds as indicators of drought resistance in shrubs from Patagonian shrublands (Argentina)

M. Celeste Varela [a], Idris Arslan [b], Mariana A. Reginato [a], Ana M. Cenzano [c], M. Virginia Luna [a, *]

[a] Laboratorio de Fisiología Vegetal, Fac. de Cs. Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina
[b] Pamukkale University, Faculty of Technology, Biomedical Eng., 20200, Denizli, Turkey
[c] Laboratorio de Ecofisiología y Bioquímica Vegetal, Instituto para el Estudio de los Ecosistemas Continentales Patagónicos-Centro Nacional Patagónico-Consejo Nacional de Investigaciones Científicas y Técnicas, Boulevard Brown 2915, 9120, Puerto Madryn, Chubut, Argentina

ARTICLE INFO

Article history:
Received 4 January 2016
Received in revised form
8 March 2016
Accepted 10 March 2016
Available online 18 March 2016

Keywords:
Plant development and life-history traits
Ecophysiology
Polyphenols
Antioxidant capacity
Lipid peroxidation
Drought stress

ABSTRACT

Summary: Plants exposed to drought stress, as usually occurs in Patagonian shrublands, have developed different strategies to avoid or tolerate the lack of water during their development. Production of phenolic compounds (or polyphenols) is one of the strategies used by some native species of adverse environments to avoid the oxidative damage caused by drought. In the present study the relationship between phenolic compounds content, water availability and oxidative damage were evaluated in two native shrubs: *Larrea divaricata* (evergreen) and *Lycium chilense* (deciduous) of Patagonian shrublands by their means and/or by multivariate analysis. Samples of both species were collected during the 4 seasons for the term of 1 year. Soil water content, relative water content, total phenols, flavonoids, flavonols, tartaric acid esters, flavan-3-ols, proanthocyanidins, antioxidant capacity and lipid peroxidation were measured. According to statistical univariate analysis, *L. divaricata* showed high production of polyphenols along the year, with a phenolic compound synthesis enhanced during autumn (season of greatest drought), while *L. chilense* has lower production of these compounds without variation between seasons. The variation in total phenols along the seasons is proportional to the antioxidant capacity and inversely proportional to lipid peroxidation. Multivariate analysis showed that, regardless their mechanism to face drought (avoidance or tolerance), both shrubs are well adapted to semi-arid regions and the phenolic compounds production is a strategy used by these species living in extreme environments. The identification of polyphenol compounds showed that *L. divaricata* produces different types of flavonoids, particularly bond with sugars, while *L. chilense* produces high amount of non-flavonoids compounds.

Synthesis: These results suggest that flavonoid production and accumulation could be a useful indicator of drought tolerance in native species.

1. Introduction

Water deficit is the dominant environmental factor that shapes the pattern of vegetation in semi-arid ecosystems. Because of this, vegetation is heterogeneously distributed forming isodiametric patches with shrubs acting as colonizers or initiators and grasses surrounding the patches, alternating with areas of bare soil (Noy-Meir, 1973). The coexisting species of these ecosystems have different strategies to survive to the different environmental factors. Under field conditions, the response to one factor, such as water stress, is complex and difficult to study in isolation because an overlapping with others environmental factors (intensity of winds, solar radiation, temperature fluctuation, etc.) occur; however, this kind of research can provide an approach about the behavior and the adaptation of the species in study.

Native plants of semi-arid ecosystems have developed different mechanisms to face drought, which involve different strategies and adaptive changes depending on the genotype (Chaves et al., 2003). Plants were classified in two main ecophysiological groups

* Corresponding author.
E-mail addresses: cvarela@exa.unrc.edu.ar (M.C. Varela), iarslan@pau.edu.tr (I. Arslan), mreginato@exa.unrc.edu.ar (M.A. Reginato), cenzano@cenpat-conicet.gob.ar (A.M. Cenzano), vluna@exa.unrc.edu.ar (M.V. Luna).

http://dx.doi.org/10.1016/j.plaphy.2016.03.014
0981-9428/

according to the strategy used: drought avoidant plants and drought tolerant plants (Levitt, 1980). Drought avoidant species are characterized by high relative growth rate, deciduous phenology, high-faster metabolism, high potential for resource capture, and low investment in secondary metabolites. Thus, in water-limited environments, avoidant plants with high stomatal conductance and good photosynthetic capacity demonstrate opportunistic and rapid growth during short periods of water availability (Hetherington and Woodward, 2003). Drought tolerant plants have the ability to maintain their normal physiological parameters even during long periods of drought by employing different strategies like osmotic adjustment, changes in cellular/tissue elasticity (elastic properties of cell walls), different antioxidant mechanisms and high production of secondary metabolites (Reddy et al., 2004; Westoby et al., 2002). Regardless the strategies used to face drought, plants exposed to long periods of abiotic stress enhance the secondary metabolites production as part of the non-enzymatic mechanisms. These compounds generally have a strong antioxidant capacity to protect cellular structures from the oxidative damage caused by the increase of reactive oxygen species (ROS) production in cells (Sies, 1993; Naczk and Shahidi, 2004; Reginato et al., 2014).

In recent years, the research about non-enzymatic components with antioxidant capacity has focused on phenolic compounds (Surweswaran et al., 2007; Krishnaiah et al., 2011). Phenolic compounds, also known as polyphenols, include a large variety of molecules and could be classified in 3 different groups: 1) Non-flavonoids, molecules that have at least one phenolic ring with different reactive groups (hydroxyl, nitrosyl, SH, etc), including simple phenolic acids, phenyl alcohols, stilbenes, chalcones and lignans; 2) flavonoids, molecules with a phenyl chromane structure of C15 formed by 2 aromatic rings bind with a carbon chain (C6–C3–C6), sub-classified into anthocyanidins, flavonols, flavones, isoflavones, flavanones, flavan-n-ols (n refers to the carbon number of the phenyl chromane structure where the hydroxyl group is bonded. Ex: flavan-3-ols, flavan-4-ols, flavan-3,4-ols), etc, depending on the amount, position and type of reactive group (Crozier et al., 2009; Motilva et al., 2013); and 3) tannins, subdivided in a) condensed tannins: flavonoids polymers type A (C7–C2 and an ether bind) and type B (C4–C8 or C4–C6) and b) hydrolyzable tannins: phenolic acids polymers bind to a 5 or 6 carbons ring (Khanbabaee and Van Tee, 2001). Phenolic compounds have antioxidant functions in response to severe abiotic stress, complementing the roles played by the enzymatic antioxidant system, with great potential to reduce ROS and to avoid cell damage (Hatier and Gould, 2008; Agati and Tattini, 2010). Fast growing plants generally have a low investment in defense and protection. Therefore, this species tend to have low phenolic compounds concentrations. Conversely, slow growing species have high phenolic compounds concentrations. This could be attributed to a long-term probability that these long-lived plant species evolved to confront both biotic and abiotic (primarily water shortage) stress factors (Karabourniotis et al., 2014).

Patagonian shrub lands are located in the North-East of Patagonian Monte, Argentina, specifically between 42 and 44° 20′S and 64–68° W, with a surface of 4200 km^2 approximately (Soriano, 1950). This region is characterized by a semi-arid climate with large daily and seasonal temperature variations and with annual rainfalls below 200 mm, generally concentrated in winter and/or spring (Cabrera, 1976). The landforms and soils of Patagonian shrub lands enable heterogeneity in vegetation distribution (from dense scrub to steppes) with large variety of species, dominated by grasses and shrubs alternating with bare soil (Bertiller et al., 2004). The most common shrub species are *Larrea divaricata*, *Chuquiraga hystrix*, *Lycium chilense*, *Junellia alatocarpa*, *Condalia microphylla*, *Prosopidastrum globosum*, *Schinusjohn stonii* and *Monttea aphylla* (Cabrera, 1976; León et al., 1998); all of these species have a variety of adaptive strategies related with water and nutrient conservation (Bertiller et al., 2005, 2006). Two of the dominant shrubs in this region are *L. divaricata* and *L. chilense*, both being considered as colonizers or initiators of the patches. The contrasting functional traits and phenologic behavior of both species were previously described (Bertiller et al., 2004; Soriano et al., 1995). *L. divaricata* is an evergreen shrub, with a perennial behavior and low leaf shedding during the whole year (Soriano and Sala, 1983; Campanella and Bertiller, 2008). *L. chilense* is a deciduous shrub with phenological activity occurring during winter–spring and sometimes until early summer when water availability in soil is greater (Soriano et al., 1995; Campanella and Bertiller, 2008).

The aim of this work was to perform a field study along the different seasons in two native species of Patagonian shrub lands with contrasting mechanisms of drought resistance, *L. divaricata* and *L. chilense*, to evaluate the oxidative damage in tissues, the levels of different groups and types of polyphenols and their antioxidant capacity. In theory, plants from semi-arid regions are classified according to their ecological niches, but in practice it becomes very difficult to catalog a plant species in one of the main ecophysiological groups because of the overlapping of different environmental factors and plant responses. It has been proposed that, independently of the ecophysiological strategies used, coexisting species in semi-arid regions have a gradient of mesophytism to xerophytism that allows them to coexist and survive in the same habitat (Cenzano et al., 2013).

