

Full Length Research Paper

Growth and anatomical characteristics of different water and light intensities on cork oak (*Quercus suber* L.) seedlings

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This study was performed to investigate the effects of water and light intensity on the growth and anatomical aspects (that is, stomatal density and chloroplasts ultrastructure) of cork oak (*Quercus suber* L.) seedlings from acorns collected from Tabarka (Northern Tunisia). Seedlings, four-month after germination, were planted at 3 L pots with artificial soil and were cultivated for 5 months at the greenhouse. The experiments were divided into two treatments: Water stress (well-watered: 25 to 35% of water contents, moderate drought stress: 15 to 20%, and severe drought stress: 7 to 10%) and light intensity stress (full sunlight: 100% of full sunlight, $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$; moderate sunlight: 65 to 70% of full sunlight, $800 \mu\text{mol m}^{-2} \text{s}^{-1}$; low sunlight: 20 to 25% of full sunlight, $300 \mu\text{mol m}^{-2} \text{s}^{-1}$; and extremely low sunlight: 3 to 4% of full sunlight, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Growth varied among water stress, but this variation was typically smaller than light intensity. Seedlings grown under well-watered treatment showed the increased above ground growth as compared with deep root growth of severe drought stress. In contrast, seedlings grown under severe drought stress increased in root growth than in the above ground part. *Q. suber* seedlings grown under the shade-exposed low light intensity indicated adaptation such as the increased leaf area and increased specific leaf area than the leaves grown in full sunlight. In different light intensity treatments, the highest specific leaf area indicated at the extremely low light. Seedlings grown under severe drought stress increased in root growth. The palisade parenchyma chloroplasts of well-watered treatment were well developed: their shape was regular, chloroplast envelope membranes and grana were distinct, and an abundance of starch grains and a scarce of plastoglobuli was also visible inside in the chloroplasts. In severe drought stress, the ultrastructural features of the abaxial leaf surface were folded and shrunken, and was observed in a higher stomatal frequency, the thicker palisade and spongy parenchyma (containing three layers of palisade parenchyma). The severe drought stress indicated a swelling and disruption of the thylakoid, large starch grains, increased plastoglobuli, and accumulation of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2). The main features in the chloroplasts ultrastructure caused by the extremely low sunlight revealed not only the higher grana stacks and the broader grana, but also the higher stacking degree of thylakoids. Furthermore, seedlings grown under the extremely low sunlight showed no starch or exhibited only little starch in the chloroplasts, occupied the intracellular space in all organelles, and presented numerous small vacuoles and lipid droplets in the cytoplasm of these cells as compared with the well-watered and the severe drought stress. These results suggested that *Q. suber* has a well-developed mechanism to drought compared with shade stress by structural adaptations such as a deeper root system, a smaller reduction in stomatal density, and a smaller leaf area.

Key words: Water stress, light intensity stress, growth, stomatal density, palisade parenchyma, starch grains, plastoglobuli, *Quercus suber* L.

INTRODUCTION

Cork oak (*Quercus suber* L.) is an evergreen tree of great importance in the Mediterranean area both for ecological

reason and for the economic value of cork (Passarinho et al., 2006). This species plays a key role such as the

maintaining biodiversity and the main socio-economic activities (Hidalgo et al., 2008). This species is a sclerophyll evergreen tree, which adapted to a 4-month hot-dry summer period with at least 450 mm mean annual rainfall or no rain, maximum temperatures approaching 35 to 40°C and midday irradiances exceeding 2,000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic active radiation (PAR) and 4 - 5°C mean temperature for the coldest month (Lumaret et al., 2005).

A great deal of attention has recently been focused that a remarkable decline in several cork oak woodlands have occurred by complex factors involving abiotic stresses such as extremely hot dry summer and the environmental contaminants (Faria et al., 1996, 1998; Otieno et al., 2006; David et al., 2007), the outbreaks of damaging *Phytophthora* species including *Phytophthora cinnamomi* (Brasier, 1996; Balci and Halmschlager, 2003; Moreira and Martins, 2005), and seed predation damage by livestock grazing during recent decades (Campos et al., 2008). In plants, the remarkable ability to adapt to adverse environmental stress such as water stress and light stress may be related to specific changes in the morphological, physiological, and ultrastructural level (e.g., chloroplast) of leaves (Meier and Lichtenthaler, 1981).

Among the environmental factors affecting plant activity, water stress was one of the most important limiting factors at the initial phase of plant growth and establishment (Yin et al., 2005). The chloroplasts of the plants grown under the water stress indicate the main damages in structural changes such as the excessive swelling of the thylakoids, the distortion of the grana, and the appearance of the lipid droplets (Riatic, 1992; Lopez-Carbonell, 1994). Chloroplasts of general mesophyll cells possess few plastoglobuli, but the number and size of these lipophilic compounds increase due to degraded thylakoids in chloroplasts during leaf senescence and in etioplasts before thylakoid formation (Steinmüller, 1985; Eymery, 1999). Munne-Bosch (2001) reported that symptoms of leaf senescence were revealed in chloroplasts at drought-induced senescence, showing an accumulation of plastoglobuli in the chloroplasts, including the disruption of thylakoids and much less grana stack.

Plants exhibit many morphological and physiological adaptations to cope with these environmental stresses (Aranda et al., 2005). Mediterranean-climate sclerophyllous trees also are typically known to possess anatomical and morphological structures in their leaves or other vegetative organs as related to an important indicator of plant strategies have high resistance to desiccation during substantial water stress (e.g., leaf mass per area indicates drought acclimation, density and thickness of leaf mesophyll, scarcity of intercellular spaces,

and thick cuticles) (Lansac et al., 1994; Castro-Díez et al., 2000; Bussotti et al., 2002).

Oak seedlings in the degraded Mediterranean environments possess several morphological traits for desiccation avoidance in open, high irradiance or xeric sites (e.g., deeper and well structured root system, greater leaf thickness, higher stomatal density and smaller guard cells than other hardwoods, and leaf curling developed in several southeastern evergreen oaks) (Abrams, 1990). The morphological traits focused to minimize the demand previously discussed help oak maintain a higher physiological activity under unfavorable conditions of drought interacting with shade (Sack and Grubb, 2002; Aranda et al., 2005).

Variation of morphological and anatomical features to the crucial environmental resources such as the water-deficit and the light gradient play a major part in the optimization of the growth and early development of seedlings (Meier and Lichtenthaler, 1981; Lichtenthaler et al., 1981; Abrams, 1990; Dickson and Tomlinson, 1996; Gregoriou et al., 2007). The shade-type chloroplasts grown under the low-light generally possess characterization such as the higher grana stacks, the higher stacking degree of thylakoids, the broader grana, and an insufficient starch as compared to those under the high-light (Meier and Lichtenthaler, 1981).

Shaded (30% of full sunlight) leaves normally display increased chlorophyll contents and are thinner thickness than those grown under natural irradiation, due to possessing two palisade cell layers instead of the three layers of the palisade parenchyma of natural irradiation (Mendes et al., 2001; Romano and Martins-Loução, 2001; Gregoriou et al., 2007). However, a detailed study of *Q. suber* trees in the morphological and anatomical characteristics during early plant growth and development of water and light gradient is lacking.

Therefore, the morphological and anatomical aspects of *Q. suber* in response to different water and light intensity will become essential for a deeper understanding of regeneration success of forestry tree species in Mediterranean-climate ecosystems.

The main objective of this study was to investigate the growth and anatomical aspects in response to different water and light intensity on *Q. suber* seedlings.

MATERIALS AND METHODS

Experimental description and plant material

The study was carried out on cork oak (*Q. suber* L.) seedlings growing in two nursery stands located in the greenhouse at the University campus (University of Seoul, Seoul). *Q. suber* seedlings were prepared with acorns collected from Ain Snoussi and Northern Tunisia.

Seedlings, four-months after germination, were planted in 3-L plastic pots in April 2008 and placed in greenhouse of University of Seoul. The 3-L pots is filled with an artificial soil (Perlite+Vermiculite+Peatmoss, 1:1:1 [v/v/v]) with soil pH 5, and electrical conductivity (EC) 15.4 μScm^{-1} . The experimental design in

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the greenhouse was performed with a completely randomized design of 18 replicates per treatment with three irrigations and four light regimes for five months.

