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Seasonal Differences in Ecophysiological Performance between Resprouters and Non-Resprouters across an Aridity Gradient in Northwest Tunisia

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Abstract: Understanding the functioning of shrub species during dry periods is necessary to forecast ecosystem responses to future climates, particularly in Mediterranean environments. We evaluated the seasonal changes in leaf gas exchange, hydraulic traits, carbon and nitrogen isotopes, and non-structural carbohydrates of seeders and resprouting shrub species typical of Aleppo pine forests across an aridity gradient in Tunisia: Djebel Zaghouan (subhumid climate), Djebel Mansour (semiarid transitional climate), and Djebel El Sarj (semi-arid climate). We monitored seven woody species: *Pistacia lentiscus*, *Erica multiflora*, *Phillyrea latifolia* (resprouters), *Cistus monspeliensis*, *Rosmarinus officinalis* (seeders), *Globularia alypum*, and *Calicotome villosa* (resprouters-seeders). The seasonal variation in leaf water content was usually higher in seeders than in resprouters and was associated with higher resistance to embolism. In contrast, the seasonal variation in non-structural carbohydrates was higher in resprouters, especially at the driest site. Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ displayed seasonal enrichment-depletion patterns, with seeders showing an overall higher $\delta^{13}\text{C}$ in summer than in spring, consistent with a water-saving strategy of increasing water use efficiency. Discriminant analysis suggested that resprouters can sustain a positive carbon balance during drought periods. The differential impact of summer droughts on water status and the ecophysiology of these plant strategies may lead to different ecosystem dynamics depending on whether climate change tips the balance towards a preponderance of stressors (drought) or disturbances (fire) in dry Mediterranean areas.

Keywords: resprouters; non-resprouters; photosynthesis; non-structural carbohydrates; carbon isotopes; nitrogen isotopes

1. Introduction

Climate change is modifying the magnitude and distribution of precipitation seasonality throughout the year and, consequently, is exacerbating the impact of warming-induced drought stress on terrestrial vegetation [1]. In this regard, the adaptation and plasticity of species to environmental fluctuations determine the structure and function of plant

communities [2]. Shrubs inhabiting the forest understory play a critical role in terms of biodiversity, carbon gain, and storage, and variations in environmental parameters influence their leaf energy balance and net productivity [3]. Photosynthetic performance, in particular, is highly dependent on the water status of leaves, which is balanced by hydraulic conductivity and water losses through transpiration. Indeed, the prevalent tight coupling between photosynthesis and water supply has been well documented [4].

In recent years, a large body of literature has highlighted the importance of trait-based ecology (functional traits) to understand plant responses to the environment and to improve predictions of climate change impacts on ecosystems [5]. Reich (2014) [6] indicates that plant functional strategies can be placed on an axis between resource acquisition (i.e., productivity) and resource conservation (i.e., persistence) and that leaf, stem, and root traits are coordinated in the whole-plant economics spectrum. Still, there are important knowledge gaps concerning how plant functional traits are integrated to yield a given degree of plasticity [7]. Adaptations to environmental conditions (e.g., climate) are thought to be the key to this coordinated evolution of traits through natural selection. Drought-resistance mechanisms in particular are diverse and include strategies such as low vulnerability to cavitation, tight stomatal control of water losses, increased carbon storage [8], and also differential access to soil water reserves, with shallow- and deep-rooted species often showing high and low levels of drought damage, respectively [9].

Resprouting and seeding are two regeneration strategies followed by Mediterranean plants after disturbances such as fire [10]. These strategies differ in resource allocation patterns [11]. While resprouters typically allocate more of their resources to the underground organs where buds are formed, seeders lack these organs and their investment is directed towards aboveground growth [12]. In addition, the seasonal variation in leaf water content is usually higher in seeders than in resprouters [13]. This is because seeders often show shallower root systems that cannot tap deep water [13], higher resistance to embolism [14], and poorer stomatal control [15]. Conversely, resprouters exhibit deeper roots but lower drought tolerance than seeders [15]. Altogether, these functional characteristics imply that seeders from the Mediterranean basin grow significantly faster and allocate more biomass to aboveground organs than resprouters [10]. In fact, such plant strategies may react differentially to the timing and intensity of warming-induced drought stress across strong aridity gradients typical of many areas of the Mediterranean basin, such as northern Tunisia. Regardless of plant strategy, current warming-induced drought stress may exacerbate carbon starvation, which should be examined through the prism of the widely accepted sink-limited way of plant functioning [16]. Hence, hydraulic dysfunction and carbohydrate depletion have been demonstrated to have different relevancies as underlying mechanisms of woody plant mortality, depending on the species and its strategy to cope with drought [17].

Being complementary to instantaneous physiological measurements related to gas exchange and plant water status, carbon isotopes of plant compounds are recognized as effective, integrative tools for assessing the coupling between carbon and water plant processes over different temporal scales [18]. The carbon isotope composition ($\delta^{13}\text{C}$) is considered an indicator of intrinsic water-use efficiency (iWUE), the ratio between leaf-level net CO_2 assimilation rate and stomatal conductance for water vapor [19]. At the interannual level, $\delta^{13}\text{C}$ is also a metric for quantifying plant responses to drought and has been used to examine the mechanisms underlying mortality [20], because it integrates plant performance under varying environmental conditions. iWUE is known to be an essential element of the survival, productivity, and fitness of individual plants [21]. Together with soil water, the availability of nitrogen is another key factor influencing ecosystem production. The natural abundance of ^{15}N (i.e., the nitrogen isotope composition, $\delta^{15}\text{N}$) in plant compounds is a potentially effective integrator of N cycling types and rates [22]. $\delta^{15}\text{N}$ can indicate whether a range of plants has access to the same N source (Robinson 2001). Additionally, plant $\delta^{15}\text{N}$ can be influenced by nitrification and mineral uptake characteristics (e.g., timing and type) [23].

In this study, we examined the effects of seasonal changes on the physiological activity of some shrub species that represent two contrasting strategies to cope with disturbances (i.e., fire) and stresses (i.e., drought) in Mediterranean forests of northern Africa (Tunisia): resprouters and seeders. The forest stands, representing an aridity gradient, are composed of *Pinus halepensis* as the main tree species. We monitored functional traits related to the plant carbon-water balance of seven shrubs that compose the understory of these stands, including three obligate resprouters (*Erica multiflora*, *Phillyrea latifolia*, and *Pistacia lentiscus*), two seeders (*Cistus monspeliensis* and *Rosmarinus officinalis*), and two facultative resprouters (*Calicotome villosa* and *Globularia alypum*). We hypothesized that: (i) seeders show higher seasonal variability in photosynthetic traits and carbon storage than obligate resprouters, because of their opportunistic performance to take advantage of rain pulses along the growing season; (ii) the contrasting strategies followed by seeders and sprouters are imprinted in $\delta^{13}\text{C}$ -derived iWUE and in $\delta^{15}\text{N}$ values, with seeders showing larger seasonal fluctuations and more extreme values than resprouters for both traits; (iii) facultative resprouters perform intermediately between resprouters and seeders; and (iv) these differences in performance are exacerbated at the driest end of the aridity gradient examined.

2. Materials and Methods

2.1. Study Sites and Species

The study was carried out at three sites located in Northeastern Tunisia (Figure 1): Djebel Zaghouan (DZ), Djebel Mansour (DM), and Djebel Serj (DS). DZ (36°22' N, 10°07' E, 340 m a.s.l.) is located near the highest point in Eastern Tunisia at 1295 m and is characterized by a sub-humid climate with mild and temperate winters [24]. The mean annual temperature is 17.0 °C, and the total annual precipitation is 493 mm, of which 14% falls in the summer (1990–2020 periods). DM is found in the vicinity of the city of El Fahs (36°15' N, 9°47' E, 405 m a.s.l.) and is characterized by a semi-arid climate with moderate winters and hot, dry summers [24]. The mean annual temperature is 17.6 °C, and the total annual precipitation is 450 mm, of which 12% falls in summer. Finally, DS (35°57' N, 9°33' E, 798 m a.s.l.) is located near the city of Siliana and is characterized by an upper semi-arid climate with cool-temperate winters and hot and dry summers [24]. The mean annual temperature is 18.0 °C, and the total annual precipitation is 430 mm, with a 7.9% fall in summer. The average climate for the study period 2019–2020 is shown in Figure 1.