Species from semi-arid ecosystems like Patagonian shrub lands, with different behavior and phenological activity to face drought, are supposed to have powerful antioxidant enzymatic and non-enzymatic mechanisms. Considering the severe stress that these species have to face during long periods, we hypothesize that polyphenols may be an important component of their antioxidant system. However, the role of polyphenols as a mechanism of drought response and their suitability as indicators of drought tolerance in native species of the Patagonian shrub lands has not been explored yet.

2. Materials and methods

2.1. Study site and plant material

This study was carried out in the wildlife refuge “La Esperanza” of Natural Patagonia Foundation, located in the northeast of Chubut province (67 km^2 surface, 42°7′ 43.92″S and 64°57′ 40.99″ W). Leaves and roots of *L. divaricata* and *L. chilense*, (five plants per specie), were randomly collected in autumn (May), winter (August), spring (November) and summer (February). Plant material was frozen immediately after collection and kept at −80 °C until biochemical analyses were performed. Samples were lyophilized previously to the extraction procedure.

2.2. Weather data

To determine seasonal changes in water availability, rainfall and temperature averages were recorded using an automatic data recorder (21× Micrologger, Campbell Scientific) located in the study site. Data were compared between the four seasons to establish the dry and wet seasons.

2.3. Soil water content (SWC)

Soil water content was measured by the gravimetric method (Peters, 1965). During each season sampling, five soil samples were taken at three different depths (10, 20 and 30 cm) from the

surrounding soil below the species under study, and placed immediately in plastic bags to prevent moisture loss. Wet weight (WW) was measured, and then samples were dried at 105 °C for 48 h to determine the dry weight (DW). The soil moisture content was calculated according the equation:

$$\text{Soil moisture content (\%)} = (WW - DW)/DW \times 100$$

2.4. Relative water content (RWC)

Relative water content was measured following the method reported by Xu and Zhou (2007). Leaves and roots samples of each species (for the four seasons) were placed into hermetic vials to avoid dehydration. RWC was calculated based on tissue fresh weight (FW), weight at full turgor (TW) after soaking the samples in distilled water for 48 h, and DW after drying the samples in oven at 80 °C for 24 h. RWC was calculated according the equation:

$$RWC = (FW - DW)/(TW - DW) \times 100$$

2.5. Oxidative damage in tissues

The level of lipid peroxidation was determined in terms of malondialdehyde (MDA) content according to Heath and Packer (1968) modified for this samples. Freeze-dried samples (0.15 g) were ground in liquid nitrogen and mixed with 1.5 ml 20% TCA (trichloroacetic acid). The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was used for MDA determination. A mixture of 0.5 ml of extract + 0.5 ml of 0.5% TBA (thiobarbituric acid) in TCA 20% was produced, heated at 95 °C for 25 min, cooled and centrifuged for 10 min. The sample was measured at 532 nm and corrected by non-specific absorption at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and taking into account the water content of the samples before their lyophilization.

2.6. Phenolic compounds extraction

0.5 g of freeze-dried samples was ground in liquid nitrogen. The material was extracted three times with 10 ml of extraction solvent (methanol/water (80:20, v/v)) on a magnetic stirrer during 30 min. After each extraction, the liquid was separated from solids by centrifugation (14,000*g*, 15 min) at 4 °C. The final volume was quantified and the final extract was reduced to 8–16 ml by rotary evaporator. The extract was then filtered with 0.45 μm filters, divided into aliquots of 4 ml and stored at −80 °C for chemical analyses. The spectrophotometric assays were performed by using a Thermo Spectronic Helios UV–visible spectrophotometer (Cambridge, UK). Concentrations of different phenolic compounds and antioxidant activity were calculated from calibration curves and expressed as mg equivalent to the corresponding standard. All tests were carried out five times.

2.6.1. Total phenols

Total phenols were determined with Folin-Ciocalteu method adapted to our samples according with Borbalán et al. (2003). 25 μl of sample, 1.25 ml of distilled water, 125 μl of Folin-Ciocalteu reagent, 0.5 ml of a solution of sodium carbonate at 20%, and 0.6 ml of distilled water, were introduced into a test tube. The solution was homogenized and after incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm. The total phenols amount was calculated as mg of gallic acid equivalents.

2.6.2. Total flavonoids

Total flavonoids were measured according to Kim et al. (2003). 100 μl of extracted sample and 60 μl of 5% $NaNO_2$ were mixed. After 5 min 40 μl of 10% $AlCl_3$ were added, and after other 5 min 400 μl of 1 M NaOH were added to the mixture. The reaction mixture was diluted with 200 μl of distilled water, mixed, and the absorbance determined at 510 nm. The total flavonoids were calculated as mg of catechin equivalents.

2.6.3. Proanthocyanidins

Proanthocyanidins were measured according to Waterman and Mole (1994). Butanol reagent was prepared by mixing 128 mg $FeSO_47H_2O$ with 5 ml of HCl and completed to 100 ml with n-butanol. 50 μl of extracted sample were mixed with 700 μl butanol reagent and heated at 95 °C in a water bath for 45 min. The sample was cooled and 250 μl of n-butanol were added. The absorbance was measured at 550 nm. Total proanthocyanidins were calculated as mg of cyanidin equivalents.

2.6.4. Tartaric acid esters and flavonols

Tartaric acid esters and flavonol contents were determined using the method described by Romani et al. (1996). An aliquot of 25 μl of extract was diluted with 225 μl of 10% ethanol and 250 μl of 0.1% HCl in 95% ethanol, and 1 ml of 2% HCl was then added. The solution was mixed, and the absorbances were determined at 320 nm for tartaric acid esters and 360 nm for flavonols. Tartaric acid esters and flavonols amounts were calculated as mg of caffeic acid and quercetin, respectively.

2.6.5. Flavan-3-ols

Total flavan-3-ols were determined with p-(dimethylamino) cinnamaldehyde (DMACA) reagent, as described by Nigel and Glories (1991). An amount of 10 μl of the sample extract was diluted with 90 μl of methanol. Next, 250 μl of HCl (0.24 N in MeOH), 250 μl of DMACA solution (0.2% in MeOH), and 250 μl of methanol were added. The absorbance was determined at 640 nm, and the total amount of flavan-3-ols was calculated as mg of catechin equivalents.

2.6.6. Antioxidant activity of phenolic extracts for spectrophotometric analysis

ABTS* (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging ability of polyphenolic extracts was determined according to the method described by Re et al. (1999). The $ABTS^+$ radical was generated by reacting an ABTS aqueous solution (7 mM L^{-1}) with $K_2S_2O_8$ (2.45 mM L^{-1}, final concentration) in the dark for 16 h and diluting with ethanol to obtain Abs = 0.700 ± 0.020 at 734 nm. 100 μl of extracted sample was mixed with 1 ml of $ABTS^+$ solution. After 4 min of reaction the absorbance was measured at 734 nm. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 2.5 mM solution was used to obtain the calibration curve. The antioxidant capacity was expressed as μmol of Trolox equivalents.

2.7. Extraction and isolation of polyphenols

Leaves of *L. divaricata* and *L. chilense* (100 g) corresponding to two treatments (dry season: autumn and wet season: spring) were crushed and powdered. The polyphenols were extracted by stirring the suspension in 70% methanol (1 L) at 40 °C for 24 h. The methanol solution was filtered with filter paper (Whatman Nº 1) to remove the remaining plant material and kept in freezer. Afterward

the solution was evaporated at 100 mbar, 40 °C and the concentrate obtained was kept at 4 °C for 24 h. To remove the low molecular compounds the solution was dialyzed (Cellu-SEp H1) against distilled water for 10 h and filter again through a Nalgene TM Disposable Filterware (0.45 μm) to obtained a pre-purified polyphenols mixture.

Analytical HPLC was performed using a Waters system, controlled by Waters millennium software, consisting of a 717 plus auto sampler, Waters 600 E pump in combination with a Waters 600 Controller with in-line degasser and Waters 996 photo diodearray detector. Waters Atlantis C18, 250 × 4.6 mm, 5 μm and Phenomenex C18 Luna-100, 250 × 10 mm, 5 μm were the HPLC-columns used for separation of pure compounds. An LC-module 1 Waters HPLC machine (Mildford, MS) was used. The binary solvent system used for elution gradient consisted of 0.01% trifluoroacetic acid in water (solvent B) and Methanol (solvent A) at a constant flow-rate of 1.5 mL min^{-1}. A linear gradient profile was applied, starting with 20% A to 70% A over 60 min. The separated polyphenols mixture, were collected and analyzed by HPLC-ESI–TOF-MS, in negative ion mode $[M-H]^-$. Each phenolic compound was detected with *m/z* values.

2.7.1. Antioxidant activity of phenolic extracts for HLPC-MS analysis

The antioxidant activity of the phenolic extracts was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, 2,2-diphenylpicrylhydrazyl (DPPH) (Brand-Williams et al., 1995). Methanolic stock dilutions (50 ml) of each extract were prepared (1, 1:2 and 1:4 v/v). 2 ml of each stock dilution were placed in a cuvette and 0.05 ml of 100 mM methanolic DPPH solution were added. The decrease in absorbance at 517 nm was determined after 30 min. A DPPH methanolic solution (100 mM) was used as blank, and Butylated hydroxytoluene (BHT) solution (100 mM) was used as positive control. All determinations were performed in triplicate. The inhibition percentage of the DPPH radical by the samples was calculated according to the following equation:

$$\%\ \text{DPPH inhibition} = [(Abs_B - Abs_E)/Abs_B] * 100$$

where Abs_B is the absorbance of the blank at $t = 0$ min and Abs_E is the absorbance of the stock dilution of the extracts at $t = 30$ min.