Seedlings were subjected to three irrigation regimes: Well-watered (WW, 25 to 35%), moderate drought stress (MDS, 15 to 20%), and severe drought stress (SDS, 7 to 10%) regime. Soil moisture was recorded three times a week by time domain reflectometry (TDR, Trase System I, Soil Moisture Equipment Corp. USA). Seedlings under four light regimes with 18 replicates were randomly divided according to the different layers of shade cloth screening. The light intensity (the photosynthetic photon flux density, PPF) of full sunlight was measured on a sunny day with a quantum sensor (type SKP 215, Skye Instruments, Powys, UK). The average photosynthetic photon flux density (PPFD, Q) under each light intensity regime during a sunny day was: FL, full sunlight (100% of full sunlight, $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$); ML, moderate sunlight (65 to 70% of full sunlight, $800 \mu\text{mol m}^{-2} \text{s}^{-1}$); LL, low sunlight (20 to 25% of full sunlight, $300 \mu\text{mol m}^{-2} \text{s}^{-1}$); ELL, extremely low sunlight (3 to 4% of full sunlight, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Seedlings were grown in a randomized block design with metal frames to produce three irrigation and four light intensity regimes.

Growth characteristics

Growth and biomass measurements

To quantify differences in growth response to water and light environment, a subset of three plants from each treatment was randomly selected for morphological and biomass measurements.

Number of leaves and height of seedlings were monitored three times with monthly intervals (June, July and August). At the end of the nursery culture, each seedling was harvested and divided into component parts, and then shoot height (cm), number of leaves, root height (cm), shoot diameter (mm), and root diameter (mm) were measured. Growth and biomass variables (that is, root, shoot, total biomass of the seedling, and shoot biomass/root biomass ratio, SRR) were also calculated for application to the analysis of growth under different water and light intensity. Leaf area was measured using an LI-3100 area meter (LI-COR Inc, Lincoln, NE, USA). In order to determine dry mass, these shoot and root biomasses were measured by weighing of final dry mass of shoot and root (after desiccation at 75°C for 48 h).

Specific leaf area (SLA)

The specific leaf area (SLA: $\text{cm}^2 \text{g}^{-1}$ dry weight) was calculated as the ratio of the fresh leaf area to its dry weight of individual leaves. SLA is pivotal features in the carbon-fixation 'strategy' of a species (Davi et al., 2008).

Leaf areas per leaf dry mass (SLA, an indicator of leaf structure characteristics) are fundamental traits in the carbon economy of plants (Wright et al., 2002). Plants grown under high light generally had thick leaves with a low SLA (Björkman, 1981).

Anatomical characteristics

Scanning electron microscopy (SEM)

Tissue samples for the microscopic observations were randomly collected from 3 to 5 mature leaves in well-watered, severe drought stress, and extremely low sunlight regimes of *Q. suber* seedlings at August 2008. All anatomical parameter data were obtained using SEM images.

The sampled leaves were placed individually in polyethylene bags and immediately transported to laboratory. Upon return to the

laboratory, field-collected tissue samples from leaves (sliced 1 to 2 mm thick) were immediately fixed by Karnovsky's fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2), and fixed overnight for 2 to 4 h at 4°C . Samples were then rinsed three times in 0.05 M sodium cacodylate buffer (pH 7.2) for 10 min at 4°C , post-fixed with 1% osmium tetroxide (OsO_4) in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C , briefly rinsed again two times with distilled water at room temperature.

The tissue was then washed, and then sections were stained *En bloc* staining with 0.5% uranyl acetate for 30 min at 4°C . In order to dry specimen, once again they are immersed two times in 100% isoamyl acetate for 15 min using critical point dryer. The specimens were then mounted on metal stubs, coated gold-palladium alloy using a sputter-coater (JFC-1100E; JEOL, Tokyo, Japan), and viewed with the scanning electron microscope (SEM, JSM-5410LV, JEOL, Tokyo, Japan) operated at an accelerating voltage of 20 kV. Scanning microscope photographs of the abaxial epidermis were used to quantify the stomatal density (the number of stomata per unit leaf area) and the leaf stomatal size.

Light microscopy (LM) and transmission electron microscopy (TEM)

For a light microscopy (LM) and a transmission electron microscopy (TEM) observation, small pieces from the middle region of the field-collected lamina were fixed overnight by immersion in Karnovsky's fixative: 2% paraformaldehyde and 2% glutaraldehyde buffered with 0.05 M sodium cacodylate buffer (pH 7.2) for 2 - 4 h at 4°C .

The specimens were rinsed three times in the same buffer, post-fixed in 1% OsO_4 (the same buffer) for 2 h at 4°C , then rinsed two times with the distilled water at room temperature, stained *En bloc* staining with 0.5% uranyl acetate for 30 min at 4°C and dehydrated through a graded series of ethanol (30, 50, 70, 80, 90, 100, 100, and 100%) for 10 min at room temperature, and transited two times in 100% propylene oxide for 15 min at room temperature, and embedded by a Spurr's resin.

The method of measuring infiltration was accomplished according to the following steps: The tissue pieces were placed in rubber embedding molds and then embedded in Spurr's resin for 4 h. After infiltration in resin, the sections were then polymerized overnight at 70°C .

The thin sections were prepared with an ultra-microtome (MT-X; RMC, Tucson, AZ, USA). Three thin transverse sections (0.5 mm) were prepared by using a microtome cryostat (HM505E; Microm, Walldorf, Germany). LM images were obtained randomly at $20\times$ $40\times$ using a light microscope (Zeiss Axioskop) with a camera (Zeiss AxioCam HRc).

The semi-thin resin sections of the stained preparations were observed with the light microscope. Then the total thickness, thickness of palisade parenchyma, spongy parenchyma, lower epidermis, and upper epidermis were measured. Ultrastructure morphology of the specimen was examined with a Transmission Electron Microscope (LIBRA 120; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The effects of water and light intensity stress on the different morphological and anatomical parameters of the seedlings were analysed separately for each extraction by a multifactor analysis of variance (ANOVA) using the version 9.2 of the SAS statistical software package (Systat 9.2, Systat Software Inc., Richmond, USA). The test for differences among arithmetic treatment means were performed by the least significant difference (LSD) multiple range test method. The significance levels were set at the $P \leq 0.05$ level.

Table 1. Effects of different water stresses on biomass parameters in *Q. suber* at the end of the experiment.

Parameter	Unit	Nursery			LSD
		WW	MDS	SDS	
Shoot fresh biomass	g/pot	6.12a*	3.96b	2.76c	1.0946
Root fresh biomass		9.04a	6.90b	7.22b	1.4120
Shoot dry biomass		2.94a	2.12b	1.44c	0.6647
Root dry biomass		4.86a	4.24 ab	3.62b	0.9390
Shoot/root ratio (SRR)		0.70a	0.56a	0.38b	0.1488

*Means ($n=5$) in a row followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. WW, well-watered; MDS, moderate drought stress; SDS, severe drought stress.

Table 2. Effects of different light intensity stresses on biomass parameters in *Q. suber* at the end of the experiment.

Parameter	Unit	Nursery				LSD
		FL	ML	LL	ELL	
Shoot fresh biomass	g/pot	6.12b*	7.14a	3.78c	2.38d	0.9514
Root fresh biomass		9.04a	9.82a	6.44b	5.66b	0.9530
Shoot dry biomass		2.94b	3.76a	1.76c	1.04c	0.7867
Root dry biomass		4.86b	6.32a	4.24b	2.54c	0.8199
Shoot/root ratio [SRR]		0.70a	0.72a	0.56b	0.42c	0.1289

*Means ($n=5$) in a row followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. FL, full sunlight; ML, medium sunlight; LL, low sunlight; ELL, extremely low sunlight.

RESULTS AND DISCUSSION

Growth and anatomical characteristics

Growth and biomass measurements

There were many studies focusing on the morphological and physiological adaptation of *Q. suber* to drought and shade stress (Abrams, 1990; Dickson and Tomlinson, 1996).

The present study was undertaken to examine effects of different water and light intensity in allometric differences of the morphological and anatomical aspects of *Q. suber* seedlings at the end of the experiment. Growth traits of *Q. suber* varied among water stress (Figure 1A and Table 1). This variation, however, was typically small compared to the pronounced differences among light stress (Figure 1B and Table 2).

The growth and morphology of *Q. suber* was affected by the levels of water (Table 3) and light intensities (Table 4). The total biomass varied among the water treatments and light treatments at the end of the experiment (Figure 1). Figure 1 showed that shoot and root biomass accumulations were greatly reduced in well-water treatments. However, the moderate drought stress

(MDS) and the severe drought stress (SDS) showed similar values of total biomass and the lowest values were observed during the moderate drought stress (MDS) period.

The root biomass within the moderate drought stress (MDS) was found in morphological aspects with smaller allocation in the response to mild drought stress as compared with the severe drought stress (SDS). But no significant difference between MDS and SDS treatments was found (Figure 1A).

