**University of Trás-os-Montes and Alto Douro**

# Strategies to Improve Olive Orchards Sustainability Under a Changing Environment

PhD Thesis

Agricultural Production Chains - From Fork to Farm

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**JURY COMPOSITION:**

The presented doctrines are the exclusive responsibility of the author.

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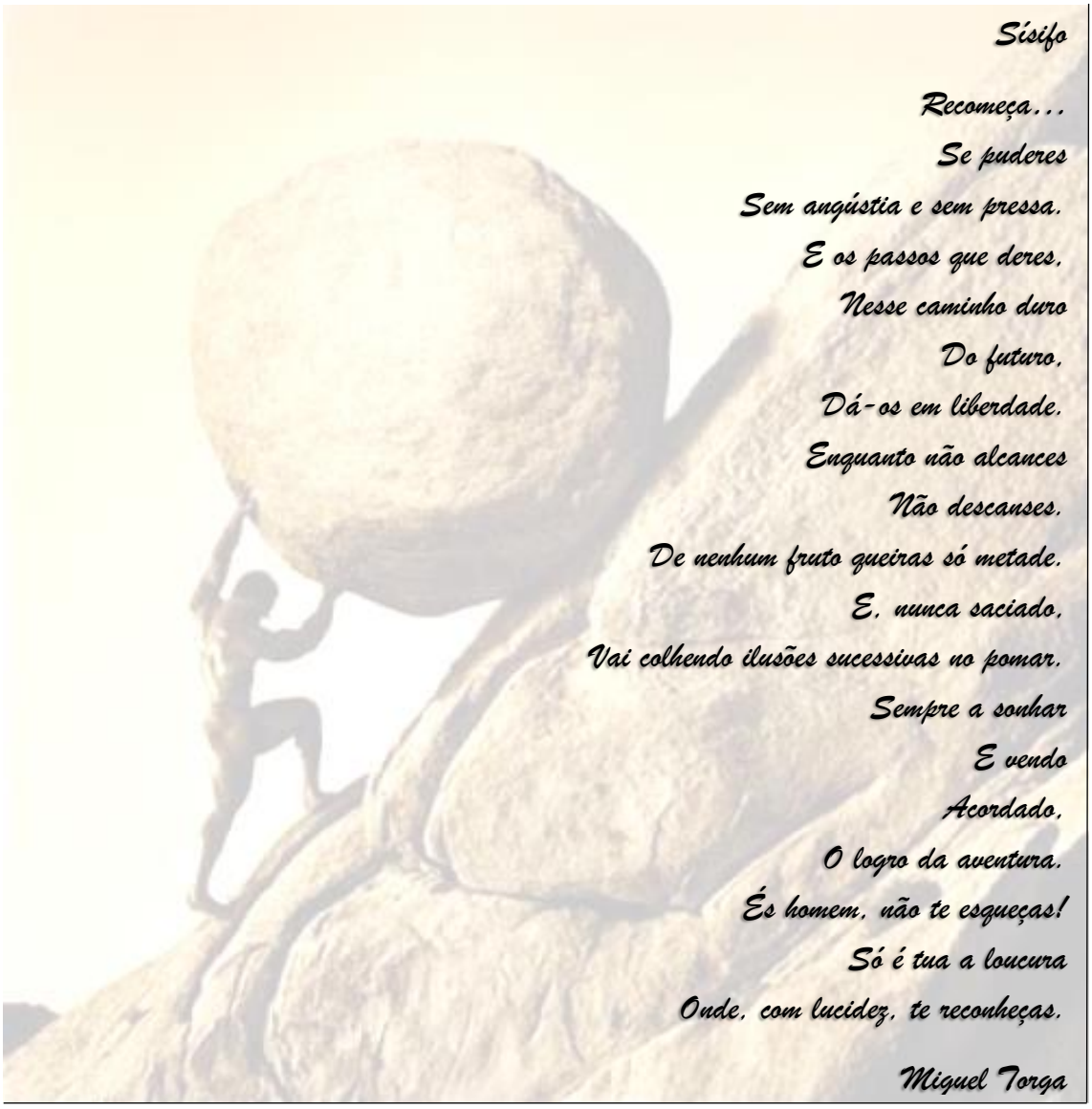

## Sísifo

Recomeça...
Se puderes
Sem angústia e sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro,
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.
E, nunca saciado,
Vai colhendo ilusões sucessivas no pomar.
Sempre a sonhar
E vendo
Acordado,
O logro da aventura.
És homem, não te esqueças!
Só é tua a loucura
Onde, com lucidez, te reconheças.

Miguel Torga

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# RESUMO

As condições ambientais são um dos maiores desafios para a agricultura. Na Região Mediterrânea, onde a oliveira é uma cultura emblemática, é muito comum o stresse estival severo que inclui défice hídrico, elevadas temperaturas e altos níveis de radiação solar, um cenário tendencialmente agravado no futuro pelas alterações climáticas. Se a esta razão acrescentarmos o aumento da demanda de azeite no mundo, torna-se evidente a necessidade de aumentar a sustentabilidade e a competitividade dos olivais tradicionais de sequeiro que contribuem para a economia de regiões remotas e para a preservação dos ecossistemas autóctones.

Dos componentes do stresse estival, o défice hídrico é talvez o mais crítico, uma vez que é extremamente agravado pelos outros. Compreender as respostas da oliveira ao défice hídrico ajuda a projetar e a selecionar práticas agronómicas que melhorem a rentabilidade da cultura. Neste particular, as respostas fisiológicas noturnas têm sido pouco exploradas pela comunidade científica. Com baixa disponibilidade de água, a oliveira aumentou a abertura estomática durante as primeiras horas da noite, bem como a proporção das perdas de água durante a noite relativamente à totalidade das perdas diárias. Embora alguns potenciais benefícios possam estar associados a este comportamento, como a redução da respiração noturna e a melhoria da absorção de minerais, elevadas perdas de água durante a noite podem ser prejudiciais em condições de seca severa.

Juntamente com a compreensão da resposta da oliveira às condições de stresse, é importante agir a curto prazo, avaliando e selecionando estratégias que aliviem e/ ou melhorem a adaptabilidade da oliveira ao stresse estival, incluindo as vertentes de capacidade de resistência e capacidade de recuperação. Neste sentido, pode considerar-se a aplicação de certos produtos naturais na superfície foliar. Neste trabalho avaliou-se o uso de caulino (KL) e ácido salicílico (SA) como agentes protetores do stresse estival. O KL forma um filme de partículas brancas que reflete o excesso de radiação, reduzindo a carga de calor, e o SA é uma hormona vegetal de sinalização com diversos papéis reguladores na resposta ao stresse. Inicialmente, a influência dos produtos foi avaliada em plantas jovens envasadas e submetidas a ciclos de seca e reidratação, de forma a avaliar a capacidade de resistência à seca e a capacidade de recuperação. No caso do SA, testaram-se três concentrações diferentes (10, 100 e 1000 µM), uma vez que a sua eficácia para diferentes genótipos depende da concentração aplicada e não há recomendação por parte do fabricante, o que acontece para o KL (5% p/v). De forma a confirmar os resultados obtidos na experiência em vasos, e para avaliar a influência na

produtividade e qualidade das colheitas, oliveiras em regime de sequeiro, em condições reais, foram pulverizadas com KL e SA a 100 µM, a concentração mais eficiente do estudo em vasos, durante dois anos.

Reunindo os resultados das experiências efetuadas, compreendeu-se melhor as respostas induzidas pelo KL e pelo SA. O KL induziu o desenvolvimento de características foliares associadas a condições de sombra, contribuiu para melhorar o estado hídrico das plantas, reduziu as limitações estomáticas e não estomáticas para a atividade fotossintética, melhorou o estado nutritivo da planta e reduziu a necessidade de investimento em metabolismo secundário durante o stresse e na reparação de danos extra durante a fase de recuperação. As principais diferenças nas duas experiências foram encontradas na restauração das trocas gasosas após o alívio do stresse, atendendo a que no ensaio de vasos a recuperação foi enfraquecida pelo sombreamento causado pelo KL, e na produção de biomassa, considerando que não se verificou influência significativa na acumulação de biomassa, enquanto que em condições de campo a aplicação de KL contribuiu para aumentar o volume da copa e a produção de azeitona. Em relação às respostas ao SA, verificou-se uma fraca influência da concentração de 10µM, enquanto a concentração de 1000 µM induziu algumas respostas negativas. Por sua vez, o SA a 100 µM melhorou o estado hídrico das plantas, em estreita associação com a acumulação de osmólitos, aumentou a concentração de proteínas solúveis totais, melhorou o estado nutritivo, a desintoxicação de ROS e capacidade fotossintética durante o stresse, contribuindo ainda para melhorar a capacidade de recuperação depois do alívio do stresse. No ensaio de vasos, a concentração de 100 µM aumentou a acumulação de biomassa, principalmente ao nível radicular, enquanto que em condições de campo aumentou a produção de frutos, não influenciando o crescimento da copa.