2.8. Statistical analysis

Infostat (2011 v.) software was used for statistical analysis. Origin Pro. 8 software was used for plots. Models were: *two-way* ANOVA (comparing between species and seasons) and *one-way* ANOVA (comparing only between seasons). *Bonferroni test* was used for comparisons and means were considered to be significant when $P < 0.05$. To test the assumptions of ANOVA *Shapiro–Wilk test* for normality and *Levene's test* for homoscedasticity were used. When the assumptions of ANOVA were not right, *Kruskal Wallis* non-parametric test was applied. The measurements were taken in 5 plants per season, and at triplicate for each plant.

Linear regressions between different variables were applied. Correlation (R) and determination (R^2) coefficients were calculated. The means were considered to be significant when $P < 0.05$.

Multivariate analysis was performed using all the data obtained from the two species, the two organs (leaves and roots) and the four seasons. Principal Component analysis was performed to visualize the distribution of the values obtained. Linear Discriminant analysis (LDA) was performed to reduce dimensionality, maximizing the variance between the categories (season/species with all the data together and season/tissue in each species) and minimizing the variance within categories, to identify differences in the phenolic compounds production between the species and their behavior.

3. Results

3.1. Weather data

Rainfall data during the sampling period did not exceed 8 mm per season along the year (Fig. 1a). Spring was considered the season with the highest water availability due to a significant increase in precipitations compared with the other seasons, and with the data obtained about the soil water content (Fig. 1b). Large seasonal and annual temperature variations were observed. The average temperatures of the different seasons were: 8.6 °C in winter, 10.8 °C in autumn, 15.9 °C in spring and 20.8 °C in summer. The seasonal variations were between 13 and 16 °C comparing between all the seasons, and the annual variation was 12.2 °C (Fig. 1a).

3.2. Soil water content (SWC)

The higher percentage of SWC was found in spring (specifically

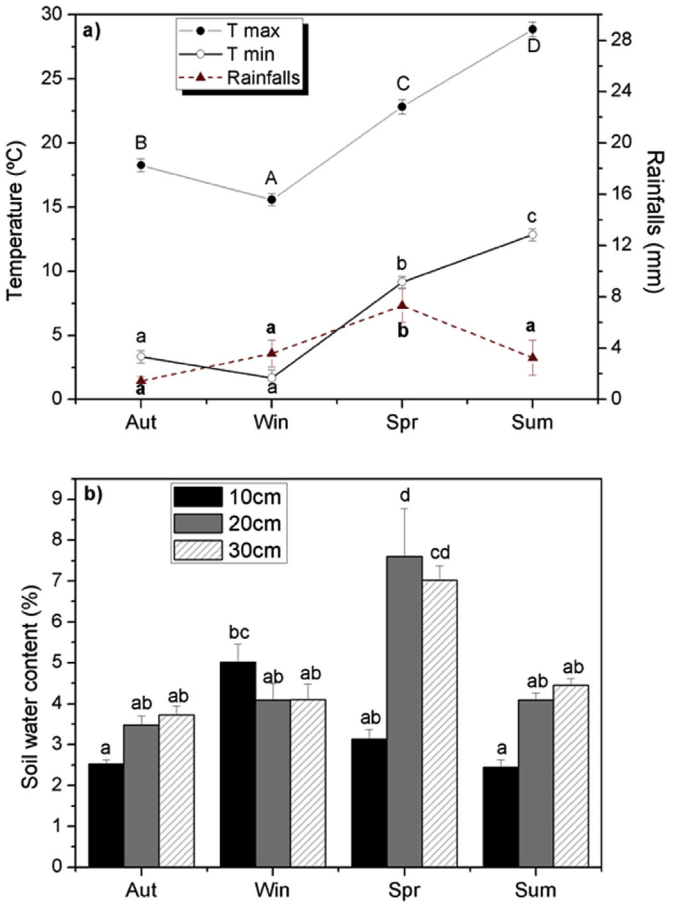

Fig. 1. a) Average values of minimum temperature, maximum temperature and rainfalls of each season. Autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Different bold letters indicate significant differences between seasons in rainfalls dates according to Kruskal Wallis test ($P < 0.05$). Different uppercase letters indicate significant differences between seasons in dates with maximum temperature, and different lowercase letters indicate significant differences between seasons in dates with minimum temperature, according to Bonferroni test ($P < 0.05$) b) Soil water content at 3 different depths (10 cm, 20 cm and 30 cm) in the four seasons: Autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Different letters indicate significant differences between seasons and depth according to Bonferroni test ($P < 0.05$).

at 20 and 30 cm depth) (Fig. 1b) in correlation with the rainfall values obtained (Fig. 1a). In winter, the highest SWC value was at 10 cm depth (Fig. 1b). However, in summer and autumn the highest SWC values were observed at 20 and 30 cm depth.

3.3. Relative water content (RWC)

L. divaricata leaves had the highest RWC in summer, while roots showed no significant differences between seasons, although the RWC of roots was slightly higher in autumn and winter (Fig. 2a). *L. chilense* lost their leaves in autumn and winter, so RWC was only measured in spring and summer, showing the highest values in the latter. There were no significant differences in RWC of *L. chilense* roots during the four seasons (Fig. 2a). In both species, RWC of roots tended to decrease from spring to summer, inversely to leaves (Fig. 2a).

3.4. Oxidative damage in tissues

Damage in cellular membranes was estimated by MDA concentrations (Fig. 2b). In *L. divaricata* leaves there were no significant differences between seasons in MDA concentrations with values between 30 and 34 nmol g^{-1} DW, while in roots MDA concentrations were high in autumn (43.68 nmol MDA. g^{-1} DW), decreased abruptly in winter (15.32 nmol MDA. g^{-1} DW) and started to increase again towards summer (Fig. 2b). *L. chilense* had lower MDA concentrations than *L. divaricata* being higher in leaves (25 and 32 nmol MDA. g^{-1} DW) than in roots (8 and 15 nmol MDA. g^{-1} DW) with no significant differences within seasons in each organ (Fig. 2b).

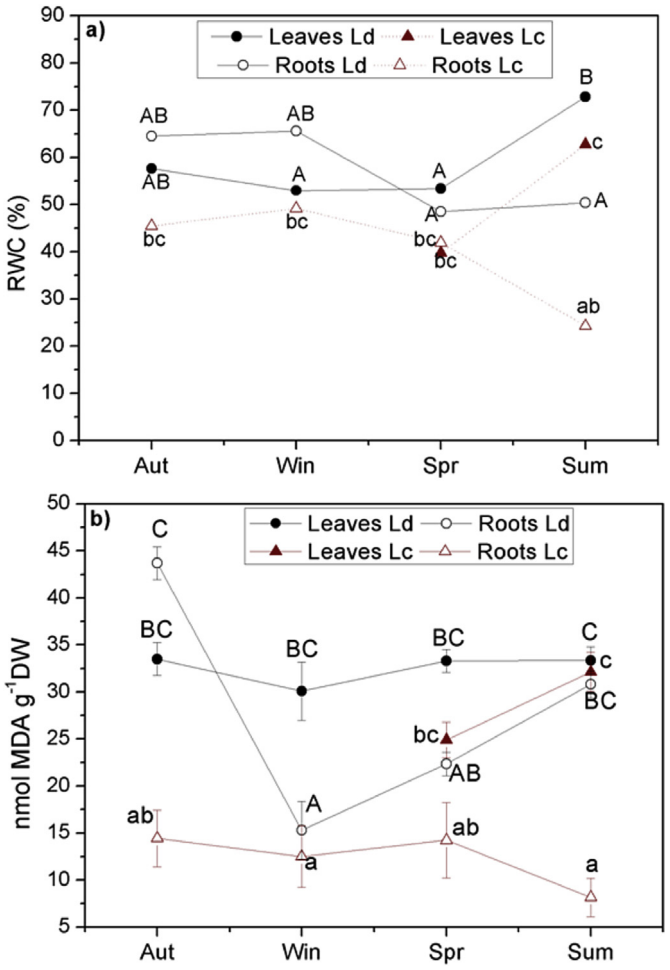

Fig. 2. a) Relative water content (RWC) in leaves and roots of *L. divaricata* (Ld) and *L. chilense* (Lc) in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). b) Lipid peroxidation in leaves and roots of *L. divaricata* and *L. chilense* in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Different letters indicate significant differences between seasons and organs according to Kruskall–Wallis test ($P < 0.05$).

3.5. Quantification of phenolic compounds by spectrophotometric assay

As mentioned above, *L. chilense* has a deciduous behavior and phenolic compounds in leaves were measured only in spring and summer; lower contents were found in comparison with *L. divaricata*. In both species the presence of flavan-3-ols was not detected with the technique employed (Figs. 3 and 4).

3.5.1. Total phenols

Total phenols concentration was higher in leaves than in roots in both species. *L. divaricata* leaves showed high concentration of total phenols, in a range of 48–78 mg g^{-1} DW, while values in roots were between 10 and 43 mg g^{-1} DW (Fig. 3). *L. chilense* leaves had a total phenol concentration in a range of 15–30 mg g^{-1} DW while in roots it was between 3.5 and 7 mg g^{-1} DW. The statistical analysis in leaves of both species did not show interaction between the two variables analyzed (specie and season), then they were analyzed separately. Total phenols concentration in *L. divaricata* leaves was significantly higher than in *L. chilense* leaves only in autumn. The statistical analysis in roots showed interaction between the two variables analyzed. Roots of *L. divaricata* had the highest total phenols concentration in autumn (Fig. 3).