Among the environmental factors affecting plant activity, water stress was one of the most important limiting factors at the initial phase of plant growth and establishment (Yin et al., 2005). Effect of water stress exhibited changes in early growth, relative biomass accumulation and allocation in response to water stress during treatment period.

There were significant differences in shoot height, shoot diameter, and shoot/root ratio of height among water treatments. *Q. suber* showed significant increase in shoot height, shoot diameter, and shoot/root ratio of height under well-watered treatments compared with water-stressed treatments. The shoot height was significantly increased in well-watered treatment (as control, WW) while the opposite trend was observed in severe

Table 3. Effects of different water stresses on *Q. suber* seedlings growth characteristics at the end of the experiment.

Nursery	Shoot height (cm)	Shoot diameter (mm)	Root height (cm)	Root diameter (mm)	Shoot/root ratio (SRR)
WW ^z	45.8 ±2.36 a ^y	4.2 ±0.17a	14.9 ±1.68	9.5 ±0.52	3.1 ±0.19a
MDS	36.3 ±1.07b	4.1 ±0.11a	14.0 ±0.12	8.9 ±1.06	2.6 ±0.08b
SDS	25.6 ±0.51c	3.3 ±0.12b	13.6 ±0.60	8.0 ±0.50	1.9 ±0.11c
LDS	4.6958	0.4217	3.1851	2.2853	0.4119

^y Means ± S.E. ($n=5$) in a column followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. ^z WW, well-watered; MDS, moderate drought stress.

Table 4. Effects of different light intensity stresses on *Q. suber* seedlings growth characteristics at the end of the experiment.

Nursery	Shoot height (cm)	Shoot diameter (mm)	Root height (cm)	Root diameter (mm)	Shoot /root ratio (SRR)
FL ^z	45.8 ±2.36 b ^y	4.2 ±0.17a	14.9 ±1.68a	9.5 ±0.52a	3.1 ±0.19b
ML	54.0 ±1.35a	4.3 ±0.09a	15.4 ±0.60a	10.4 ±0.47a	3.5 ±0.08ab
LL	44.8 ±3.14b	2.8 ±0.16b	11.7 ±0.70b	7.5 ±0.15b	3.8 ±0.27a
ELL	41.9 ±2.67b	2.2 ±0.15c	10.6 ±0.10b	7.8 ±0.54b	3.9 ±0.22a
LDS	7.4049	0.4339	2.8794	1.3484	0.6067
FL ^z	45.8 ±2.36 b ^y	4.2 ±0.17a	14.9 ±1.68a	9.5 ±0.52a	3.1 ±0.19b
ML	54.0 ±1.35a	4.3 ±0.09a	15.4 ±0.60a	10.4 ±0.47a	3.5 ±0.08ab
LL	44.8 ±3.14b	2.8 ±0.16b	11.7 ±0.70b	7.5 ±0.15b	3.8 ±0.27a
ELL	41.9 ±2.67b	2.2 ±0.15c	10.6 ±0.10b	7.8 ±0.54b	3.9 ±0.22a
LDS	7.4049	0.4339	2.8794	1.3484	0.6067

^y Means ± S.E. ($n=5$) in a column followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. ^z FL, full sunlight; ML, moderate sunlight; LL, low sunlight; ELL, extremely low sunlight.

drought stress (SDS) (Table 3 and Figure 2A). The growth traits focused to minimize the demand as previously discussed help oak maintain a higher physiological activity under unfavorable conditions of drought interacting with shade (Aranda et al., 2005; Jimenez et al., 2009). In addition, the watering regimes significantly affected all these morphological properties, and the effect was also significant except for root height and root diameter.

On the other hand, there were not significant difference in root height and diameter among water treatments (WW, MDS, and SDS) at the end of the experiment. Root height and root diameter indicated lower values (13.6 ± 1.3 , 8.0 ± 1.1) in SDS than in WW (14.9 ± 3.8 , 9.5 ± 1.1) and in MDS (14.0 ± 0.3 , 8.9 ± 2.4), respectively, although the difference was not statistically significant (Table 3).

They also showed higher shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, and shoot/root biomass ratio in well-watered (WW) treatment compare with water-stressed treatments (MDS and SDS). Consequently, the seedlings grown under well-watered (WW) showed higher aboveground and belowground biomass

than those of the MDS and SDS seedlings.

However, the direction of the relationship in shoot/root ratio of biomass indicated relatively high root system in water-stressed plants compared with the well-watered control plants. The results suggest root-morphological adaptations against drought on the more arid SDS. A deep and thick root system, with deep root length density, is the important factor contributing drought avoidance mechanisms to resistance to drought stress (Abrams, 1990; Dickson and Tomlinson, 1996).

Previous studies have suggested that drought avoidance mechanisms (deep root system, leaf curling, smaller leaf area, etc.) and drought tolerance mechanisms (osmotic adjustment, stomatal control to maintain moderate photosynthetic rates, etc.) are two drought strategies of different oak species indicated in response to water stress (Dickson and Tomlinson, 1996; Loewenstein and Pallardy, 1998). The values of shoot/root biomass ratio and height showed an increase of 1.8- and 1.7-fold, respectively, in well-watered (WW) as compared to values in severe drought stress (SDS) seedlings (Tables 1 and 3).

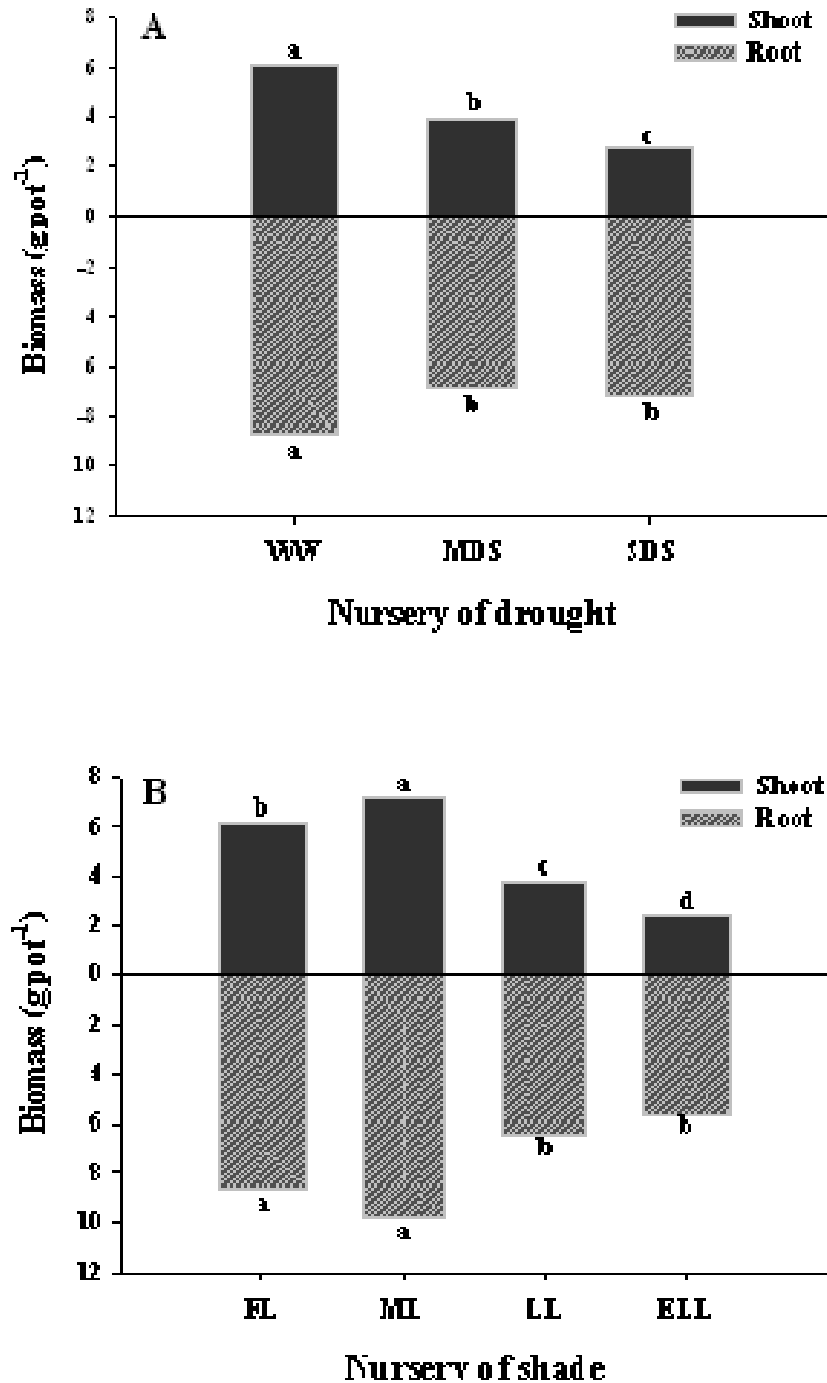


Figure 1. Allometric analysis of shoot and root biomass allocation in the growth response of seedlings to different levels of water (A) and light intensities (B) on *Q. suber* at the end of the experiment. Means ($n=5$) designated with the same letter are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. WW, well-watered; MDS, moderate drought stress; SDS, severe drought stress; FL, full sunlight; ML, moderate sunlight; LL, low sunlight; ELL, extremely low sunlight.