A influência do KL e do SA na qualidade das colheitas dependeu da severidade do stresse. Com stresse estival moderado, o KL e o SA aumentaram as concentrações de antioxidantes e a capacidade antioxidante das azeitonas e dos azeites, enquanto que o oposto se verificou com o aumento da severidade do stresse. O KL e o SA também contribuíram para reduzir a degradação dos compostos fenólicos durante os eventos de geadas. Por outro lado, estes produtos não afetaram significativamente os índices de qualidade do azeite, acidez, índice de peróxidos e o coeficiente de extinção relacionado com a formação de dienos conjugados.

Resumindo, o trabalho desenvolvido no âmbito desta tese revelou que o balanço hídrico noturno deve ser incluído na gestão agronómica. Além disso, este estudo expôs estratégias

rentáveis que atenuam o efeito negativo do stresse estival em oliveiras e contribuiu para aumentar a compreensão do modo de ação do KL e do SA na mitigação do stresse.

**Palavras-chave:** Ácido salicílico, balanço hídrico noturno, caulino, oliveira, stresse estival, seca.

## ABSTRACT

One of the major challenges for agriculture are the environmental conditions. In the Mediterranean Region, where olive is an emblematic crop, is commonly notice a severe summer stress that includes drought, heat and high irradiance levels, a scenario tendentially worsened by climate change. In addition, as the demand for olive oil is increasing all over the world, urge the necessity to increase the sustainability and competitiveness of the traditional rainfed olive orchards that highly contribute to the economy of remote regions and for the autochthone ecosystems preservation.

Among the constituents of summer stress, drought is perhaps the most critical, as is highly exacerbated by the others. A better understanding of how olive tree responds to drought help to design and select agronomic practices to improve the crop profitability. In this regard, nighttime physiological responses has been less discussed. Under drought, olive tree increases both nighttime stomatal open in the first hours of night and the proportion of nighttime water loss in relation to whole-day losses. Although some potential benefits to plants can be associated to this response, as the reduction in nighttime respiration and the improvement in minerals uptake, a continuous nighttime water loss might be detrimental under severe drought conditions.

Along with the understanding of olive tree response to stress conditions, it is important to act in the short-term, by evaluating and selecting strategies that contribute to alleviate and/or to improve olive tree adaptability (stress resistance and recovery capacity) to summer stress. The application of exogenous products on leaf surface can be considered in this sense. The use of kaolin (KL) and salicylic acid (SA) as summer stress mitigating agents was evaluated in this thesis. KL forms a white protective particle film which increases the reflection of excess radiation, reducing the heat load, and SA is a signaling phytohormone with diverse regulatory roles in stress response. The influence of these products was primarily evaluated in young potted plants subjected to repeated cycles of drought-rewatering, to properly assess the drought resistance and the recovery capacity. In the case of SA, three different concentrations were tested (10, 100 and 1000 µM) since concentration highly determine its effectiveness and no manufacture recommended dosage is available, as for KL (5% w/v). To confirm the pots experiment results under realistic field conditions, and to evaluate the influence on yield and harvests quality, rainfed olive trees were sprayed with KL and the most effective SA concentration, 100 µM, for two consecutive growing seasons.

Pulling up together the results of both experiments, a better understanding about KL and SA induced responses was achieved. KL particle film induced shade-related leaf characteristics

and contributed to improve plant water status, reduced the stress-induced stomatal and non-stomatal limitations to photosynthesis, improved plant mineral status and reduced the necessity to invest in secondary metabolism during stress and in extra repair damages under stress recovery. The major differences of KL effects between experiments were found in gas exchange restauration after stress relief, as under pot conditions the recovery was weakened by the KL shaded effect, and also in biomass yield as no influence was recorded in biomass accumulation, while in field conditions KL contributed to higher canopy volume and crop yield. Regarding SA, although the influence of 10µM SA was less noticeable and 1000 µM SA induced some negative responses, 100 µM SA improved water status in line with osmolites accumulation, increased total soluble proteins concentration, plant mineral status and ROS detoxification and photosynthetic capacity during stress, contributing also to improved recovery capacity after stress relief. In pots experiment, 100 µM SA increased biomass accumulation, mainly at root level, while under field conditions enhanced crop yield and no influence was noticed in canopy growth.

KL and SA influenced harvests quality depending on the severity of stress conditions. Under moderate summer stress, KL and SA improved antioxidants and antioxidant capacity of olives and olive oil, while the opposite was verified with higher stress severity. The effects of KL and SA was also associated with phenolics protection against severe frost events. By other side, both products did not significantly affect the oil quality indices, free acidity, peroxide value and the extinction coefficient related with the formation of conjugation diene compounds.

In summary, the work developed in the scope of this thesis revealed that nighttime water balance is worth being considered in agronomic management. Moreover, this study exposes cost-effective strategies to attenuate the negative effects of summer stress in olive trees, and contributed to increase the understanding about KL and SA action mode in stress mitigation.

**Keywords:** drought, kaolin, nighttime water balance, olive tree, salicylic acid, summer stress.

# LIST OF PUBLICATIONS DRIVED FROM THIS THESIS

## Scientific Articles

1. Brito, C., Dinis, L.-T., Ferreira, H., Moutinho-Pereira, J., Correia, C., 2018. The role of nighttime water balance on *Olea europaea* plants subjected to contrasting water regimes. Journal of Plant Physiology, 226:56-63. https://doi.org/10.1016/j.jplph.2018.04.004

2. Brito, C., Dinis, L.-T., Ferreira, H., Rocha, L., Pavia, L., Moutinho-Pereira, J., Correia, C., 2018. Kaolin particle film modulates morphological, physiological and biochemical olive tree responses to cyclic water deficit. Plant Physiology and Biochemistry, 133: 29-39. https://doi.org/10.1016/j.plaphy.2018.10.028

3. Brito, C., Dinis, L.-T., Meijón M., Ferreira, H., Pinto, G., Moutinho-Pereira, J., Correia, C., 2018. Salicylic acid modulates olive tree physiological and growth responses to drought and rewatering events in a dose dependent manner. Journal of Plant Physiology, 230: 21-32. https://doi.org/10.1016/j.jplph.2018.08.004
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4. Brito, C., Dinis, L.-T., Ferreira, H., Coutinho, J., Moutinho-Pereira, J., Correia, C. Salicylic acid increases drought adaptability of young olive trees by changes on redox status and ionome (submitted).

5. Brito, C., Dinis, L.-T., Luzio, A., Silva, E., Gonçalves, A., Meijón, M., Escandón, M., Arrobas, M., Rodrigues, M.A., Moutinho-Pereira, J., Correia, C., 2019. Kaolin and salicylic acid alleviate summer stress in rainfed olive orchards by modulation of distinct physiological and biochemical responses. Scientia Horticulturae, 246: 201-211. https://doi.org/10.1016/j.scienta.2018.10.059

6. Brito, C., Dinis, L.-T., Silva, E., Gonçalves, A., Matos, C., Rodrigues, M.A., Moutinho-Pereira, J., Barros, A., Correia, C., 2018. Kaolin and salicylic acid foliar application modulate yield, quality and phytochemical composition of olive pulp and oil from rainfed trees. Scientia Horticulturae, 237:176-183. https://doi.org/10.1016/j.scienta.2018.04.019

## Invited Speaker

1. Aplicação de Agentes Protetores Alivia os Efeitos Negativos do Stresse Estival na Olivicultura. VII Feira do Azeite e do Figo, seminário: Impactos das alterações climáticas na agricultura, 14 October, Lombo, Portugal.

## Oral communications in conferences

### International

1. Brito, C., Dinis, L.T., Ferreira, H., Gonçalves, A., Coutinho, J., Moutinho-Pereira, J., Correia, C. M., 2018. Salicylic acid increases drought adaptability of olive trees by changes on redox status and ionome. In 11th International Conference "Plant Functioning Under Environmental Stress", Abstract Book, pp. 38, 12-15 September, Krakow, Poland. **ISBN 978-83-86878-37-6**

2. Brito, C., Dinis, L-T., Ferreira, H., Moutinho-Pereira, J.M., Correia, C., 2016. Salicylic Acid Modulates Physiological and Biochemical Responses of Olive Tree to Water Stress. In 19th Symposium of Biology Students in Europe, Abstract Book, pp. 51, 27 July-05 August, Vila Real, Portugal.

