

UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

**Influence of kaolin application on physiological behavior
of olive trees (*Olea europaea* L.) submitted to water deficit**

Dissertação de Mestrado em Biologia

Bárbara Catarina Borges do Forno

Orientadores: Professor Doutor Carlos Manuel Correia
Professor Doutor José Manuel Moutinho Pereira



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Disertação de Mestrado elaborada para a obtenção de grau de Mestre em Biologia, sob a orientação científica do Professor Doutor Carlos Manuel Correia e Professor Doutor José Manuel Moutinho Pereira da Universidade de Trás-os-Montes e Alto Douro.

Green was my birthday

But dressed in mourning

To give light to the world

A thousand torments I suffered

“Popular guess”

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I thank God for helping me in this journey.

Resumo

O clima futuro na maior parte das regiões onde se cultiva a oliveira (*Olea europaea* L.) será caracterizado por condições mais severas durante o verão, com menor disponibilidade de água, calor excessivo e alta irradiância fotônica, o que poderá provocar alterações significativas na fisiologia da árvore, na produtividade e na qualidade da produção. Assim, é crucial a implementação de estratégias de adaptação eficientes e de baixo custo para garantir a sustentabilidade dos olivais de sequeiro. Neste sentido, foram estudadas as alterações induzidas na anatomia, estado hídrico, trocas gasosas, fluorescência da clorofila, composição química e indicadores de stresse oxidativo em folhas de plantas jovens de oliveira (cv. Cobrançosa) submetidas a regimes hídricos contrastantes e à aplicação foliar (5%; p/v) de caulino, um aluminossilicato branco, não abrasivo, não poroso e não tóxico que se dispersa facilmente em água. Os resultados revelaram que a aplicação foliar de caulino aliviou os efeitos adversos do déficit hídrico na oliveira. O caulino contribuiu para melhorar o estado hídrico das plantas, tendo em consideração os maiores valores de RWC, grau de suculência, condutância estomática e taxa de transpiração, e para reduzir os sintomas de stresse oxidativo, como comprovado pela maior concentração de clorofilas, carotenoides e tióis totais. Aquelas respostas, conjuntamente com a diminuição da razão parênquima em paliçada/parênquima lacunoso, que reduz a limitação à difusão de CO₂ no mesófilo clorofilino, permitiram o aumento da taxa fotossintética líquida nas plantas tratadas com caulino. Assim, estes resultados suportam a hipótese de que a aplicação de caulino poderá ser uma prática agrícola promissora para aliviar os efeitos do déficit hídrico nos olivais de sequeiro.

Palavras-chave: Caulino, *Olea europaea*, stresse hídrico, stresse oxidativo.

Abstract

Future climate in most of the olive (*Olea europaea* L.) growing regions will be characterized by severe summer conditions, with lower water availability, excessive heat load and high daily irradiance, which may bring relevant changes in tree physiology, productivity and yield quality. Thus, is crucial the implementation of efficient and cost-effective adaptation strategies in order to guarantee the sustainability of olive rainfed orchards. With this in mind, changes in anatomy, water status, gas exchange, chlorophyll fluorescence, chemical composition and oxidative stress indicators were studied in leaves of young plants of olive (cv. Cobrançosa) submitted to contrasting water regimes and to 5% (w/v) foliar kaolin, a white, non-abrasive, non-porous, non-toxic aluminosilicate clay mineral that disseminate easily in water. The results revealed that kaolin foliar spray alleviates the adverse effects of drought stress on olive tree. Kaolin contributed to better tree water status, taking into account the higher values of RWC, degree of succulence, stomatal conductance and transpiration rate, and to lower oxidative stress, as is proved by the higher concentration of chlorophylls, carotenoids and total thiols. All together, these responses jointly with the lower palisade/spongy parenchyma ratio, which reduces the diffusional limitation related to mesophyll conductance, allow the enhancement of net photosynthetic rate in kaolin treated plants. Thus, these results support the hypothesis that kaolin application could be a promising agricultural practice to relieve water stress in rainfed olive orchards.

Keywords: Drought stress, kaolin, *Olea europaea*, oxidative stress.

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Abbreviations

A/g_s	Intrinsic water use efficiency
A	Net CO ₂ assimilation rate
AA	Ascorbic acid
ABA	Abscisic acid
AQPs	Aquaporins
Car	Carotenoids
Chl_{a+b}	Total chlorophyll
Ci/Ca	Ratio of intercellular to atmospheric CO ₂ concentration
Ci	Intercellular carbon concentration
Cvs	Cultivars
E	Transpiration rate
E_c	Cuticular transpiration rate
ETR	Electron transport rate
ε	Elastic modulus
F_m	Maximal fluorescence
F_v/F_m	Maximal photochemical efficiency of PSII
g_s	Stomatal conductance
H₂O₂	Hydrogen peroxide
HSPs	Heat-shock proteins
KL	Kaolin
LA	Leaf area
LTD	Leaf tissue density
NPQ	Nonphotochemical quenching
OA	Osmotic adjustment
PAR	Photosynthetically active radiation
PE	Fraction of PPFD absorbed in PSII antenna
P_n	Net photosynthetic rate
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate
RWC	Relative water content
-SH	Total thiols
SLW	Specific leaf weight
SS	Total soluble sugars
St	Starch
TP	Total phenols
VPD	Vapour pressure deficits
WC	Water content
WCS	Water content at saturation
WS	Water stressed plants
WUE	Water use efficiency
WW	Well-watered plants
Φ_{PSII}	Photochemical efficiency of PSII
Ψ	Water potential
Ψ_π	Osmotic potential

1. General Introduction and objectives

Olive (*Olea europaea* L.), one of the most important cultivated fruit tree species in the Mediterranean region, is an evergreen tree of great longevity, and a sclerophyllous and hypostomatous species with a higher degree of drought tolerance and a higher specific transpiration rate compared with other kind of fruit tree species in both ideal and water scarcity conditions (Wahbi *et al.*, 2005; Díaz-Espejo *et al.*, 2006; Boussadia *et al.*, 2008; Roussos *et al.*, 2010). The great ability of the root system to water absorption, as well as the maintenance of turgor have been proposed as possible explanations for this adaptation (Chartzoulakis *et al.*, 1999).

The genesis of the olive tree is unknown, but is presumed to have coincided with the expansion of Mediterranean civilizations that for centuries have left their mark on Western culture (Chiappetta and Muzzalupo, 2012). Its cultivation is very old, and olive cultivation and olive oil production from the mature drupe (fruit) remain a fundamental part of farming practices (Owen *et al.*, 2000). It is a woody fruit crop largely widespread in the Mediterranean regions and includes many economically important cultivars and wild olive genotypes (Loureiro *et al.*, 2007). There are many different genotypes of *Olea europaea*, each one with unique physical and biochemical characteristics, providing olive oils with typical compositions and performances (Matos *et al.*, 2007). In Portugal, the cultivars (cvs.) “Galega”, “Cobrançosa”, “Carrasquenha”, “Cordovil” and “Verdeal” are dominant throughout the country, whereas “Verdeal Transmontana”, “Madural”, “Cobrançosa” and Negrinha de Freixo” are the main cultivars in Trás-os-Montes (Northeast Portugal) (Brito, 2012). The cvs. most commonly grown in the region are considered to be well adapted to drought (Bacelar *et al.*, 2004).

There is growing interest in the olive tree and its products in many regions of the world (Fernández and Moreno, 1999). Currently, olive cultivation is extended outside the Mediterranean area, including southern Africa, South and North America, Australia, Japan and China, but the main area still remains there (Brito, 2012). The motives for this popularity of the species are not merely agronomic and economic, but also related to the environment and human health (Fernández and Moreno, 1999). World demand for olive products, both oil and fruit, is increasing due to its nutritional properties. Effectively, the low saturated-to-unsaturated fatty acid ratio and the presence of natural antioxidants could avoid certain human diseases (Bacelar, 2006), especially coronary heart diseases and determined cancer tumours (Valderrama *et al.*, 2006). In environmental terms, the olive tree crop minimize

erosion/desertification, improves the carbon sequestration of these areas, decrease the air dryness with its transpiration and allows the presevation of a high biodiversity (Díaz-Espejo *et al.*, 2006; Sofo *et al.*, 2007; Fernández and Moreno, 1999).

Mediterranean climate during the growing season is characterized by elevated temperatures, reduced rainfall, high light levels and elevated vapour pressure deficits (VPD) (Angelopoulos *et al.*, 1996; Roussos *et al.*, 2010; Khaleghi *et al.*, 2015). The prolonged summers are hot, cloudless and dry, while winters are wet and mildly cold (Karabourniotis and Bornman, 1999). The diversity of climatic conditions in these areas is due to the specific configuration of land surface (topography) and the distance from the sea (Sofo *et al.*, 2007). These environmental conditions highly influence the growth and productivity of crop plants, as they all exposure additive and interactive effects on plant physiology (Roussos *et al.*, 2010), and thus cause significant yield losses (Bacelar *et al.*, 2004; Boussadia *et al.*, 2008).

Climate change emerged in recent years as one of the most serious phenomena affecting our planet (Bacelar *et al.*, 2013). Portugal, like other southern European regions is a place of mild Mediterranean climate, but with well known vulnerability, mainly to drought during spring, summer and autumn, and desertification (Bacelar, 2006; Bussotti *et al.*, 2014). Actually, many countries face significant problems of water shortage and quality deterioration. It is expected that these problems will worsen in the coming decades as the world population will increase from 6.6 billion to around 8 billion by 2030 and to more than 9 billion by 2050 (Petousi *et al.*, 2015). The limit of available water in the Mediterranean ecosystems and the current and expected decrease of water resources as stated above, are driving to the urgent need to reduce water use for irrigation in the arid and semiarid regions (Wahbi *et al.*, 2005). At the same time, for all models, in all scenarios, a significative increase in the average temperature is expected for all Portuguese regions by the end of the 21st century (Bacelar, 2006). Thus, such hard alterations in environmental conditions might be a real problem for this crop. Therefore, acclimation and adaptation to restricted water supply are extremely important for the growth and survival of plants (Galle *et al.*, 2011).