Light is one of the most important environmental factors regulating growth and development (Ma et al., 2001). Seedlings grown under shade treatments had significantly different growth than that of seedlings grown

under water regimes (Tables 2 and 4). Shoot height and diameter, root height and diameter were greatly increased in moderate sunlight (ML) treatment compared with other light treatments (full sunlight, FL; low sunlight,

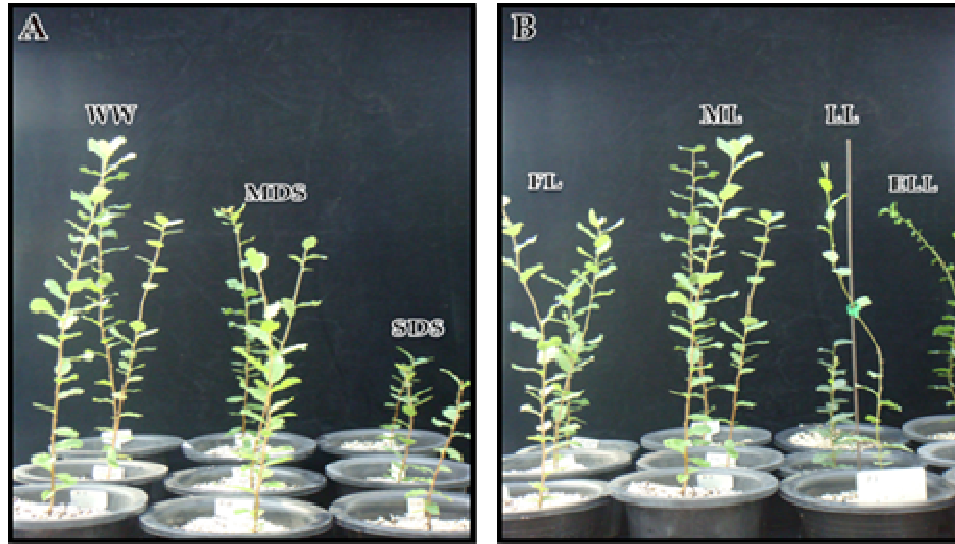


Figure 2. Growth characteristics of different water (A) and light intensities (B) on *Q. suber* seedlings at the end of the experiment. WW, well-watered; MDS, moderate drought stress; SDS, severe drought stress; FL, full sunlight; ML, moderate sunlight; LL, low sunlight; ELL, extremely low sunlight.

LL; extremely low sunlight, ELL).

The allometry of biomass such as shoot height, shoot diameter, and root height was significantly decreased as gradually dense shade (Table 4 and Figure 2B). Within a given shade treatment, seedlings of the moderate sunlight (ML) conditions showed highly increase than other shade-grown seedlings (LL and ELL). However, shoot/root ratio of height showed opposite result under shade treatment. Root height per unit shoot height (SRR, shoot/root ratio) indicated more increase in shade conditions (ML, LL, and ELL) compared with the full sunlight (FL) treatment, and showed highly in ML than LL and ELL in different shade treatments (Table 4).

In spite of the differences found in morphological shoot height and diameter, similar allocation patterns of root height and diameter were found in FL and ML, LL and ELL, respectively, which belonged to the same group. The moderate sunlight (ML) condition showed a tendency to allocate more biomass to shoot height. Nevertheless, there were no statistically significant differences in shoot diameter, root height, and root diameter between the full sunlight (FL) and the moderate sunlight (ML) treatment (Table 4). Different oak species have developed both morphological and physiological adaptations to survive and grow on their growing environmental sites.

Morphological adaptations such as a lower above ground and deeper belowground in response to water stress is commonly observed in both drought avoidance and drought tolerance (Dickson and Tomlinson, 1996). Shade-tolerant species are generally manifested as an enhanced phenotypic plasticity in leaf morphological variations in comparison with physiological variations

(Valladares et al., 2005). In the present study, cork oak seedlings grown under shade-induced stress indicated adaptation strategy such as the increased leaf area and the increased specific leaf area (SLA).

Specific leaf area (SLA)

Specific leaf area (SLA) reflects alternatively the leaf thickness and the relative rates of assimilative tissues in leaves (Rhizopoulou et al., 1991).

The specific leaf area (SLA) was described as the ratio of leaf area to leaf dry mass (in $\text{cm}^2 \text{g}^{-1}$). The different light intensity (as shade, Figure 3B) treatments showed statistically significant differences, while no significantly differences in the specific leaf area (SLA) were found among all water stress (Figure 3A) treatments in cork oak (*Q. suber* L.).

As shown in Figure 3, shade-exposed leaves of *Q. suber* were found to a higher specific leaf area (SLA) than the leaves grown in the full sunlight (FL). In different shade treatments, the highest specific leaf area (SLA) indicated the extremely low sunlight (ELL) treatment (Figure 3B). The differences of biomass allocation of leaf tissue, as reflected by the increased specific leaf area (SLA) at the extremely low sunlight (ELL), suggested the decreased leaf thickness (Table 6).

The reliable effect of shade has approximately the strongest negative relationships on leaf area, which is a major factor in determining the leaf photosynthesis (Gregoriou et al., 2007). Moreover, the most common morphological plasticity in response to shade was to