**National**

1. Brito, C., Dinis, L.-T., Silva, E., Gonçalves, A., Rodrigues, M. A., Moutinho-Pereira, J., Barros, A., Correia, C., 2018. Weather year-to-year variations determine the influence of kaolin and salicylic acid in olive fruits and oil phenolic composition. In Congresso Nacional sobre as Alterações Climáticas 2018. Abstract Book, pp. 34, 19-21 February, Vila Real, Portugal. **ISBN:978-989-704-259-1**

2. Brito, C., Dinis, L.-T., Silva, E., Gonçalves, A., Pavia, I., Arrobas, M., Rodrigues, M.A., Moutinho-Pereira, J., Correia, C.M., 2017. Salicylic acid improves the tolerance of olive trees against the Mediterranean adverse summer conditions. In Ciência e Cidadania-UTAD, Abstract Book, pp. 107, 21-24 November, Vila Real, Portugal. **ISBN: 978-989-704-252-2**

3. Brito, C., Dinis, L-T., Ferreira, H., Moutinho-Pereira, J.M., Correia, C., 2016. Physiological Responses Induced by Kaolin and Salicylic Acid in Droughted Olive Trees are Related with Anatomical Changes. In Microscopy and Microanalysis in Materials and Life Sciences - 50th Meeting of the Portuguese Microscopy Society, Abstract Book, pp. 123, 29-30 June, Porto, Portugal.

4. Brito, C., Dinis, L-T., Ferreira, H., Moutinho-Pereira, J.M., Correia, C., 2016. Drought-induced variations in nighttime transpiration of olive tree, Vila Real, Portugal. In X Jornadas de Biologia da UTAD, Abstract Book, pp. 18, 12-13 October, Vila Real, Portugal.

**Flash-talk communications in conferences**

**International**

1. Brito, C., Dinis, L.-T., Pinto, G., Ferreira, H., Meijón, M., Valledor, L., Moutinho-Pereira, J., Correia, C., 2017. Exogenous salicylic acid as an olive tree physiological regulator during drought and post-drought recovery. In XV Spanish Portuguese Congress of Plant Physiology, Abstract Book, pp. 98, 26-29 June 2017, Barcelona, Spain.

**Panel communications in conferences**

**International**

1. Brito, C., Dinis, L.-T., Luzio, A., Silva, E., Gonçalves, A., Meijón, M., Escandón, M., Arrobas, M., Rodrigues, M. A., Moutinho-Pereira, J., Correia, C., 2018. Kaolin and salicylic acid alleviate summer stress effects on rainfed olive orchards through distinct physiological and biochemical processes. In Plant Abiotic Stress Tolerance V, 5-6 July, Viena, Austria.

2. Brito, C., Dinis, L.-T., Ferreira, H., Moutinho-Pereira, J., Correia, C.M., 2017. Salicylic acid modulates physiological and biochemical responses of olive tree to drought and recovery events in a dose dependent manner. In VIII Congresso Ibérico de Ciências Hortícolas, Abstract Book, pp. 179, 7-9 June, Coimbra, Portugal. **ISBN 978-972-8936-27-3**

3. Brito, C., Dinis, L-T., Silva, E., Ferreira, H., Rocha, L., Barros, A., Matos, C., Ferreira, I., Moutinho-Pereira, J., Rodrigues, M.A., Correira, C., 2016. Potential Benefits of kaolin and salicylic acid on olive tree performance and mineral composition of fruits under rainfed conditions. In 24th International Symposium of the International Scientific Centre of Fertilizers, Plant nutrition and Fertilizer issues for specialty crops, Abstract Book, pp. 3-4, 6-8 September, Coimbra, Portugal. https://doi.org/10.5073/berjki.2016.185.000

**National**

1. Brito, C., Dinis, L.-T., Silva, E., Gonçalves, A., Rocha, L., Rodrigues, M. A., Moutinho-Pereira, J., Barros, A., Correia, C., 2018. Use of kaolin as a summer stress alleviating product in olive orchards under rainfed conditions. In Congresso Nacional sobre as Alterações Climáticas 2018. Abstract Book, pp. 56, 19-21 February, Vila Real, Portugal. **ISBN:978-989-704-259-1**

2. Brito C, Dinis L-T, Luzio A, Meijón M, Escandón M, Silva E, Gonçalves A, Rodrigues MA, Moutinho-Pereira J, Correia C. 2017. Kaolin spray induces changes in ABA and IAA immunodistribution in olive leaves. In Ciência e Cidadania-UTAD, Abstract Book, pp. 123, 21-24 November, Vila Real, Portugal. **ISBN: 978-989-704-252-2**

3. Brito, C., Dinis, L-T., Pinto, G., Ferreira, H., Meijón, M., Valleador, L., Moutinho-Pereira, J.M., Correia, C., 2016. ABA and IAA Immunolocalization in Olive Leaves During Post-Drought Stress Recovery. In Microscopy and Microanalysis in Materials and Life Sciences - 50th Meeting of the Portuguese Microscopy Society, Abstract Book, pp. 135 pp. 29-30 June, Porto, Portugal.

4. Brito, C., Dinis, L.-T., Gerós, H., Moutinho-Pereira, J., Correia, C.M., 2015. Exogenous salicylic acid ameliorates drought tolerance of olive tree by modulating water status and oxidative stress. In VIII Jornadas de Bioquímica UTAD, Abstract Book, pp. 39, 15-16 April, Vila Real, Portugal

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**CHAPTER 6**

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## 6.1. Kaolin and salicylic acid foliar application modulate yield, quality and phytochemical composition of olive pulp and oil from rainfed trees

## LIST OF ABBREVIATIONS

**A**

**ABA –** Abscisic acid
**ABA-GE –** Glucose-conjugated abscisic acid
**AC –** Antioxidant capacity
**$A_n$ –** Net photosynthetic rate
**$A_n/g_{day}$ –** Daytime intrinsic water use efficiency
**$A_n/g_s$ –** Intrinsic water use efficiency
**ATP –** Adenosine triphosphate
**Aux –** Auxins

**B**

**BI –** Plant biomass increase

**C**

**C –** Control
**Car –** Total carotenoids
**$Chl_{(a+b)}$ –** Total chlorophylls
**$Chl_a$ –** Clorophyll a
**$Chl_b$ –** Chlorophyll b ratio
**$C_i$** – Intercellular $CO_2$
**$C_i/C_a$ –** Ratio of intercellular/ atmospheric $CO_2$
**$C_{i\text{-}night}$ –** Concentration of $CO_2$ in intercellular spaces at night
**CVI –** Canopy volume increase

**D**

**D –** Droughted plants
**D0 –** Droughted plants sprayed with distilled water
**D10 –** Droughted plants sprayed with 10 µM of salicylic acid
**D100 –** Droughted plants sprayed with 100 µM of salicylic acid
**D1000 –** Droughted plants sprayed with 1000 µM of salicylic acid
**DNA –** Deoxyribonucleic acid
**DP –** Drought period
**DW –** Dry weight

**E**

**E –** Leaf transpiration
**$E_{cuticular}$ –** Leaf cuticular transpiration
**$E_{day}$ –** Daytime leaf transpiration
**EL –** Electrolyte leakage
**$E_{night}$ –** Nighttime leaf transpiration
**$E_{night}$ –** Nighttime leaf transpiration
**ETR –** Electron transport rate
**$E_{W\text{-}night}$ –** Whole nighttime leaf transpiration

**F**

**$F'_v/F'_m$** – Capture efficiency of excitation energy by open PSII reaction centers
**FA –** Free acidity
**Fl –** Flavonoids
**$F_m$ –** Maximal fluorescence
**$F_0$ –** Minimal fluorescence
**$F_s$ –** Fluorescence yield
**$F_v$ –** Variable fluorescence
**$F_v/F_m$** – Maximum quantum efficiency of photosystem II
**FW –** Fresh weight

**G**

**$g_{cuticular}$ –** Cuticular conductance
**$g_{day}$ –** Daytime stomatal conductance
**$g_m$ –** Mesophyll conductance
**$g_{night}$ –** Nighttime stomatal conductance
**$g_s$ –** Stomatal conductance

**H**

**H1 2015 –** 9th November 2015 harvest
**H1 2016 –** 9th December 2016 harvest
**H2 2015 –** 30th November 2015 harvest
**HSPs –** Heat shock proteins

**I**

**IAA –** Indoleacetic acid
**IR –** Infrared radiation

**K**

**$K_{232}$ –** excitation coefficient at 232 nm
**$K_{270}$ –** excitation coefficient at 270 nm
**KL –** Kaolin

**L**

**LA –** Leaf area
**LAR –** Leaf area ratio
**LE –** Lower epidermis
**LMA –** Leaf mass area
**LMF –** Leaf mass fraction
**LPP –** Lower palisade parenchyma

**M**

**mid –** Midday
**mo –** Morning

**N**

**NAR –** Net assimilation rate
**NPQ –** Non-photochemical quenching
**NSC –** Non-structural carbohydrates

**O**

**OA –** Osmotic adjustment
**o-DP –** *Ortho*-diphenols

**P**

**PAR –** Photosynthetic active radiation
**$PE_{W\text{-}daytime}$ –** Plant whole-daytime transpiration
**$PE_{W\text{-}night}$ –** Plant whole-nighttime transpiration
**PP –** Palisade parenchyma
**PPFD –** Photosynthetic photon flux density
**PSII** – Photosystem II
**PV –** Peroxide value