Soils are shallow and nutrient-poor at the study sites. The soil at DZ is marly-limestone with the presence of hard limestone bars, whereas the soil at DM is marly to marly-limestone and underwent a very developed limestone encrustation during the Quaternary period. Finally, the soil at DS is almost entirely composed of Cretaceous limestone, whose raised strata are exposed to erosion. In the three sites, the vegetation is a 2–3 m high maquis dominated by *E. multiflora*, *P. latifolia* (only present in DS), and *P. lentiscus* (obligate resprouters), *C. monspeliensis* and *R. officinalis* (seeders), and *C. villosa* and *G. alypum* (facultative resprouters; after a fire, they can regenerate by resprouting and also by recruiting new individuals from seed). This understory vegetation, therefore, consists of persistent and sclerophyllous species, which are usually present in the lowlands of the Mediterranean basin (Table 1). The arboreal cover is dominated by Aleppo pine (*P. halepensis*), either natural or planted, with the scattered presence of maple (*Acer monspessulanum* L.), mountain ash (*Fraxinus angustifolia*), and Kermes oak (*Quercus coccifera*). Field measurements were carried out from March 2019 to January 2020 in three plots per site. The plots were about 100 m² in size (10 × 10 m²) and approximately square. They were chosen as representative of the forest ecosystem of each study site in terms of tree cover and the regular presence of the studied shrub species. The largest tree crown cover corresponded to DS (52%), followed by DZ (45%) and DM (28%).

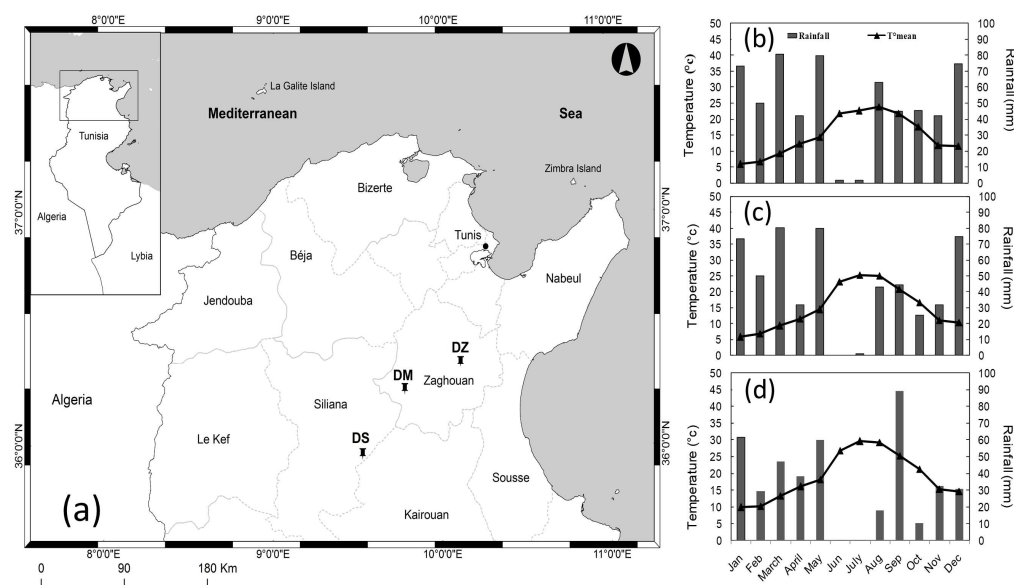


Figure 1. Study sites (a) located in Tunisia (Djebel Zaghouan (DZ), Djebel Mansour (DM), and Djebel Serj (DS)) and their weather conditions over the study period (2019–2020): monthly mean temperature (°C) and rainfall (mm) (DZ, (b); DM, (c); DS, (d)). Data were obtained from the nearest meteorological station to each study site (monthly mean).

Table 1. Shrub species and their density at three forest stands of Aleppo pine in Tunisia (Djebel Zaghouan, DZ; Djebel Mansour, DM; and Djebel Serj, DS). Plant density was obtained from six independent plots located in the studied areas. R = Resprouter, S = Seeder.

Species	Strategy	Code	Family	Plant Density (Individuals ha ⁻¹)		
				DZ	DM	DS
<i>C. villosa</i>	R-S	CV	Fabaceae	1900 ± 404	2900 ± 250	-
<i>C. monspeliensis</i>	S	CM	Cistaceae	4100 ± 152	4200 ± 692	2800 ± 416
<i>E. multiflora</i>	R	EM	Ericaceae	5100 ± 100	8400 ± 953	16,200 ± 2402
<i>G. alypum</i>	R-S	GA	Globulariaceae	6500 ± 251	2900 ± 152	63,100 ± 3350
<i>P. latifolia</i>	R	PL	Oleaceae	-	-	2000 ± 208
<i>P. lentiscus</i>	R	PH	Anacardiaceae	6400 ± 416	3000 ± 200	-
<i>R. officinalis</i>	S	RO	Lamiaceae	9200 ± 152	55,900 ± 1517	36,600 ± 814

2.2. Soil Water Content

Soil water content (SWC) was monitored at each site by Time Domain Refractometry (TDR 30, Trase System I, Soil Moisture Equipment Corp., Goleta, CA, USA). SWC was measured in ten points at each site spaced about 2.5 m at a depth of 30 cm in spring (25–27 March), summer (1st–2nd July and 1st August), and autumn (31st October and 1st–2nd November).

2.3. Ecophysiological Parameters

2.3.1. Gas Exchange

Photosynthetic traits were measured in situ on mature leaves of three plants per plot of each species (one leaf per plant) during mid-morning (09:00–11:00 h solar time). Gas exchange measurements were carried out in attached intact leaves using a Li-6400 (Li-Cor, Lincoln, NE, USA) in spring (25–27 March), summer (1st–2nd July and 1st August), and autumn (31st October and 1st–2nd November). Leaf temperature was maintained at 25.0 °C, photon flux density at 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, ambient CO₂ partial pressure (*Ca*) at 400 ppm, and a leaf vapor pressure deficit of around 1 kPa. Photosynthetic parameters were recorded when photosynthesis (*An*) and stomatal conductance (*gs*) stabilized. Instantaneous water-

use efficiency (WUE, $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$) was calculated as the ratio between A_n and transpiration (E), A_n/E .

2.3.2. Leaf Water Potential

Midday leaf water potential (ψ_{leaf} , MPa) was obtained directly after completing gas exchange measurements. Thus, three mature leaves per species and replicate plot were detached and rapidly pressurized (within 15 s) with nitrogen using a Scholander-type pressure chamber (SKPM 1400[®], Skye Instruments Ltd., Powys, UK). ψ_{leaf} was recorded on the same days as for gas exchange measurements.

2.3.3. Percent Loss of Conductivity

The xylem hydraulic conductivity was obtained using the high-pressure flowmeter method [25,26]. This method infuses degassed water at positive pressure (~ 2 MPa) into the stem segment and quantifies the flow rate at the inlet. Three plants were collected per species and plot during the spring (25–27 March) and summer of 2019 (30–31 July and 1 August). The maximum hydraulic capacity was used to estimate xylem cavitation as follows:

$$PLC = 100 \times 100 \left(\frac{K_{in}}{K_{max}} \right)$$

where PLC is the percent loss of hydraulic conductivity, K_{in} is the initial conductivity, and K_{max} is the maximum conductivity measured after removing the trapped gas from the pipes [27].

