Original article

Study of functional and physiological response of co-occurring shrub species to the Mediterranean climate

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A B S T R A C T

The Mediterranean basin is characterised by increasingly dry summers and the study of the adaptive traits developed by plants living in this stressful environment is of great interest, also in relation to climate projections for this area.

Cistus monspeliensis, *Myrtus communis* and *Phillyrea angustifolia* are three co-occurring shrubs typical of the Mediterranean maquis. Their functional and physiological parameters were studied in spring, summer and autumn in order to highlight adjustments of these traits and to test eventual different adaptive strategies.

Soil and leaf chemical characteristics were determined in the different seasons. Leaf area, specific leaf area, leaf dry matter content, succulence index, pigment contents hydric status and main markers of oxidative stress and antioxidant response were detected.

The stressful summer season induced disturbance in hydric balance, decrease in succulence index and chlorophyll content and high contents of hydrogen peroxide. Thanks to higher enzymatic activities and total glutathione content, in the two evergreen species *M. communis* and *P. angustifolia* oxidative damage remained at levels equal to or lower than the other seasons. Only in the semideciduous *C. monspeliensis* both functional and biochemical traits showed a higher stress condition in summer. The higher stability of functional traits in the two evergreen species may be explained by the sclerophyllous nature of their leaves. Four environmental variables – Tmax, Tmin, soil conductivity and organic matter – mostly influenced NMDS segregation of these species.

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1. Introduction

Cistus monspeliensis L. (Cistaceae), *Myrtus communis* L. (Myrtaceae), and *Phillyrea angustifolia* L. (Oleaceae) are among the most widely diffused vascular plant species in the Mediterranean maquis. *Cistus monspeliensis* is a small semideciduous shrub, distributed in south Europe, growing in very different soils below 700 m a.s.l. (Pignatti, 1982). This species, similarly to other drought semideciduous taxa, is expected to be characterised by a seasonal leaf dimorphism: small leaves at the end of spring-beginning of summer, and larger and thinner leaves in autumn-winter (Werner et al., 1999; Aronne and De Micco, 2001; De Micco and Aronne, 2009; Catoni et al., 2012). *Myrtus communis* and *Phillyrea angustifolia* are tall evergreen shrubs distributed in southwestern Mediterranean region, growing in very dry and hot habitats below 500 m a.s.l. (Pignatti, 1982). Ecophysiological studies on *Phillyrea angustifolia* have shown the importance of photo- and antioxidative protection for the survival of drought-stressed plants in the field (Peñuelas et al., 2004).

Summer drought in which the combined action of water deficit, high air temperatures and excess of light could be very stressful, is generally considered the primary constraint to plant growth in Mediterranean Basin (Werner et al., 1999). Taking into account that climate change is expected to increase temperatures and the intensity and/or frequency of drought in the Mediterranean basin (IPCC 2013) the characterization of plants living in this stressful environment and of their response to constraints related to climate results of great interest.

Mediterranean plants have developed different mechanisms to cope with summer drought involving morphological, biochemical and physiological changes (Gratani et al., 2013). Many species are

sclerophyllous shrubs with small, cutinized, and vertically oriented leaves to reduce heat loading and promote stem flow when rain occurs. Environmental constraints of this habitat can induce oxidative stress on plants and the ability to cope with these restrictions depends also on the capacity to activate an adequate antioxidant response. Moreover, plant functional traits are useful features to study ecological strategies and to determine how plants respond to environmental factors (Perez-Harguindeguy et al., 2013).

Due to the useful combination of morphological and physiological strategies in plant adaptation (Gratani et al., 2013) both functional and physiological traits were studied in *Cistus monspeliensis*, *Myrtus communis* and *Phillyrea angustifolia* in the stressful summer period. To better highlight the modulation of the response of the three co-occurring taxa, these parameters were also studied in spring and autumn. To our knowledge, few reports exist about oxidative stress related to environmental changes and functional data (Gil et al., 2014). As a consequence, in this work functional traits, stress markers and antioxidant response have been studied in detail.

The principal aim was to point out the eventual different strategies in the three co-occurring species during the selected seasons and to assess if there is a correlation between physiological and functional traits depending on seasonal conditions.

Other purpose was to determine which, among the selected physiological and functional parameters, are mostly influenced in stressful conditions.

2. Materials and methods

2.1. Study area and plant species

The study was carried out on selected adult shrubs of *Cistus monspeliensis* L., *Myrtus communis* L., and *Phillyrea angustifolia* L., growing in the Mediterranean maquis at Castellare Mount (43°45′N, 10°27′E, Pisa, Italy) in the period 2015–2016 (Fig. 1). The soil texture is characterised by a predominance of sand (∼60%) and low percentages of clay and silt (∼8% and ∼32%, respectively). The climate is Mediterranean sub-humid type; according to Rapetti (2003), its mean annual temperature is about 15 °C and the mean rainfall is 800–900 mm. Climatic data were available from Tuscany Region (Sviluppo Rurale Settore "Rete dati agrometeo-climatici", www.consiglio.regione.toscana.it/artico-lazioni/Strutture.aspx?cmu=04172) and they were relative to the meteorological station of Pisa (43°42′N, 10°24′E; 6 m a.s.l.), 8 km south west from the study site (Table 1).