**Q**

**qP** – Photochemical quenching

**R**

**R –** Respiration
**RAB –** Relative aboveground biomass increase
**RAI –** Relative alleviation index
**RBB –** Relative belowground biomass increase
**RMF –** Root mass fraction
**$R_{night}$ –** Nighttime dark respiration
**ROS –** Reactive oxygen species
**RP –** Recovery period
**RTI –** Relative tolerance index
**Rubisco –** Ribulose-1,5-bisphosphate carboxylase/oxygenase
**RWC –** Relative water content

**S**

**SA –** Salicylic acid
**-SH –** Total thiols
**SLA –** Specific leaf area
**SP –** Spongy parenchyma
**SS –** Soluble sugars
**St –** Starch
**STM –** Stem mass fraction

**T**

**TAC –** Total antioxidant capacity

**TEAC –** Trolox equivalent antioxidant capacity
**TL –** Trichome layer
**TLA –** Total leaf area
**TP –** Total phenolics
**TPC –** Total phenolic compounds
**TS –** Total section
**TSP –** Total soluble proteins
**TSS –** Total soluble sugars
**TW –** Turgid weight

**U**

**UC –** Upper cuticle
**UE –** Upper epidermis
**UPP –** Upper palisade parenchyma
**UV –** Ultraviolet radiation
**UV-B –** Ultraviolet B radiation

**V**

**VPD –** Vapor pressure deficit
**$VPD_{leaf\text{-}air}$ –** Leaf-to-air vapor pressure deficit

**W**

**WS –** Water stressed plants
**WUE –** Water use efficiency
**$WUE_i$ –** Intrinsic water use efficiency
**$WUE_{WP}$ –** Whole-plant water use efficiency or water use efficiency of biomass production
**WW –** well watered plants

**Symbols**

**ΔK –** Variation of the specific extinction
**ΦPSII –** Effective quantum efficiency of photosystem II
**Ψ –** Water potential

# CHAPTER 1

## General Introduction

Climate change scenarios predict the temperature rising and shifts in precipitation patterns, leading to higher evaporative demand and decreased soil water availability in many areas of the world (IPCC, 2013). In addition, the frequency and severity of drought and heat waves spells are likely to increase. Mediterranean region, where olive is the iconic tree, is well known by the severe summer conditions, including low rainfall, excessive heat load and high daily irradiances (both photosynthetic active radiation, PAR and ultraviolet radiation, UV). Moreover, due to its location, is particularly vulnerable to climate change (IPCC, 2013).

The projected climate changes are of utmost relevance for olive tree, although is a well-adapted species to harsh conditions, a considerable expense of energy resources will be used in defense mechanisms, compromising plant growth and productivity (Fernández, 2014). Those environmental factors cause adverse pleiotropic effects on plant growth and development at molecular, physiological and biochemical levels (Correia *et al*., 2005; Bacelar *et al*., 2006, 2007a,b; Guo *et al*., 2006; Moutinho-Pereira *et al*., 2009). The negative influence of these abiotic stresses on plants, as well the known mechanisms that enable olive tree to survive under such hard conditions are deeply reviewed in **Chapter 2.1**.

Although the drought effects in olive tree are widely evaluated, the nighttime processes, namely transpiration and respiration are less discussed. The processes that occur at night can have more influence in plants than we usually assume. A substantial nighttime stomatal open and, thus, transpiration affect plant water balance and water use efficiency (Escalona *et a*l., 2013). Furthermore, respiration is a determining factor related to growth and productivity, particularly in conditions in which photosynthesis is negatively affected (Flexas *et al*., 2005; Galmes *et al*., 2007). Thus, as the temperature increase will be more marked during the night period (IPCC, 2013), rise the necessity to understand the response of these processes to drought conditions. Furthermore, drought adaptability integrates much more than drought resistance, playing recovery capacity a key role (Chen *et al*., 2016). This concept takes special importance in Mediterranean-type ecosystems that are commonly exposed to repeated cycles of drought-rewatering (Munné-Bosch and Peñuelas, 2003). However, the capacity for recovery after successive drought and rewatering cycles have poorly been studied.

The predicted climate change scenarios might compromise the economic viability of the olive sector. This may lead to the abandonment of traditional groves, with devastating socioeconomic (*e. g.* income and employment reduction in marginal regions) and environmental (*e. g.* soil erosion, increased risk of wildfires, changes in wildlife communities) consequences. To cope with climate change effects, it is required the implementation of

agronomic strategies to alleviate the adverse effects of summer stress and offset the loss of productivity. Given the high natural limitations in water resources and the rugged topography of some Mediterranean areas, the systems of water captation and distribution in large scale involve high costs and are environmentally unsustainable (Conde *et al*., 2016). On the other hand, attempts to improve tolerance through plant breeding methods are time-consuming and laborious, and rely on existing genetic variability (Kaya *et al*., 2009). Moreover, it is difficult to modify single traits that are probably multigene controlled. Without disregarding the importance that irrigation and genetics can have on improving olive performance under climate change, it is crucial to act on short-term adjustments. The foliar application of kaolin (KL) and salicylic acid (SA) can mitigate the influence of abiotic stresses in due season. KL, once sprayed on leaf surface, leaves a white protective particle film after water evaporation. This film increases the reflection of excess radiation (ultraviolet, visible and infrared), reducing the risk of leaf and fruit damage from heat load accumulation and solar injury (Glenn *et al*., 2005). However, although KL has been studied in fruit trees, the plant induced changes are still not fully understood. In addition, there is still no consensus about its effectiveness in different crops and under stress prevalence and/or intensity (Denaxa *et al*., 2012; Boari *et al*., 2015; Nanos *et al*., 2015; Brillant *et al*., 2016; Dinis *et al*., 2016). The characterization and mode of action, as well the effects of KL on crops are deeply reviewed in **Chapter 2.2**. SA is a signaling phytohormone with diverse regulatory roles in plant metabolism, such as the antioxidant defense system activation, secondary metabolites production, osmolytes synthesis modulation and optimization of mineral nutrients status (Khan *et al*., 2015). However, its effectiveness is highly dependent on applied concentration, being generally low concentrations more efficient than the higher ones (Korkmaz *et al*., 2007; Kang *et al*., 2012). Moreover, although the knowledge about its action mode is wider, the investigation remains essentially in herbaceous species (Umebese *et al*., 2009; Kang *et al*., 2012; Kabiri *et al*., 2014). The characterization and mode of action, as well the effects of SA on crops are deeply reviewed in **Chapter 2.3**. The **overall objective** of this thesis is to contribute to enhance the sustainability and competitiveness of the rainfed olive sector, commonly found in marginal areas of the Mediterranean Region. In consequence, the specific objectives of the present thesis are:

1) to get a more comprehensive information about the olive tree response to drought, specifically the role of nighttime water balance (**Chapter 3**);

2) to evaluate the influence of exogenously applied KL on olive tree physiological and growth responses to drought and rewatering events (**Chapter 4**);
3) to evaluate the influence of exogenously applied SA on olive tree physiological and growth responses to drought and rewatering events (**Chapter 5**);
4) to evaluate how different SA concentrations, determine olive tree physiological and growth responses to drought and rewatering events (**Chapter 5**);
5) to gain a greater understanding of the KL and SA effects on physiological, biochemical, growth and yield responses of rainfed olive orchards (**Chapter 6**);
6) to test how long KL and SA effects are prolonged in rainfed conditions (**Chapter 6**);
7) to evaluate the influence of KL and SA on fruit and olive oil quality (**Chapter 6**).

To accomplish these objectives two distinct experiments were implemented and a holistic analysis was performed, being described and discussed across the following Chapters. The present thesis is organized in 9 chapters and the thesis outline is summarized in Figure 1.