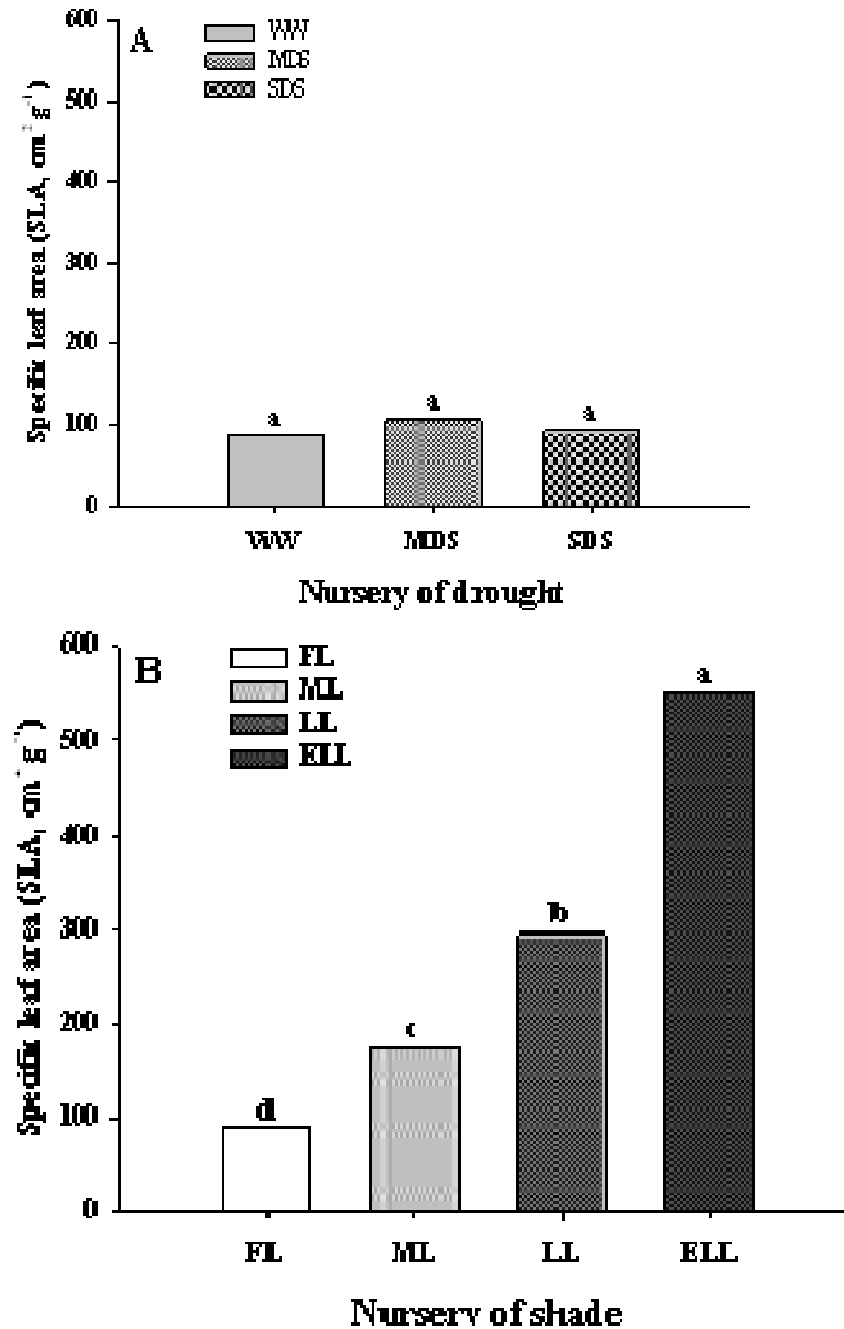


Figure 3. Effects of different water (A) and light intensities (B) for the specific leaf area (SLA, $\text{cm}^2 \text{g}^{-1}$) on *Quercus suber* seedlings at the end of the experiment. Bars represent mean values ($n=15$); the same column letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. WW, well-watered; MDS, moderate drought stress; SDS, severe drought stress; FL, full sunlight; ML, moderate sunlight; LL, low sunlight; ELL, extremely low sunlight.

possess the higher specific leaf area (SLA) (Puértolas et al., 2008). Sun and shade-exposed leaves of trees indicate complete differences in their leaf size, morphology, composition, chloroplast structure and photosynthetic capacity. As compared to shade-induced

leaves, sun-exposed leaves are often characterized by thicker leaves and smaller leaf area (Lichtenthaler et al., 1981).

Sack and Grubb (2002) reported that the specific leaf area (SLA) in high irradiance declined across species with

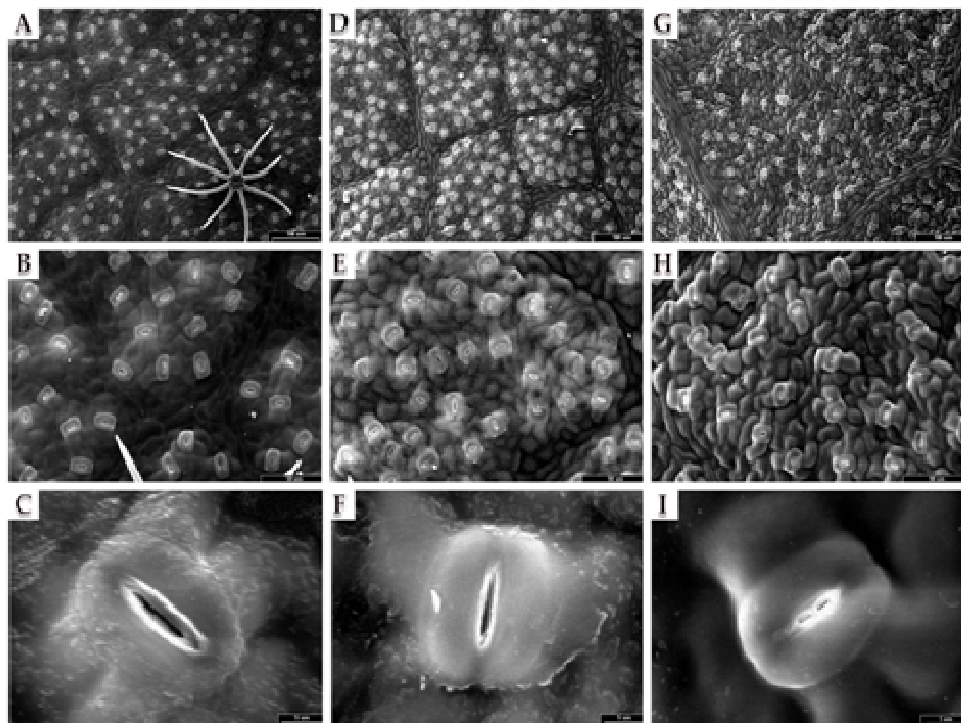


Figure 4. Stomatal frequency and shape on the abaxial surface view using a scanning electron microscopy (SEM) of the well-watered (WW, A-C), the severe drought stress (SDS, D-F), and the extremely low sunlight (ELL, G-I) leaves of *Q. suber* seedlings. Note that the leaf was severely folded in severe drought stress (SDS). Magnification: A, D, and G ($\times 200$, Scale bar = $100\ \mu\text{m}$); B, E, and H ($\times 500$, bar = $50\ \mu\text{m}$); C, F, and I ($\times 35,000$, bar = $5\ \mu\text{m}$).

with increasing drought tolerance, from *Viburnum opulus* to *V. lantana*, to *Hedera* and *V. tinus*. In addition, each study in response to severe shade was found to possessing a higher SLA.

Anatomical characteristics

Scanning electron micrography (SEM)

The measurement of stomatal frequency and size was conducted after the preparation of the adaxial surface of leaf epidermis. Stomatal control in response to different water stress is the first and one of the most important steps in maintaining some degree of photosynthesis by the minimal water loss (Dickson and Tomlinson, 1996). Although the length of the guard cells was approximately equal in leaves among treatments, the number of stomata per unit leaf area was significantly different among treatments (Figure 4 and Table 5).

In the severe drought stress (SDS) was observed higher stomatal frequency as compared with the well-watered (WW) treatment, while the extremely low sunlight (ELL) treatment showed the opposite trend (Figure 4 G and H). Leaves of seedlings grown under the severe drought stress (SDS) had approximately 19% more

stomata per unit area than those grown under well-watered (WW) treatment without stress, on the other hand, those grown under the extremely low sunlight (ELL) treatment had about 34% fewer stomata per unit surface (Figure 4 and Table 5). Therefore the stomatal frequency of epidermal cells was significantly higher in the severe drought stress (SDS) than in the well watered (WW) and in the extremely low sunlight (ELL).

In the severe drought stress (SDS) leaves, the increase of stomatal frequency reflected the overall reduction in epidermal cell size (Quarrie and Jones, 1977).

Moreover, Mishra (1997) suggested that epidermal cell size indicated the main factor reflecting stomatal frequency.

The degree of epidermal surface folding was clearly evident in micrograph of the severe drought stress (SDS) leaves (Figures 4D and E). Furthermore, the severe drought stress (SDS) also indicated the shrunken abaxial surface and showed a deeply folded epidermal tissue compared with well-watered (WW) and the extremely low sunlight (ELL) treatment. Curtis et al. (1996) have represented that the evergreen tree *Cordeauxia edulis* was indicated the deeply folded, shrunken aspect of the concertina cells in dried (not rehydrated) leaves during dry periods. Therefore, the development of smaller leaves

Table 5. The stomatal frequency and the size of guard cell from abaxial epidermal surface by a scanning electron microscopy (SEM) survey on effects of water and light intensity of *Q. suber* seedlings.

Nursery	Guard cells length (μm)	Guard cells width (μm)	Stomatal frequency per mm^2
WW ^z	18.9 \pm 0.74 ^y	13.1 \pm 0.48	593 \pm 13.2 b
SDS	17.8 \pm 0.94	14.6 \pm 0.52	705 \pm 22.4 a
ELL	19.1 \pm 0.81	12.9 \pm 0.54	394 \pm 5.9 c
LSD	2.4255	1.5031	53.362

^y Means \pm S.E. ($n=5$) in a column followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. ^z WW, well-watered; SDS, severe drought stress; ELL, extremely low sunlight.

Table 6. Effects of water and light intensity stress on the leaf tissue thickness present in cross-section of *Q. suber* leaves.

Nursery	Upper epidermis (μm)	Lower epidermis (μm)	Palisade parenchyma (μm)	Spongy parenchyma (μm)	Total thickness (μm)
WW ^z	50.2 \pm 0.98 a ^y	26.9 \pm 2.71	170 \pm 0.9 b	90 \pm 3.0 c	337 \pm 6.0 b
SDS	40.8 \pm 1.45 b	30.0 \pm 1.55	218 \pm 6.4 a	138 \pm 7.0 a	426 \pm 3.3 a
ELL	38.2 \pm 1.46 b	30.2 \pm 1.78	67 \pm 2.9 c	115 \pm 2.4 b	250 \pm 5.9 c
LSD	4.0472	6.3773	12.605	14.208	16.069

^y Means \pm S.E. ($n=5$) in a column followed by the same letters are not significantly different at $P \leq 0.05$ according to the least significant difference (LSD) multiple range test. ^z WW, well-watered; SDS, severe drought stress; ELL, extremely low sunlight.

might reduce water loss at the whole-plant level, and the higher stomatal density probably avoided water stress by possessing flexible regulation (Bacelar et al., 2004).