Fig. 1. Photo of the experimental field: Castellare Mount (43° 45′ N, 10° 27′ E, Pisa, Italy).

Table 1
Temperature and rainfall values in spring, summer and autumn (2015–2016) at Castellare Mount (43°45′N, 10°27′E, Pisa, Italy).

	Spring	Summer	Autumn
Mean Rainfall (mm)	1.78 ± 0.80 ab	0.15 ± 0.13 b	4.39 ± 1.85 a
Total Rainfall (mm)	55.20	4.60	131.80
Tmax (°C)	22.15 ± 0.44 b	30.10 ± 0.38 a	15.91 ± 0.53 c
Tmin (°C)	12.91 ± 0.37 b	19.56 ± 0.36 a	8.61 ± 0.81 b
Tmean (°C)	17.53 ± 0.28 b	24.83 ± 0.34 a	12.26 ± 0.65 c

Data are mean of daily registration ± SE for each parameter (except for total rainfall). Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test.

2.2. Determination of soil characteristics

Soil chemical characteristics were determined in spring, summer and autumn using standard soil analysis methodology. Each sample represented approximately 3 kg of soil randomly collected from 10 soil cores (20 mm diameter, 0–10 cm depth) in each season. Specifically, soil pH was determined in water as detailed by McLean (1982). Electrical conductivity was determined by fixed-ratio extract (Rhodes, 1982). Soil nitrogen (N) was obtained by micro-Kjeldahl digestion (Allen 1989). Available phosphorus (P) was obtained by Olsen et al. method (1954). The organic matter content was estimated through the Walkley–Black method following the protocol of SISS (1985).

2.3. Functional traits

For each species four morphological traits were determined: leaf area, specific leaf area, leaf dry matter content, and succulence index. Each trait was quantified by measuring at least 20 replicate samples from ten different individuals, randomly selected at the time of the first sampling. For leaves, sample storing and processing followed the standardized methodologies detailed by Pérez-Harguindeguy et al. (2013). Leaf area (LA) is the one-sided projected surface area of a fresh leaf, expressed in mm^2. Specific leaf area (SLA) is LA divided by its oven-dry mass, expressed in $mm^2\ mg^{-1}$. Leaf dry matter content (LDMC) is the proportion of the water-saturated fresh mass of a leaf accounted for by its oven-dry mass, expressed in %. Succulence index (SI) is the ratio of the difference between the oven-dry mass of a leaf and its water-saturated fresh mass to the leaf surface area, expressed in mg cm^{-2} (Read et al., 2005). Leaf projected area was acquired with a CanoScan LiDE 90 (Canon) and determined by CompuEye, Leaf and Symptom Area software (available at www.ehabsoft.com/CompuEye/LeafSArea/).

Leaf N content (LNC), leaf P content (LPC), and leaf C content (LCC) were determined using conventional methods (Allen, 1989) and expressed on a dry matter basis (%): C was determined by the Springeer–Klee method, N using the micro-Kjeldahl digestion method, P was determined spectrophotometrically using ammonium molybdate, after acid digestion.

Chlorophylls (a, b and total) and carotenoids were extracted in 80% acetone and determined according to Hassanzadeh et al. (2009) and to Lichtenthaler (1987) respectively. Pigment contents were expressed as mg $g^{-1}DW$.

2.4. Relative water content

Leaf relative water content (RWC) was determined according to Balestri et al. (2014) and calculated with the formula:

$$RWC = [(FW - DW)/(TW - DW)] * 100$$

FW = Fresh weight
DW = Dry weight
TW = Turgid weight

Leaf fresh weight was obtained by weighing the fresh leaves. The leaves were then immersed in water over night, blotted dry and then weighed to record the turgid weight. The leaves were then dried in an oven to constant weight and reweighed to obtain the dry weight.

2.5. Oxidative stress and antioxidant response

Hydrogen peroxide content was determined using 0.1% titanium chloride in 20% (v/v) H_2SO_4 according to Jana and Choudhuri (1982). The amount of H_2O_2 in the extracts, expressed as $\mu mol\ g^{-1}DW$, was calculated from a standard curve.

Lipid peroxidation in leaves was estimated as the amount of TBARS, determined by the thiobarbituric acid (TBA) reaction, according to Hartley-Whitaker et al. (2001) with minor modifications. Leaves were mixed with TBA reagent (10% w/v trichloroacetic acid + 0.25% w/v thiobarbituric acid), heated (95 °C for 30 min), cooled and centrifuged (2000g for 15 min). The content of TBARS was measured as specific absorbance at 532 nm by subtracting the non-specific absorbance at 600 nm and calculated using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$. TBA-reactive materials were expressed in nmol g^{-1} DW.