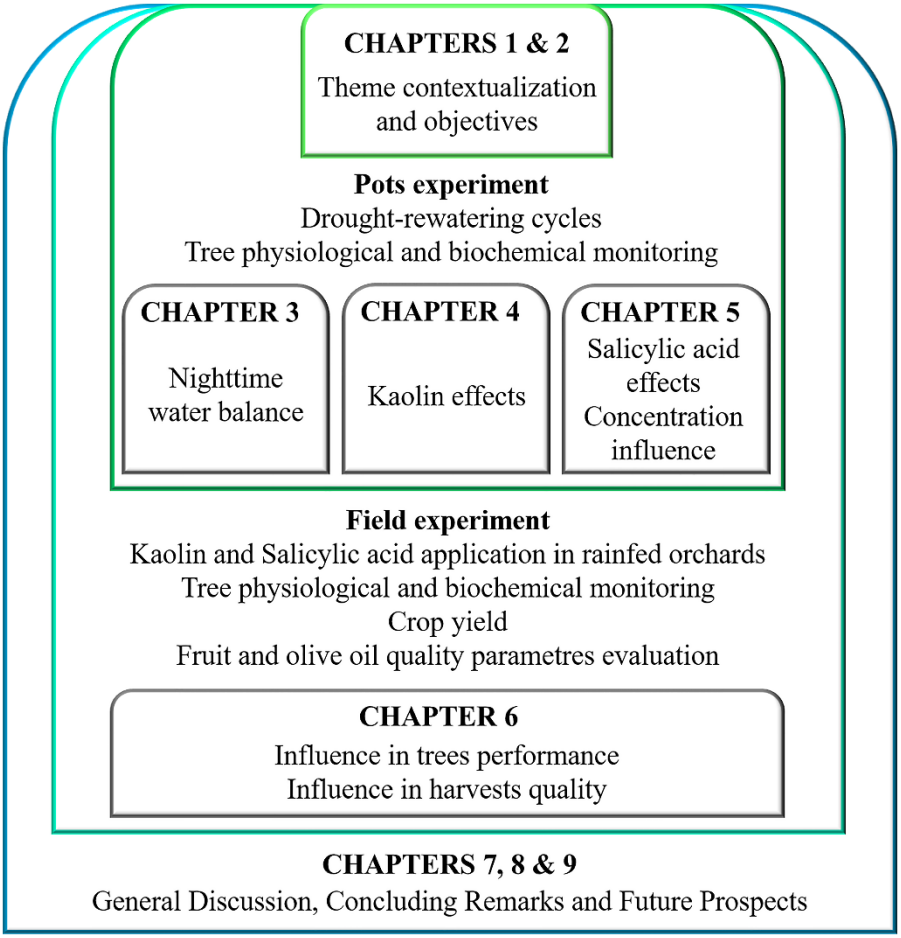


**Figure 1.** Schematization of the thesis outline.

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# CHAPTER 2

State of the Art

## 2.1. Olive crop behavior in a changing world

### 2.1.1. Olive tree cultivation

Olive tree (*Olea europaea* L.), belonging to the botanical family Oleaceae and genus *Olea* (Therios, 2009), is one of the oldest cultivated plants native of the Mediterranean basin (Connor and Fereres, 2005), contributing to the economy, health, nutrition, culture and sustainability of the region. Nowadays, its cultivation is extended outside the Mediterranean area, including southern Africa, South and North America, Australia, Japan and China, although the region of origin remains the main area of cultivation (IOC, 2017). The olive cultivation worldwide is limited by edapho-climatic factors to Mediterranean isoclimatic zones lying between the parallels 30 and 45 of the northern and southern hemispheres (Böhm and Antunes, 2013). The Mediterranean climate is typically mild and wet during the winter and hot and dry during the summer (Giorgi and Lionello, 2008), being the Mediterranean region usually also exposed to high daily irradiances, including UV radiation.

The growing awareness about the olive oil nutritional value has been leading to the expansion in olive tree cultivation (IOC, 2017). The world area harvested in 2016 was over 10.6 million ha, with a production of 192 674 93 tons (FAOSTAT, 2017). In 2016, Spain was the country with higher area, followed by Tunisia, Italy, Morocco and Greece. In terms of production, Spain remains in the 1$^{st}$ place, followed by Greece, Italy, Turkey and Morocco (FAOSTAT, 2017). About 90% of the world olives production is for oil extraction, and the remaining 10% for table olives (Gucci and Fereres, 2012). About 93% of world olive oil production is obtained in the Mediterranean region, being the European Union countries (Spain, Italy, France, Greece and Portugal) responsible for 69% of global production (IOC, 2017). To increase production, large areas were irrigated and fertilized, trees were adjusted to mechanical pruning and harvesting, and new orchards were planted in high and super-high density (Connor and Fereres, 2005; Sanzani *et al*., 2012).

### 2.1.2. Climatic change scenarios and implications in olive cultivation

The effects of weather and climate in agriculture can be felt at different levels, as changes in $CO_2$ atmospheric concentration, temperature and water resources availability, among other factors, affect plants development and productivity, the possibility to execute agriculture operations, the incidence of pests and diseases and the geographical distribution of crops.

Impacts from recent climate-related extremes, such as heat waves, droughts, floods, cyclones, and wildfires, revealed significant vulnerability and exposure of some ecosystems to

current climate variability (IPCC, 2013). However, while the above records are concerning, the forecasted scenarios can be worse, accounting with global temperatures rising, with special prominence at nighttime, and increase in extreme events intensity and frequency (IPCC, 2013). Due to its unique geographic location, in a transition zone between the arid climate of North Africa and the temperate and rainy climate of central Europe, Mediterranean basin is particularly vulnerable to present and future climate variability and climate change (Giorgi and Lionello, 2008), being expected an intensification of the typical olive stressors.

The interrelations between olive yield and future climatic scenarios, including changes in rainfall, solar radiation, temperature, relative humidity and wind speed, revealed that $CO_2$ assimilation and olive yield will decrease substantially (Viola *et al*., 2013). However, the potential assimilation increases with an increase in atmospheric $CO_2$ concentration and consequently the overall productivity, even if reduced water availability controls and limits this tendency (Viola *et al*., 2014). In fact, it is known that stomatal conductance decreases with increasing $CO_2$ levels, while the photosynthesis is improved, leading to greater water use efficiency of several Mediterranean species, including olive tree (Chartzoulakis and Psarras, 2005; Moutinho-Pereira *et al*., 2009). On the other hand, higher temperatures will accelerate phenological development and increase evapotranspiration and the demand of water. In addition, the predicted decrease in water availability and increase in saline water use for irrigation will enhance the co-occurrence of several stressing factors that will be very difficult to be overcome by the increase in atmospheric $CO_2$ (Chartzoulakis and Psarras, 2005).

Warmer conditions will also determine a possible north range expansion of cropping activities into regions were lower temperature was limitative in the past (IPCC, 2013). Conversely, will be expected a reduction in the southerly cropping areas and crop yields. Potentially cultivable areas for olive growing are expected to extend northward and to higher altitudes (Orlandi *et al*., 2013; Tanasijevic *et al*., 2014), increasing by 25% in 50 years (Tanasijevic *et al*., 2014). Following, will be discussed in more detail the impact of the referred stressors on olive morphological characteristics and physiological and biochemical mechanisms, which compromise its development, growth and yield (sections 2.1.3 and 2.1.4).

### 2.1.3. Summer stress effects

Plants that grown in typical semi-arid and arid conditions are affected by multiple environmental constraints factors, since drought stress is commonly associated with high

temperatures and high irradiation levels. These stresses are considered some of the most limiting factors for agricultural productivity worldwide, especially drought.

#### 2.1.3.1. Drought

With drought imposition, as plant water content decreases, the cells shrink and the cell wall relax, resulting in loss of turgor (Taiz and Zeiger, 2006), causing a reduction in leaf water potential and in cell division and expansion (Farooq *et al.*, 2009). If water deficit is imposed early in the development, the inhibition of cell expansion results in a reduced leaf area, while if it is imposed after a substantial leaf area has developed, leaves will senesce and can fall off (Taiz and Zeiger, 2006). The number of leaves can also be affected, associated with a decrease in the number of branches and growth rate (Taiz and Zeiger, 2006; Farooq *et al.*, 2012). These responses limit the photosynthetic area and thus contribute to the decline in whole-canopy photosynthesis (Farooq *et al.*, 2012).

One of the primary drought consequences is the regulation of stomatal aperture to restrict water losses (Hernandez-Santana *et al.*, 2016). Olive tree present a tight control of stomatal behavior to maintain water potential (Ψ) within an adequate level, avoiding critical values and keep them in a safe range to avoid embolism (Torres-Ruiz *et al.*, 2013; Torres-Ruiz *et al.*, 2015). Stomatal regulation is influenced by both hydraulic and chemical signals (abscisic acid, ABA), but may also vary under increasing drought and recovery conditions (Torres-Ruiz *et al.*, 2015). As stomatal aperture decreases, the entering of $CO_2$ into the mesophyll also decrease, with negative consequences on photosynthesis ($A_n$) (Boussadia *et al.*, 2008; Boughalleb and Hajlaoui, 2011). Moreover, the mesophyll compactness increases under drought conditions to restrict water diffusion, what also restrict the supply of $CO_2$ to the carboxylation sites (Tognetti *et al.*, 2007; Tomas *et al.*, 2013). Thus, both diffusional limitations, *i. e.* stomatal ($g_s$) and mesophyll ($g_m$) conductance, contribute to $A_n$ decline, showing a close relationship between each other (Diaz-Espejo *et al.*, 2007; Perez-Martin *et al.*, 2009, 2014). Nonetheless, diffusional limitations to photosynthesis were not exclusively associated with leaf anatomical traits. In fact, especially in harsh environmental conditions, changes in leaf biomechanical and biochemical traits can lead to a reduction in mesophyll conductance to $CO_2$ (Perez-Martin *et al.*, 2009; Fernández, 2014), *e. g.* carbonic anhydrase and aquaporins (Perez-Martin *et al.*, 2014).