As a result, stomatal closure significantly occurs when plants are exposed to drought, in some cases (e.g., severe drought stress) photosynthesis may be more controlled by the chloroplast's capacity for CO_2 fixation (Yordanov et al., 2000).

Light micrography (LM)

The leaf area was significantly smaller under severe drought stress than those under the well-watered (WW) and the extremely low sunlight (ELL) treatment, whereas the leaf thickness showed the opposite trend (Table 6). Thicker upper epidermis was slightly found under well-watered (WW) as compared to the severe drought stress (SDS) and the extremely low sunlight (ELL) leaves of *Q. suber*, while no significant differences in thickness of low epidermis were found among all treatments. Epidermal thickness under well-watered (WW) treatment seedlings was approximately 10 μm , and 12 μm larger on the upper epidermal of leaf surfaces, respectively, compared with in severe drought stress (SDS) and in the extremely low sunlight (ELL) treatment seedlings. Total leaf thickness was significantly lower in the extremely low sunlight (ELL, 250.3 \pm 13.3 μm) treatment than in well-watered (WW,

337.1 \pm 13.3 μm) treatment and in severe drought (SDS, 426.4 \pm 7.5 μm) treatment (Table 6). This difference may be attributable to the lower layer of palisade parenchyma, due to possessing only single layer of palisade parenchyma compared to the sun-leaf structure with three layers of palisade parenchyma (Figure 5E).

Under severe drought stress (SDS) treatment with drought stress leaves, the total leaf thickness was extremely thicker, due to the thicker palisade parenchyma (containing three layers of mesophyll), also due to the higher spongy and epidermal thickness (Figures 5B and E). The parenchyma of the leaves under well-watered (WW) and severe drought stress (SDS) treatment was more densely packed in the palisade parenchyma layer than in the spongy parenchyma, whereas a significantly increased thickness of the spongy parenchyma layer was found in leaves of the extremely low sunlight (ELL) seedlings. A leaf structure under the extremely low sunlight (ELL) was observed in cross-sections, with the greater proportion of spongy parenchyma by possessing three to four layers of spongy parenchyma, but with an only single layer of the palisade parenchyma (Figures 5C and F).

Plants grown under shaded conditions (that is, a low quantum flux density) normally have a higher SLA and thinner thickness, due to only shaded leaf consists of a single layer of the palisade parenchyma than those grown under natural irradiation (Young and Smith, 1980;

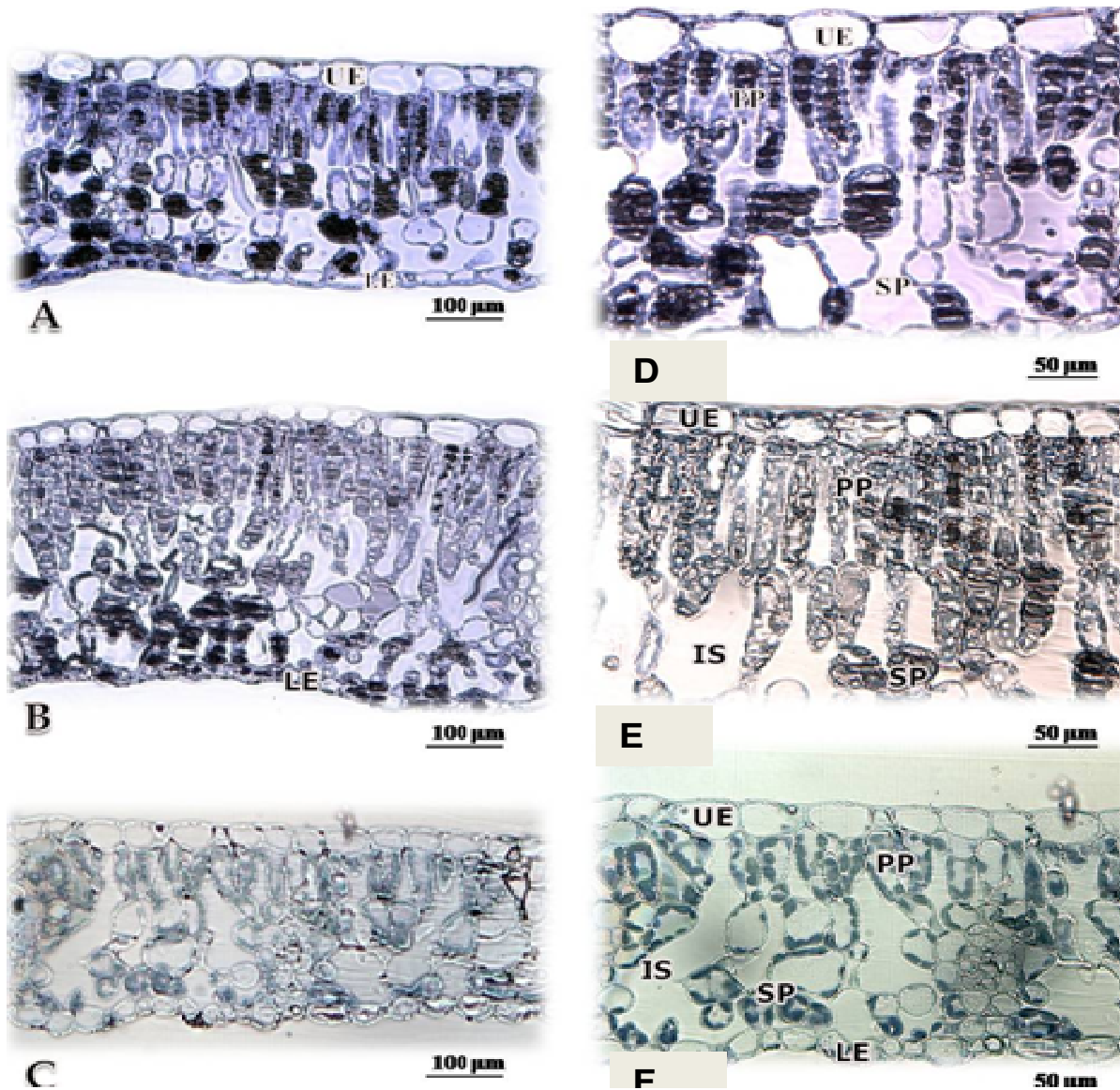


Figure 5. Epidermal morphology of cross-sections using light microscopy (LM) through the leaf blade region of fully expanded leaf middle region in the well-watered (WW, A), the severe drought stress (SDS, B), and the extremely low sunlight (ELL, C) leaves. Notice differences in total leaf thickness, and size, shape and arrangement of the mesophyll tissues (UP, upper epidermis; LE, lower epidermis; PP, palisade parenchyma; SP, spongy parenchyma; IS, intercellular air spaces). Note that the palisade parenchyma had only one layer in the extremely low sunlight (ELL). Magnification: A-C, $\times 20$, bar = 100 μm ; 1-3, $\times 40$, bar = 50 μm .

Mendes et al., 2001; Terashima et al., 2006; Gregoriou et al., 2007).

In the extremely low sunlight (ELL) treatment, intercellular air spaces were greatly observed in the extent of present in the spongy parenchyma by the elevated shade effect. The effect of shade also led to the collapse of the palisade parenchyma. Evans et al. (1977) reported that the epidermal and palisade parenchyma cells frequently collapsed adjacent to spongy parenchyma cells. DeLucia et al. (1996) also concluded that the greater proportion of spongy parenchyma was

displayed in shade leaves. The greater proportion and rounded shape of the spongy parenchyma of shade leaves may increase intercellular light scattering and thus absorptance, and increase the mean pathlength of light in leaves.