Total phenols were measured using Folin-Ciocalteu reagent according to Arezki et al. (2001). Phenolic extracts were obtained after centrifugation of frozen leaf samples homogenised in HCl 0.1 N. After incubation at 100 °C for 1 min in the presence of Na_2CO_3 (20% w/v), samples were cooled and the absorbance at 750 nm was read. Level of phenolic compounds was calculated as equivalent of gallic acid (GAE mg g^{-1} DW) on the base of a standard calibration curve.

Ascorbate, reduced form (ASA) and oxidized form (dehydroascorbate, DHA), extraction and determination were performed according to Kampfenkel et al. (1995) with minor modifications (Spanò et al., 2011b). Briefly, leaves were ground in a chilled mortar and homogenised with 5% (w/v) TCA. The homogenate was centrifuged at 12,000g for 10 min at 4 °C and the supernatant was used for the determination at 525 nm. Total ascorbate was determined after reduction of DHA to ASA by dithiothreitol and DHA level was estimated on the basis of the difference between total ascorbate and ASA value. Calculations were made on the base of a standard curve and ascorbate content was expressed as mg g^{-1}DW.

Glutathione was extracted and determined according to Gossett et al. (1994). Total glutathione (reduced form, GSH + oxidized form, GSSG) was determined by the 5,5′-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure, monitoring the rate of change in absorbance at 412 nm. Calculations were made on the base of a standard curve and content was expressed as nmol g^{-1}DW.

For enzyme extraction and assays, leaves were ground in liquid nitrogen with a mortar and pestle. Extraction was made as in Spanò et al. (2013). Supernatants were collected and stored in liquid nitrogen until their use for enzymatic assays.

Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981) with modification as in Spanò et al. (2011a). Enzyme activity was assayed from the decrease in absorbance at 290 nm (extinction coefficient 2.8 $mM^{-1}cm^{-1}$) as ascorbate was oxidized and the unit of activity was expressed as micromole of ascorbic acid oxidized min^{-1}. Correction was made for non-enzymatic oxidation of ascorbate by hydrogen peroxide (blank).

Glutathione peroxidase (GPX) activity was determined according to Navari-Izzo et al. (1997) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 $mM^{-1}\ cm^{-1}$), one unit being defined as 1 μmol of NADPH oxidized min^{-1}.

Catalase (CAT) activity was determined as described by Aebi (1984) following the H_2O_2 consumption at 240 nm (39.4 mM^{-1} cm^{-1} extinction coefficient), one unit of activity being defined as the amount of enzyme that catalyses the conversion of 1 mM of H_2O_2 min^{-1}. A blank containing only the enzymatic solution was made.

All enzymatic activities were determined at 25 °C and expressed as U mg^{-1} protein. Protein measurement was performed according to Bradford (1976), using BSA as standard and expressed mg g^{-1}DW.

2.6. Statistical analyses

As data did not show a Gaussian distribution and the variances were not homogeneous, functional and physiological traits recorded in each season were compared by the non-parametric test of Kruskal-Wallis to verify if there were significant differences. Moreover, a matrix of species x traits recorded in the three seasons was analysed using Cluster Analysis (CA) and Non-metric Multidimensional scaling (NMDS). Cluster analysis is a classification technique that enables the natural groupings among the samples that are characterised by the dataset to be disclosed. The aim of NMDS is to represent samples and/or species in a low-dimensional ordination space by optimizing the correspondence between original dissimilarities and distances in the ordination (Økland, 1996). In both cases, the matrix was prior standardized and square-root transformed, then was subjected to CA and NMDS analysis using the Euclidean distance. The Spearman product-moment correlation coefficient was also calculated in order to point out which environmental variables was more correlated to the NMDS axes. All statistical tests were performed using R 2.14.1 software (2012, vegan package, Oksanen et al., 2012).

3. Results and discussion

Our results suggest that the three coexisting plants under study - *Cistus monspeliensis*, *Myrtus communis*, and *Phillyrea angustifolia* - have evolved different sets of functional and physiological traits to survive in the Mediterranean maquis. These traits differed not only between the species but also across the seasons.

Soil chemical characteristics were different between the three seasons (Table 2): pH and conductivity were significantly the lowest and the highest in summer respectively. Organic matter showed significant different values between seasons reaching the highest levels during summer. The soil was alkaline, with low levels of salinity, while available phosphorous content was significantly the lowest in spring (Table 2). In accordance with previous literature (Fabre et al., 1996), P leaching from fresh litter (leaf fall) and the limitation of mineralization processes during low temper-

Table 2
Soil characteristics in spring, summer and autumn at Castellare Mount (43°45′N, 10°27′E, Pisa, Italy).