In response to moderate stressful conditions, $g_s$ and $g_m$ are the main limitations to $A_n$ (Guerfel *et al.*, 2009a; Fernández, 2014), but at severe stress levels the biochemical component of photosynthesis can also be inhibited (Sofo *et al.*, 2008; Bacelar *et al.*, 2009; Boughalleb and

Hajlaoui, 2011). Meanwhile, limitations in net $CO_2$ assimilation might lead to an overexcitation and subsequent photoinhibitory damage of photosystem II (PSII), as demonstrated in several studies by reduction of relevant photochemical traits as photosynthetic electron transport rate (ETR), effective quantum efficiency of photosystem II (ΦPSII), maximal quantum efficiency of photosystem II ($F_v/F_m$), capture efficiency of excitation energy by open PSII reaction centers ($F'_v/F'_m$), and photochemical quenching (qP), and by the increase of non-photochemical quenching (NPQ) (Bacelar *et al.*, 2007a; Boussadia *et al.*, 2008; Guerfel *et al.*, 2009b; Boughalleb and Hajlaoui, 2011; Sofo, 2011; Petridis *et al.*, 2012; Ben Abdallah *et al.*, 2017).

The decrease in $CO_2$ assimilation, under conditions of drought and high light stresses, is usually also associated with increases in photorespiration rate, owing the due nature of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Farooq *et al*., 2012). Nonetheless, under severe drought, photorespiration is involved in energy dissipation, reducing photoinhibition (Flexas and Medrano, 2002). Severe drought can also cause the impairment of Rubisco and other photosynthetic enzymes activities (Flexas and Medrano, 2002; Diaz-Espejo *et al.*, 2006; Flexas *et al.*, 2012), as well the photosynthetic pigments degradation (Bacelar *et al*., 2006; Guerfel *et al*., 2009a; Boughalleb and Hajlaoui, 2011).

Although unexpected, because there is no opportunity for carbon gain (Caird *et al*., 2007), a substantial nighttime stomatal conductance ($g_{night}$) and leaf transpiration ($E_{night}$) were observed in a wide range of species from different functional groups and ecosystems (Snyder *et al*., 2003; Dawson *et al.*, 2007; Ogle *et al*., 2012; Resco de Dios *et al*., 2015), suggesting potential benefits related with the continue water loss during the night. For instance, it was proposed that may improve nutrients uptake (Snyder *et al*., 2003; Scholz *et al*., 2007), may prevents excess cell turgor at night after stress relief, when leaves still contain substantial contents of osmoticants (Donovan *et al*., 1999, 2001), and may supplies $O_2$, enhancing the capacitance of the trunk and stem (Daley and Phillips, 2006). Although the implication of nighttime water loss in physiological processes remain unclear (Ogle *et al*., 2012; Escalona *et al*., 2013; Coupel-Ledru *et al*., 2016), the occurrence of this phenomenon affects plant water balance and water use efficiency (Escalona *et al*., 2013), what might be detrimental under limited water conditions. Still, as far as we know the occurrence of this phenomenon in olive tree is less explored.

Respiration (R) and $A_n$ are strongly coupled and intrinsically interdependent because $A_n$ provides photosynthetic substrates to R, and R supplies adenosine triphosphate (ATP) and carbon skeletons to sustain plant energy requiring processes (Cannell and Thornley, 2000). However, in response to drought the R trend is still not clear. It was suggested that R varies

generally from inhibition, with low to moderate stress (due to the decrease of energy demand for plant growth and the impairment of some enzymes involved in R), to stimulation, with severe stress (due to changes in metabolism to extra repair costs to offset serious damage) (Flexas *et al.*, 2005; Atkin and Macherel, 2009; Varone and Gratani, 2015). Additionally, because phloem transport depends on turgor, the decrease in water potential in the phloem under severe drought might inhibit the amount of assimilates exported (Taiz and Zeiger, 2006), and sugars accumulation in leaves might control photosynthesis by feedback processes (Paul and Pellny, 2003).

Derived by changes in $A_n$ and $g_s$, the intrinsic water use efficiency ($WUE_i$), defined as $A_n/g_s$, will be eventually affected by water availability. Under mild to moderate drought, $WUE_i$ typically increases, while might decrease under severe drought conditions, as illustrated in olive tree by Bacelar *et al.* (2007a). However, improving $WUE_i$ might not necessarily result in improving whole plant water use efficiency ($WUE_{WP}$) (Flexas *et al.*, 2010), the whole plant carbon and biomass acquisition per amount of transpired water (Bacelar *et al.*, 2012). The difference in timescale of both processes (from seconds to months) and non-accounted energy expenses in growth and maintenance in the long-term water use can justify such differences (Maroco *et al.*, 2000). Effectively, Bacelar *et al*. (2007b) reported an absence of a significant association between $WUE_i$ and $WUE_{WP}$ in different cultivars of olive trees under drought conditions.

Reduced water availability also results in limited minerals uptake and diminished tissue concentrations, impairing crop plants development (Silva *et al.*, 2011). Transpiration drives water and nutrients uptake, so its drought-induced decline also impair roots nutrient absorbing power (Farooq *et al.*, 2012). In addition, drought declines soil-water potential, slowing the diffusion rate of nutrients between the soil matrix and root surface (Farooq *et al.*, 2009), and impairs the activity of enzymes involved in nutrient assimilation, disturbing nutrient acquisition (Farooq *et al*., 2012).

Drought is also associated with increased generation of reactive oxygen species (ROS) due to energy accumulation in drought-stressed plants, which increases the photo-oxidative effect. Usually, under moderate water deficit, drought tolerant plants increase the concentration of enzymatic and non-enzymatic antioxidants (Bacelar *et al*., 2007a; Flexas *et al.*, 2012), but when stress imposition increases occur an imbalance between ROS production and antioxidant defense (Farooq *et al*., 2012), damaging lipids, proteins, carbohydrates, pigments and

deoxyribonucleic acid (DNA) (Bacelar *et al*., 2006, 2007a; Farooq *et al*., 2009; Guerfel *et al*., 2009b; Petridis *et al*., 2012).

Stress conditions often stimulate changes in the production, distribution or signal transduction of phytohormones. In fact, through the action of these molecules plants responds to the adverse conditions modifying their physiology and biochemistry (Colebrook *et al.*, 2014). As already mentioned, ABA biosynthesis and accumulation was stimulated by drought, in association to a key role in $g_s$ regulation, but drought also influences other stress signaling pathways (Pantin *et al*., 2013; Vishwakarma *et al.*, 2017). Although ABA is considered the main stress hormone, there are other important hormones that play important roles in abiotic stresses responses, namely auxins (Aux). The Aux response to drought usually contradict with each other, varying from decrease (Man *et al.*, 2011; Du *et al.*, 2013) to increase (Pustovoitova *et al.*, 2004; De Diego *et al.*, 2012). Wang *et al.* (2008) reported a markedly decrease in this hormone with a transient increase during the initial stage of drought adaptation. Some studies have shown that drought influence the local Aux concentration and distribution by changes in Aux transport, allowing to maintain a balance between vegetative growth and survival (Shen *et al.*, 2010; Shojaie *et al.*, 2015). In fact, it was well demonstrated that Aux was unequally distributed within and between the different plant organs (De Diego *et al.*, 2013). Additionally, it was suggested that Aux could act as a stress hormone, direct or indirectly, once was observed that several auxin-responsive genes operate during stress signaling (Sharma *et al.*, 2015). However, hormone action cannot be considered in isolation, as the cross-talk between the different plant hormones results in synergetic or antagonic interactions that play crucial roles in response of plants to abiotic stress (Peleg and Blumwald, 2011).

#### 2.1.3.2. Heat and high irradiance

The presence of other abiotic stresses, such as heat and high irradiances, can exacerbate the effects of water deficit. With high temperature, plants might increase transpiration to heat load dissipation by evaporative cooling. However, involves negative consequences for plant water status and if water becomes limiting, evaporative cooling decreases and tissue temperature increase (Taiz and Zeiger, 2006). One of the main deleterious effects of high temperature is the imbalance between $A_n$ and R, as $A_n$ drops before R rates carbohydrate reserves decline and were not replaced (Taiz and Zeiger, 2006). Indeed, the negative influence of heat in $g_s$ and $A_n$ was already reported in olive trees (Koubouris *et al*., 2015). Heat stress also causes membrane damage and lipid peroxidation in olive leaf (Cansev, 2012; Koubouris *et al.*, 2015), enhancing

its permeability and, consequently, electrolyte leakage (Wahid *et al*., 2007). With high temperatures, protein denature and lose activity, and when temperatures rise above a critical value, the production of heat shock proteins (HSPs) is favored over the synthesis of many normal cellular proteins (Bray *et al.*, 2000).