2.3.4. Non-Structural Carbohydrates

To assess the investment in reserves of each species, the concentration of non-structural carbohydrates (NSC) was quantified in the stems and roots of the same plants previously used for gas exchange and ψ_{leaf} . Three plants per species and plot (i.e., three main stems and three main roots for each species) were collected during spring (25–27 March) and summer of 2019 (30–31 July and 1 August), transported in a portable cooler to the laboratory, and stored at -20 °C until freeze-dried. Then, they were weighed and ground into a homogeneous powder in a ball mill (Retsch Mixer MM301, Retsch GmbH, Haan, Germany). Soluble sugars (SS) were extracted with ethanol- H_2O 4:1 (v/v), and their concentration was determined colorimetrically using the phenol-sulfuric method [28]. Enzymatic digestion was then used to degrade the starch and complex sugars that remained following ethanol extraction, as described by Colangelo et al. [29]. Total NSC concentration (% dry matter) was calculated as the sum of SS and starch concentrations.

2.3.5. Carbon and Nitrogen-Stable Isotopes

The $^{13}\text{C}/^{12}\text{C}$ ratio was evaluated in aqueous extracts (mainly represented by sucrose) obtained from leaves previously evaluated in the field for gas exchange parameters (three leaves per replicate plot) in the spring (25–27 March) and summer of 2019 (30–31 July and 1 August). Dried and ground leaves were mixed, boiled for 15 min in distilled water, and filtered. The filtrate was freeze-dried and powdered for $^{13}\text{C}/^{12}\text{C}$ mass spectrometry analysis (AN-CAIRMS Europa Scientific Integra at UC-Davis Stable Isotope Facility, Davis, CA, USA). The results were expressed as the carbon isotope composition of soluble organic matter ($\delta^{13}\text{C}_{\text{SOM}}$) [30], where:

$$\delta^{13}\text{C} (\text{‰}) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

being R the $^{13}\text{C}/^{12}\text{C}$ molar ratio. The standard for comparison was a secondary standard calibration against Pee Dee Belemnite (PDB) carbonate. Sample sizes of about 1 mg were used and placed into tin capsules. Replicate samples differed by less than 0.10‰.

In addition, three leaves per replicate plot were sampled. Oven-dried leaf samples (72 h at 110 °C) from the three leaves were mixed, ground, and used for the carbon isotope

composition of bulk leaf material (i.e., total organic matter, $\delta^{13}\text{C}_{\text{TOM}}$), as described above for water-soluble leaf extracts. The same three leaves per replicate plot as those used for $\delta^{13}\text{C}_{\text{TOM}}$ were employed to determine the nitrogen isotope composition ($\delta^{15}\text{N}$). In this case, the standard used was N_2 in air. Isotopic standards of known $^{15}\text{N}/^{14}\text{N}$ ratios (IAEA N1 and IAEA N_2 ammonium sulfate and IAEA NO_3 potassium nitrate) were used as secondary standards.

2.4. Statistical Analysis

2.4.1. Analysis of Variance

A mixed-effects model was fitted to the data to test for the significance of different factors. The analysis of variance (ANOVA) used was (random effects appear underlined):

$$Y_{ijklm} = \mu + L_i + S_j + T_m + \underline{TE_{(m)k}} + LS_{ij} + ST_{jm} + LT_{jm} + LST_{ijm} + \underline{e_{ijklm}}$$

where Y_{ijklm} refers to the response of the l th plant of the k th species of the m th strategy in the j th season and the i th site, μ is the overall mean, L_i is the fixed effect of the i th site, S_j is the fixed effect of the j th season, T_m is the fixed effect of the m th strategy, $\underline{TE_{(m)k}}$ is the random effect of the k th species nested to the m th strategy, LS_{ij} is the fixed interaction between the i th site and j th season, ST_{jm} is the fixed interaction between the j th season and the m th strategy, LT_{jm} is the fixed interaction between the i th site and the m th strategy, LST_{ijm} is the fixed interaction between the i th site, the j th season and the m th strategy, and $\underline{e_{ijklm}}$ is the random residual term. Treatment means were compared using the Student-Newman-Keuls test. Residual normality was evaluated using Shapiro-Wilk's test, while Levene's test was used to check the homogeneity of variances. Differences were considered statistically significant at the $p < 0.05$ level. The results were expressed as a mean $\pm$ standard error.

2.4.2. Discriminant Analysis

To pinpoint the functional differences between the shrub species that compose the understory of the studied stands (seeders, resprouters, and resprouters-seeders) during the dry season, the ecophysiological dataset was subjected to discriminant analysis [31]. First, a stepwise discriminant analysis was used to ascertain the traits that best discriminated between strategies during peak summer. The following traits showing a significant season-by-strategy interaction were considered to be informative of differential physiological performance under drought: ψ_{leaf} , An , gs , E , WUE , sugar and starch content (stem), sugar and starch content (root), and PLC. We also included the available isotopic data ($\delta^{13}\text{C}$, $\delta^{13}\text{C}_{\text{TOM}}$, $\delta^{15}\text{N}$). The significance level corresponding to the F -value for entering or excluding a specific variable was set at $p = 0.10$. All the variables that remained in the model once the stepwise regression process stopped were considered to discriminate significantly between shrub strategies. Second, Mahalanobis distances were obtained, and Hotelling's T^2 statistics were calculated to test for the significance of between-class differences. Finally, canonical discriminant analysis based on the selected variables was used to create a graphical representation of the classification of strategies.

3. Results

3.1. Soil Water Content

The soil water content (SWC) showed significant differences ($p < 0.001$) among sites and seasons and also a significant site-by-season interaction, indicating that seasonal SWC changes depended on the site (Figure 2). Overall, the highest SWC was recorded in spring, followed by a drop in summer, when SWC reached values around 5%, and a later recovery in autumn (Figure 2). The highest seasonal SWC was recorded in DZ in both spring (26.6%) and autumn (15.0%), and the lowest SWC was recorded in DS in summer (4.4%). Therefore, the most favorable soil water status was observed in DZ, followed by DM, although all sites shared very low SWC values in summer (i.e., below 10%).

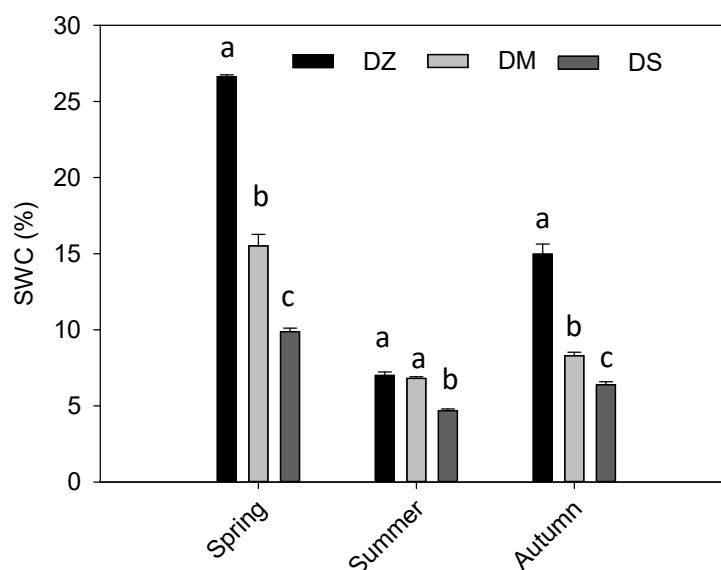


Figure 2. Seasonal soil water content of the studied sites: Djebel Zaghouan (DZ), Djebel Mansour (DM), and Djebel Serj (DS). Data are mean $\pm$ SE of four independent measurements. For each season, different letters indicate significant differences between sites ($p < 0.05$).