3.5.2. Total flavonoids

Total flavonoids concentration was higher in leaves than in roots, in both species. Flavonoids in *L. divaricata* were in a range of 11–24 mg g^{-1} DW in leaves and between 1 and 4 mg g^{-1} DW in roots. Flavonoids in *L. chilense* did not exceed 4 mg g^{-1} DW. The statistical analysis in leaves did not show interaction between the

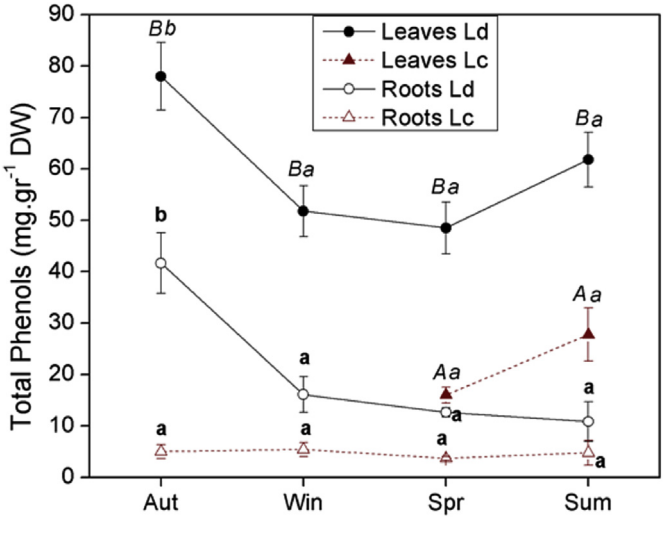

Fig. 3. a) Total Phenols in leaves and roots of *L. divaricata* (Ld) and *L. chilense* (Lc) in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Seasons and species were compared by Two-way ANOVA. Different bold letters indicate interaction and significant differences. Without interaction, different uppercase letters indicate significant differences between species and different lowercase letters indicate significant differences between seasons according to Bonferroni test ($P < 0.05$).

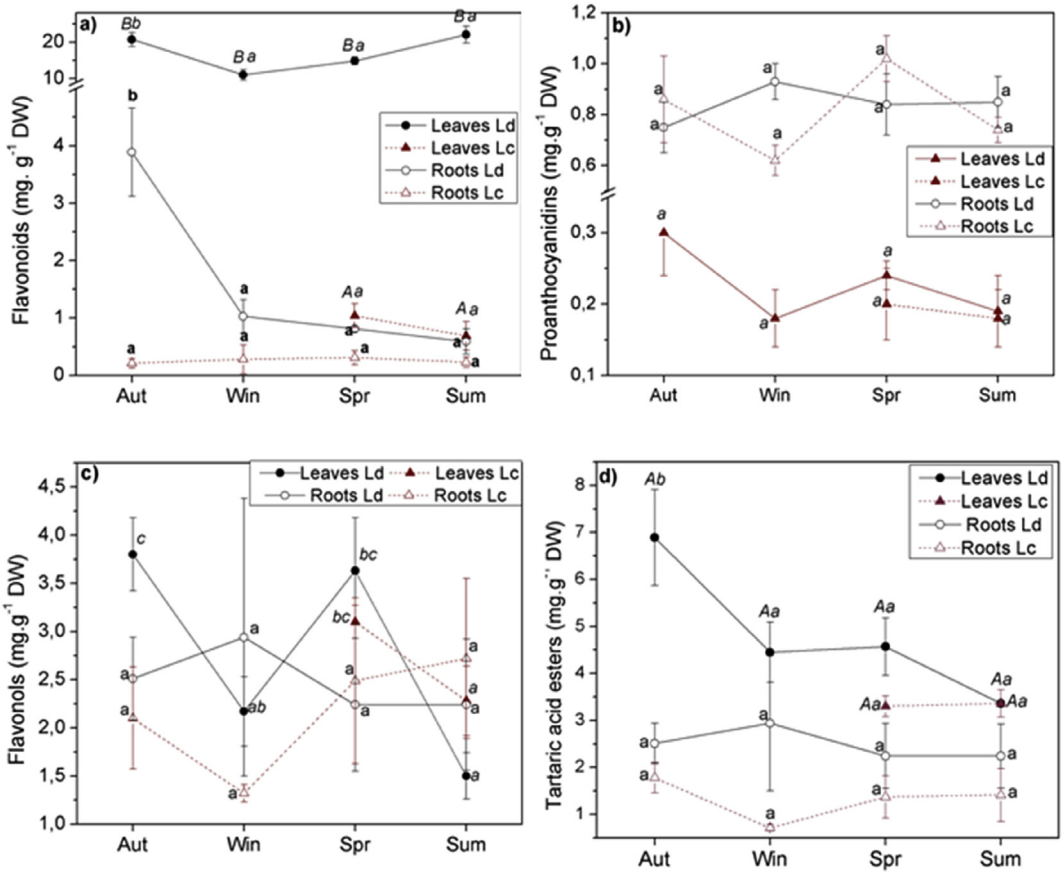

Fig. 4. a) Flavonoids, b) Proanthocyanidins, c) Flavonols and d) Tartaric acid esters content in leaves and roots of *L. divaricata* (Ld) and *L. chilense* (Lc) in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Seasons and species were compared by Two-way ANOVA. Different bold letters indicate interaction and significant differences. Without interaction, different uppercase letters indicate significant differences between species and different lowercase letters indicate significant differences between seasons according to Bonferroni test ($P < 0.05$).

two variables analyzed (specie and season). Flavonoids in leaves were higher in *L. divaricata* than in *L. chilense*, and only in autumn the flavonoids concentration was significantly higher than in the other seasons. In roots, the statistical analysis showed interaction between the two variables and the flavonoids concentration was only significantly higher in autumn for *L. divaricata* (Fig. 4a).

3.5.3. Proanthocyanidins

The results obtained for proanthocyanidins showed that the values did not exceed 2 mg g^{-1} DW in both species analyzed. The highest proanthocyanidin concentrations were found in roots. The statistical analysis did not showed significant differences between the species and seasons. Proanthocyanidins values in *L. divaricata* roots showed a similar tendency to that observed for total phenols and flavonoids (Fig. 4b).

3.5.4. Flavonols and tartaric acid esters

Total flavonols did not exceed 4.5 mg g^{-1} DW in all samples. The statistical analysis in leaves did not show interaction between the variables, and the data obtained presented great variability between individuals. Flavonols concentration in leaves was increased in spring for both species and in autumn only for *L. divaricata*, while in roots there were not differences between seasons. No differences were found in leaves and roots between both species (Fig. 4c).

Tartaric acid esters concentration showed values between 6.8 and 3.3 mg g^{-1} DW in leaves and lower values in roots (between 2.9 and 0.7 mg g^{-1} DW). There was no interaction between the variables and no significant differences between species. Only leaves had significant differences between the seasons, showing an increase in tartaric acid esters concentration for *L. divaricata* in autumn (Fig. 4c).

3.5.5. Antioxidant capacity of phenolic extracts

L. divaricata has a greater antioxidant capacity than *L. chilense* as shown in Fig. 6. The values obtained for *L. divaricata* were in a range between 400 and 1800 μmol g^{-1} DW, while in *L. chilense* the range was between 90 and 270 μmol g^{-1} DW. The statistical analysis in leaves did not showed interaction between the variables. The antioxidant capacity of *L. divaricata* leaves was higher than *L. chilense* leaves. When values were compared between seasons, in *L. divaricata* the lower activity was found in spring, increased in summer and winter, and was highest in autumn. The antioxidant capacity in roots showed interaction between the variables, and a significant difference was only found in autumn for *L. divaricata* roots (Fig. 5).

3.6. Relationship between different variables

3.6.1. Simple lineal regression analysis

Simple linear regression was applied in order to establish different correlations between variables. In *L. divaricata* leaves a strong direct correlation between antioxidant capacity (ABTS) and total phenols (TPh) was detected ($R = 0.85$ and $R^2 = 0.91$). Comparing antioxidant capacity vs. soil water content (SWC) a

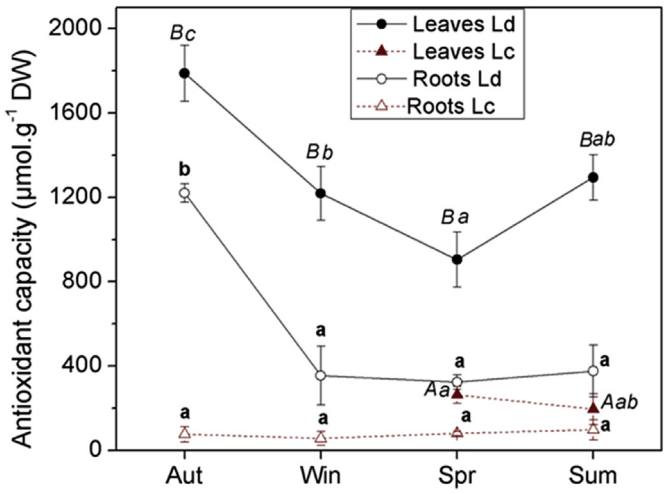

Fig. 5. Antioxidant capacity of the phenolic extract in leaves and roots of *L. divaricata* (Ld) and *L. chilense* (Lc) in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Seasons and species were compared by Two-way ANOVA. Different bold letters indicate interaction and significant differences. Without interaction, different uppercase letters indicate significant differences between species and different lowercase letters indicate significant differences between seasons according to Bonferroni test ($P < 0.05$). Italic letters indicate comparison between leaves and non-italic letters indicate comparison between roots.