This study aims to evaluate the possible ameliorating effects of a foliar spray of kaolin on physiological behavior of olive trees subjected to water stress, and thus on water use efficiency and productivity. The work present methodologies centered on the evaluation of the olive tree physiology, including leaf anatomy, water status, gas exchange, chlorophyll a, fluorescence, photosynthetic pigments/metabolites and indicators of oxidative stress.

2. Water stress effects and symptoms

Water stress, one of the summer stress components, is regarded as the main environmental factor that restrict photosynthesis, plant growth, and yield in the world, particularly in semiarid areas, where olive tree is well adapted (Perez-Martin *et al.*, 2014). Often, large reductions in photosynthetic activity are associated with such alterations in water status, as well as a reduction in leaf expansion, precocious leaf senescence and associated decrease in food production (Bacelar *et al.*, 2007; Farooq *et al.*, 2009). Cell growth is one of the physiological processes more drought sensitive due to the reduction in turgor pressure (Farooq *et al.*, 2009). As the plant water content decreases, the cells shrink and relax the cell walls. This decrease in cell volume originates lower hydrostatic pressure, or turgor, which increase the solutes concentrations in the cytosol and extracellular matrices (Bacelar, 2006; Lisar *et al.*, 2012). Increasing the concentration of solutes, which leads to an increase in viscosity of the cytoplasm, may become toxic and can be harmful to the functioning of enzymes, including those of the photosynthetic machinery (Farooq *et al.*, 2009).

If the quantity of water available in the soil is limited, the first response of plants to acute water deficit is the regulation of stomatal aperture (or even stomatal closure), in order to decrease the loss of water by transpiration (Farooq *et al.*, 2009). Stomatal closure decreases significantly the transpiration rate and, therefore, contributes to the maintenance of positive turgor pressure of cells (Boughalleb and Hajlaoui, 2011). The stomatal activity also influence the internal concentration of CO₂ and, consequently, affects photosynthesis and plant growth (Figure 1). Moreover, under severe drought, the carboxylation efficiency by Rubisco decreases, and acts more as a oxygenase than carboxylase, leading to an increase in photorespiration rate (Figure 1) (Farooq *et al.*, 2009).

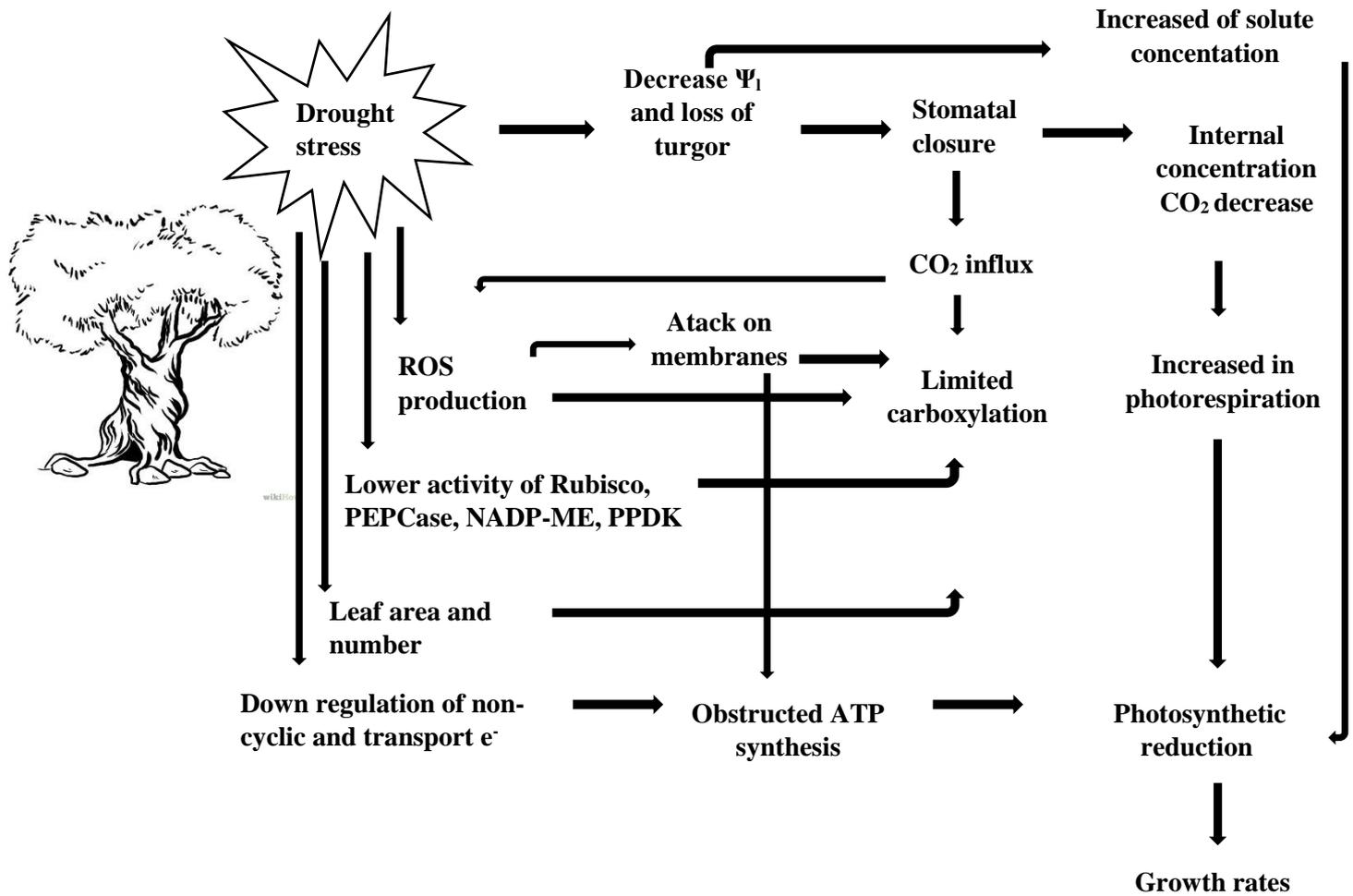


Figure 1 - Scheme of possible mechanisms in which photosynthesis is reduced under stress (Adapted from: Farroq *et al.*, 2009).

The close association between photosynthetic rate (A) and stomatal conductance (g_s) showed that the decline in net photosynthesis during the day was mostly a consequence of stomatal limitation (Bacelar *et al.*, 2007). Nevertheless, as the stress advances, photosynthetic CO_2 fixation might be restricted most directly by non-stomatal factors, including reductions on mesophyll conductance, perturbations on biochemical CO_2 assimilation and inhibition of photochemical reactions (Bosabalidis and Kofidis, 2002; Tognetti *et al.*, 2004; Tognetti *et al.*, 2007; Ahmed *et al.*, 2010). Besides Rubisco (ribulose-1.5-bisphosphate carboxylase/oxygenase), water stress may reduce the activity of other photosynthetic enzymes to different levels such as fructose-1.6-bisphosphatase (FBPase), NADP-malic enzyme (NADP-ME),

phosphoenolpyruvate carboxylase (PEPCase), and pyruvate orthophosphate dikinase (PPDK) (Figure 1) (Farooq *et al.*, 2009; Lisar *et al.*, 2012).

It is also known that hormones play a remarkable role in the response of plants to water stress status (Zingaretti *et al.*, 2013). The plant hormone, abscisic acid (ABA) builds up under water drought conditions and plays an important role in response and tolerance to dehydration (Lisar *et al.*, 2012). This hormone moves in the xylem from the root to other parts of the shoot, where controls water loss by transpiration and leaf growth (Hartung *et al.*, 2002). During drought, endogenous ABA is produced quickly setting off a cascade of physiological reactions, such as stomatal closure (Osakabe *et al.*, 2014). Various mechanisms, like as hydropassive closure and chemical signals, work together to close the stomata from the plant stress hormone ABA (Arve *et al.*, 2011). The accumulated ABA in the vascular tissue, during drought stress, is carried to guard cells via passive diffusion in response to pH modifications and by specific transporters (Osakabe *et al.*, 2014). An increase in the concentration of ABA around the guard cells induce stomatal closure, helping to save water (Luan, 2002; Lisar *et al.*, 2012). Furthermore, high ABA levels also causes an increase in hydraulic conductivity at the roots and xylem, allowing the plants carrying more water and, thus, to recover quickly after water stress (Arve *et al.*, 2011). Concerning to the production of ABA, occurs frequently under persistent drought conditions and may be considered as a “slower” pathway for the regulation of stomata under stress conditions (Luan, 2002).

In Mediterranean olive growing areas, water stress is usually accompanied by other environmental limitations, namely high light intensity and high temperature during the summer season, and it has been shown that the conjunction of these factors, make plants more vulnerable to photoinhibition and to reduction in carbon assimilation (Boussadia *et al.*, 2008). The synergistic action of high level of irradiance and water deficit decreases the ability of photosynthetic systems to use the incident radiation, inducing a higher degree of photodamage. Under environmental conditions, where the photon energy exceeds the CO₂ assimilation, photosystem II (PSII) is the first target for photoinhibition, while PSI is more stable than the PSII, receiving an injury generally less significant and closely related to the rate of electron flow from PSII and the presence of oxygen (Sofa *et al.*, 2004). During the evolution of summer stress events, usually may occur the reduction of photosynthetic and transpiration rate, stomatal conductance, the PSII maximal photochemical efficiency (F_v/F_m), the intrinsic efficiency of open PSII reaction centres (F_v'/F_m'), the effective photochemical efficiency of PSII (Φ_{PSII}) and the total electron flow (Boussadia *et al.*, 2008; Boughalleb and Hajlaoui, 2011). Moreover,

decreased leaf chlorophyll and carotenoids concentrations, particularly in severe water deficit conditions may also occur (Boughalleb and Hajlaoui, 2011). Regarding the non-photochemical quenching (q_N), usually this variable increase in the response to summer stress (Boussadia *et al.*, 2008). Furthermore, the exposition of leaves to elevated doses of UV-B, also a habitual stress in Mediterranean region, may lead the loss and activity of Rubisco, perturbations on photochemically capable PSII complexes and induce stomatal closure (Nogués and Baker, 2000).