3.2. Leaf Water Potential, Gas Exchange, and Percentage Loss of Conductivity

The midday leaf water potential (ψ_{leaf}) showed significant differences ($p < 0.001$) among seasons (Table 2). In particular, there was a clear seasonal ψ_{leaf} pattern in all study sites (Figure 3), which approximately followed SWC fluctuations (i.e., highest ψ_{leaf} values in spring, lowest in summer). ψ_{leaf} also showed a significant site-by-season interaction, indicating that ψ_{leaf} remained lower during spring in DS and DM (the driest sites) compared to DZ, regardless of plant strategy. Although there were no significant differences in ψ_{leaf} among strategies, a significant season-by-strategy interaction was observed, with seeders usually showing the lowest ψ_{leaf} and resprouters the highest ψ_{leaf} across sites in the summer, but not in spring or autumn (Figure 3).

Table 2. *F*-values (and their significance) of the analysis of variance (fixed effects only) for different ecophysiological traits of seven shrub species categorized into three different strategies (seeder, resprouter, and resprouter-seeder) and evaluated during three seasons at three stands (sites) of Aleppo pine in Tunisia. The traits are: leaf water potential (ψ_{leaf}), net photosynthesis (An), stomatal conductance (gs), transpiration (E), instantaneous water-use efficiency (WUE), non-structural carbohydrates (NSC), and percentage loss of conductivity (PLC).

Source of Variation	ψ_{leaf}	An	gs	E	WUE	NSC (Stem)	NSC (Root)	PLC
Site	28.38 ***	0.51 ns	2.00 ns	12.00 ***	0.75 ns	4.24 **	12.10 ***	22.55 ***
Season	73.68 ***	320.17 ***	47.78 ***	343.51 ***	64.66 ***	130.42 ***	75.72 ***	50.11 ***
Site $\times$ Season	3.22 **	1.05 ns	0.55 ns	1.34 ns	1.08 ns	0.30 ns	3.35 **	2.35 ns
Strategy	1.23 ns	1.20 ns	3.10 *	7.76 **	9.66 ***	12.16 ***	11.31 ***	2.44 ns
Site $\times$ Strategy	0.08 ns	0.43 ns	0.50 ns	2.08 ns	0.31 ns	3.08 *	1.89 ns	12.59 ***
Season $\times$ Strategy	1.18 ns	14.64 ***	5.15 **	1.91 ns	15.70 ***	2.25 ns	0.40 ns	1.39 ns
Season $\times$ Strategy $\times$ Site	1.21 ns	7.16 ***	0.16 ns	0.91 ns	2.64 **	1.75 ns	1.55 ns	0.49 ns

***: significant at $p < 0.001$, **: significant at $p < 0.01$, *: significant at $p < 0.05$, ns: non significant.

The gas exchange traits (An , gs , and E) also showed significant differences ($p < 0.001$) among seasons. There were also significant site-by-season and, particularly, season-by-strategy interactions, the latter being contingent on site (i.e., significant second-order interactions; Table 2). In spring and autumn, net CO_2 uptake (An) and stomatal conductance (gs) were higher in comparison to those of summer for all strategies and sites (Figure 4). As expected, the summer drought caused a generalized decrease in An and gs regardless of site. Overall, the highest An and gs values were observed in DZ, followed by DM and DS. This ranking resembled well the existing aridity gradient across sites. Variations in An followed

those of g_s in resprouters, resprouters-seeders, and seeders at the three sites. In addition, E followed variations in g_s at the different sites regardless of plant strategy (Figure 4). In the autumn season, all three strategies showed a fast recovery of photosynthetic performance, but the recovery was significantly higher in the case of seeders at all sites. In particular, the best post-drought recovery of photosynthetic activity was observed in seeders at DZ (with increases of 297% for An , 313% for E , and 600% for g_s) (Figure 4).

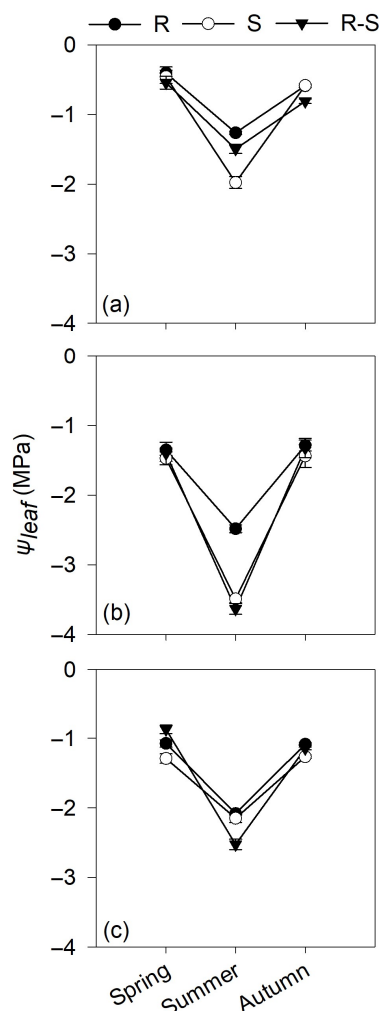


Figure 3. Seasonal variation of midday leaf water potential (ψ_{leaf}) for three plant strategies (seeder (S), resprouter (R), and resprouter-seeder (R-S)) at three forest stands of Aleppo pine in Tunisia: Djebel Zaghuan, DZ (a); Djebel Mansour, DM (b); Djebel Serj DS (c). Data are mean $\pm$ SE of four independent measurements.

The instantaneous water-use efficiency (WUE) showed significant differences ($p < 0.001$) among seasons, and there were also significant differences among plant strategies as well as significant interactions involving strategy, site, and season (Table 2). WUE tended to increase during the summer in most strategies at all three sites, except for seeders at DZ and DM sites (Figure 5). The most pronounced increase in WUE was observed in resprouters-seeders for the summer, regardless of site.

We also found significant differences ($p < 0.001$) in PLC among sites and seasons, and there was also a significant strategy by site interaction (Table 2). For all three strategies, PLC was relatively low during moderate water stress (i.e., spring), but higher at the driest (DS) site compared with the other (wetter) sites (Figure 5). When water stress became severe in summer, PLC increased particularly for resprouters at DZ and DM sites (74% and 81% respectively), reaching significantly higher values compared with the other strategies. At

the driest (DS) site, however, PLC reached 76% for both resprouters-seeders and seeders but showed values <60% for resprouters. Similarly, in the autumn season, the resprouters were more vulnerable to embolism at DZ and DM than at the driest DS site, while the opposite occurred for resprouters-seeders and seeders (Figure 5).