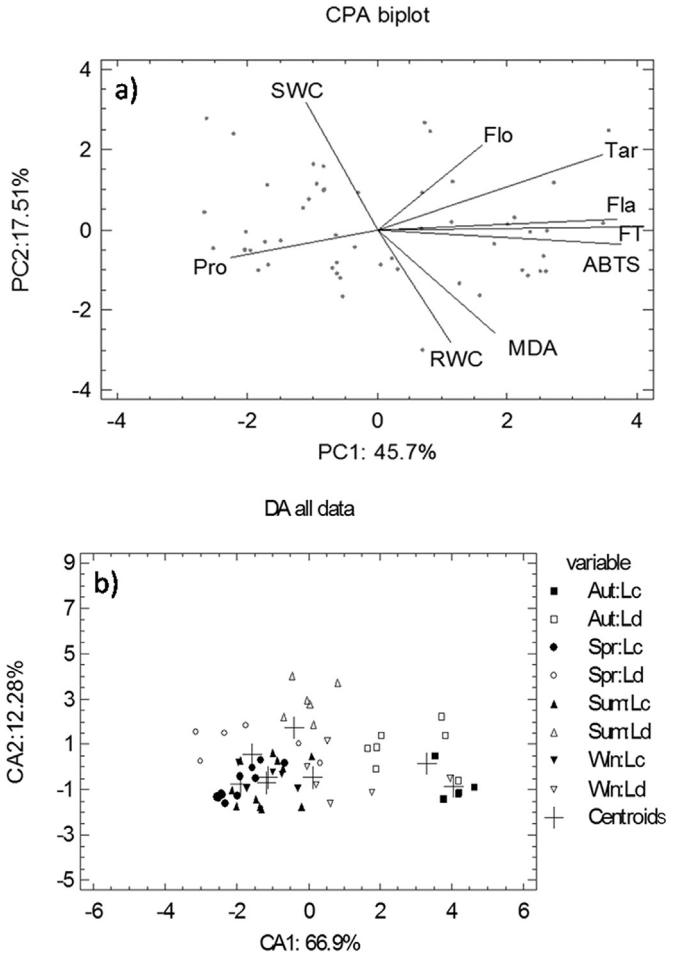

Fig. 6. a) Principal component analysis (PCA) of the values obtained from the different variables analyzed (Antioxidant capacity (ABTS), Lipid peroxidation (MDA), Total Phenols (FT), Flavonoids (Fla), Flavonols (Flo), Proanthocyanidins (Pro), tartaric acid (Tar), soil water content (SWC) and, relative water content (RWC)). b) Linear Discriminant analysis (LDA) of all data. The combination of categories is season and species. *L. divaricata* (Ld) and *L. chilense* (Lc) in autumn (Aut), winter (Win), spring (Spr) and summer (Sum).

significant inverse correlation was detected, assuming that when SWC increases, antioxidant capacity decreases ($R = -0.45$ and $R^2 = 0.2$). There was no significant correlation comparing antioxidant capacity vs. RWC ($R = 0.12$ and $R^2 = 0.014$) and vs. MDA concentration ($R = 0.11$ and $R^2 = 0.013$), assuming that RWC and lipid peroxidation did not affect antioxidant activity directly. The influence of TPh, SWC and RWC over MDA concentration was also analyzed. There were no significant relationships between MDA and SWC ($R = 0.26$ and $R^2 = 0.068$), MDA and RWC ($R = 0.01$ and $R^2 < 0.01$) and MDA and TPh ($R = 0.07$ and $R^2 = 0.06$).

In *L. divaricata* roots there were correlations between ABTS and TPh ($R = 0.94$ and $R^2 = 0.88$), ABTS and MDA content ($R = 0.65$ and $R^2 = 0.42$) and MDA vs. TPh ($R = 0.53$ and $R^2 = 0.28$), but there were no correlations between antioxidant capacity with SWC ($R = -0.36$ and $R^2 = 0.13$) and RWC ($R = 0.33$ and $R^2 = 0.11$) and between MDA content with SWC ($R = -0.41$ and $R^2 = 0.17$) and RWC ($R = 0.33$ and $R^2 < 0.01$).

In *L. chilense* leaves there were no correlation between any of the variables measured: ABTS vs. TPh ($R = 0.38$ and $R^2 = 0.14$), antioxidant capacity vs. SWC ($R = 0.29$ and $R^2 = 0.18$), antioxidant capacity vs. RWC ($R = 0.29$ and $R^2 = 0.086$), antioxidant capacity vs. MDA ($R = 0.04$ $R^2 < 0.01$), MDA vs. SWC ($R = 0.48$ and $R^2 = 0.23$), MDA vs. RWC ($R = 0.11$ and $R^2 = 0.12$) and MDA vs. TPh ($R = 0.53$ and $R^2 = 0.28$).

There was no correlation between the variables analyzed in *L. chilense* roots: antioxidant capacity vs. total phenols ($R = 0.38$ and $R^2 = 0.14$), antioxidant capacity vs. MDA ($R = 0.33$ and $R^2 = 0.11$), antioxidant capacity vs. SWC ($R = 0.13$ and $R^2 = 0.018$), antioxidant capacity vs. RWC ($R = 0.29$ and $R^2 = 0.08$), MDA vs. SWC ($R = -0.41$ and $R^2 = 0.17$) and MDA vs. RWC ($R = 0.05$ and $R^2 < 0.01$).

3.6.2. Multivariate analysis

A PCA analysis was performed to identify which variable was the most affected by seasonal changes Fig. 6a. The first principal component (PC1) explained 45.7% of the variation in the data set. This variation is strongly influenced by the following variables: antioxidant capacity (ABTS), total phenols (TPh) and flavonoids (Fla). The second principal component (PC2) explained 17.5% of the total variance and it is influenced by the variables related to water availability. Soil water content (SWC) influenced positively while relative water content (RWC) negatively. The third principal component which contributes 12.8% of the total variance (data not shown) is influenced by the variables: proanthocyanidins (Pro) and flavonols (Flo). The individuals with high concentration of total phenols and flavonoids and consequently, antioxidant capacity, were located towards the positive end of the PC1 axis, and the individuals with high values of RWC were located towards the negative end of the PC2 axis. The higher values of SWC were located towards the positive end of the PC2 axis. It was not possible to appreciate clusters between seasons and/or organs analyzed.

The first Linear Discriminant Analysis (LDA) was carried out considering the combination between seasons and species as a category (Fig. 6b). The LDA shows that the different clusters of both species overlap in each season (which was expected in view of the CPA), with higher values in *L divaricata* than *L. chilense*, but both species have similar behavior in each season. The canonical axis 1 (CA1) (66.96%) separates autumn (in the positive axis) from the other seasons assuming differences in each variable analyzed for this seasons (described above), while the canonical axis 2 (CA2) (12.28%) separates both species, locating *L. divaricata* in the positive

axis and *L. chilense* in the negative axis. The second and third LDA were carried out considering the combination between seasons and organ of each species as a category (Fig. 7). For *L. divaricata* the CA1 (79.92%) separates between organs, locating roots in the positive axis and leaves in the negative axis, while CA2 (13.90%) separates autumn (negative axis) from the other seasons, with the exception of winter: leaf that have similar values than that of autumn: leaf (Fig. 7a). For *L. chilense* the CA1 (71.89%) separates between the organs while CA2 (23.90%) separates autumn roots from all the other groups (Fig. 7b).

3.6.3. Extraction and isolation of polyphenols by HPLC-MS

Leaf samples of two contrasting seasons were selected for each species: autumn (dry season) and spring (wet season). Table 1 summarize the different molecules identify in these samples. In *L. divaricata* were found, as different molecules from the extract of the wet season, procyanidin dimer B1, kaempferol conjugated with sugar (see Fig. S1 in Supporting Information) and dihydrobenzoic acid under the dry season. Non-flavonoids were gallic acid and oleuropein, while the flavonoids were nobiletin, quercetin, kaempferol, epicatechin and catechin (the last two being part of the procyanidin dimer B1 structure). In *L. chilense* were found different flavonoids (quercetin and luteolin) and non-flavonoids (scopoletin, resveratrol and oleuropein), some of them conjugated with sugars, under the dry season. During the wet season only oleuropein was detected (see Fig. S2 in Supporting Information).