In addition to the above induced effects, under summer stress the leaf transpiration frequently exceeds the water transport capacity of the xylem (Fernández, 2014). The water is transported in the xylem under strain, and under drought (low Ψ_1) is vulnerable to cavitation and the fast expansion of gas filled spaces (embolisms) inside individual xylem conduits (Connor, 2005). Moreover, the low level of water in the soil induces a limited availability of mineral nutrient for the growth of plants, as well as a decrease in dry weight and a reduced absorption of nutrients (Celano *et al.*, 1999). The lowered uptake of the inorganic nutrients may result from interference in nutrient absorption, the discharge mechanism, and decreased xylem water flow (Farooq *et al.*, 2009).

Water and co-occurring stresses often leads to oxidative stress (Figure 1) (Liu *et al.*, 2011). In stressed plants the limitation of CO₂ assimilation results in a decrease along the photosynthetic electron chain. This overage power reduction establishes a photonic energy redirection in processes that facilitate the creation of reactive oxygen species (ROS), especially in the photosynthetic and mitochondrial electron transport chains (Sofó *et al.*, 2004; Bacelar *et al.*, 2007). Concerning the main intracellular producers of ROS following stand out chloroplasts, mitochondria, and peroxisomes (Ahmed *et al.*, 2010). The generation of such ROS (hydroxyl radical (OH•), hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and singlet oxygen (¹O₂)) can induce oxidative damage, including oxidation of lipids, nucleic acids, and proteins, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes, when the accumulation of ROS exceed the removal capacity of the antioxidant system (Figure 1) (Bacelar *et al.*, 2006). Usually, leaves growing under rainfed conditions showed symptoms of oxidative stress, such as the decrease in chlorophylls and the increased levels of lipid peroxidation (Bacelar *et al.*, 2007). For this reason, the cell membrane stability is a largely used indicator of plant tolerance to severe drought (Ennajeh *et al.*, 2009). As a consequence of all above effects, Table 1 summarized the water necessities of olive tree and the main general effects of water stress on the growth period.

Table 1 - The effects of water stress on the development stages of olive tree (Tangu, 2014).

Phenological event	Period	Effect of water stress
Shoot growth	Late winter-early summer	Shoot growth decrease
Flower bud formation	February-April	Flower bud is reduced
Blooming	April-May	Abortive flowers occur
Fruit set	May-June	Fruit set is reduced, periodicity increases
Fruit growth	June-July	Cell division decreases, fruit volume becomes smaller
Fruit enlargement	August-harvest	Cell elongation is reduced, smaller fruits occur
Fat accumulation	September-harvest	Fruit oil ratio decreases

3. Olive tree strategies to withstand water stress

Under conditions of water stress, even during extended drought periods in summer, olive tree subsist through the development of defence mechanisms (Stefanouadaki *et al.*, 2001). Some of them presented below are, at the same time, also defence mechanisms against high solar irradiation and high temperatures. The elevated capacity of olive growing in adverse conditions is related to their morphological characteristics, as well as physiological and biochemical mechanisms, associated with escape, avoidance and tolerance components of stress resistance (Faraloni *et al.*, 2011; Fernández, 2014). Drought tolerance is the final drought strategy (Connor, 2005) and it refers to the adaptations that enable the plant to withstand the stress, with or without a decreased in performance (Bacelar *et al.*, 2013). The physiological response of plants to drought at the whole plant level is extremely complex and includes deleterious and/or adaptive changes (Lisar *et al.*, 2012). Indeed, plants have developed many molecular mechanisms to lower their consumption of resources and regulate its growth to become acclimated to unfavourable environmental conditions (Osakabe *et al.*, 2014).

Olive cultivars well adapted to dry conditions exhibit enhanced sclerophylly, with elevated density of the foliar tissue and the existence of thick cuticle and trichome layers (Bacelar *et al.*, 2006). Dense trichome layers change leaf optical properties, as well as determinate physiological processes through various definable interactions. For instance, they

can protect against high water loss by influencing the water diffusion boundary layer of the leaf surface (Karabourniotis and Bornman, 1999). In hot environments, the peltate trichomes layers also decrease the leaf thermal stress through the sunlight reflection, decreasing transpiration of the leaves (Palliotti *et al.*, 1994; Bacelar, 2006).

The leaves of xeromorphic plants are characterized by a short surface/volume ratio as a consequence of modifications in cell number and cell size, and by a higher density of the vascular system and stomata (Bacelar *et al.*, 2004). They have also higher specific leaf weight (SLW), as a result of increased density and thickness, which can contribute to tolerance to drought (Fernández, 2014). Moreover, other morphological and anatomical characteristics that contribute to mitigate the loss of water during drought comprises lower leaf size, leaf rolling, paraheliotropic movements, dense leaf pubescence, thick cuticle and epicuticular wax layer, smaller mesophyll cells, fewer intercellular spaces and heavily lignified tissue (Bacelar *et al.*, 2013). That combination of characteristics limits the rise of temperature in leaves with small latent heat exchange when transpiration is restricted by the closure stomata (Connor, 2005; Bacelar, 2006).

Pubescent leaf surfaces may also result in significant changes in spectral characteristics of the leaf in the PAR (photosynthetically active radiation, 400-700 nm) region of the spectrum, reducing leaf absorptance and increasing leaf reflectance (Karabourniotis and Bornman, 1999). Hair layers also absorb in the ultraviolet-B (UV-B) region of the spectrum, safeguarding leaves against UV-B radiation damage (Karabourniotis *et al.*, 1998). The presence of hairs on leaves and young stems are usually considered as an adaptation to arid conditions (Karabourniotis and Bornman, 1999).

Olive trees are usually regarded as drought tolerant, and leaves may achieve highly low values of leaf water potential and RWC (-3.5 MPa and 75-80%, respectively), before missing turgor (Wahbi *et al.*, 2005). A number of strategies work synergistically in this species to protect against water stress, such as the emergence of leaf morphological and anatomical changes presented above, the efficient regulation of stomatal aperture and of the antioxidant system, the capacity to develop osmotic adjustment, the capacity to extract water from the soil due to a deep root system, as well as a high water potential gradient between canopy and root system (Sofa *et al.*, 2007).

An efficient stomatal control is considered an important physiological factor for optimizing water use in olive under drought conditions (Giorio *et al.*, 1999). The stomata of olive are small, dense and occur on the abaxial surface (hypostomatous), under dense layers of

peltate trichomes (or peltate scales), who prevents dehydration (Bacelar, 2006). The capacity to control the opening and closure of stomata is important to prevent drought stress damage. While a few plants close their stomata in the first stages of stress and reduces stomatal conductance, some might not be capable to do this and can be injured by the stress (Saei *et al.*, 2006). The increasing stomatal resistance under stress denote the effectiveness of a species to preserve water (Lisar *et al.*, 2012).

Other relevant mechanism adopted by olive tree to address water stress is osmotic adjustment (OA) (Sofo *et al.*, 2007). This physiological process is an important mechanism of drought tolerance that enables plants to tolerate temporary or extended periods of water scarcity (Dichio *et al.*, 2005; Farooq *et al.*, 2009; Bacelar *et al.*, 2013). It is an adaptive process, which may relieve some of the harmful effects of lack of water, and during the last years it has drawn much attention (Hessini *et al.*, 2009). For instance, the accumulation of organic osmolytes in cytosolic compartment of cells, like proline and sugars, is a well known adaptive mechanism in plants against stressed conditions (Sofo *et al.*, 2007; Ahmed *et al.*, 2010). During periods of water stress, the active and passive osmotic adjustment plays a fundamental role in the maintenance of cell turgor and leaf activities, due to accumulation of sugars (particularly mannitol and glucose), proline and others organic and inorganic osmolytes (Sofo *et al.*, 2007). Furthermore, elevated concentrations of such compatible solutes contribute to reduce the osmotic potential ($\Psi\pi$) and enable the movement of water into the cells, thus keeping turgor (Ψ_p) and increasing tissue tolerance to low soil water potentials (Boussadia *et al.*, 2013). In addition, these solutes also sequester water molecules, safeguard cell membranes and protein complexes and enable the metabolic machinery to continue operating (Dichio *et al.*, 2005). In olive tree, the contribution of passive osmotic adjustment differs according to the physiological conditions of the plant and genotypes, but express about a half of the total osmotic adjustment, while the other half is because of the *ex novo* synthesis of osmotically active compounds (Dichio *et al.*, 2009). Additionally, olive tree triggers the active and passive osmotic adjustment under water stress conditions, not just in leaves, as well as in roots, raising their capacity to remove water from the soil at water potential beneath the wilting point (Sofo *et al.*, 2007). Moreover, the osmotic adjustment in the root system enables the maintaining of cell turgor, preventing or retarding the segregation of roots from soil particles (Bacelar, 2006). On the other hand, the accumulation in cells of elevated concentration of proline has also been related with the prevention of protein denaturation, conservation of enzyme structure and activity, like

Rubisco, and protection of membranes from damage by ROS produced under high light conditions and drought (Bacelar, 2006).

Cell wall elasticity it is also regarded as one of the most important physiological mechanisms of adaptation to water deficit (Hessini *et al.*, 2009). The composition and structure of the cell wall influences the elasticity and plasticity of cells (Sofa *et al.*, 2007). Under drought conditions, in species such as olive tree, grapevine, wheat, and ordinary beans, amongst others, an increase in the elasticity of the cell wall may also contribute to turgor or cell volume maintenance (Chartzoulakis *et al.*, 1999; Hessini *et al.*, 2009). A inelastic cell wall should operate to increase the absorption of water from drying soil, because most negative water potentials would originate from a relatively low reduce in water content. Conversely, a more flexible cell should permit shrinkage during water loss, thereby maintaining the elevated turgor for a given variation in water content (Bacelar, 2006; Bacelar *et al.*, 2013). Furthermore elevated values of elastic modulus (ϵ), simultaneously with reduced values of $\Psi\pi$ may be responsible for the observed high gradients of water potential between leaves and soil and, therefore, may facilitate water extraction from the soil (Sofa *et al.*, 2007). A study with olive (Bacelar *et al.*, 2009) demonstrated that cultivars which employing a prodigal water use strategy showed high tissue elasticity, while conservative water use strategy cultivars showed high tissue inelasticity (Aroca, 2012).