As compared to shaded leaves, sun-type leaves are typically thicker with multiple and longer layers of palisade parenchyma, followed by relatively thinner spongy parenchyma (Lichtenthaler et al., 1981). Oak species have been suggested that possess various effective morphological adaptations such as thicker leaf

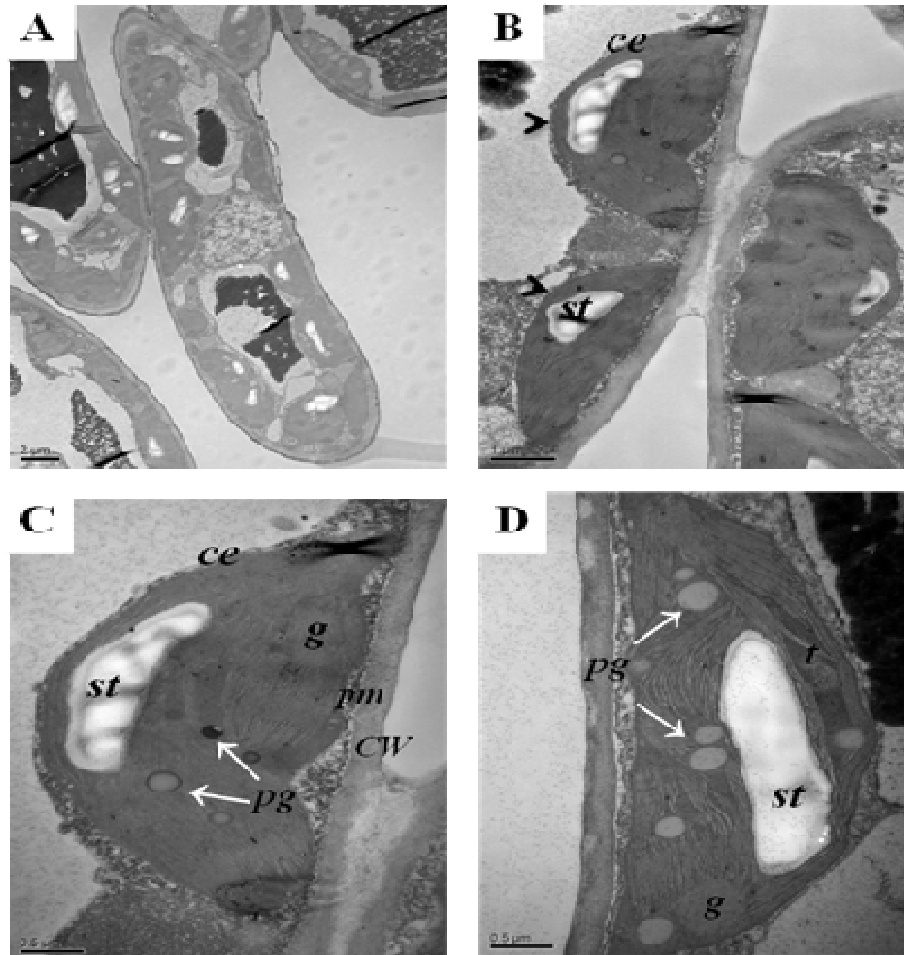


Figure 6. Ultrastructure by a transmission electron micrography (TEM) of the *Q. suber* palisade parenchyma cells submitted to well-watered (WW) leaves. Well organized chloroplasts with the distinctive thylakoids (*t*), grana (*g*), and scarce plastoglobuli (*pg*) from well-watered leaves. *st*, starch grains; *pg*, plastoglobuli; *g*, grana; *t*, thylakoid; *ce*, chloroplast envelope; *cw*, cell wall; *pm*, plasmalemma; arrowhead = chloroplasts. Magnification: A, $\times 6,000$, bar = 2 μm ; B, $\times 20,000$, bar = 1 μm ; C-D, $\times 40,000$, bar = 0.5 μm .

thickness, thicker cuticle thickness, greater stomatal density and decreased stomatal size that improve drought resistance, decrease photochemical damage, and contribute to minimal degree of photosynthesis under drought stress (Abrams, 1990; Dickson and Tomlinson, 1996).

As a result, *Q. suber* seedlings may possess the microscale morphological adaptations against drought on the more arid severe drought stress, which have well developed palisade parenchyma, whereas leaf thickness generally had negative effects on the shade effect.

Transmission electron micrography (TEM)

An environmental stress can severely disrupt the ultrastructural features of chloroplasts and parenchyma

cells (Ristic, 1992). The plant's ability to adapt to environmental stress such as water stress and shade stress may be related to specific changes in the morphological, physiological, and ultrastructural level (e.g., chloroplast) of leaves (Meter and Lichtenthaler, 1981).

At the ultrastructural level, the palisade parenchyma chloroplasts of leaves of well-watered (WW) treatment were well developed (Figure 6A): their shape was regular; chloroplast envelope membranes and grana were distinct; and abundance of starch grains and scarce plastoglobuli were also visible inside in the chloroplasts (Figures 6B, to D).

In contrast, severe drought stress (SDS) treatment led to marked transformation to the structure of chloroplasts included swelling and disruption of the thylakoid and aberration of the chloroplast shape (Figure 7). In some

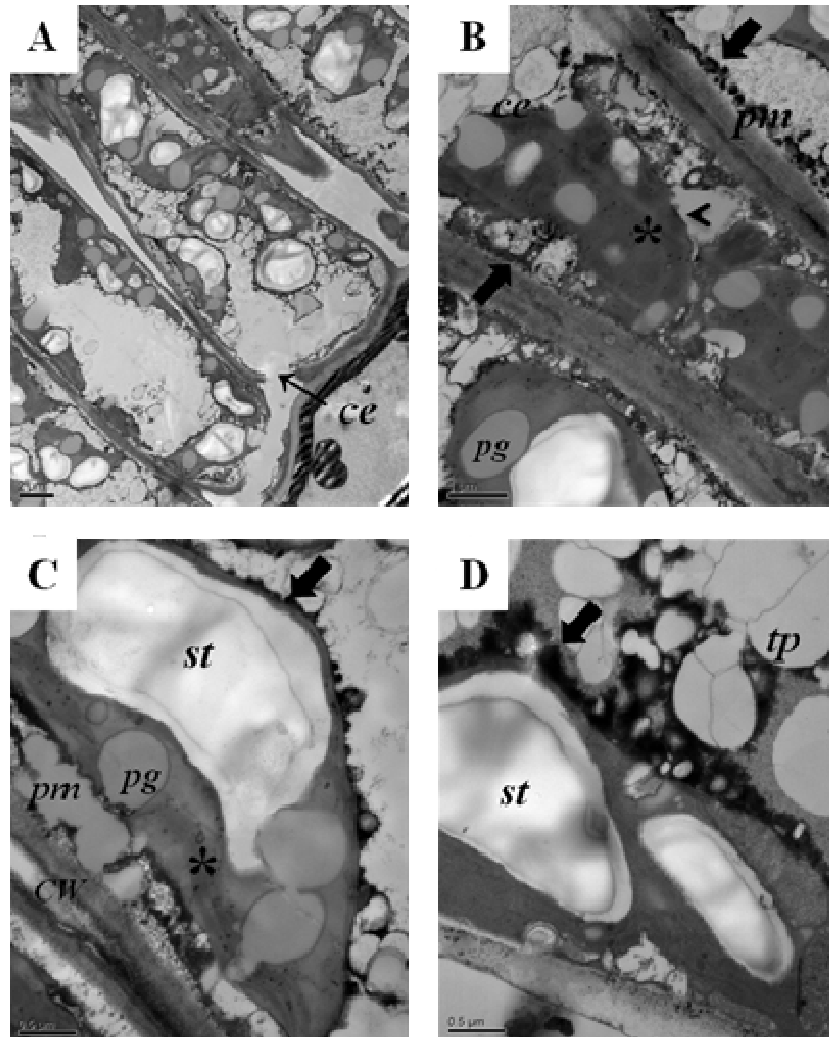


Figure 7. Ultrastructure by a transmission electron micrography (TEM) of the *Q. suber* palisade parenchyma cells submitted severe drought stress (SDS) leaves. Note the numerous plastoglobuli, the distortion of chloroplast envelope and the swelling of thylakoids. *st*, starch grains; *pg*, plastoglobuli; *ce*, chloroplast envelope; *cw*, cell wall; black arrow = localization of H_2O_2 in tonoplast (*tp*) and plasmalemma (*pm*); arrowhead = chloroplasts; asterisk = swollen thylakoid. Magnification: A, $\times 6,000$, bar = $2 \mu m$; B, $\times 20,000$, bar = $1 \mu m$; C-D, $\times 40,000$, bar = $0.5 \mu m$.

cases, the chloroplast envelope membranes were broken and not distinctive (Figure 7A).

Despite the structural change of the reduced grana and thylakoid, their shape was even deformed morphologically, the chloroplasts were observed not only large starch grains but also an increased number and a larger size plastoglobuli within their interior (Figure 7B). Moreover, leaves of severe drought stress (SDS) showed the obvious symptoms of senescence due to accumulation of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), in tonoplast and plasmalemma (Figure 7B to D).

The chloroplasts of the plants grown under the water stress indicate the main damages in structural changes

such as the excessive swelling of the thylakoids, the distortion of the grana, and the appearance of the lipid droplets (Riatic, 1992; Lopez-Carbonell, 1994).