	Spring	Summer	Autumn
pH	8.11 ± 0.01 a	7.57 ± 0.01b	8.01 ± 0.01 a
Conductivity (mS cm^{-1})	0.40 ± 0.00b	0.72 ± 0.01 a	0.40 ± 0.00b
N (g Kg^{-1})	6.70 ± 0.16 a	7.47 ± 0.18 a	6.51 ± 0.13 a
Available P (mg Kg^{-1})	2.01 ± 0.05b	6.06 ± 0.12 a	6.00 ± 0.12 a
Organic matter (%)	10.22 ± 0.07b	12.68 ± 0.08 a	9.40 ± 0.08c

Data are mean of three replicates ± SE for each season. Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test.

Table 3

Morphological and biochemical traits of spring, summer and autumn leaves of *Cistus monspeliensis*, *Myrtus communis* and *Phillyrea angustifolia* plants collected at Castellare Mount (43°45′N, 10°27′E, Pisa, Italy).

Leaf traits	*C. monspeliensis*			*M. communis*			*P. angustifolia*		
	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
LA (mm^2)	294.98 ± 12.91 a	201.74 ± 13.38 b	308.51 ± 11.04 a	377.31 ± 11.96 a	391.71 ± 14.80 a	304.51 ± 23.01 b	429.31 ± 19.21 a	365.08 ± 15.71 b	376.37 ± 10.29 ab
SLA (mm^2 mg^{-1})	8.94 ± 0.42 a	6.70 ± 0.26 b	8.90 ± 0.23 a	7.52 ± 0.22 c	8.38 ± 0.11b	9.20 ± 0.24 a	6.78 ± 0.48 a	6.21 ± 0.10 ab	5.59 ± 0.31 b
LDMC (%)	34.37 ± 1.33 b	42.45 ± 0.74 a	31.05 ± 0.62 b	43.96 ± 1.13 b	48.53 ± 0.70 a	41.45 ± 0.69 b	41.71 ± 1.89 b	47.97 ± 0.59 a	51.29 ± 0.74 a
SI (mg cm^{-2})	22.66 ± 1.15 ab	20.82 ± 0.77 b	25.46 ± 0.83 a	17.19 ± 0.35 a	12.72 ± 0.23 c	15.54 ± 0.29 b	21.94 ± 0.41a	17.58 ± 0.35 b	17.57 ± 0.49b
LNC (%)	1.52 ± 0.01a	1.18 ± 0.01 b	1.54 ± 0.00 a	1.14 ± 0.00 b	1.05 ± 0.01 c	1.38 ± 0.01 a	1.55 ± 0.01 a	1.15 ± 0.01b	1.55 ± 0.01 a
LPC (%)	0.10 ± 0.01 b	0.11 ± 0.0 1b	0.18 ± 0.01 a	0.08 ± 0.01 a	0.06 ± 0.01 a	0.07 ± 0.01 a	0.09 ± 0.01 a	0.06 ± 0.01 a	0.08 ± 0.01 a
LCC (%)	49.80 ± 0.19 a	35.60 ± 1.80 b	48.18 ± 0.18 a	45.50 ± 0.13 a	43.00 ± 1.53 a	46.23 ± 0.26 a	52.69 ± 0.44 a	35.03 ± 0.96 b	51.32 ± 0.32 a
Total Chl (mg g^{-1}DW)	4.55 ± 0.23 a	1.36 ± 0.07 c	3.35 ± 0.24 b	2.49 ± 0.10 b	1.38 ± 0.15 c	3.89 ± 0.30 a	2.73 ± 0.20 a	1.43 ± 0.09b	3.26 ± 0.23 a
Chl *a/b*	3.52 ± 0.34 a	2.58 ± 0.06 b	2.61 ± 0.05 b	2.63 ± 0.09a	2.33 ± 0.07b	2.46 ± 0.06 ab	3.17 ± 0.12a	2.49 ± 0.04 b	2.08 ± 0.13c
Carotenoids (mg g^{-1}DW)	0.74 ± 0.07 a	0.47 ± 0.02b	0.56 ± 0.04b	0.52 ± 0.05 a	0.56 ± 0.05 a	0.67 ± 0.04 a	0.63 ± 0.05 a	0.60 ± 0.02 a	0.56 ± 0.04 a
Car/Total Chl	0.16 ± 0.01 b	0.34 ± 0.01 a	0.17 ± 0.00 b	0.21 ± 0.02 b	0.40 ± 0.01 a	0.17 ± 0.01 b	0.23 ± 0.00 b	0.42 ± 0.01 a	0.17 ± 0.01 c

Data are mean of twenty replicates ± SE for each species. Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test. Comparisons are made within each individual species.
Abbreviations: Car, carotenoids; Chl, chlorophyll; LA, leaf area; LCC, leaf carbon content; LDMC, leaf dry matter content; LNC, leaf nitrogen content; LPC, leaf phosphorous content; SI, succulence index; SLA, specific leaf area.