For overcoming oxidative stress, plants have evolved enzymatic and non-enzymatic antioxidant defense mechanisms to capture ROS (Liu *et al.*, 2011). Phenolic compounds, including flavonoids, carotenoids, ascorbic acid and tocopherols belong to the non-enzymatic antioxidant groups (Elhami *et al.*, 2015). Between the enzymatic components ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) play an important role in protecting olive leaf tissue against oxidative stress induced by water stress (Ennajeh *et al.*, 2009; Farooq *et al.*, 2009). After the exposure to abiotic stresses, tolerant cells stimulate their enzymatic antioxidant system, who begins to quenching ROS and safeguarding the cell (Farooq *et al.*, 2009). Regarding the capacity for energy dissipation and metabolic protection (constitutive or induced) this is considered a key element for the success of plants under drought against the harmful effects of ROS (Chaves and Oliveira, 2004).

The root system of olive tree appears to be conceived to absorb the water of the light and periodic rainfall common in this habitat, instead of taking the water from deep layers (Fernández and Moreno, 1999). Indeed, a great root portion, grows approximately parallel to

the ground surface, thereby allowing to explore top soil layers (Fernández, 2014). In drought stress conditions olive tree terminates shoot growth, but not the photosynthetic activity (Xiloyannis *et al.*, 1999). This enables continued production of assimilates and their accumulation in the different parts of the plant, specifically in the root system, generating a higher root/leaf ratio comparatively with the well watered plants (Xiloyannis *et al.*, 1999; Sofo *et al.*, 2007). The large capacity of the root system water absorption, as well as maintaining a positive turgidity pressure under the extreme conditions of water scarcity was proposed as a key of a special adaptation to drought (Wahbi *et al.*, 2005; Guerfel *et al.*, 2007). Furthermore, the roots with short hydraulic conductance or some but extensive roots could allow a tardy but sustainable supply of water to the plant (Bacelar *et al.*, 2013). In short, it is proven that olive trees are efficient users of water in the soil thanks to the capacity to keeping significant rates of gas exchange even during water stress and its xylem sap transport (Sofo *et al.*, 2007).

Aquaporins (AQPs) have been suggested as the main facilitators for the circulation of water across membranes (Bacelar *et al.*, 2013). In plants AQPs are abundantly present in the plasma membrane and in the vacuolar membrane (Farooq *et al.*, 2009). Plant AQPs are implicated in the opening and closing of cellular gates, that play major roles in the physiology of water balance and water use efficiency (Li *et al.*, 2015). As AQPs accumulate in cells around stomatal cavities and in guard cells, they might also be related with the regulation of g_s (Perez-Martin *et al.*, 2014). Moreover, they can promote the circulation of water in drought stressed tissues and facilitate the fast recovery of turgor on watering (Bacelar *et al.*, 2013). For example, they are widely expressed in roots where they mediate the water uptake from the soil (Farooq *et al.*, 2009).

There is growing evidence that other class of proteins, the small heat-shock proteins (HSPs), can play a role in cell protection during dehydration (Bacelar *et al.*, 2013) and high temperatures. The synthesis of stress proteins is a ubiquitous response to handle with the predominant stressful conditions including water stress. Most of the HSPs are soluble in water and consequently contribute to the stress tolerance phenomena through hydration of cell structures (Farooq *et al.*, 2009). These molecular chaperones play an important role in the stabilization of proteins and membranes, as well as in assisting protein refolding under stress conditions, especially elevated temperature (Farooq *et al.*, 2009; Lisar *et al.*, 2012).

4. Kaolin properties and plant effects

4.1. Kaolin characterization

Kaolin (KL) formation is due to erosion of aluminous rocks such as feldspats (Jordão *et al.*, 2002). The major mineral component of kaolin is kaolinite, which can normally contain quartz and mica (Panda *et al.*, 2010).

The use of particle film technologies (i.e., spraying canopies with a suspension of particles of several kinds of clay, leaving a film on the leaves) was recently introduced as new approach to suppress arthropod pests (Barker *et al.*, 2006; Rosati *et al.*, 2006). Kaolin, the basis of these particle films, is a white, non-abrasive, non-porous, non-toxic aluminosilicate ($\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$) clay mineral that disseminate easily in water (Showler, 2002; Cantore *et al.*, 2009). Over a wide pH range, the material is chemically inert and consequently has no direct toxicity to animals or plants (Barker *et al.*, 2006). Is commercialized as Surround® WP (BASF, Research Triangle Park, NC; previously Engelhard Corporation, Iselin, NJ) and was formulated as a wettable powder for use by conventional spray equipment (Cantore *et al.*, 2009). Actual technology enables this extracted, aluminosilicate mineral to be formed into particles having various degrees of size, shape and surfaceproperties (Lalancette *et al.*, 2005). Sprayed on the foliage as an aqueous formulation, the suspension dries to leave a uniform particle film, by giving the plant surface a white aspect (Barker *et al.*, 2006).

An efficient particle film in plant tissues has determinate characteristics: particle diameter $< 2\mu\text{m}$; chemically inert mineral particles; a special formulation to disperse and create a uniform film; reflect ultraviolet (UV) and infrared (IR) radiation, while it transmits PAR; porous film does not interfere with leaf gas exchange; control of insect/pathogen behaviour on the plant, and easy removal from harvested commodities (Cantore *et al.*, 2009).

4.2. KL action mode

Several products have been used to improve the adverse effects of water deficit in plants, since various environmental factors like extreme high temperature, drought and elevated irradiance negatively affect plant growth and development, targeting at different physiological or biochemical functions (Roussos *et al.*, 2010; Denaxa *et al.*, 2012). One not so often used is kaolin particle film, a natural compost, without toxic effects, that have been utilized to minimize the negative impacts of environmental stresses on crop plants (Khaleghi *et al.*, 2015). It is used

lately in agricultural crops especially to avoid injuries from excessive heat load on leaves and vulnerable fruits, also as a biological insecticide with good results against olive fruit fly (*Bactrocera oleae*) and other pests control (Roussos *et al.*, 2010; Denaxa *et al.*, 2012; Chamchaiyaporn *et al.*, 2013). Kaolin is considered a potential alternative pest management product with greater safety for pesticide handlers and reduced environmental impact. Because of these characteristics, as well as their mode of action not be susceptible to development of resistance, resulted in the consent of their use in organic agriculture (European Economic Community (ECC) 1991) (Pascual *et al.*, 2010).

Kaolin keep safe plants from oviposition and insect feeding by covering their surfaces with a protective mineral barrier (Tubajika *et al.*, 2007). Hydrophilic or hydrophobic kaolin particles, when applied to produced a uniform film over the whole surface of the plant, might change the crops microclimate and repulse arthropod pests (Lalancette *et al.*, 2005). In plants coated with hydrophobic particle films, were observed repellency, ovipositional deterrence, and limited survival of insects (Braham *et al.*, 2007). Moreover, it has been reported that foliar applications of kaolin particle film decrease the negative impact of stress, that is important for optimal plant growth and development (Khaleghi *et al.*, 2015). Since KL increase the reflection of solar radiation (both PAR and UV radiation), kaolin reduce leaf temperature and, thus, the water pressure deficit from leaf to air, which would alleviate the effect of drought by reducing evaporative demand, contributing to better plant water status. Nevertheless, kaolin action mechanisms are not yet fully clear. In fact, the effects on leaf and canopy temperatures, gas exchange, and yield is still under debate (Cantore *et al.*, 2009).

4.3. Plant effects

Many of the horticultural advantages of kaolin particle films are connected with the coating effect on light intensity (Lalancette *et al.*, 2005). Additionally, provoke light reflection on the leaf surface that contributes to a lower leaf temperature (Chamchaiyaporn *et al.*, 2013), and, therefore, resulting in a reduced transpiration rate (Denaxa *et al.*, 2012). Kaolin applications equally decrease the leaf thickness, particularly in plants under reduced irrigation conditions (Segura-Monroy *et al.*, 2015). Furthermore, the concentrations of chlorophyll and carotenoids on olive trees increased after spraying kaolin particle film (Khaleghi *et al.*, 2015).

Canopy temperatures have been shown to be lower than on horticultural kaolin treated crops like olive, apple, grapefruit tree, peach, pomegranate and pecan, mainly due to an increase in reflectance of ultraviolet, visible and infrared radiation (heat) that reach the plant surface (Glenn *et al.*, 2003; Novasource Reporter, 2009; Segura-Monroy *et al.*, 2015). However, other studies on the effects of kaolin in leaf and canopy temperature of tomato differ with those obtained for many tree species (Cantore *et al.*, 2009). So, kaolin particle films can help reduce sunburn damage, heat and water stress (Lalancette *et al.*, 2005; Denaxa *et al.*, 2012; Chamchaiyaporn *et al.*, 2013). In addition, kaolin can relieve the incidence of pests (Correia *et al.*, 2013). As a consequence of all these effects, several studies revealed benefits on leaf gas exchange traits (Jifon and Syvertsen, 2003; Rosati *et al.*, 2006; Denaxa *et al.*, 2012; Chamchaiyaporn *et al.*, 2013). Moreover, Correia *et al.* (2013) reported that kaolin application had a notable effect on T_{leaf} , P_n , g_s , intrinsic water use efficiency (A/g_s), photosystem II functioning, photosynthetic pigments concentration and yield in grapevines, acting as a summer stress alleviator. Furthermore, Glenn *et al.* (2001, 2002) have also shown that heat stress and solar injury were decreased when kaolin was applied to apple trees (Lombardini *et al.*, 2005). In apple, KL was also discovered to ameliorate yield, fruit size and colour, as well as instantaneous rate of net photosynthesis of leaves at saturating light (A_{max}) (Rosati *et al.*, 2006). The attenuation of fruit temperature by kaolin could also grant an increment in medium fruit weight and improving some qualitative characteristics of fruits as total soluble solids, redness, anthocyanins and lycopene concentration (Boari *et al.*, 2015; Ćosić *et al.*, 2015). In addition, it has been proposed that under drought conditions kaolin applications on mature rainfed olive trees increased fruit production and oil content (Nanos, 2015).