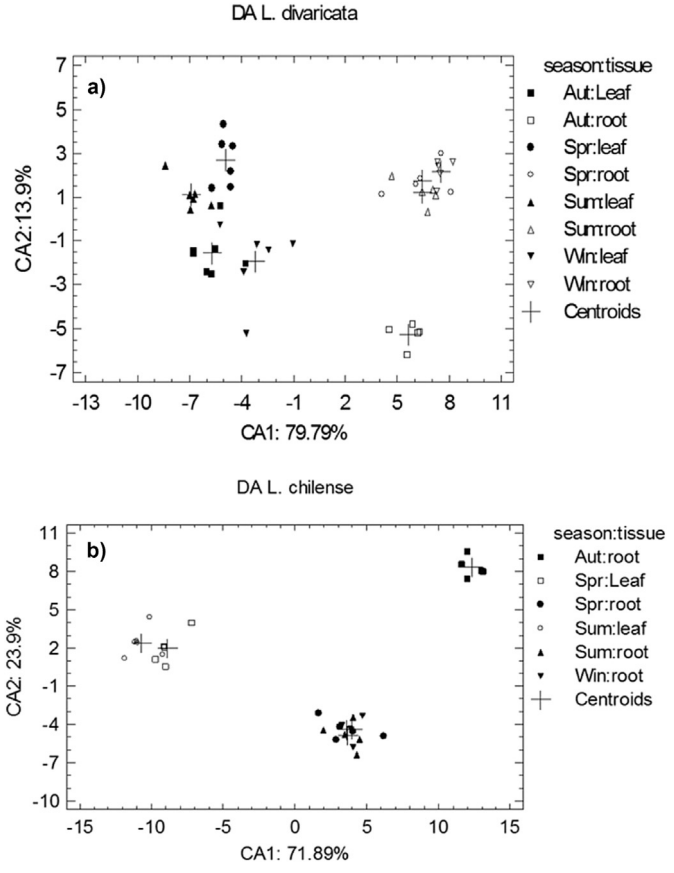

Fig. 7. Linear Discriminant analysis (LDA) of a) *L. divaricata* and b) *L. chilense*. The combination of categories is season and tissue. Leaf and root in autumn (Aut), winter (Win), spring (Spr) and summer (Sum). Canonic axis 1 (CA1) vs. canonic axis 2 (CA2).

3.6.4. Antioxidant capacity of phenolic extract for HPLC-MS and relationship between antioxidant capacity and reactive groups of polyphenols

The antioxidant capacity of the H_2O/MeOH extract study by HPLC-MS is showed in Fig. 8. In *L. divaricata* extract were not found differences between the two seasons analyzed. Comparing the different compounds (Table 1a) and their chemical structure (supplementary data), the presence of dihydrobenzoic acid, procyanidin dymer B1 and kaempferol-3-o-glucosyl-rhamnosyl-glucoside enhance the amount of reactive groups (OH^-), which posses high antioxidant capacity, however, these differences were not detected by the DPPH assay.

L. chilense had a higher antioxidant capacity under the dry seasons. Comparing the phenolic compounds (Table 1b) a higher amount of flavonoids under drought conditions was found, with a 5-fold increase in the amount of hydroxyl groups (supplementary data).

4. Discussion

The Patagonian shrub lands are semi-arid regions with daily and seasonal temperature variations and with seasonal rainfalls not exceeding 200 mm per year, generally concentrated in winter and/or spring (Cabrera, 1976). The results obtained in this study indicate that during the sampling period along the year, rainfalls were concentrated in spring, and the highest average temperatures occurred in spring and summer. This could affect the soil water availability producing high rates of evapotranspiration. Spring was the season with the highest water availability as shown by the SWC values. These results could be interpreted according to Grayson et al. (1997) who determined different states in spatial soil moisture patterns. The state taken as local control (predominant in semi-arid region) establishes that in periods when evapotranspiration continually exceeds precipitation, the soil moisture pattern is influenced by temporary elevation of soil water content following rainfalls. This establishment could also explain the results obtained in SWC in winter, due to rainfall occurrence in the days before to sample collection, indicating that water infiltration into greater depth had not yet occurred.

Reddy et al. (2003) proposed that drought resistant species typically show small decreases in RWC due to osmotic adjustment as a tolerance mechanism that allows the maintenance of a positive turgor potential during the stress period. Our results showed that RWC in leaves and roots of *L. divaricata* had no significant differences between seasons, being in a range of 52 and 75%. These results support the idea proposed by Reddy et al. (2003) suggesting that this species would be well adapted to extreme environments and display a tolerance mechanism which helps to maintain normal physiological parameters even during long period of stress. On the contrary, due to the avoidance mechanism described for *L. chilense*, the RWC in leaves were higher in summer (when leaves were mature) than in spring. Otherwise, the RWC in roots did not change along seasons, supporting the idea that *L. chilense* leaf loss is the main avoidance mechanism, while roots maintain their physiological activity during the whole year. RWC values were between 40 and 60% for leaves and 20–40% for roots, showing that *L. chilense* is a species with a partial xerophytic behavior adapted to a semi-arid environment that uses an avoidance mechanism to survive.

Several experiments have shown that plants exposed to drought stress produce higher concentration of phenolic compounds such as total phenols (de Abreu and Mazzafera, 2005; Jaafar et al., 2012), flavonoids (de Abreu and Mazzafera, 2005; Hernández et al., 2006) and proanthocyanidins (Arteaga et al., 2005; Jaafar et al., 2012). Specifically, evergreen desert shrubs may accumulate large amounts of secondary compounds, including polyphenols (Carrera

Table 1
Phenolic compounds found by HPLC-MS in leaves of a) *L. divaricata* and b) *L. chilense* during autumn (dry season) and spring (wet season). ESI Scan (negative mode) molecules from H_2O/MeOH extract.

		Molecule	m/z value (negative mode)
a)			
L. divaricata leaves	Autumn	Dihydrobenzoic acid	154
	(dry season)	Gallyc Acid (Galloyl ester)	170
		Gallyc acid-3-O-Gallate	323
		Nobiletin (3-methoxysinensetin)	401
		Oleuropein	540
		Quercetin-O-3-O-(6″-acetyl-galactoside-7-O-rhamnoside)	650
		Procyanidin dimer B1	577
		Kaempferol 3-O-glucosyl-rhamnosyl-glucoside	751
	Spring	Gallyc Acid (Galloyl ester)	170
	(wet season)	Gallyc acid-3-O-Gallate	323
		Nobiletin (3-methoxysinensetin)	401
		Oleuropein	540
		Quercetin-O-3-O-(6″-acetyl-galactoside-7-O-rhamnoside)	650
b)			
L. chilense leaves	Autumn	Phenol group	96
	(dry season)	Scopoletin	190
		Resveratrol	227
		Oleuropein-aglycone	378
		Quercetin-3-O-acetyl rhamnoside	489
		Luteolin-7-O-diglucuronide	637
	Spring	Phenol group	96
	(wet season)	Oleuropein-aglycone	378

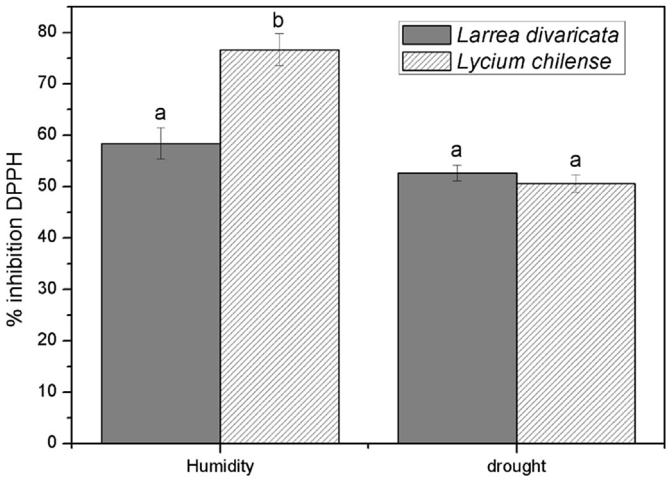

Fig. 8. Antioxidant capacity of the phenolic extract for HPLC-MS assay in *L. divaricata* leaves and *L. chilense* leaves in Humidity (spring) and drought (autumn). Seasons were compared by one-way ANOVA for each species. Different letters indicate significant differences between seasons according to Bonferroni test ($P < 0.05$).

and Bertiller, 2010; Campanella and Bertiller, 2011), which could be used as a strategy to tolerate drought (Westoby et al., 2002; Reddy et al., 2004) due to their powerful antioxidant activity able to control excessive ROS formation in tissues (Hatier and Gould, 2008; Agati and Tattini, 2010). Hyder et al. (2002) showed that *Larrea tridentata* (a native species of the semi-arid regions in North America closely related to *L. divaricata*) contains several lignans, flavonoids, condensed tannins, triterpenes, saponins and waxes. In this study we found that *L. divaricata* had a higher production of total phenols and flavonoids compared with *L. chilense*; the production of these compounds was higher in leaves than in roots, and their concentration was enhanced in autumn. The antioxidant capacity of the phenolic extracts (spectrophotometric assay) was also higher in *L. divaricata* than in *L. chilense*, and increased approximately 95% from spring to autumn. The relationship between variables was analyzed by univariate way: SLR (Simple Linear Regression) and multivariate way PCA and LDA (Principal Component Analysis and Linear Discriminant Analysis). As we expected the SLR shows that *L. divaricata* had significant relationship between total phenols and antioxidant capacity, supporting the idea that this evergreen shrub produces secondary metabolites that function as non-enzymatic antioxidants in a mechanism to tolerate drought stress naturally imposed by the arid environment (Quideau et al., 2011; Gill and Tuteja, 2010). Only *L. divaricata* roots had a significant relationship between MDA content and antioxidant capacity and between MDA content and total phenols. When roots sense drought-induced risk of oxidative stress, secondary metabolites production increases in order to reinforce the enzymatic antioxidant mechanism to avoid cellular damage. On the other hand, SLR of the variables measured in *L. chilense* had no significant relationship between them; the differences between this two species could be explained by the classification proposed by Karabourniotis et al. (2014) in slow growing species like the evergreen *L. divaricata*, and fast growing plants like the deciduous *L. chilense*.