Table 4

Antioxidant response in spring, summer and autumn leaves of *Cistus monspeliensis*, *Myrtus communis* and *Phillyrea angustifolia* plants collected at Castellare Mount (43°45′N, 10°27′E, Pisa, Italy).

	C. monspeliensis			*M. communis*			*P. angustifolia*		
	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
Total Ascorbate (mg g^{-1}DW)	18.06 ± 0.51 b	11.84 ± 0.08 c	29.49 ± 0.32 a	17.65 ± 0.16 b	10.06 ± 0.03 c	29.99 ± 0.28 a	8.53 ± 0.29 b	5.77 ± 0.08 c	11.98 ± 0.16 a
ASA/DHA	2.56 ± 0.29 b	8.31 ± 1.43 a	7.90 ± 1.01 a	1.31 ± 0.02 c	5.03 ± 0.20 a	3.95 ± 0.14 b	1.67 ± 0.18 b	2.16 ± 0.10 b	2.85 ± 0.17 a
Total glutathione (nmol g^{-1}DW)	166.02 ± 4.56 a	120.84 ± 12.03 b	149.47 ± 5.17 a	11.18 ± 0.41b	96.69 ± 2.68 a	nd	4.12 ± 0.00 b	21.12 ± 1.98 a	nd
Phenols (mg GAE g^{-1}DW)	67.33 ± 1.77 a	44.32 ± 0.89 c	56.77 ± 1.16 b	85.28 ± 1.95 b	102.66 ± 2.65 a	94.92 ± 2.54 a	48.92 ± 0.50 a	34.76 ± 0.21 b	22.62 ± 0.65 c
Soluble proteins (mg g-1DW)	21.72 ± 2.26 a	22.89 ± 3.07 a	24.03 ± 0.99 a	15.44 ± 2.42 b	34.16 ± 0.67 a	15.78 ± 1.55 b	21.73 ± 2.85 b	37.16 ± 2.15 a	19.52 ± 1.37 b
APX (U mg^{-1}protein)	0.40 ± 0.02 ab	0.49 ± 0.05 a	0.35 ± 0.01 b	0.37 ± 0.03 a	0.39 ± 0.05 a	0.29 ± 0.02 a	0.87 ± 0.03 a	0.93 ± 0.04 a	0.63 ± 0.05 b
GPX (U mg^{-1}protein)	1.49 ± 0.12 ab	1.72 ± 0.01 a	1.28 ± 0.08 b	0.80 ± 0.02 b	1.38 ± 0.03 a	0.84 ± 0.02 b	0.67 ± 0.00 b	3.94 ± 0.31 a	0.79 ± 0.01 b
CAT (U mg^{-1}protein)	3.58 ± 0.14 c	5.08 ± 0.19 a	4.29 ± 0.26 b	7.47 ± 0.30 a	7.02 ± 0.66 a	4.87 ± 0.34 b	5.11 ± 0.42 a	6.56 ± 0.74 a	5.85 ± 0.32 a

Data are mean of 3–9 replicates ± SE for each species. Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test. Comparisons are made within each individual species.
Abbreviations: APX, ascorbate peroxidase; ASA, ascorbate; CAT, catalase; DHA, dehydroascorbate; GPX, glutathione peroxidase.

atures season could help to explain the higher soil phosphorous content recorded in autumn than in spring when actively growing plants could deplete soil available phosphorous content. On the other hand, the high phosphorous content during summer could derive from organic matter return to soil due to semideciduous plants. The high phosphorus soil contents were not related to high phosphorous content in autumn leaves and the slight increase recorded only in the leaves of the semideciduous *C. monspeliensis* could be due to a translocation of this element from older leaves before their fall (Yavitt et al., 2004). Leaf nitrogen content (LNC) always showed the significant lowest values in summer leaves (Table 3) characterised however by protein contents significantly higher than in the other two seasons in the two evergreen species (Table 4) capable to invest more nitrogen in protein synthesis. Just in summer, in addition, *C. monspeliensis* and *P. angustifolia* plants showed the significant lowest values of LCC (Table 3).