In olive tree, particles of kaolin clay had a significant positive effect on leaf water content (WC), leaf tissue density (LTD), succulence, and leaf temperature under drought and good irrigated conditions (Denaxa *et al.*, 2012), whereas in drought-stressed olive trees the KL application resulted in reduce LTD and elevated RWC and WC values (Roussos *et al.*, 2010; Denaxa *et al.*, 2012). In addition, Khaleghi *et al.* (2015) reported that the oil extracted from kaolin treated olive trees has a greater oxidative stability and shelf life than untreated ones.

In soybean, kaolin equally demonstrated positive effects on vegetative and reproductive characteristics under restricted irrigation. The data revealed a notable growth concerning the number of pods/number of seeds per plant, node number, stem height/diameter, seed yield, thousand seed weight, biological yield and harvest index in treated plants, but the number of seeds per pod was unaffected (Javan *et al.*, 2013).

At the fruit quality level, several studies also showed positive effects of KL. In fact, kaolin contributed to an increase in red fruit color of apple and mango (Glenn *et al.*, 2005; Chamchaiyaporn *et al.*, 2013), increased soluble solids (SS) concentrations, titratable acidity (TA) and weight (Shellie and Glenn, 2008) in grape berries, as well as increased the tomato fruit lycopene content (Cantore *et al.*, 2009). However, not all studies share the same opinion concerning the positive response to kaolin particle film (Glenn, 2009). Conflicting findings about the effects of kaolin on canopy temperature and gas exchange have been reported for herbaceous crops. Kaolin induces a decrease in transpiration of tea plant (Anandacoomaraswamy *et al.*, 2000), while Russo and Díaz-Pérez (2005) discovered that kaolin application in pepper did not affect yield, net CO₂ assimilation, stomatal conductance, T_{leaf} or transpiration (Cantore *et al.*, 2009). Similarly, Lombardini *et al.* (2005) ascertained in pecans that the use of kaolin did not affect E, g_s and A. Moreover, the study by Rosati *et al.* (2006) concluded that the applications of kaolin were not sufficient to attenuate the harmful effects of water stress and heat on photosynthesis in walnut and almond trees, while KL decreased T_{leaf} and VPD_{leaf-air}, but did not influence g_s (Chamchaiyaporn *et al.*, 2013; Segura-Monroy *et al.*, 2015). Furthermore, the utilization of KL leads to divergences in the composition of photosynthetic pigments. For example, Shellie and King (2013) reported that leaves with particle film had lower chlorophyll-a to chlorophyll-b ratio, and higher ratio of chlorophylls to carotenoids than those without particle film. Identical alterations were observed in pigments content of shaded leaves. On the other hand, other studies reported no significant effect on chlorophyll concentration (Glenn *et al.*, 2003; Roussos *et al.*, 2010; Denaxa *et al.*, 2012) and decreased leaf carotenoids under standard irrigation, although increased carotenoids under reduced irrigation (Shellie and King, 2013). In the same way, the effects of kaolin in WUE differ. Some studies have shown increases of WUE (Jifon and Syvertsen, 2003; Glenn and Puterka, 2005; Glenn *et al.*, 2010; Boari *et al.*, 2015; Segura-Monroy *et al.*, 2015), while others have demonstrated reductions (Glenn *et al.*, 2003; Roussos *et al.*, 2010; Shellie and King, 2013), or no effects (Denaxa *et al.*, 2012). Thus, the results of the literature on the advantages of kaolin are occasionally conflicting and differ according to species, plant architecture, scale of measurement (leaf or canopy scale) and environmental conditions (Boari *et al.*, 2015). However, the majority of the studies on treated plants with kaolin demonstrated positive effects and no significant adverse effect were reported. In this way, particle film technology might be one of the options in order to attenuate drought impact, decreases the dependence of agriculture

on expensive screens and saves water in semiarid regions like those found in the Mediterranean (Boari *et al.*, 2015; Ćosić *et al.*, 2015).

5. Materials and methods

5.1. Geographic localization, plant material and experimental conditions

The experiment was carried out from June to September at the University of Trás-os-Montes e Alto Douro, Vila Real, Portugal (41°19'N, 7°44'W, 450 meters altitude). The climate of the study site is warm and temperate. Mean annual rainfall is 1023 mm, most of which falls in the winter with negligible rainfall during the summer months. The warmest months are July/August and the coldest are December/January, with mean daily temperatures of 21.3/21.7 and 6.8/6.3 °C, respectively (IPMA, 2016). It was used as plant material potted plants (one plant per pot) of olive (*Olea europaea* L. cv. Cobrançosa), with three years old. The pots were coated with plastic film and aluminum foil to decrease evaporation from the soil surface and to reduce the temperature inside the same.

Plants were divided in three groups of 12 individuals: the first group was sprayed with distilled water and kept under well-watered conditions (control), receiving water every second day to field capacity throughout the entire experimental period (WW); the second group was sprayed with distilled water and was subjected to 3 successive drought-recovery cycles by withholding water until natural rainfall occur in the first 2 cycles and, in the third cycle, until the stomatal conductance for water vapor (g_s) during mid-morning (at the peak photosynthetic activity) dropped below $50 \text{ mmol m}^{-2} \text{ s}^{-1}$ (WS); The third group was sprayed with 5% kaolin (w/v) (Surround WP; Engelhard Corp., Iselin, NJ) and kept on the same conditions of WS (KL). Between each drought cycle WS and KL plants were re-watered to field capacity in the evening during the following days until net photosynthesis (A) were almost restored to control values. The volume of water needed to reach field capacity was determined gravimetrically in each pot. The 1st drought-recovery cycle had the duration of 12-6 days, the 2nd 9-3 days and the 3rd 21-16 days. The position and block of each plant was changed periodically to minimize effects deriving from spatial variation of conditions. Plant size and leaf area were similar in all treatments at the onset of the experiment.

Each group of 12 individuals was divided in two subgroups, (i) one was maintained for periodic physiological and biochemical analysis and the other (ii) was kept intact throughout the experiment to biomass accumulation analysis. In the present work only data from the first subgroup was presented. All the physiological, histological and biochemical measurements detailed below were taken in 6 healthy, full expand mature leaves of each treatment. The physiological and biochemical data were obtained at the end of the 3rd drought cycle, while the histological data was obtained at the end of the experiment.

5.2. Leaf anatomy

Anatomical tissue measurements were performed on mature leaves. Histological sections of leaf tissue were made to measure the total thickness of the blade and tissue constituents (upper cuticle, upper and lower epidermis, palisade and spongy mesophyll and trichome layer).

The sections were then examined and photographed with an inverted optical microscope Olympus IX51 (Olympus Biosystem, Munich, Germany) equipped with a digital camera (Color ViewIII, Soft Imaging System GmbH, Münster, Germany). The image processing program utilized was, Olympus Cell[^]A.

The thickness of the entire leaf blade, were obtained using the program Digimizer 4 Image Analysis.

5.3. Morphology, sclerophylly and leaf water status

For studying these variables, leaf samples collected were immediately placed in sealed cups to avoid losses of water by transpiration. Thereafter the following parameters were evaluated: leaf area (LA) using a leaf area measurer connected to a computer with the image processing program WinDIAS 1 (Delta T Devices Ltd., Cambridge, UK); fresh weight (FW); turgid weight (TW), measured after immersion of leaf petioles in distilled water for 48 hours in the dark at 4° C and dry weight (DW) measured after the leaves are dried at 70° C in a drying oven until reaching a constant weight. For the same leaves were also calculated various indexes

of leaf water status: relative water content [$RWC=(FW-DW)/(TW-DW)100$]; succulence [$S=(FW-DW)/LA$] and water content at saturation [$WCS=(TW-FW)/DW$].

Cuticular transpiration rate (E_c) was determined based on the leaf weight loss during dehydration. Excised leaves were kept on the dark at ambient room temperature and were allowed to dry and wilt. Leaves were weighted approximately every 15 minutes and, after initial rapid loss of water during which time stomata presumably closed, the linear relationship of water loss and time was used to estimate E_c .

5.4. Gas Exchange and chlorophyll a fluorescence

The measurement of gas exchange was performed at natural incident photosynthetic photon flux density (PPFD) in the field, using an infrared gas analyzer (IRGA LCpro + ADC, Hoddesdon, UK) in a differential mode and open circuit. Measurements were made at solar midday, under environmental conditions and natural irradiance with PPFD higher to $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, in well exposed adult leaves.

The calculation of the net CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E) and the ratio of the concentration of intercellular and atmospheric CO_2 (C_i/C_a), was made based on measurements of gas exchange using the equations developed by von Caemmerer and Farquhar (1981). In relation to the intrinsic efficiency of water use was determined by the ratio A/g_s .