Palacio et al. (2012) reported the presence of two compounds in *L. divaricata* leaves, quercetin and kaempferol (flavonols). Our analysis showed that flavonols accumulation in *L. divaricata* was higher in leaves than in roots, and that their production increased in autumn (dry season) and spring. Flavonols may not only play an antioxidant role in drought stress, but are also important compounds for leaf tissues protection from UV absorption, thermal damage and defense against herbivores and/or pathogens (Aerts and Chapin, 2000; Souto et al., 2000; González-Rodríguez, 2004).

The lower polyphenol concentration in *L. chilense* and the lack of correlation with the antioxidant capacity in leaves, suggest that the production of secondary metabolites in this species is not a very important trait in drought avoidance (Westoby et al., 2002; Reddy et al., 2004). However, total phenols and flavonoids in roots had positive correlation with the antioxidant capacity, which suggests their importance for cellular structure protection from the damage caused by ROS during long periods of drought (Surweswaran et al., 2007; Krishnaiah et al., 2011).

Proanthocyanidins concentration was higher in roots than in leaves in both species; however, a positive correlation with the

antioxidant capacity was only found in *L. divaricata* leaves. Similarly, Hyder et al. (2002) and Holechek et al. (1990) found higher levels of proanthocyanidins in roots than in leaves of *L. tridentata*. Taken together, these results suggest the possibility that high levels of proanthocyanidins in roots could play an allelopathic role against other plants species (Bais et al., 2002, 2003), insects (Thelen et al., 2005) and/or microorganism (Li et al., 2010) instead of an antioxidant role. Additionally, it has been proposed that these compounds may play a role in strengthening the cell walls under stressing conditions (Field et al., 2006).

The isolation of specific polyphenols showed different patterns among both species and even among the two seasons contrasted. *L. divaricata* presented several flavonols, mostly bonded with sugars. The bond between sugar and phenolic compounds increase their solubility in the sap vacuole (Peshev et al., 2013), may be part of the redox system (Bolouri-Moghaddam et al., 2010) and/or could be involved as intermediaries of different biosynthetic pathways (Karabourniotis et al., 2014).

As mentioned above, Palacio et al. (2012) identified two flavonoids in *L. divaricata*, quercetin and kaempferol. According to our analysis, the flavonoids identified were also quercetin, kaempferol and a novel compound, nobiletin, in its free form. The structure of this molecule suggests a potential antioxidant capacity due to the presence of several reactive groups (Kaur and Kapoor, 2001). The presence of condensed tannins in *L. divaricata* during the dry season, specifically procyanidin dimer B1, could be related to the structural role proposed for these compounds by giving structural support by increasing cell wall rigidity and helping to this species to deal with the drought season (Saraví Cisneros et al., 2013). The levels of flavonoids identified in *L. chilense* were lower than in *L. divaricata*, supporting the idea that *L. chilense* has an avoidance mechanism to tolerate drought, with a deciduous behavior instead of investing high amount of energy in these complex antioxidant molecules. Independently of the season analyzed, a greater production and accumulation of flavonoids in the evergreen species than in the deciduous species contributes to the concept that flavonoids have an important antioxidant role in native perennial plants to cope with these adverse environments, mainly for protection of the photosynthetic apparatus (Tattini et al., 2005; Heruiyot et al., 2007).

5. Conclusion

Native species of Patagonian shrublands can use different mechanism to resist long periods of drought stress in order to survive and coexist in the same habitat. In this study we showed that accumulation of secondary metabolites, specifically flavonoids, is an important feature within the mechanisms employed by xerophytic species to avoid oxidative damage, and could be used as an indicator of drought tolerance.

Acknowledgments

This research was funded by the National Research Council of Argentina (Projects D3148-CONICET and PIP-191-CONICET) to A.C. and CONICET PIP 5628 and PICT-ANPCYT 02232/07 to V.L. CONICET also founded the Doctoral Fellowship to C.V. We thank to Fundación Patagonia Natural who allowed us to harvest plant material on his Wildlife Reserve “La Esperanza”, to Dr. Analía Carrera (Centro Nacional Patagónico: CENPAT-CONICET) for providing the weather data for this study and to Şefika Emine Arslan and Fatma Gül Akar for helping in polyphenols determinations at the Dr. Arslan's laboratory at Pammukale University.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.03.014.

Contributors

Sample collection was carried out by C.V. and A.C. Polyphenols quantification by spectrophotometry and their antioxidant activity were conducted by C.V., aided by M.R. Extraction and isolation of polyphenols by HLPC-MS and their antioxidant activity were conducted by C.V. and I. A. Statistical analyses were conducted by C.V.

C.V. wrote the manuscript; A.C., I.A., M.R., and V.L edited the manuscript.

I.A. was the tutor of C.V. in an Internship in Turkey.

V.L. is the Head of the Plant Physiology Laboratory where the project is currently being carried on.

All authors approved the final version of this article.

Conflicts of interest

No conflicts of interest.

Authors contributions

- María Celeste Varela and Ana María Cenzano carried out sample collection in the study site.
- María Celeste Varela conducted polyphenols quantification by spectrophotometry and their antioxidant activity, aided by Mariana Andrea Reginato.
- María Celeste Varela and Idris Arslan conducted together Extraction and isolation of polyphenols by HLPC-MS and their antioxidant activity.
- María Celeste Varela conducted statistical analyses
- María Celeste Varela wrote the manuscript. Idris Arslan, Mariana Andrea Reginato, Ana María Cenzano and María Virginia Luna edited the manuscript.
- Idris Arslan was the tutor of María Celeste Varela in an Internship in Turkey.
- María Virginia Luna is the Head of the Plant Physiology Laboratory where the project is currently being carried on.

All authors approved the final version of this article.

References

Aerts, R., Chapin III, F.S., 2000. The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. Adv. Ecol. Res. 30, 1–67.
Agati, G., Tattini, M., 2010. Multiple functional roles of flavonoids in photoprotection. New Phytol. 186, 786–793.
Arteaga, S., Andrade-Cetto, A., Cárdenas, R., 2005. *Larrea tridentata* (Creosote bush), an abundant plant of Mexican and US-American deserts and its metabolite nordihydroguaiaretic acid. Review J. Ethnopharmacol. 98, 231–239.
Bais, H.P., Vepachedu, R., Gilroy, S., Callaway, R.M., Vivanco, J.M., 2003. Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science 301, 1377–1380.
Bais, H.P., Walker, T.S., Stermitz, F.R., Hufbauer, R.A., Vivanco, J.M., 2002. Enantiomeric-dependent phytotoxic and antimicrobial activity of (±)-catechin. A rhizosecreted racemic mixture from spotted knapweed. Plant Physiol. 128, 1173–1179.
Bertiller, M.B., Mazzarino, M.J., Carrera, A.L., Diehl, P., Satti, P., Gobbi, M.J., Sain, C.L., 2006. Leaf strategies and soil N across a regional humidity gradient in Patagonia. Oecologia 148, 612–624.
Bertiller, M.B., Sain, C.L., Carrera, A.L., Vargas, D.N., 2005. Patterns of nitrogen and phosphorus conservation in dominant perennial grasses and shrubs across an aridity gradient in Patagonia, Argentina. J. Arid Environ. 62, 209–223.
Bertiller, M.B., Bisigato, A.J., Carrera, A.L., Del Valle, H.F., 2004. Estructura de la vegetación y funcionamiento de los ecosistemas del Monte Chubutense. Bol. Soc. Argent. Bot. 39 (3–4), 139–158.
Bolouri-Moghaddam, M.R., Le Roy, K., Xiang, L., Rolland, F., Van den Ende, W., 2010.