Under excessive irradiances the use of absorbed light in both A and photorespiration and the thermal dissipation are not enough to cope with excess of excitation energy. Thus, the development of reducing power may result in ROS formation and photoinhibition (Connor and Fereres, 2005). Meanwhile, olive may cope well with ultraviolet B radiation (UV-B) in the absence of additional stressors (Koubouris *et al*., 2015), but a synergetic effect with heat impairs photosynthesis. High UV-B radiation also induce oxidative damage in olive leaf membranes, which may lead to higher activities of antioxidant enzymes, as guaiacol peroxidase and, specially, superoxide dismutase (Koubouris *et al*., 2015).

### 2.1.4. Olive tree strategies to withstand summer stress

The capacity of olive to grow under harsh conditions is due to the development of certain morphological, anatomical, physiological and biochemical adaptations (Fernández, 2014), benefiting from the memory effects caused by stress pre-exposure (Ben Abdallah *et al*., 2017). However, these mechanisms are activated at considerable expense to the plant in terms of energy, what causes a decrease in current-season production and compromise the vegetative development, impairing the next year production. Olive tree can slow the onset of stress (avoidance) by the ability to extract water from the soil and to restrict loss of water to the atmosphere. Moreover, tolerance is the ultimate drought strategy by the ability to sustain large internal water deficit and maintain sufficient metabolic activity for survival (Connor and Fereres, 2005). However, as defended by Chen *et al.* (2016) drought adaptability integrate much more than drought resistance (drought escape, drought avoidance and drought tolerance), playing recovery capacity a fundamental role to plants growth and survival. This take special importance in Mediterranean-type ecosystems, where plants are continuously exposed to repeated cycles of drought-rewatering during their life. Nevertheless, compared to the developing drought, the study of recovery has been neglected. Although drought is considered the primary stressor, others, as heat and high irradiance, especially in association, also impair plant functions and therefore different adaptative mechanisms are adopted by plants.

#### 2.1.4.1. Drought adaptability

Olive leaves are small, with high mesophyll compactness, grouped long sclereids in spongy parenchyma and two palisade layers, the major one associated with the upper epidermis (Bacelar *et al*., 2004; Ennajeh *et al.*, 2010), being the thickness and density especially marked under adverse conditions (Guerfel *et al*., 2009a; Tognetti *et al.*, 2009; Ennajeh *et al*., 2010; Boughalleb and Hajlaoui, 2011). This particular structure reduces the internal conductance to water vapor transport (Chartzoulakis *et al.*, 1999) and provide a great resistance to physical damage driven by desiccation (Mediavilla *et al.*, 2001). Olive leaves also present a thick cuticle that prevents water diffusion through the cuticular layer. In fact, cuticular conductance ($g_{cuticular}$) is negligible when compared with $g_s$ being the transpiration essentially limited to the stomata (Connor and Fereres, 2005). Moreover, leaf surface, especially the abaxial surface, are covered with a waxy layer and peltate trichomes (Bacelar *et al*., 2004), which increase water use efficiency (WUE), by increasing leaf boundary-layer resistance, and allowing leaves to take advantage of light rain or water condensation (Savé *et al.*, 2000).

Stomata of olive leaves are small and present only on abaxial surface (hypostomatous), being even smaller and denser in water shortage situations, increasing the control of water loss by transpiration (Ennajeh *et al*., 2010; Boughalleb and Hajlaoui, 2011). Moreover, the stomatal closure helps to maintain xylem water potential values above the safety threshold for loss of hydraulic conductance (Torres-Ruiz *et al*., 2013; Fernández, 2014). Although strong evidences showed that $g_s$ decreases as plant $\Psi$ becomes more negative (Guerfel *et al*., 2009a; Tognetti *et al*., 2009; Boughalleb and Hajlaoui, 2011), under severe conditions, the stomatal control over transpiration is not enough to prevent loss of hydraulic conductance (Tognetti *et al*., 2009). For some plant species, the permanent wilting point is reached when $\Psi$ = -1.5 MPa (Veihmeyer and Hendrickson, 1928), but since olive tissues can withstand very negative values of $\Psi$ (Xiloyannis and Dichio, 2003) the wilting point for olive range approximately between -2.5 MPa (Dichio *et al.*, 2003) and -3.5 MPa (Dichio *et al.*, 2006), or even have the huge capacity to sustain values below -8 MPa (Moriana *et al.*, 2003). In fact, Moriana *et al.* (2003) reported that rainfed olive trees with $\Psi$ around -8 MPa extracted more 40 mm of water below the conventional wilting point (-1.5 MPa). To rainfed orchards, in arid regions, this amount has significant importance since represent around 10-15% of annual transpiration (Orgaz and Fereres, 2008). During recovery, olive tree typically shows a conservative behavior, restoring rapidly the water status but exhibiting a slow recovery of $g_s$ (Torres-Ruiz *et al*., 2013; Perez-Martin *et al*., 2014). Torres-Ruiz *et al*. (2015) found that neither hydraulic nor non-hydraulic factors were able to

explain the delay in the full recovery of $g_s$. These authors proposed two explanations, one involves the restoration of certain aquaporins activity, not affecting leaf hydraulic conductance directly, but the balance of osmolytes in the cells; and the other involves the occurrence of a metabolic limitation, as the increase in ABA in guard cells under drought induces the expression of hexokinases, which accelerates the stomatal closure. On the other hand, the hexokinases are also involved in sugar sensing and stimulation of the osmolytes balance that should be restarted after the recovery of water status. Olive trees pre-exposed to drought also recovers $A_n$ faster than $g_s$ after stress relief (Ben Abdallah *et al*., 2017).

Olive tree show a high resistance to drought-induced embolism, essentially due to the small diameter of the xylem vessels and high density, leading to low xylem hydraulic conductivity that limit transpiration (Bacelar *et al*., 2007b; Dichio *et al.*, 2013; Torres-Ruiz *et al*., 2013). Furthermore, olive root system grows quite parallel to the soil and the highest root density is found close to the trunk surface being more suitable to absorb the light and intermittent rainfall, typical of its habitat, than water from deep layers (Fernández and Moreno, 1999). In addition, under low water potential, olive tree also slows or even stops canopy growth, but still presented some net photosynthesis, allowing the production of photoassimilates that are particularly accumulated in root system (Xiloyannis *et al.*, 1999; Sofo *et al*., 2008). So, it is usual an increase in root/canopy ratio (Xiloyannis *et al*., 1999; Ben Abdallah *et al*., 2017), adjusting the demand for transpiration and soil water uptake. Olive tree also benefits from hydraulic redistribution, the ability of deep roots to uptake water in moist soil layers to maintain transpiration during the hot dry season and to redistribute soil water through different root types, reducing the intensive drying of the upper soil layers (Nadezhdina *et al.*, 2015).

To ensure the hydraulic conductance, maintaining water flow from roots to leaves, olive tree decreases the water potential of their tissues in relation to the soil, establishing a particularly high gradient between leaves and roots (Sofo *et al*., 2008; Dichio *et al.*, 2009). Under drought conditions olive tree display a strong capacity to osmotic adjustment (OA), the accumulation of solutes, both in leaves and roots (Dichio *et al*., 2003, 2006; Sofo *et al.*, 2004, 2008; Ben Abdallah *et a*l., 2017). This mechanism decreases the osmotic potential, creating a soil-plant water gradient, which enables the extraction of water from soil at water potential below the wilting point (Dichio *et al*., 2006). OA is linked with passive and active osmotic regulation mechanisms, an increase in solute concentration resulting from symplastic water loss (Dichio *et al*., 2006), and an accumulation or *de novo* synthesis of solutes within cells (Sanders and Arndt, 2012), respectively. Two major classes of solutes can lower the osmotic potential of

tissues: inorganic cations and anions and organic compatible solutes, such as sugars, sugar alcohols, amino acids (notably proline), and quaternary ammonium compounds (notably glycine betaine) (Patakas *et al*., 2002; Sanders and Arndt, 2012). Some of the organic solutes can also protect cellular proteins, enzymes and cellular membranes and allow the metabolic machinery to continue functioning (Sanders and Arndt, 2012).