Concerning the chlorophyll fluorescence variables, were measured in the same leaves used for gas exchange, through a PAM fluorometer (FMS 2, Hansatech Instruments, Norfolk, UK), as described by Wass and Oquist (1988). After 30 min of dark adaptation, the minimum fluorescence (F_0) was measured by applying a weak-intensity modulated light. The maximum fluorescence (F_m) was measured after applying, during 0.7s, a saturating pulse of white light ($> 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Then, leaves were adapted to light during 30 min and after this period steady-state fluorescence (F_s) was averaged over 2.5s, followed by exposure to saturating light ($5000 \mu\text{mol m}^{-2} \text{s}^{-1}$, during 0.7s) to establish the maximal fluorescence in the light (F'_m). After turned off the actinic light, the minimal fluorescence (F'_0) was determined. The maximum quantum efficiency of photosystem II (PSII) – F_v/F_m , efficiency of excitation energy capture by open PSII reaction centres – F'_v/F'_m , relative quantum efficiency of PSII – Φ_{PSII} , and nonphotochemical quenching – NPQ (Bilger and Schreiber, 1986; Genty *et al.* 1989).The

electron transport rate ($ETR = (A_f / F_m) \times 0.5 \times 0.84 \times PPF$) was determined according to Krall and Edwards (1993).

5.5. Photosynthetic pigments and metabolites

For quantification of chlorophylls and carotenoids, the leaf disks were subjected to a treatment with 80% acetone (v/v). The quantification of total chlorophyll (Cl_{a+b}) was determined according to Arnon (1949) and Sesták *et al.* (1971). Concerning the quantification of carotenoids (Car) this was effected according Lichtenthaler (1987).

Total soluble sugars (SS) were extracted by placing the leaf discs in 80% ethanol (v/v) for 1 hour at 80° C, according to a procedure adapted from Irigoyen *et al.* (1992), and subsequently quantification was performed by the colorimetric method of anthrone. Relatively to starch extraction (St), this was made from the same solid phase with perchloric acid at 30%, and St concentration was taken by anthrone method according to a procedure adapted of Osaki *et al.* (1991). For both measurements (SS and St) was used as a standard glucose.

Total phenolic compounds, ascorbate and total thiols were evaluated as indicators of oxidative stress. The concentration of total phenolics (TP) was measured following the method of Folin-Ciocalteu (Singleton and Rossi, 1965), whereas ascorbic acid concentration was measured according to Klein and Perry (1982). Finally, the concentration of total thiols was performed according to a procedure adapted from Ellman (1959) and Suzuki *et al.* (1990). Quantification was made by using a molar extinction coefficient of $13.600 \text{ M}^{-1}\text{cm}^{-1}$ (Ellman, 1959).

5.6. Statistical analysis

Statistical analysis was performed in SuperAnova program (Abacus Concepts Inc., USA). The effect of the treatments was tested by analysis of variance (ANOVA) and the separation of means was performed by Tukey test. According to the Tukey test, significant differences between values were determined at P-values < 0.05.

6. Results and discussion

The cross sections of olive (cv. Cobrançosa) leaves revealed an asymmetric/heterogeneous structure (Figure 2). The palisade parenchyma appears in two parts, one is in contact with the upper epidermis (palisade parenchyma I) and the other (palisade parenchyma II) in contact with the lower epidermis. The upper palisade parenchyma is composed by three compacted layers of elongated cells and the lower one is constituted by one layer of relatively elongated cells. This pattern (presence of palisade parenchyma in contact with the upper and lower epidermis) is common in olive leaves under dry conditions, as reported by Bacelar *et al.* (2004).

Cobrançosa is well protected against water loss, as evidenced by the thickness of the cuticle and the well developed trichome layer covering the abaxial surface (Figure 2). Peltate trichomes are present in both leaf surfaces, but their number is higher on the abaxial surface than on the adaxial. Similar response was observed in other studies with an average of two to three layers of peltate trichomes (Bacelar *et al.*, 2004; Moreno-Alías *et al.*, 2009).

Another xeromorphic feature visible in Figure 2 is the location of stomata on the abaxial surface, below the trichome layer, which delay the dehydration process (Bosabalidis and Kofidis, 2002; Guerfel *et al.*, 2009; Moreno-Alías *et al.*, 2009), and may also improve the external CO₂ supply (Ennajeh *et al.*, 2010).

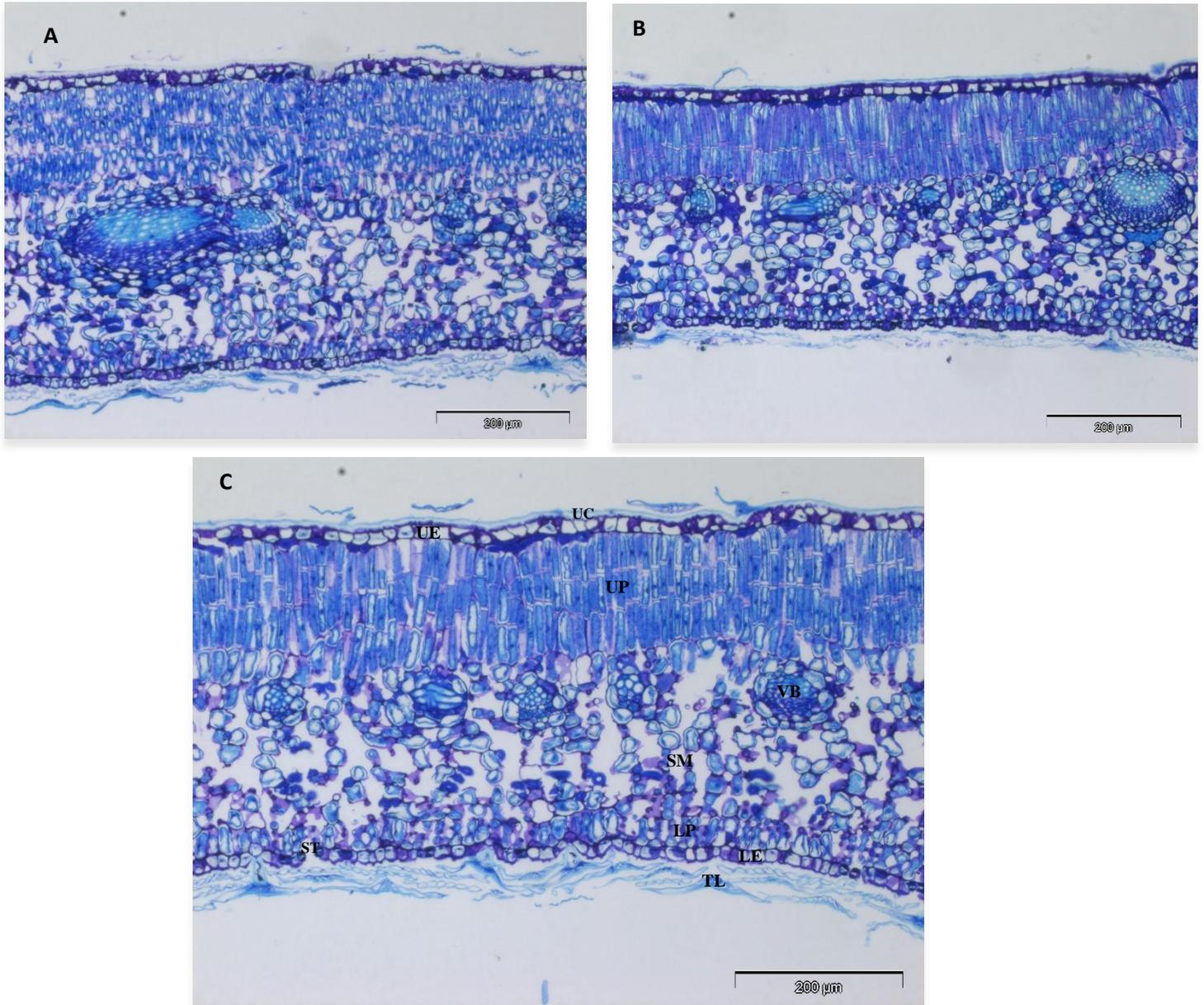


Figure 2 - Transverse sections of the leaf of olive cultivar Cobrançosa. Panel A – WW; Panel B – KL and Panel C – WS. UC = upper cuticle; UE = upper epidermis; UP = upper palisade parenchyma (composed by three layers of elongated cells); SM = spongy mesophyll; LP = lower palisade parenchyma (composed by one layer of short cells); LE = lower epidermis; TL = Trichome layer; VB = vascular bundle; ST = stomata.

Since the leaves are the major organs of internal water removal, olive plants with water scarcity undertake anatomical changes, particularly in the leaf, in order to save water (Boughalleb and Hajlaoui, 2011). Thus, the evaluation of the foliar anatomy is important to understand the adjustment of olive to different environmental conditions (Brito, 2012). In this study the total lamina thickness differ significantly between the treatments, being lower in kaolin treated leaves, due to lower thickness of upper cuticle and upper palisade (Table 2). It is possible that the plants treated with foliar applications of kaolin did not invest so much in foliar

structures to protect against heat, high light and drought stresses, since kaolin performs this function through their excellent reflective properties of solar radiation. As a result of substantial differences in upper palisade, KL plants had a lower palisade/spongy parenchyma ratio than WS plants, which reduces the diffusional limitation related to mesophyll conductance, allowing a more efficient supply of CO₂ to carboxylation sites (Boughalleb and Hajlaoui, 2011; Tomás *et al.*, 2013), and thus to higher photosynthetic rate. Conversely, the trichome layer is higher in kaolin treated leaves than in well-watered plants, which disagree with the study of Segura-Monroy *et al.* (2015). Leaf pubescence improves water use efficiency through increasing leaf boundary layer resistance, contributing to lower loss of water by transpiration (Boughalleb and Hajlaoui, 2011). Additionally, trichomes operate as an efficient filter, safeguard subjacent tissues from damage caused by solar radiation, including ultraviolet-B radiation, and allow olive leaves to benefit of light rain or condensation of water and thus increase the chance of water uptake by the leaves (Bacelar *et al.*, 2004).