Sugar signalling and antioxidant network connections in plant cells. FEBS J. 277, 2022–2037.
Borbalán, A.M.A., Zorro, L., Guillen, D.A., Barroso, C.G., 2003. Study of the polyphenol content of red and white grape varieties by liquid chromatography-mass spectrometry and its relationship to antioxidant power. J. Chromatogr. 1012, 31–38.
Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol. 28, 25–30.
Cabrera, A.L., 1976. Las regiones fitogeográficas Argentinas. (Enciclopedia Argentina de Agricultura, Jardinería y Horticultura. ACME). Argentina, pp.85.
Campanella, M.V., Bertiller, M.B., 2008. Plant phenology, leaf traits and leaf litterfall of contrasting life forms in the arid Patagonian Monte, Argentina. J. Veg. Sci. 19, 75–85.
Campanella, M.V., Bertiller, M.B., 2011. Is N-resorption efficiency related to secondary compounds and leaf longevity in coexisting plant species of the arid Patagonian Monte, Argentina? Austral Ecol. 36, 395–402.
Carrera, A.L., Bertiller, M.B., 2010. Relationship among plant litter, fine roots, and soil organic C and N across an aridity gradient in northern Patagonia, Argentina. Ecoscience 17, 276–286.
Cenzano, A., Varela, M.C., Bertiller, M., Luna, V., 2013. Effect of drought on morphological and functional traits of Poa ligularis and Pappostipa speciosa, native perennial grasses with wide distribution in Patagonian rangelands, Argentina. Austral J. Bot. 61, 383–393.
Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant response to drought: from genes to the whole plant. Funct. Plant Biol. 30, 239–264.
Crozier, A., Jaganath, I.B., Clifford, M.N., 2009. Dietary phenolics: chemistry, bioavailability and effects on health. Nat. Product Rep. 8, 1001–10043.
de Abreu, I.N., Mazzafera, P., 2005. Effect of water and temperature stress on the content of active constituents of Hypericum brasiliense Choisy. Plant Physiol. Biochem. 43, 241–248.
Field, B., Jordan, F., Osbourn, A., 2006. First encounters-deployment of defence-related natural product by plants. New Phytol. 172, 193–207.
Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.
González-Rodríguez, L., 2004. Relaciones alelopáticas. In: Reigosa, M.J., Pedro, l.N., Sánchez, A. (Eds.), La Ecofisiología Vegetal. Una Ciencia de Síntesis. Thomson, Madrid, pp. 527–576.
Grayson, R.B., Western, A.W., Chiew, F.H.S., 1997. Preferred states in spatial soil moisture patterns: local and nonlocal controls. Water Resour. Res. 22, 1786–1897.
Hatier, J.H.B., Gould, K.S., 2008. Foliar anthocyanins as modulators of stress signals. J. Theor. Biol. 253, 625–627.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.
Hernández, I., Alegre, L., Munné-Bosch, S., 2006. Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water- stressed tea plants. Phytochemistry 67, 1120–1126.
Heruiyot, Erick K.C., Umera Louis, M.M., Etich Wilson, K.N.G., Assanali Ahmed, H., Achira Francis, W., 2007. Polyphenols as potential indicators for drought tolerance in tea (*Camellia sinensis L.*). Biosci. Biotechnol. Biochem. 71 (9), 2190–2197.
Hetherington, A.M., Woodward, F.I., 2003. The role of stomata in sensing and driving environmental change. Nature 424, 901–908.
Holechek, J.L., Munshikpu, A.V., Saiwana, L., Nunez-Hernandez, G., Valdez, R., Wallace, J.D., Cardenas, M., 1990. Influences of six shrub diets varying in phenol content on nitrogen retention by goats. Trop. Grassl. 24, 93–98.
Hyder, P.W., Fredrickson, E.L., Estell, R.E., Tellez, M., Gibbens, R.P., 2002. Distribution and concentration of total phenolics, condensed tannins, and nordihydroguaiaretic acid (NDGA) in creosote bush (*Larrea tridentata*). Biochem. Syst. Ecol. 30, 905–912.
Jaafar, H.Z., Ibrahim, M.H., Fakri, N.F.M., 2012. Impact of soil field water capacity on secondary metabolites, phenylalanine ammonia- lyase (PAL), malondialdehyde (MDA) and photosynthetic responses of Malaysian Kacip Fatimah (*Labisia pumila* Benth). Molecules 17, 7305–7322.
Karabourniotis, G., Liakopoulos, G., Nikolopoulos, D., Bresta, P., Stavroulaki, V., Sumbele, S., 2014. "Carbon gain vs. water saving, growth vs. defence": two dilemmas with soluble phenolics as a joker. Plant Sci. 227, 21–27.
Kaur, C., Kapoor, H.C., 2001. Antioxidants in fruits and vegetables—the millennium's health. Int. J. Food Sci. Technol. 36, 703–725.
Khanbabaee, K., Van Tee, T., 2001. Tannins: classification and definition. Nat. Product Rep. 18, 641–649.
Kim, D., Chun, O.K., Kim, Y.J., Moon, H.-Y., Lee, C.Y., 2003. Quantification of phenolics and their antioxidant capacity in fresh plums. J. Agric. Food Chem. 51, 6509–6515.
Krishnaiah, D., Sarbatly, R., Nithyanandam, R., 2011. A review on the antioxidant potential of medicinal plant species. Food Bioprod. Process. 89, 217–233.
León, J.J.C., Bran, D., Collantes, M., Paruelo, J.M., Soriano, A., 1998. Grandes unidades de la Patagonia extra andina. Ecol. Austral 8, 125–144.
Levitt, J., 1980. Responses of Plants to Environmental Stresses. Water, Radiation, Salt and Other Stresses, vol. II. Academic Press, New York, pp. 93–128.
Li, Z.-H., Qiang, W., Xiao, R., Cun-De, P., De-An, J., 2010. Phenolics and plant allelopathy. Review Molecules 15, 8933–8952.
Motilva, M.J., Serra, A., Macià, A., 2013. Analysis of food polyphenols by ultra high-performance liquid chromatography coupled to mass spectrometry: an overview. J. Chromatogr. A 1292, 66–82.
Naczk, M., Shahidi, F., 2004. Extraction and analysis of phenolics in food. J. Chromatogr. A 1054, 95–111.
Nigel, C.W., Glories, Y., 1991. Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. Am. J. Enol. Vitic. 42, 364–366.
Noy-Meir, E., 1973. Desert ecosystems: environment and producers. Annu. Rev. Ecol. Syst. 4, 3–51.
Palacio, L., Cantero, J.J., Cusidó, R.M., Goleniowski, M.E., 2012. Phenolic compound production in relation to differentiation in cell and tissue cultures of Larrea divaricata (Cav.). Plant Sci. Int. J. Exp. Plant Biol. 193–194, 1–7.
Peshev, D., Vergauwen, R., Moglia, A., Hideg, E., Van den Ende, W., 2013. Towards understanding vacuolar antioxidant mechanisms: a role for fructans? J. Exp. Bot. http://dx.doi.org/10.1093/jxb/ers377.
Peters, D.B., 1965. Water availability. In: Black, C.A., Clark, F.E., Dinauer, R.C., Ensminger, L.E., White, J.L. (Eds.), Methods of Soil Analysis. Part I. Physical and Mineralogical Properties, Includind Statistics of Measurement and Sampling. American Society of Agronomy, Inc., Publisher, USA, pp. 279–285.
Quideau, S., Deffieux, D., Douat-Casassus, C., Pouységu, L., 2011. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew. Chem. Int. Ed. 50, 586–621.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237.
Reddy, A.R., Chaitanyaa, K.V., Vivekanandan, M., 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 161, 1189–1202.
Reddy, T.Y., Reddy, V.R., Anbumozhi, V., 2003. Physiological responses of groundnut (Arachis hypogea L.) to drought stress and its amelioration: a critical review. Plant Growth Regul. 41, 75–88.
Reginato, M.A., Castagna, A., Furlán, A., Castro, S., Ranieri, A., Luna, V., 2014. Physiological responses of a halophytic shrub to salt stress by Na_2SO_4 and NaCl: oxidative damage and the role of polyphenols in antioxidant protection. AoB Plants 6. http://dx.doi.org/10.1093/aobpla/plu042 plu042.
Romani, A., Mancini, P., Tatti, S., Vincieri, F., 1996. Polyphenols and polysaccharides in Tuscan grapes and wines. Ital. J. Food Sci. 1, 13–24.
Saraví Cisneros, H., Bertiller, M.B., Carrera, A.L., Larreguy, C., 2013. Diversity of phenolic compounds and plant traits in coexisting Patagonian desert shrub species of Argentina. Plant Ecol. 214 (11), 1335–1343.
Sies, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215, 213–219.
Soriano, A., 1950. La vegetación del Chubut. Rev. Argent. Agron. 17, 30–66.
Soriano, A., Nogués-Loza, M., Burkart, S., 1995. Plant biodiversity in the extraandean Patagonia: comparisons with neighbouring and related vegetation units. In: Montes, L., Oliva, G.E. (Eds.), Actas del taller internacional sobre recursos fitogenéticos, desertificación y uso sustentable. CRPS INTA, Río Gallegos, Argentina, pp. 36–45.
Soriano, A., Sala, O.E., 1983. Ecological strategies in a Patagonian arid steppe. Vegetation 56, 9–15. UNESCO, 1979. Carte de la répartitionmondiale des régionsarides. 1/25.000.000. Notice explicative. UNESCO, Paris, 55pp.
Souto, X.C., Chiapusio, G., Pellissie, F., 2000. Relationships between phenolics and soil microorganisms in spruce for- ests: significance for natural regeneration. J. Chem. Ecol. 26, 2025–2034.
Surweswaran, S., Cai, Y.Z., Corke, H., Sun, M., 2007. Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. Food Chem. 102, 938–953.
Tattini, M., Guidi, L., Morassi-Bonzi, L., Pinelli, P., Remorini, D., Degl'Innocenti, E., Giordano, C., Massai, R., Agati, G., 2005. On the role of flavonoids in the integrated mechanisms of response of Ligustrum vulgare and Phillyrea latifolia to high solar radiation. New Phytol. 167, 457–470.
Thelen, G.C., Vivanco, J.M., Newingham, B., Good, W., Bais, H.P., Landers, P., Caesar, A., Callaway, R.M., 2005. Insect herbivory stimulates allelopathic exudation by an invasive plant and the suppression of natives. Ecol. Lett. 8, 209–217.
Waterman, P.G., Mole, S., 1994. Analysis of phenolics plant metabolites. In: Lawton, J.H., Likens, G.E. (Eds.), Methods in Ecology. Blackwell Scientific Publications Oxford pp.238.
Westoby, M., Falster, D.S., Moles, A.T., Vesk, P.A., Wright, I.J., 2002. Plant ecological strategies: some leading dimensions of variation between species. Annu. Rev. Ecol. Syst. 33, 125–159.
Xu, Z.Z., Zhou, G.S., 2007. Photosynthetic recovery of a perennial grass Leymus chinensis after different periods of soil drought. Plant Production Sci. 10, 277–285.