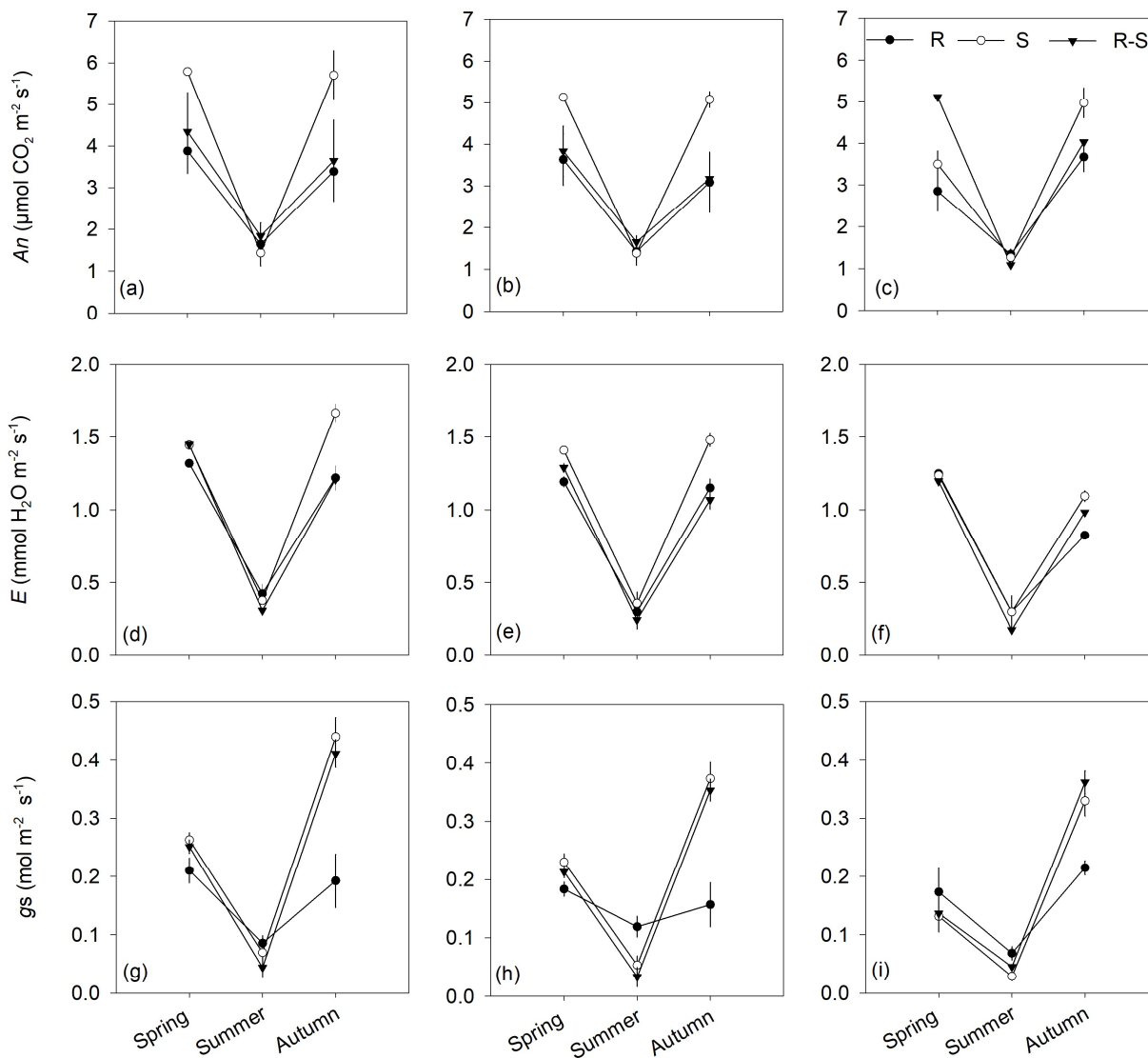


Figure 4. Seasonal variations of net photosynthesis (An), transpiration (E), and stomatal conductance (gs) for three plant strategies (seeder (S), resprouter (R), and resprouter-seeder (R-S)) at three forest stands of Aleppo pine in Tunisia: Djebel Zaghouan, DZ (a,d,g); Djebel Mansour, DM (b,e,h); and Djebel Serj, DS (c,f,i). Data are mean $\pm$ SE of four independent measurements.

3.3. Non-Structural Carbohydrates in Stems and Roots

There were significant differences ($p < 0.001$) in non-structural carbohydrates (NSC) of stems and roots among strategies, sites, and seasons (Table 2). The amount of NSC was larger in roots than in stems, regardless of strategy (Figure 6). The NSC of both stems and roots was higher in DZ compared with DM and DS, regardless of strategy and season (Figure 6). The highest NSC values were systematically observed in spring, with significant decreases in summer across strategies and sites in both stems and roots (Figure 6).

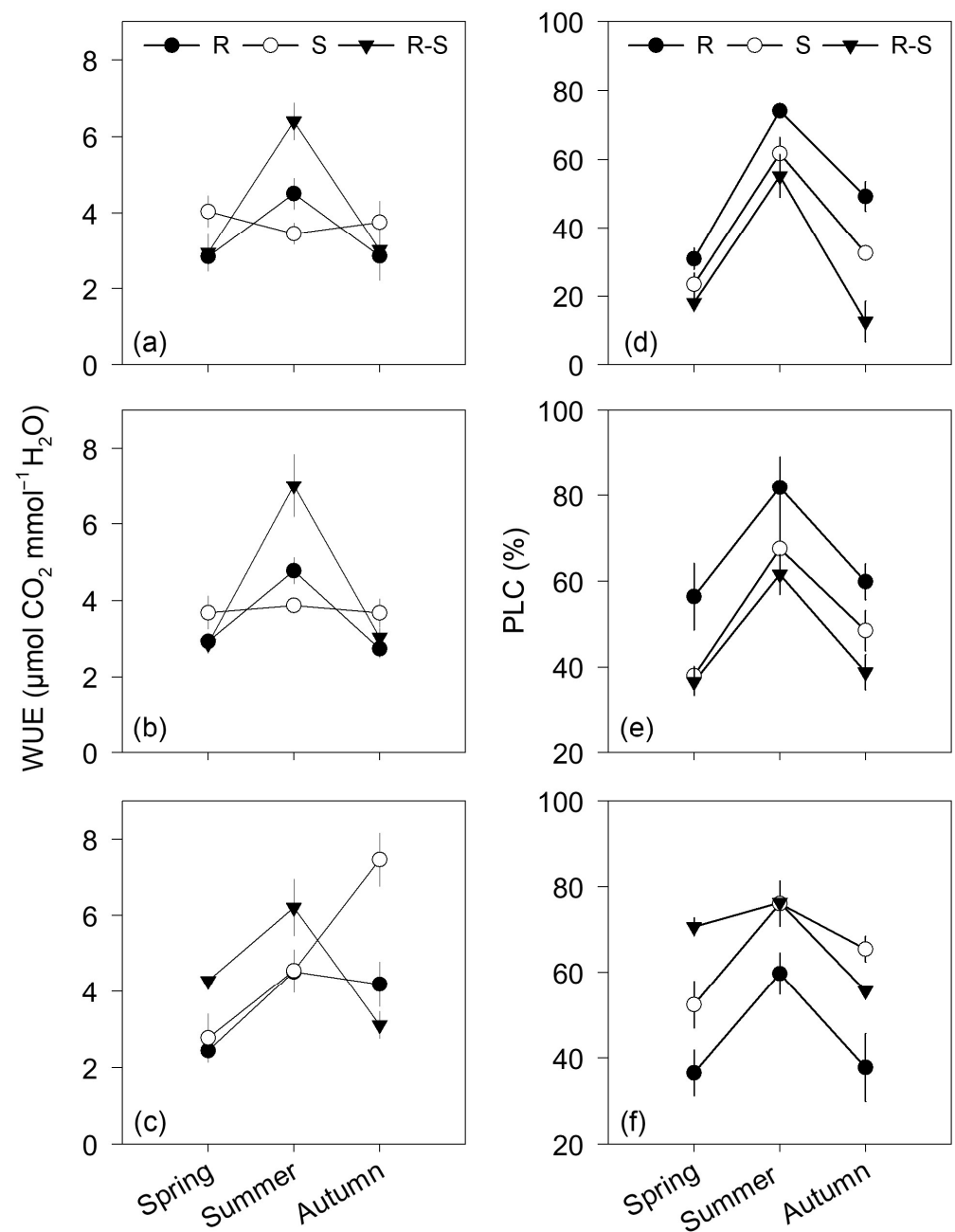


Figure 5. Seasonal variations of instantaneous water-use efficiency (WUE) (a–c) and percentage of loss conductivity (PLC) (d–f) for three plant strategies (seeder (S), resprouter (R), and resprouter-seeder (R-S)) at three forest stands of Aleppo pine in Tunisia: Djebel Zaghouan, DZ (a,d); Djebel Mansour, DM (b,e); and Djebel Serj, DS (c,f). Data are mean $\pm$ SE of four independent measurements.

Overall, resprouters showed lower stem NSC at the driest (DS) site compared with seeders and resprouters-seeders, but higher stem NSC at the wettest (DZ) site, which translated into a significant strategy by site interaction (Table 2).