From a morphological point of view, we confirmed the expected seasonal leaf dimorphism of *C. monspeliensis* (Werner et al., 1999; Aronne and De Micco, 2001; De Micco and Aronne, 2009; Catoni et al., 2012). In summer, *C. monspeliensis* had the significant lowest LA and SLA values, while, just as *Myrtus communis*, showed the significant highest value of LDMC (Table 3). Myrtle was also characterised in this season by the lowest SI value. Spring values of LDMC and SI were significantly the lowest and the highest, respectively, in *P. angustifolia* samples (Table 3). In autumn leaves *M. communis* displayed the significant lowest LA and highest SLA, respectively. Its leaf size traits (LA and SLA) were significantly different between summer and spring-autumn. Anyway, the avoidance strategy of *C. monspeliensis*, which is a semideciduous plant partially losing its leaves during summer, was confirmed by generally lower values of LA and LDMC in comparison with the other plants, that indicate lower investments in mechanical tissues (Perez-Harguindeguy et al., 2013). *M. communis* displayed a set of functional and physiological traits different to those of *P. angustifolia*. In myrtle, the significant lowest LA, and the highest SLA and LNC found in autumn leaves with respect to spring and summer samples (Table 3) might be connected with leaf structural alterations by cool temperatures, as suggested by other studies on similar seasonal variations of SLA (Bermúdez and Retuerto, 2014; Ciccarelli et al., 2016). On the other hand, leaves of *P. angustifolia* with higher LDMC were tougher and more resistant to physical hazards than myrtle leaves (Perez-Harguindeguy et al., 2013).

As expected summer drought induced significant decreases in relative water content (RWC, Fig. 2); nevertheless, the recovery detected in autumn, in the three species, was a clear signal of their ability to counteract seasonal restrictions of the Mediterranean area (Jubany-Marí et al., 2009). The lowest value of RWC detected in *C. monspeliensis* (32%) was comparable to that recorded in
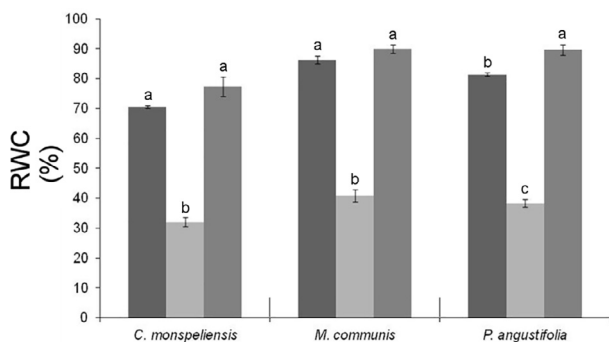
C. albidus under water stress condition (33%, Brossa et al., 2015). Total chlorophyll contents (Table 3), comparable to data reported in literature (Díaz-Barradas et al., 2017), reached the minimum values in summer in each species and had an opposite trend in *C. monspeliensis* and in *M. communis*, with a higher content in spring and in autumn leaves, respectively. Carotenoid contents (Table 3) in *C. monspeliensis* had in spring the highest value. The lowest chlorophyll contents detected in summer leaves of the plants under study, associated with the lowest leaf nitrogen and carbon contents and RWC, might be markers of damage, with a decrease in photosynthetic capacity during this season (Grant et al., 2014). These could be symptoms of summer leaf senescence that nevertheless is recognized as favouring plant survival in the Mediterranean habitat (Munné-Bosch and Peñuelas, 2003). The values of the Chl *a/b* ratio (Table 3), on the average lower in summer and in autumn than in spring, could help to increase the ability to harvest incident light. As expected, the highest Car/Total Chl ratios were always registered in summer leaves (Table 3), when photoprotection due to the nonphotochemical quenching activity of carotenoids, was particularly necessary (Arena et al., 2013).

The study of oxidative stress and antioxidant response can give an insight into the ability of plants to cope with environmental stressors. As already highlighted, due to the combined action of water deficit, high air temperatures and excess of light, summer is generally considered the primary constraint to plant growth in the Mediterranean area (Werner et al., 1999). These environmental constraints can induce ROS overproduction (Munné-Bosch et al., 2003; Sofo et al., 2015). Accordingly, just in summer hydrogen peroxide reached the highest content in all the species, in particular in *C. monspeliensis* (Fig. 3); however, these high values did not correspond to a greater oxidative damage in terms of TBARS indicative of lipid peroxidation and membrane damage (Fig. 3). Similar trends, with values however significantly lower, have been recorded in *C. albidus* during summer period (Jubany-Marí et al., 2009). While in *M. communis* and *P. angustifolia* the lowest values
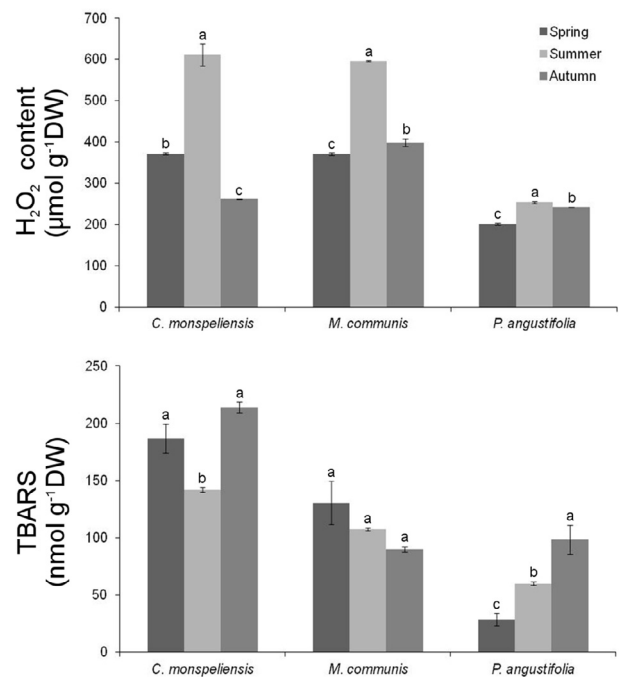
Fig. 3. Hydrogen peroxide and TBA-reactive substances (TBARS) in spring, summer and autumn leaves of *Cistus monspeliensis, Myrtus communis* and *Phillyrea angustifolia*. Data are mean of 3–9 replicates ± SE for each species. Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test.