Table 2 - Mean values of leaf tissue thickness (μm) of olive cultivar Cobrançosa grown under different treatments (KL – foliar kaolin applications; WS – water stress plants; WW – well-watered plants). TL: Total lamina; UC: upper cuticle; UE: upper epidermis; UP: upper palisade; SM: spongy mesophyll; LP: Lower palisade; LE: Lower epidermis; Tric. layer: Trichome layer. Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	Thickness (μm)								
	TL	UC	UE	UP	SM	LP	LE	Tric. layer	Palisade/spongy
KL	454.3 b	6.30 b	17.2 a	132.3 b	204.5 a	27.8 a	15.7 a	47.4 a	0.650 b
WS	490.5 a	7.17 a	16.0 a	164.4 a	211.2 a	28.1 a	15.6 a	45.6 ab	0.780 a
WW	496.4 a	7.17 a	16.8 a	166.7 a	220.9 a	29.2 a	15.9 a	39.1 b	0.756 ab
Significance	0.0267	0.0458	0.5174	0.0093	0.0875	0.8139	0.8418	0.0428	0.0425

Several studies reported changes of leaf structure under contrasting water availability conditions (Chartzoulakis *et al.*, 2002; Bacelar *et al.*, 2004; Bacelar, 2006; Ennajeh *et al.*, 2010), but in the present study no significant differences were reported between WW and WS leaves (Table 2). Different experimental conditions, namely related with the intensity and duration of water stress, and probably the inclusion of three recovery periods along the experiment may have contributed to explain the similar leaf structure.

Leaf water status indices are presented in Table 3. As can be seen, WW olive trees had higher values of RWC (94%) than the other treatments, mainly than on WS plants (65%), the

treatment in worst water status conditions. This such low value of RWC is an indicator of severe water stress, with significative consequences on plant function. In fact, Jorba *et al.* (1985) discovered that a decrease in RWC from 96 to 65% caused a reduction of 85% in photosynthesis of potted olive trees. So, the low values of RWC in WS plants leads to a decline in photosynthesis (Table 4). Interestingly, kaolin treated leaves showed a much better water status than WS leaves, with a RWC of 90%, being able to maintain an identical succulence as in well watered plants (Table 3), although their lower leaf thickness. This special form of water conservation may be related with the binding of water to mucilage in cells and ducts and intercelular cavities (Margaris, 1981). Water reserves of this kind can protect the plant from sudden wilting and severe leaf shrinkage (Bacelar *et al.*, 2004). Meanwhile, leaf WCS was not affected by the treatments. The low values, ranging from 1.25 to 1.33, are in accordance with previous studies with the cultivar Cobrançosa (Bacelar *et al.*, 2004, 2006), denoting that this genotype have the capacity to withstand arid environments.

After the stomata, the principal place of water loss by transpiration is the cuticle. Elevated cuticular permeability not only affects the non-stomatal transpiration pathway, but also water loss from guard cells and, thus, guard cell water status and stomatal aperture (Bacelar, 2006). Through the data presented in Table 3, it was visible that there were no significant differences at the level of cuticular transpiration among the different treatments. Nevertheless, exists a tendency to significance ($p = 0.057$), with a trend to higher values in WW and KL treatments. The lower thickness of upper cuticle of KL leaves, as well differences in cuticle structure or composition, or both, may explain the tendency to higher cuticular transpiration in those treatments.

Table 3 - Relative water content (RWC), water content at saturation (WCS), succulence and cuticular transpiration of olive cultivar Cobrançosa grown under different treatments (KL – foliar kaolin applications; WS – water stressed plants; WW – well-watered plants). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	RWC (%)	WCS (g H ₂ O/g DW)	Succulence (mg H ₂ O cm ⁻²)	Cuticular transpiration (g H ₂ O m ⁻² h ⁻¹)
KL	89.8 ± 1.4 b	1.25 ± 0.09 a	26.6 ± 0.8 a	4.67 a
WS	64.7 ± 1.2 c	1.27 ± 0.01 a	18.1 ± 1.1 b	2.88 a
WW	94.0 ± 0.8 a	1.33 ± 0.04 a	26.6 ± 0.5 a	4.31 a
Significance	0.0001	0.493	0.0001	0.057

Gas exchange measurements showed reductions in net CO₂ assimilation rate in WS olive plants that were associated with increased stomatal resistance (Table 4), which also

restricted water loss through transpiration. The closely relationship between A and g_s suggests that stomatal diffusion was an important limiting factor for photosynthesis under water stress. Nonetheless, in this study the decrease of A may also be associated with mesophyll limitations, since there was no significant differences in C_i/C_a , despite the substantial decrease (88%) of g_s . The non-stomatal limitations to photosynthesis were also evident in leaves treated with kaolin, that presented similar C_i/C_a ratio and a decrease of 65% in stomatal conductance relatively to WW plants. Nevertheless, as kaolin particle film increase the reflection of solar radiation and prevent some thermal effects, and as a consequence induce a better water status than on WS plants, KL plants had higher transpiration and photosynthetic rate than WS plants, reaching 74 and 62%, respectively. Similar positive effects of kaolin were reported in other studies (Denaxa *et al.*, 2012; Boari *et al.*, 2015).

The data here presented shows that the performed water-stressed treatments induced different water-use behaviours by the final of the 3rd drought cycle. Passioura (1982) pointed out that two types of water-use behaviour may be employed in woody plants. The prodigal water-use behaviour is beneficial in conditions where water supply is interrupted for short periods only. In this situation, there is little danger of serious desiccation despite rapid water-use, and it enables a plant to grow quickly. The conservative water-use behaviour is beneficial in conditions, where a long, dry period prevails, enabling the plant to use the available water efficiently. According to this theory of plant water-use behaviour, KL plants with high g_s and high C_i/C_a and low A/g_s that is positively correlated with A , appears to employ a prodigal or non-conservative strategy, whereas WS plants, with high A/g_s , appear to employ a conservative strategy in the use of water.

Table 4 - Leaf net CO₂ assimilation rate (A), stomatal conductance (g_s), ratio of intercellular to atmospheric CO₂ concentration (C_i/C_a), intrinsic water use efficiency (A/g_s) and transpiration rate (E) of olive cultivar Cobrançosa grown under different treatments (KL – foliar kaolin applications; WS – water stressed plants; WW – well-watered plants). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	g_s ($\text{nmol m}^{-2} \text{s}^{-1}$)	C_i/C_a	A/g_s ($\text{nmol m}^{-2} \text{s}^{-1}$)	E ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
KL	4.85 ± 0.56 b	78.5 ± 2.3 b	0.706 ± 0.036 a	62.1 ± 8.1 b	1.83 ± 0.06 b
WS	2.99 ± 0.66 b	27.4 ± 2.5 c	0.500 ± 0.068 b	106.2 ± 15.5 a	1.05 ± 0.11 c
WW	16.6 ± 0.98 a	226.6 ± 17.9 a	0.620 ± 0.138 ab	74.0 ± 3.6 b	4.67 ± 0.20 a
Significance	0.0001	0.0001	0.0418	0.0257	0.0001

Concerning chlorophyll a fluorescence, it can be a very powerful tool in the study of the photosynthetic performance of plants (Brito, 2012). Is a rapid, nondestructive and relatively

simple technique for detecting energetic/metabolic imbalance of photosynthesis because of water or heat stress or both (Araus *et al.*, 1998). Through the analysis of data presented in Table 5, it can be observed that there were no significant changes in Fv/Fm of olive dark-adapted leaves in the different treatments performed, although there is a tendency to be lower in WS and KL plants. Therefore, these data indicates no occurrence of photodamage to PSII reaction centers induced by drought. The Fv/Fm values are close to the reference value (0.830) considered optimal for non stressed plants (Faraloni *et al.*, 2011). However, there are significant differences in all other parameters under study, with decreases of Φ_{PSII} , ETR and F'v/ F'm under both water stress treatments and an increase of NPQ, with a higher extent in WS plants (Table 5), indicating that total electron flow through PSII was inhibited in these plants, and thus photochemical factors are also responsible for the decrease in photosynthesis, being this behavior in line with leaf gas exchange results. Regarding to nonphotochemical quenching, was noted that WS plants have the highest value (Table 5), which can be associated with an increased need to protect the PSII against oxidative damage. Therefore, this means that KL plants had lower photochemical risks, and thus the above changes in PSII photochemistry may represent a down-regulation mechanism. An increase in NPQ induced by water stress was also reported in wheat (Subrahmanyam *et al.*, 2006).

Table 5 - Maximal quantum efficiency of photosystem II (Fv/Fm), effective quantum efficiency of photosystem II (Φ_{PSII}), electron transport rate (ETR), capture efficiency of excitation energy by open PSII reaction centers (F'v/ F'm) and nonphotochemical quenching (NPQ) of olive cultivar Cobrançosa grown under different treatments (KL – foliar kaolin applications; WS – water stressed plants; WW – well-watered plants). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	Fv/Fm	Φ_{PSII}	ETR ($\mu\text{molm}^{-2}\text{s}^{-1}$)	F'v/F'm	NPQ
KL	0.812 a	0.262 b	154.1 b	0.479 b	2.04 b
WS	0.818 a	0.300 b	176.5 b	0.495 b	3.04 a
WW	0.865 a	0.523 a	307.7 a	0.691 a	1.06 c
Significance	0.1567	0.0002	0.0002	0.0015	0.0001

Photosynthetic pigments play a relevant role in survival and adaptation of plants to drought because they control the absorption energy by chlorophylls and dissipate excess energy, via carotenoids (Baquedano and Castillo, 2006). Significant differences in Chl_{a+b} and Car concentrations, as well as in Chl_a/Ch_b and Chl_{a+b}/Car ratio were recorded between treatments (Table 6). Higher concentrations of Chl_{a+b} and Car were observed in the leaves of WW and KL

plants. The WW plants have higher Chl_a/Ch_b ratio than the other treatments, while the Chl_{a+b}/Car ratio was higher on KL than on WS plants.

The sensitivity of chlorophyll to environmental stresses, such as drought, have been reported by several researchers (Bacelar *et al.*, 2006; Bacelar *et al.*, 2007; Guerfel *et al.*, 2009; Boughalleb and Hajlaoui, 2011). In this experiment the WS olive trees exhibited a decrease in total chlorophyll concentration (Chl_{a+b}) and Car comparatively to the other treatments, which is consistent with a study accomplished by Bacelar *et al.* (2006). Decreased chlorophyll concentration is a symptom of oxidative stress and could be a result of degradation of chlorophyll and/or due to deficient synthesis of chlorophyll together with structural changes of thylakoid membranes (Bacelar, 2006; Bacelar *et al.*, 2007). However, it may also be interpreted as an acclimation mechanism to prevent excessive energy absorption (Brito, 2012), particularly useful in high light intensity situations (Rei, 2015).