In drought-stressed *Solanum tuberosum* plants, a higher number of plastoglobuli exhibiting a larger size was detected in chloroplasts compared with well-watered plants (Eymery, 1999). Chloroplasts of general mesophyll cells possessed a few plastoglobuli, but the number and size of these lipophilic compounds increased due to degraded thylakoids in chloroplasts during leaf senescence and in etioplasts before thylakoid formation (Steinmüller, 1985; Eymery, 1999). Munne-Bosch (2001) reported that symptoms of leaf senescence were revealed in chloroplasts at drought-induced senescence,

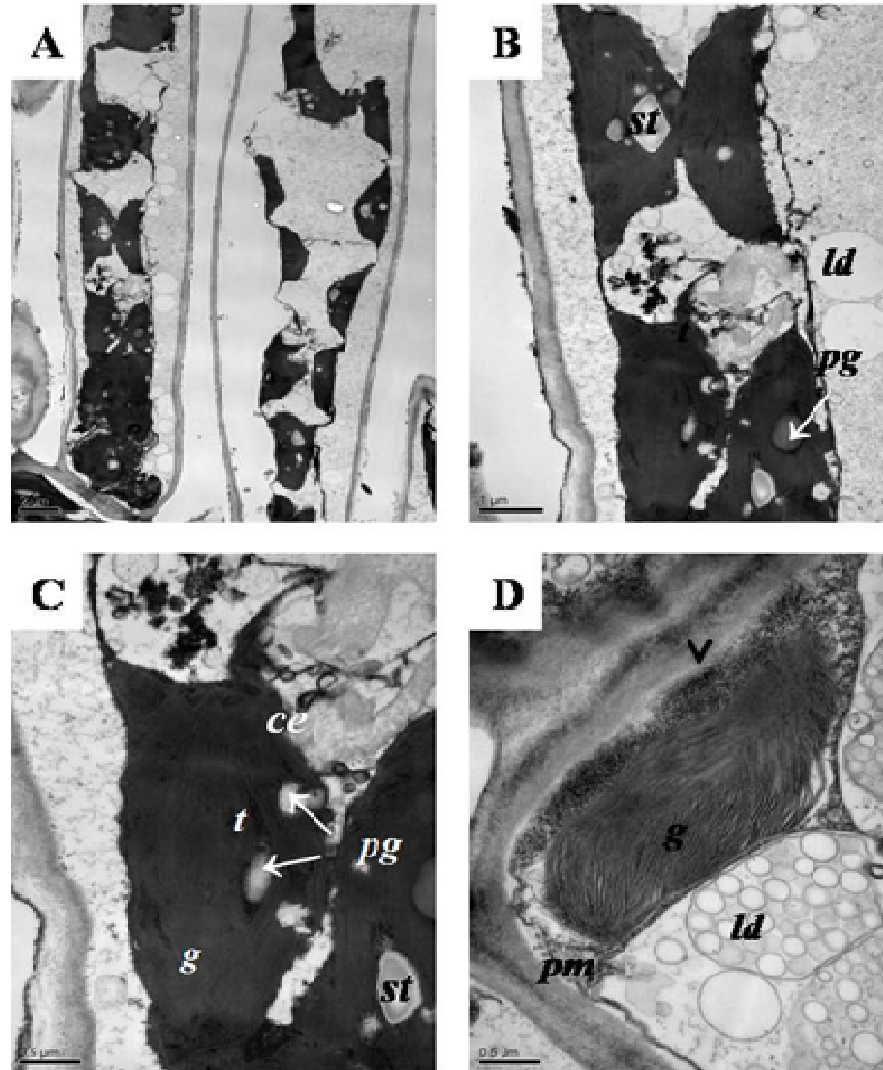


Figure 8. Ultrastructure by a transmission electron micrography (TEM) of the *Q. suber* palisade parenchyma cells submitted the extremely low sunlight (ELL) leaves. Chloroplasts with higher stacking degree and cytoplasm with lipid droplets from the extremely low sunlight (ELL) leave. *pg*, plastoglobuli; *g*, grana; *t*, thylakoid; *ld*, lipid droplet; *ce*, chloroplast envelope; *pm*, plasmalemma; arrowhead = chloroplasts. Magnification: A, $\times 6,000$, bar = 2 μm ; B, $\times 20,000$, bar = 1 μm ; C-D, $\times 40,000$, bar = 0.5 μm .

showing an accumulation of plastoglobuli in the chloroplasts, including the disruption of thylakoids and much less grana stack. The increased number and larger size of plastoglobuli in chloroplasts may result from storage of thylakoid decreased during the severe drought stress (SDS) (Eymery, 1999; Munne-Bosch, 2001).

Hydrogen peroxide (H_2O_2) accumulation was observed first in the xylem vessels, in the cell walls, and in the plasma membrane of mesophyll cells of severely stressed plants, and increased during the drier and hotter summer (Munne-Bosch, 2001). Romero-Puertas et al. (2004) also suggested that the presence of H_2O_2 in cadmium-treated pea leaves indicate the accumulation in

the plasma membrane and tonoplast of different types.

On the other hand, the extremely low sunlight (ELL) followed by severe shade stress (SDS) may lethally suffer from severe damage in chloroplasts (Figure 8A): Their shape was irregular and the chloroplast envelope membranes were not apparent. The main damage to the chloroplasts by the extremely low sunlight (ELL) revealed not only the higher grana stacks and the broader grana, but also the higher stacking degree of thylakoids (Figure 8C). Furthermore, another distinct difference in the extremely low sunlight (ELL, Figure 8A) possessed no starch or exhibited only little starch in the chloroplasts as compared with the well-watered (WW) and the severe

drought stress (SDS) (Figure 6 and 7). In addition to chloroplasts, the intracellular space occupied in nearly all organelles, otherwise numerous small vacuoles and lipid droplets were present in the cytoplasm of these cells (Figure 8D).

The ultrastructure of chloroplasts grown under low light exhibited the structural differences. The shade-type chloroplasts from radish seedlings (*Rapbanus sativus* L.) grown under the low-light possessed characterization such as the higher grana stacks, the higher stacking degree of thylakoids, the broader grana, and an insufficient starch as compared to those under the high-light (Meier and Lichtenthaler, 1981). The restraint of starch in the chloroplasts of low light leaves may be related to the low rate of photosynthesis at the low light intensities. Chloroplasts of *Euphorbia forbesii* was discovered a greater thylakoid membrane development in low light than in high light (Percy, 1986). Percy (1986) also suggested that *E. forbesii* as a result may possess a very flexible photosynthetic apparatus to survive in the understory of shaded forests.

Conclusions

This study examined the growth and anatomical aspects to examine the response of *Q. suber* to water and light intensity. Specifically, it focused on processes affecting drought and shade in *Q. suber*.

Seedlings of *Q. suber* grown under the severe drought stress lead to an increased stomatal frequency due to morphological plasticity such as the deeply folded, shrunken, and the overall reduction in epidermal cell size, a decreased shoot/root ratio due to stimulation of the deeper root growth. Moreover, in severe drought stress with drought stress leaves, the total leaf thickness was extremely greater, due to the thicker palisade parenchyma (containing three layers of mesophyll), also due to the higher spongy and epidermal thickness. The seedlings rapidly develop deep root system by stress effects due to soil water deficit. Furthermore, the response of its physiological root system is ability to avoid water stress in water stress-induced seedlings.

Severe drought stress (SDS) led to a marked transformation to the structure of chloroplasts included swelling and disruption of the thylakoid and aberration of the chloroplast shape. The chloroplasts were observed not only the large starch grains but also the increased number and the larger size plastoglobuli within their interior. Moreover, leaves of severe drought stress (SDS) showed the obvious symptoms of senescence as accumulation of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), in tonoplast and plasma-lemma. Specially, seedlings grown under the extremely low sunlight possess one palisade cell layers instead of three layers, decreased shoot/root ratio due to stimulation of deeper root growth and increased in specific leaf area (SLA) due to the lower leaf thickness (thinner). The

extremely low sunlight (ELL) may lethally suffer from the severe damage in chloroplasts: Their shape was irregular and the chloroplast envelope membranes were not apparent. The main damage to the chloroplasts by the extremely low sunlight (ELL) was revealed not only the higher grana stacks and the broader grana, but also the higher stacking degree of thylakoids.

As a result, *Q. suber* seedlings may possess the microscale morphological adaptations against water-deficit on the more drought stress in Mediterranean-climate ecosystems, which have the deeper root growth and the well developed palisade parenchyma, whereas the shade stress generally has negative effect in the growth and the physio-biochemical aspect.

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