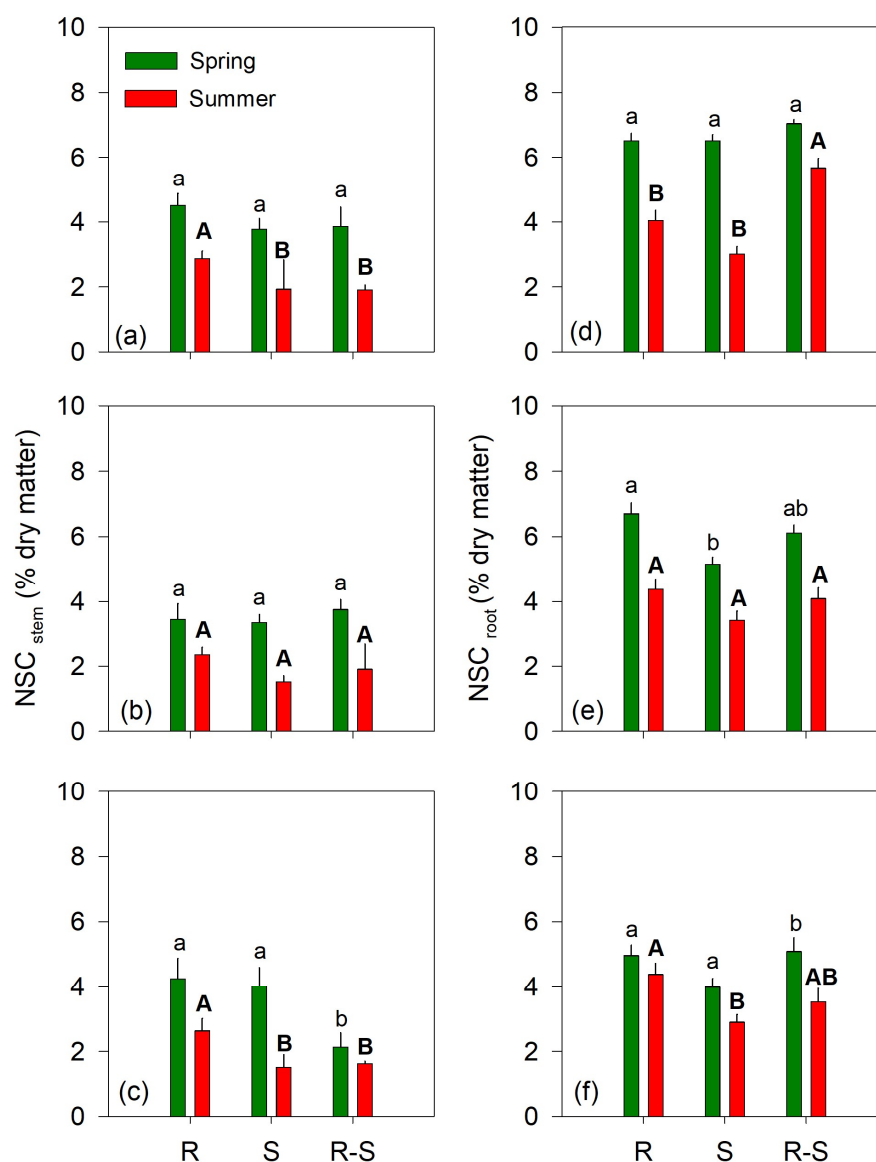


Figure 6. Spring and summer concentrations of non-structural carbohydrates (NSC) in stems (a–c) and roots (d–f) for three plant strategies (seeder (S), resprouter (R), and resprouter-seeder (R-S)) at three forest stands of Aleppo pine in Tunisia: Djebel Zaghouan, DZ (a,d); Djebel Mansour, DM (b,e); and Djebel Serj, DS (c,f). Different letters indicate significant differences between plant strategies for spring (lower case) and summer (capital letters) ($p < 0.05$).

3.4. Carbon and Nitrogen Isotopes

We found significant season-by-strategy interactions ($p < 0.05$) for both $\delta^{13}\text{C}_{\text{TOM}}$ and $\delta^{13}\text{C}_{\text{SOM}}$ (results not shown). In both cases, seeders showed an overall higher $\delta^{13}\text{C}$ in summer than in spring, which is consistent with a water-saving strategy of increasing water-use efficiency under dry summer conditions. Conversely, resprouters and resprouters-seeders show higher $\delta^{13}\text{C}$ values in the spring than in the summer, implying greater water-use efficiency in spring. However, these differences among strategies were site-dependent, being inexistent at the driest (DS) site (Figure 7).

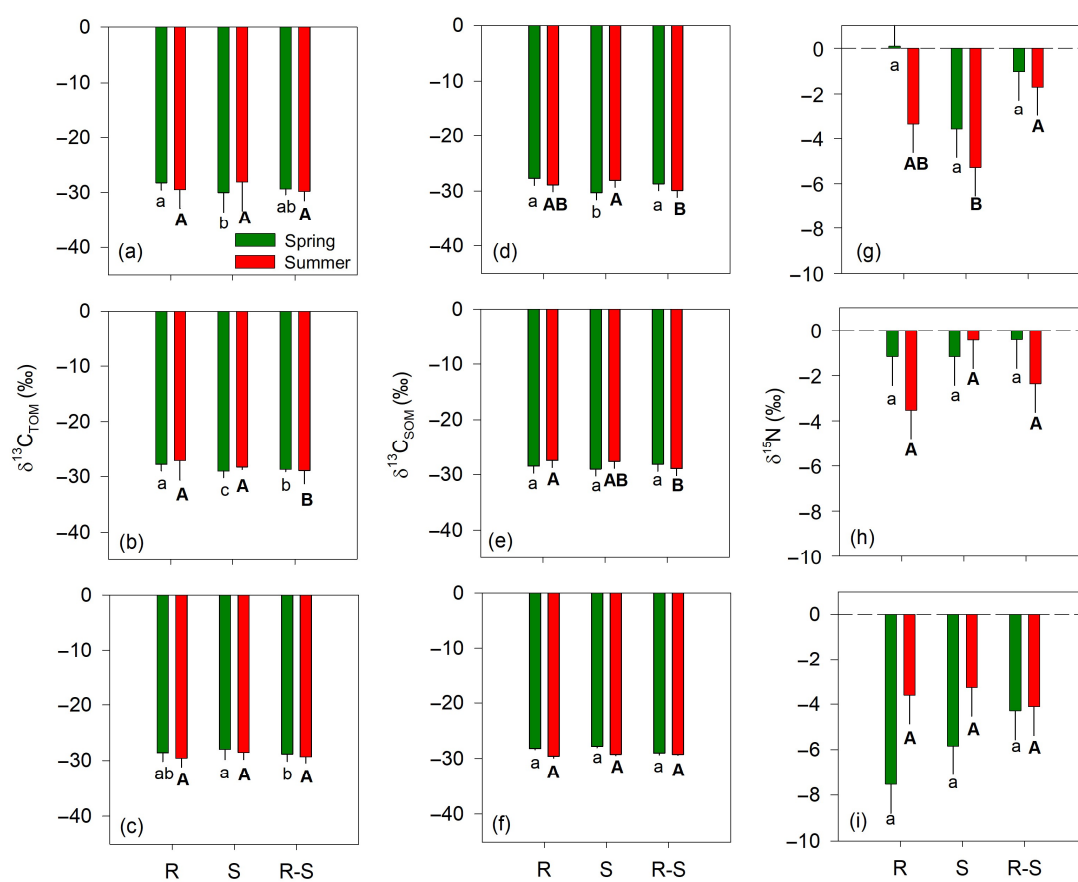


Figure 7. Spring and summer values of carbon isotope composition in total organic matter of leaves ($\delta^{13}\text{C}_{\text{TOM}}$) and water-soluble leaf extracts ($\delta^{13}\text{C}_{\text{SOM}}$), and nitrogen isotope composition in total organic matter of leaves ($\delta^{15}\text{N}$) strategies for three plant strategies (seeder (S), resprouter (R), resprouter-seeder (R-S)) at three forest stands of Aleppo pine in Tunisia: Djebel Zaghouan, DZ (a,d,g); Djebel Mansour, DM (b,e,h); Djebel Serj, DS (c,f,i). Different letters indicate significant differences between plant strategies for spring (lower case) and summer (capital letters) ($p < 0.05$).