Fig. 2. Relative water content (RWC) in spring, summer and autumn leaves of *Cistus monspeliensis, Myrtus communis* and *Phillyrea angustifolia*. Data are mean of 3–9 replicates ± SE for each species. Means followed by the same letters are not significantly different at 5% according to the Kruskal-Wallis test.

were recorded in spring leaves, in *C. monspeliensis* the lowest content of hydrogen peroxide was detected in autumn leaves (Fig. 3). In *P. angustifolia* and *C. monspeliensis* the highest values of TBARS content were registered in autumn leaves, while the minimum TBARS value characterised spring leaves of *P. angustifolia* (28.58 nmol g^{-1}DW, Fig. 3). The overall lack of positive correlation between H_2O_2 content and lipid peroxidation in the different seasons could indicate a membrane damage not completely dependent on hydrogen peroxide. On the other hand, these results are also consistent with the role of signalling molecule envisaged for H_2O_2, able to trigger the antioxidant response necessary for protecting plants from environmental-induced stress (Jubany-Marí et al., 2009). In fact, the high contents of hydrogen peroxide recorded in summer leaves were accompanied in the two evergreen species by increases in total glutathione content, not detectable in autumn leaves (Table 4) and high GPX activities (in *P. angustifolia* the activity of this enzyme was about fivefold higher than in the other seasons, Table 4).

In *C. monspeliensis* and *M. communis*, the lowest CAT activity values were typical of spring (3.58 U mg^{-1} protein) and autumn (4.87 U mg^{-1} protein) leaves respectively (Table 4). In accordance with literature (Shoshtari et al., 2017), in *M. communis,* summer leaves were also characterised by high phenol content and high reducing power of the ASA/DHA couple (Table 4). The maximum reducing power of the couple ASA/DHA (8.31) was however recorded in the semideciduous species, that showed in this season high levels of APX, GPX and in particular CAT activities (Table 4). Interestingly, in *C. monspeliensis* all non-enzymatic antioxidants reached in summer their lowest contents (Table 4). This decrease in antioxidant content could be linked to the semideciduous character of this species (Vitale et al., 2014) that losses part of its leaves in this season.

Further information may derive from the different modulation of the antioxidant response in spring and autumn. Antioxidant machinery generally reached its minimum in these seasons (Table 4): APX had the lowest values of activity in autumn leaves of *C. monspeliensis* and *P. angustifolia*. In *C. monspeliensis* and *M. communis*, the lowest CAT activity values were typical of spring (3.58 U mg^{-1}protein) and autumn (4.87 U mg^{-1}protein) leaves respectively. On the whole, in accordance with literature (Rice-Evans et al., 1997), present data highlight the important protective action of phenols. In fact, high levels of hydrogen peroxide were not accompanied by high oxidative damage only when high levels of phenols were also recorded (Table 4): in spring leaves of
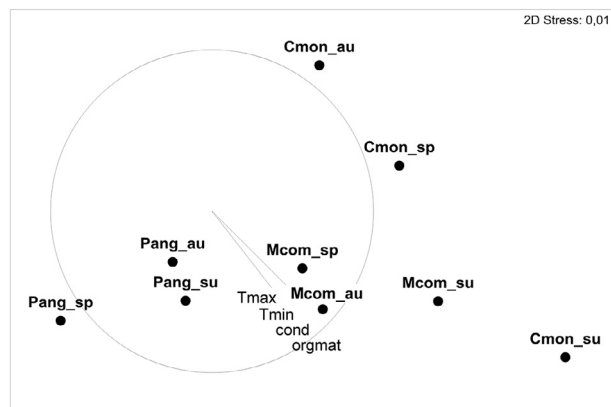

Fig. 5. NMDS diagram based on the dissimilarity (measured by the Euclidean distance) occurring in the three species measuring morphological, biochemical, and physiological traits. Four environmental variables have a Spearman correlation coefficient >0.6 with the two axes. Variables abbreviations: cond, conductivity; orgmat, organic matter content; Tmax, maximum temperature; Tmin, minimum temperature. Species abbreviations: Cmon, *Cistus monspeliensis*; Mcom, *Myrtus communis*; Pang, *Phillyrea angustifolia*; au, autumn leaves; sp, spring leaves; su, summer leaves.