This study also revealed changes in the proportions of chlorophylls a e b. In fact, plants under water stress had lower Chl_a/Ch_b , which reflects the relative increase in the light harvesting chlorophyll a/b proteins, at the expense of the chlorophyll a containing reaction centre complexes (Evans, 1993). In addition, the lower Chl_a/Ch_b is probably associated with a decline in cytochrome f content (Watanabe *et al.*, 1994), which causes the reduction in electron transport capacity, and may also help to explain the low A of these treatments.

Carotenoids are photosynthetic pigments of particular importance, since in addition to absorption of PAR also protect the plants against photoinhibition, by dissipating the excess of energy by thermal processes through the xanthophyll cycle (Brito, 2012). Moreover, it is well documented that carotenoids are implicated in protecting the photosynthetic apparatus against photoinhibitory damage by singlet oxygen (O_2), which is generated by the excited triplet state of chlorophyll (Bacelar *et al.*, 2006). Therefore, any reduction in carotenoids may have serious consequences to the chlorophyll levels (Reshmi and Rajalakshmi, 2012). So, the lower chlorophyll concentration in WS leaves may also demonstrates an increased need for photoprotection of chlorophylls by carotenoids (Bacelar *et al.*, 2006). As a consequence, WS leaves had lower chlorophyll/carotenoids ratio than kaolin treated plants (Table 6).

Interestingly, the application of kaolin in olive leaves under water stress conditions have a significant positive effect on photosynthetic pigments, since KL leaves presented higher concentrations of pigments that control plants under water stress and similar values as well watered plants. This higher content of chlorophylls and carotenoids could be related to the fact that kaolin reduce the heat, high light and drought stresses, thereby preventing the activation of

chlorophyllase enzymes that degrade chlorophyll. The results of this study are in accordance with those obtained by Khaleghi *et al.* (2015) and Dinis *et al.* (2016). Carotenoids retention has been proposed as a mechanism of photoprotection and is broadly used to diagnose the physiological state of the plant during the development, acclimation, as well as adaptation to different environments and stresses (Dinis *et al.*, 2014).

Table 6 - Total chlorophyll (Chl_{a+b}), chlorophyll a/b ratio, total carotenoids (Car) and chlorophyll/carotenoid ratio of olive cultivar Cobrançosa grown under different treatments (KL – foliar kaolin applications; WS – water stressed plants; WW – well-watered plants). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	Chl _{a+b} (mg dm ⁻²)	Chl _a /Chl _b	Car (mg dm ⁻²)	Chl _{a+b} /Car
KL	8.40 a	2.90 b	1.85 a	4.55 a
WS	6.89 b	2.96 b	1.61 b	4.27 b
WW	8.01 a	3.11 a	1.81 a	4.42 ab
Significance	0.003	0.000	0.017	0.005

The protection of plant tissues against stress factors is a combined action of diverse enzymatic and non-enzymatic antioxidant mechanisms. Non-enzymatic antioxidant network, for example, includes the synthesis of phenolic compounds (specially flavonoids), carotenoids and ascorbic acid (Rajabbeigi *et al.*, 2013). Such as carotenoids, ascorbic acid (AA) plays an important role in safeguarding photosynthetic apparatus from the harmful effects of light and ROS (Šircelj *et al.*, 1999), being one of the most studied and powerful antioxidants (Blokhina *et al.*, 2003). The capability to donate electrons in a broad range of enzymatic and non-enzymatic reactions makes this molecule the major ROS detoxifying compound in the aqueous phase. AA can directly remove superoxide, hydroxyl radicals and singlet oxygen and reduce H₂O₂ in water via ascorbate peroxidase reaction (Blokhina *et al.*, 2003). The results obtained in the present work revealed significant differences of AA concentrations among treatments (Table 7). In fact, ascorbic acid concentration was higher in WS olive plants compared to KL and WW treatment. The increase of this antioxidant in WS plants may be due to the need to minimize the effects of oxidative stress, suggesting that the plants of the other treatments are under lower oxidative stress conditions. Such indication was also confirmed by the total thiols data (Figure 3). WW olive trees present the highest values, meaning that well-watered plants are more protected against oxidative stress. Similar results were found by Bacelar *et al.* (2007). Furthermore, the leaves of olive trees with kaolin particle film exhibited higher total thiols than

WS plants, probably due to lower oxidation of non-proteic – SH groups (e. g. glutathione), which decreases the susceptibility to membrane damage and the negative effects on cellular functions.

Under diverse abiotic stress conditions plants accumulate phenolic compounds (Ennajeh *et al.*, 2009). The accumulation of these compounds, that include flavonoids, are relevant against several types of stresses, due to their function as UV screens, antioxidants and agents of dissipation energy (Reshmi and Rajalakshmi, 2012). This study revealed significant differences in the concentration of total phenols among treatments (Table 7). Higher values are shown in the KL and WS treatments, comparatively to WW plants. Similar results were obtained by Bacelar *et al.* (2006) and Ennajeh *et al.* (2009) in olive plants under water stress and by Dinis *et al.* (2016) in berries and leaves of grapevine with kaolin exogenous application. According to Petridis *et al.* (2012), the higher content of phenolic compounds is in agreement with the literature data indicating that it is a response to the production of ROS. Some of those secondary metabolites, like the polyphenols, possess ideal chemistry for free radical scavenging actively acting as plant antioxidants (Blokchina *et al.*, 2003). Another mechanism underlying the antioxidant properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al.*, 2000).

Concerning the non-structural carbohydrates concentrations, no statistically significant differences were observed among treatments (Table 7), although exists a tendency for higher soluble sugars in KL than in WS leaves, at the expenses of leaf starch. The similar carbohydrates concentrations in leaves of water stressed plants as in leaves of well watered plants, in spite of the lower A of the formers, must be attributed to the reduced utilization of carbohydrates, due to a lower sink demand. At the same time, the accumulation of non-structural carbohydrates under water stress, namely soluble sugars, could be interpreted as a strategy of acclimation. In fact, soluble sugars, act as osmoprotectants, facilitating water retention in the cytoplasm (Ahmed *et al.*, 2009), as well as sources of carbon for maintenance and re-growth during recovery (Chaves *et al.*, 2002) and also protect cells from extreme oxidative stress (Rajabbeigi *et al.*, 2013). Therefore, for all these reasons, we can assume that, as result for the tendency for higher concentration of soluble sugars in KL than in WS leaves, KL plants are better prepared to withstand drought stress.

Table 7 - Total phenols (TP), vitamin C, total soluble sugars (SS) and starch (St). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

Treatments	TP (mg dm ⁻²)	Vit. C (mg dm ⁻²)	SS (mg dm ⁻²)	St (mg dm ⁻²)
KL	110.1 a	3.56 b	604.0 a	108.2 a
WS	109.5 a	4.14 a	528.5 a	158.2 a
WW	98.6 b	3.48 b	582.9 a	138.2 a
Significance	0.000	0.002	0.116	0.148

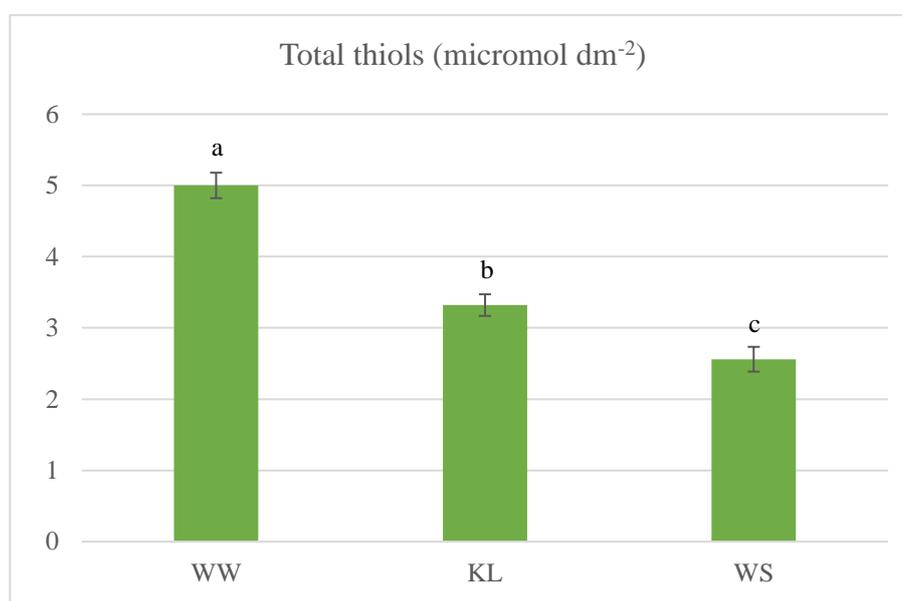


Figure 3 - Concentration of total thiols of olive leaves submitted to different water regimes (WW and WS) and foliar kaolin application (KL). Means inside the same column followed by different letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$).

7. Conclusions

The results of the present study revealed that kaolin foliar spray alleviates the adverse effects of drought stress on olive tree, through the improvement of physiological and biochemical processes. Kaolin contributed to better tree water status, taking into account the higher values of RWC, degree of succulence, stomatal conductance and transpiration rate, and to lower oxidative stress, as is proved by the higher concentration of chlorophylls, carotenoids and total thiols. All together, these responses jointly with the lower palisade/spongy parenchyma ratio, which reduces the diffusional limitation related to mesophyll conductance, allow the enhancement of net photosynthetic rate in kaolin treated plants. Thus, these results support the hypothesis that kaolin application could be a promising agricultural practice to relieve water stress in rainfed olive orchards.

8. References

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