There was a marginally significant site-by-strategy interaction ($p = 0.11$) for $\delta^{15}\text{N}$, but a lack of significant differences among strategies or season-by-strategy interaction (results not shown). Resprouters and resprouters-seeders consistently showed the lowest $\delta^{15}\text{N}$ at the driest (DS) site, but seeders displayed the lowest $\delta^{15}\text{N}$ at both the driest and wettest (DZ) sites (Figure 7).

3.5. Discriminant Analysis

Seven physiological traits contributed to differentiation among plant strategies in the summer. These were (ranked by order of inclusion in the stepwise discriminant analysis): instantaneous water-use efficiency (WUE), the percentage loss of conductivity (PLC), net photosynthesis (An), starch concentration in stems, sugar concentration in stems, $\delta^{13}\text{C}_{\text{TOM}}$ and $\delta^{15}\text{N}$ (Table 3). Hotelling's T^2 -statistics testing for between-class differences was significant (results not shown), and over 90% of the between-class to within-class variability was explained by the first two axes of a canonical discriminant analysis (Table 3).

The discriminant loadings for each ecophysiological trait are shown in Figure 8. The first dimension correlated positively with WUE and negatively with PLC and An and separated two distinctive strategies satisfactorily: resprouters (with high PLC and An and low WUE) and resprouters-seeders (with low PLC and An and high WUE). The second dimension correlated positively with a starch concentration in stems and negatively with a sugar concentration in stems and $\delta^{13}\text{C}_{\text{TOM}}$. This dimension indicated that seeders had high $\delta^{13}\text{C}_{\text{TOM}}$ and sugar content but low starch, whereas resprouters exhibited the opposite

pattern. Resprouters-seeders showed values for these traits somewhat closer to resprouters than to seeders. The mean values for each physiological trait and plant strategy in summer are shown in Table 3. $\delta^{15}\text{N}$ was scarcely relevant for differentiating among strategies.

Table 3. Summary of discriminant analysis for summer ecophysiological traits. Only variables that explain a significant proportion of the variance are included. For all three strategies, all variables were significant; net photosynthesis (An), instantaneous water-use efficiency (WUE), the percentage loss of conductivity (PLC), starch (stem), sugar (stem), $\delta^{13}\text{C}_{\text{TOM}}$, and $\delta^{15}\text{N}$.

Stepwise Discriminant Analysis					Physiological Traits							
Variable	Step	Partial R-Square	Pr > F	Average Squared Canonical Correlation	Mean Values per Functional Group							
					Mean	SD	Min	Max	Resprouter	Seeder	Resprouter-Seeder	
WUE ($\mu\text{mol CO}_2$ mmol H_2O)	1	0.46	0.013	0.23	4.94	1.42	3.19	7.98	4.70	3.94	6.14	
PLC (%)	2	0.59	0.003	0.43	67.7	17.6	33.5	85.8	79.5	68.4	57.2	
An ($\mu\text{mol CO}_2$ m $^{-2}$ s $^{-1}$)	3	0.51	0.013	0.55	1.47	0.29	1.09	2.12	1.50	1.36	1.56	
Starch (stem) (%)	4	0.47	0.029	0.73	2.50	0.84	1.51	4.64	3.02	1.95	2.63	
Sugar (stem) (%)	5	0.48	0.037	0.80	1.43	0.59	0.56	2.52	1.25	1.28	1.72	
$\delta^{13}\text{C}_{\text{TOM}}$ (‰)	6	0.52	0.036	0.87	−28.8	0.8	−30.0	−27.1	−28.6	−28.7	−29.2	
$\delta^{15}\text{N}$ (‰)	7	0.58	0.031	0.92	−3.0	2.3	−7.5	0.4	−3.0	−2.9	−3.1	

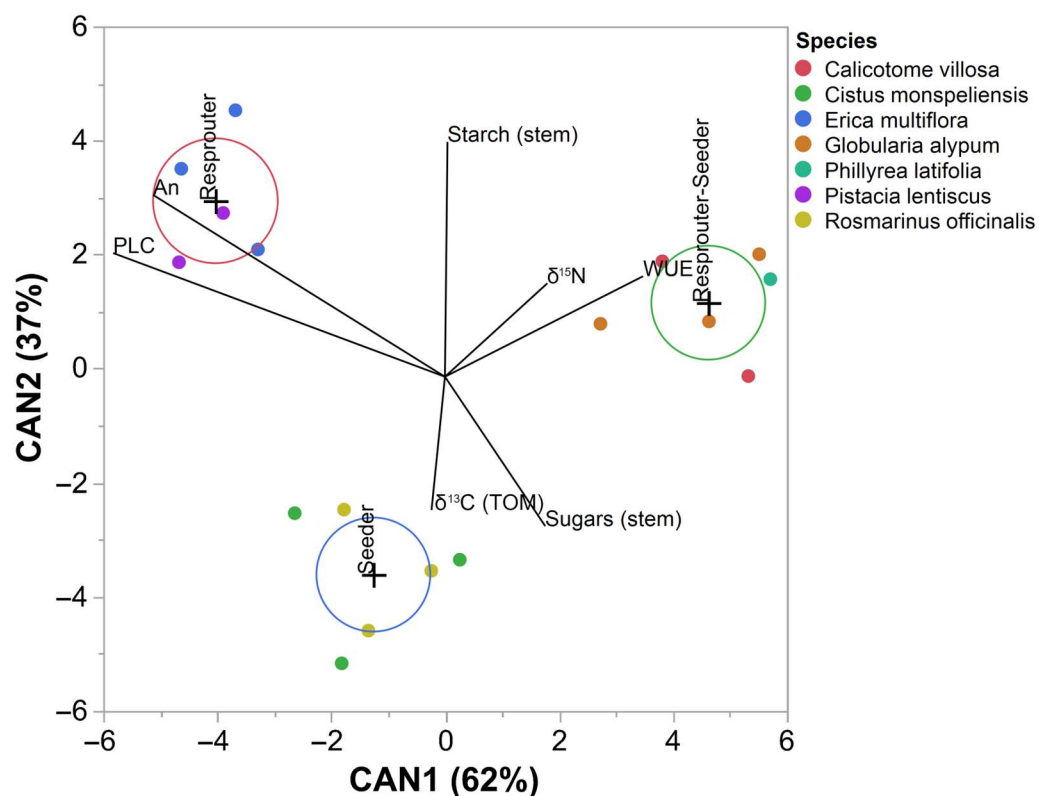


Figure 8. Plot of the centroids (indicated with crosses) and their 95% confidence ellipses for the first two canonical variables (CAN 1 and CAN2), for three plant strategies: seeder, resprouter, and resprouter-seeder. Rescaled discriminant loadings of the explanatory physiological traits in summer are included in the plot (WUE = instantaneous water-use efficiency; PLC = percentage loss of conductivity; An = net photosynthesis; Starch (stem) = starch concentration in stems; Sugar (stem) = sugar concentration in stems; $\delta^{13}\text{C}_{\text{TOM}}$ = carbon isotope composition of total organic matter; $\delta^{15}\text{N}$ = nitrogen isotope composition of total organic matter. Colors indicate the different shrub species (previously classified by plant strategy).