C. monspeliensis and *P. angustifolia* and in autumn leaves of *M. communis*, where the maximum content of this antioxidant molecule (102.66 mg GAE g^{-1}DW) was recorded.

Based on a Euclidean distance matrix, the CA separated summer samples of *C. monspeliensis* from the other samples with a distance >25% (Fig. 4). The other samples were classified into two groups with a distance ∼20%: i.e., Cluster I, composed of all samples of *P. angustifolia*; and Cluster II, which separated *C. monspeliensis* and *M. communis* into two subgroups with a distance ∼18% (Fig. 4). This classification was supported by the NMDS (Fig. 5), which resulted in a clear separation of the three species in the bidimensional space. The stress value of 0.01 corresponds to an excellent ordination with no prospect of a misleading interpretation. Four environmental variables showed a high correlation with the first two NMDS axes (Spearman's coefficient > 0.6): two climatic variables – Tmax (F = 3.053, p-value = 0.044) and Tmin (F = 3.250, p-value = 0.048), and two soil parameters – conductivity (F = 3.242, p-value = 0.036) and organic matter content (F = 3.130, p-value = 0.037). The samples that seemed to be most affected by the environmental variables were *M. communis* leaves collected
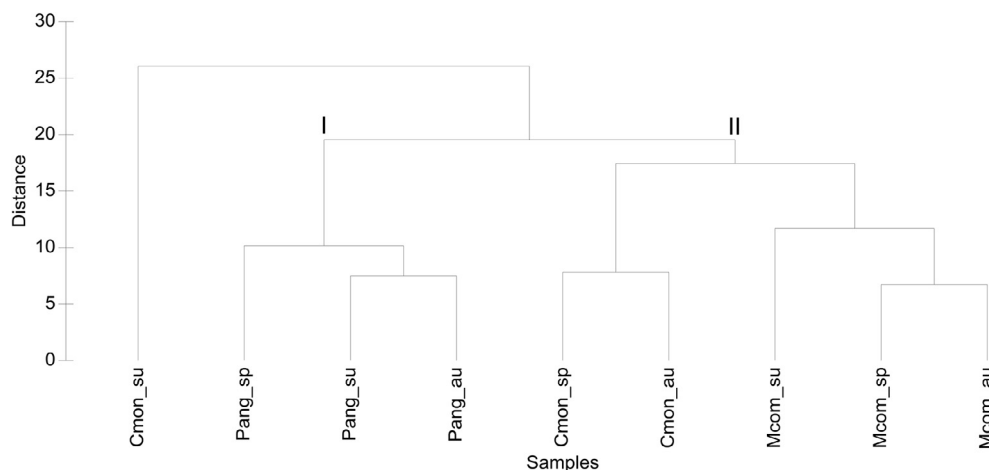

Fig. 4. Dendrogram obtained by average-linkage cluster analysis based on the Euclidean distance of the matrix samples x morphological, biochemical, and physiological traits. Species abbreviations: Cmon, *Cistus monspeliensis*; Mcom, *Myrtus communis*; Pang, *Phillyrea angustifolia*; au, autumn leaves; sp, spring leaves; su, summer leaves.

in autumn. Multivariate analysis highlighted that summer samples of *C. monspeliensis* segregated in a different cluster with respect to spring and autumn samples of the same taxon and the other samples of the two sclerophylls. *Myrtus communis* was confirmed a peculiar sclerophyll, as evidenced in other studies (Gratani et al., 2013; Ciccarelli et al., 2016), especially during autumn when the plant was strongly influenced by two climatic variables - Tmax and Tmin – and two soil parameters – conductivity and organic matter content. The four environmental variables showed a high correlation with the first two NMDS axes, highlighting their importance in samples segregation in the two-dimensional space.

4. Conclusions

In conclusion, this study underlines the different responses of three co-occurring species to the climatic stress factors in the Mediterranean maquis. The stressful summer season induced, as expected, disturbance in hydric balance, decrease in succulence index and in chlorophyll content and high contents of hydrogen peroxide, important signalling molecule able to trigger antioxidant response. Thanks to higher enzymatic activities, accompanied in the two evergreen species by an increase in total glutathione, oxidative damage remained at levels equal to or lower than the other seasons. An important protective role can be confirmed for phenols, as underlined by the study of the antioxidant response in spring and autumn. Differently from the two evergreen species, only in the semideciduous *C. monspeliensis* both functional and biochemical traits showed a higher stress condition in summer. The sclerophyllous nature of leaves of *M. communis* and *P. angustifolia* may explain the higher stability of functional traits across seasons than physiological characters, which are more efficient to fine-tune the response of plants to the environmental changes. The combination between physiological and functional adjustments is therefore crucial for plant adaptation.

Acknowledgements

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