4. Discussion

Coexisting resprouters and seeders differed in terms of plant water relations, with marked differences in gas exchange and hydraulic traits that elicited contrasting summer drought responses. These differences have also been partly observed in previous studies [32], thereby supporting the existence of distinct adaptive syndromes characterized by different functional characteristics at the whole plant level. Particularly, we found that obligate resprouters are physiologically dampened against strong fluctuations in surface soil water availability compared with seeders in drought-prone environments. As the summer drought progressed, seeders and facultative resprouters experienced a decline in tissue water status, which was followed by a rapid recovery with the arrival of autumn rains. In contrast, resprouters had little seasonal change in water status and showed a delayed response to autumn rains [33].

The three plant strategies showed the highest water potentials at the more favorable DZ site compared with the DM and DS sites. Indeed, these differences can be attributed to the higher soil water availability in DZ (i.e., higher SWC) compared to the other sites, particularly during the dry summer season. However, under peak summer conditions, water potentials (ψ_{leaf}) became much lower for seeders and resprouters-seeders than for resprouters at all three sites. These differences can be explained by the typical rooting pattern of resprouters, which provides immediate access to deep water sources or groundwater, thereby allowing these species to avoid the summer drought. Indeed, rooting depth is known to have a strong influence on species' abilities to avoid drought in shrubby, dry ecosystems [34]. On the other hand, seeders displayed relatively high levels of cavitation resistance, but only at the driest DS site (Figure 5). This better hydraulic efficiency allowed seeders to maintain a high capacity to supply water to the leaves and, therefore, a high stomatal conductance. Thus, our analysis showed that the xylem of seeders species is more resistant to cavitation than that of resprouters species. This result is in agreement with the results found for other Mediterranean-type climate shrubs [35].

It is normally assumed that obligate resprouters contain greater reserves than seeders and resprouters-seeders, especially in their resprouting organs [36]. In our case, the root was the tissue having the highest sugar and starch concentration, in agreement with the function of roots as reservoirs of carbohydrates and nutrients to ensure rapid regrowth after disturbances [37]. Cruz and Moreno [38], for instance, found that most NSC is stored in the roots of the Mediterranean shrub *Erica australis* compared with stem tissues. On the other hand, seeders are reported to have lower reserves of starch and sugar in underground parts and instead accumulate more NSC in stems [12]. We did not find empirical support for this; instead, we observed that NSC decreased following an aridity gradient (DZ > DM > DS) and that reserves in either stems or roots were kept near spring levels at the driest DS site in resprouters only. The overall decrease in NSC detected from spring to summer across strategies may be associated with an increase in phloem sugars, suggesting that starch hydrolysis may increase cell osmolarity and help maintain phloem transport during the period of water stress [39]. However, the NSC of resprouters decreased comparatively less in summer at the driest DS site in comparison to other sites. This trend was previously observed in Mediterranean species, as shown by Klein et al. [40]. The overall lower NSC concentration during summer in the studied species occurred when drought decreased photosynthetic performance [40]. In this regard, NSC depletion was similar in both tissues, suggesting that carbon demand or mobilization was also similar above and below ground.

The overall reverse $\delta^{13}C_{TOM}$ and $\delta^{13}C_{SOM}$ patterns exhibited by seeders and resprouters when comparing their spring and summer values indicate that resprouters display the least conservative water-use strategy (i.e., the lowest water-use efficiency) under drought (summer) conditions. $\delta^{13}C_{SOM}$ in particular can be considered a tracer of intra-annual variation in the carbon-water balance of the different species strategies. The low intrinsic water-use efficiency exhibited by resprouters is likely due to poor control of stomatal closure in these species, especially in highly water-limited ecosystems such as those of northern Tunisia [41]. This is in contrast with other studies reporting the highest

$\delta^{13}\text{C}$ signatures in evergreen sclerophylls during the summer drought [42]. In spring, seeders showed the lowest $\delta^{13}\text{C}$ values among strategies (i.e., the lowest intrinsic water-use efficiency), thereby pointing to their opportunistic ecophysiological performance. These results were supported by measurements of instantaneous photosynthesis.

Resprouters and resprouters-seeders consistently showed the lowest $\delta^{15}\text{N}$ at the driest (DS) site, but seeders displayed the lowest $\delta^{15}\text{N}$ at both the driest and wettest (DZ) sites. The interpretation of $\delta^{15}\text{N}$ leaf values is far more complex than that of $\delta^{13}\text{C}$ due to the existence of a network of multiple processes and factors that determine foliar $\delta^{15}\text{N}$ values [43], including the nitrogen source, multiple assimilative processes such as organ-specific N loss or reabsorption/retranslocation, and different mycorrhizal associations that can cause $\delta^{15}\text{N}$ deviation from the main N source. The overall trend of higher spring foliar $\delta^{15}\text{N}$ values at DZ and DM sites is probably coherent with the higher soil N losses after N cycling processes are activated by spring rains and increasing temperatures [44]. In summer, conversely, the lower $\delta^{15}\text{N}$ of leaves could indicate minimal losses in the system across strategies, given the absence of N mineralization and water leaching [44]. Since older needles were found to be enriched in $\delta^{15}\text{N}$ relative to younger needles in previous studies involving evergreen tree species [45], the higher values of $\delta^{15}\text{N}$ values of leaves in spring do not appear to be caused by a reallocation of plant-stored N to newly formed leaves. Mycorrhiza was another possible source of $\delta^{15}\text{N}$ variation [46], since the studied species have different types of mycorrhizal associations (e.g., *C. villosa* is a legume with associated arbuscular mycorrhiza species). In any case, we did not observe obvious differences in $\delta^{15}\text{N}$ -derived N metabolism among functional strategies in peak summer.

Overall, *P. lentiscus*, *E. multiflora*, and *P. latifolia* are considered dehydration-avoiding species (sensu) [14]. Based on the outcome of our discriminant analysis, they show largely decoupled plant water relations from soil moisture, with low water potentials that can sustain gas exchange (and probably growth) during dry periods. These typical resprouter species maintained a substantial positive carbon balance in summer (high photosynthesis), a high starch concentration in the stem, a high WUE, and low ^{13}C isotopic discrimination [42]. In any case, xylem vulnerability was high during severe water stress (i.e., high PLC in summer). Conversely, *R. officinalis* and *C. monspeliensis* showed a higher WUE and a less favorable (i.e., more drought-sensitive) water status. These seeder species had a high ^{13}C isotopic discrimination, a high sugar concentration in the stem, and low PLC. In turn, seeders exhibited low photosynthesis in summer. These differences are overly consistent with similar differences reported for these functional groups in other drought-prone environments [47] and are therefore substantiated in this study for the severe water-limiting conditions typical of northern Africa. Interestingly, facultative resprouters showed an ecophysiological performance somewhat closer to obligate resprouters than to seeders.

5. Conclusions

In the present study, we evaluated the seasonal changes in the physiological activity of some shrub species representative of two contrasting strategies for coping with disturbance (i.e., fire) and stress (i.e., drought) in Mediterranean forests of North Africa (Tunisia): resprouters and non-resprouters. We examined the functional traits related to the carbon-water balance of seven shrubs that comprise the understory of these stands, including three obligate resprouters (*E. multiflora*, *P. latifolia*, and *P. lentiscus*), two seeders (*C. monspeliensis* and *R. officinalis*), and two optional resprouters (*C. villosa* and *G. alypum*). We conclude that: (i) seeders show greater seasonal variability in photosynthetic traits and carbon storage than obligate resprouters, as a consequence of their opportunistic strategy to take advantage of rain pulses during the growing season; (ii) these contrasting strategies are imprinted in $\delta^{13}\text{C}$ -derived iWUE and in $\delta^{15}\text{N}$ values, with seeders exhibiting greater seasonal fluctuations than resprouters in both traits; (iii) facultative resprouters perform intermediately between resprouters and seeders; and (iv) the information that can be gained on drought adaptations was particularly limited for resprouters growing in the wettest site, Djebel Zaghouan (DZ), which overall showed the lowest range of seasonal fluctuations across the aridity gradient.

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