Finally, the regulation of the antioxidant system is one of the most relevant mechanisms against oxidative stress caused by ROS. ROS plays a double role in plant physiology, but whether ROS would act as signaling molecules or might cause oxidative stress to the tissues depend on the refined balance between its production and scavenging (Mattos and Moretti, 2015). In a study conducted by Ben Abdallah *et al*. (2017) it was demonstrated that upon rewatering olive trees still exhibited higher levels of hydrogen peroxide ($H_2O_2$), a known signaling ROS, possibly to maintain the antioxidative system in alert. Moreover, olive trees that were drought-primed showed an alleviation in oxidative stress in relation to plants exposed to drought for the first time. Indeed, in response to drought the increment in some antioxidant enzymes activities, as ascorbate peroxidase, catalase, superoxide dismutase and glutathione reductase and/or in non-enzymatic antioxidant mechanisms, as the accumulation of phenolic compounds, tocopherols, carotenoids, ascorbate and glutathione, were commonly described in olive tree (Bacelar *et al*., 2006, 2007a; Sofo *et al*., 2008; Guerfel *et al*., 2009b; Petridis *et al*., 2012; Ben Abdallah *et al*., 2017).

#### 2.1.4.2. Heat and high irradiance adaptability

Many of the mechanisms developed to counteract drought stress are also responsible for protection against co-occurring irradiance and heat stresses. Nevertheless, there are some specific resistance mechanisms associated.

The silver-white color conferred by trichomes to the abaxial surface of the leaves and the extremely bright upper surface, due to the presence of a thick cuticle, also limits sunlight absorption by solar radiation reflection (Rapoport, 2008) and protects against UV-B radiation damage (Sebastiani *et al.*, 2002). Such high reflectivity capacities assist with the dissipation of sensible heat, which takes special importance under drought conditions with stomatal closure (Connor and Fereres, 2005). Additionally, the small size and the adoption of vertical position (paraheliotropism) by the leaves help to reduce solar radiation interception, and the high sclerophylly also reduce the transmissivity of PAR within the leaf (Mariscal *et al.*, 2000). To minimize damage by photoinhibition olive tree also regulate thermal dissipation directly in the

chlorophyll carotenoid-binding antennae complex of PSII, involving the xanthophyll cycle (Demmig-Adams and Adams, 1996; Connor and Fereres, 2005). The xanthophyll cycle has a specific photoprotection function since these pigments can accept excitation energy from excited chlorophylls, preventing the oxidative disruption of the photosynthetic process (Demmig-Adams *et al.*, 1996). Under excess light, violaxanthin is converted rapidly into zeaxanthin, the de-epoxidized form that better dissipates energy, via the intermediate antheraxanthin by the enzyme violaxanthin de-epoxidase (Demmig-Adams and Adams, 1996; Taiz and Zeiger, 2006).

Olive, like other plant species, also respond to heat stress by producing HSPs, a family of stress proteins thought to be involved in chaperone functions and repair, solving the problem of misfolding and aggregation (Al-Whaibi, 2011; Assab *et al.*, 2011).

## 2.2. Summer stress mitigation strategies

As discussed in the previous section, in a climate change context is realistic to expect harmful consequences to the olive crop. Therefore, it is required to adopt agronomic strategies to maintain and improve the olive sector sustainability and competitiveness in the Mediterranean Region. There is a large set of measures that can and should be adopted in this regard. The long-term measures, as cultivars selection (Therios, 2009), breeding (Pais, 2013) and appropriate orchard design (Bacelar *et al*., 2012), are of utmost importance. However, these measures are time consuming, and the short-term ones must be evaluated in parallel. The resource to irrigation is the most effective short-term strategy (Fernández, 2014), but with the Mediterranean Region characteristics it is difficult to implement in olive groves, being rainfall often the only water support (Pastor, 2008). Other short-term strategies may include soil management (e.g. cover crops) (Pastor, 2008), canopy management (e.g. pruning intensity) (Lopes *et al*., 2009) and the application of mitigating products (e.g. kaolin and salicylic acid) (Glenn and Puterka, 2005; Khan *et al*., 2015). Meeting the main objective of this thesis, in the following subsections the use of kaolin (KL) and salicylic acid (SA) will be reviewed in detail.

### 2.2.1. Kaolin

#### 2.2.1.1. Characterization and action on abiotic stress alleviation

KL ($Al_2Si_2O_5(OH)_4$) is a white mineral, chemically inert, non-abrasive and non-toxic that easily disperses in water. Once sprayed as suspension on the leaf surface, water evaporates leaving a protective particle film (Glenn and Puterka, 2005; Cantore *et al.*, 2009). Produced as

particle film technology, is a combined synthesis of knowledge on mineral technology, insect behavior, and light physics applied to pest control and plant physiology (Glenn and Puterka, 2005). KL particle film increases the reflection of excess radiation (photosynthetically active, PAR; ultraviolet, UV; infrared, IR), reducing the risk of leaf and fruit damage from heat load accumulation and solar injury (Glenn and Puterka, 2005; Glenn, 2012), thereby reduces water loss trough transpiration and improves plant water status (Figure 1). To obtain the desired results on plant tissues, an effective particle film must have certain characteristics, especially the particles must have a diameter < 2 μm, must be formulated to spread and create a uniform film, must transmit PAR and UV and IR radiation to some degree, do not interfere with gas exchange from the plant organs, and it is fundamental that can be removed from harvested commodities (Glenn and Puterka, 2005).

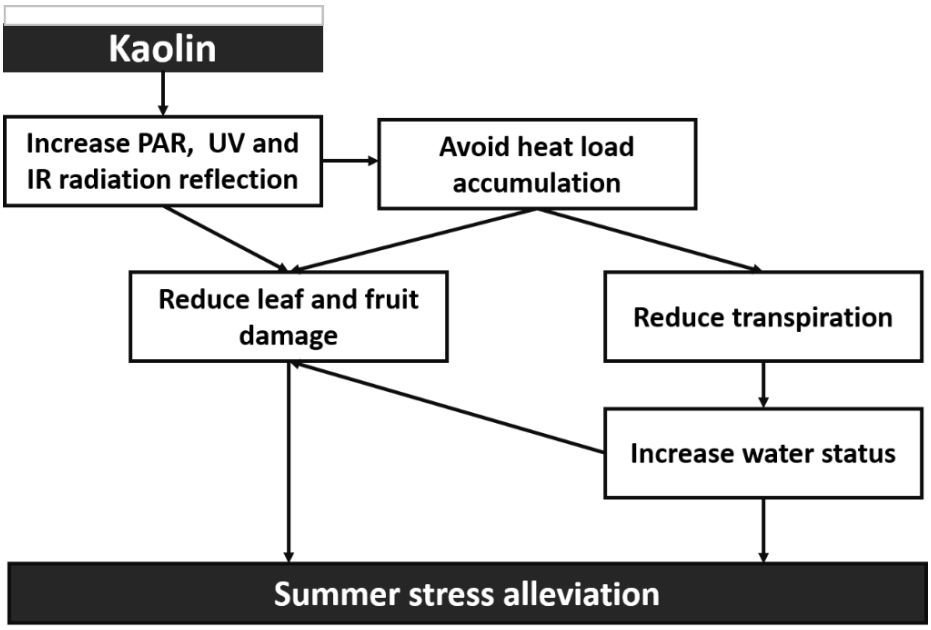


**Figure 1.** Schematization of kaolin action on the alleviation of summer stress.

#### 2.2.1.2. Effects on crops

KL effects on leaf and canopy temperatures, gas exchange, metabolic processes, growth, yield and plant products quality is not fully understood. In fact, it depends on several factors that may act in combination or in isolation: (i) applied concentration, as high concentration reduce the availability of PAR (Rosati *et al.*, 2006; Shellie and King, 2013); (ii) plant species and cultivars (Glenn *et al.*, 2005; Shellie and Glenn, 2008; Glenn, 2009; Roussos *et al.*, 2010;

Denaxa *et al.*, 2012); (iii) environmental conditions, as usually KL improve plant performance under harsh conditions, when plants cannot use all PAR that reach the leaves (Glenn *et al.*, 2003; Jifon and Syvertsen, 2003; Glenn, 2009; Denaxa *et al*., 2012; Boari *et al.*, 2015; Brillante *et al.*, 2016) (iv) dimension of the canopy/plant age, as different responses are expected when open are compared with dense canopies, related with changes on light distribution within the canopy and on amount of carbon consumed in respiration process (Rosati *et al.*, 2007; Cantore *et al*., 2009; Glenn, 2009) and (v) duration of the experiments, as in the long-term the influence of factors iii and iv are higher, as well the capacity to develop resistance mechanisms against stress factors may be different, including recover capacity, and thus dissimilar results can occur (Shellie and Glenn, 2008; Nanos, 2015).

KL particle film technology has been tested as a tool for saving water and improve crop performance since can alter the microclimate around sprayed leaves. Although some negative effects are recorded in specific conditions (Rosati et al., 2006; Roussos et al., 2010; Brilliant *et al.* 2016), a positive influence is more generalized. It is recurrent to find a decrease in KL-sprayed organs temperature, as already reported in a substantial number of crops (Rosati *et al*., 2006; Glenn, 2009; Denaxa *et al*., 2012; Correia *et al.*, 2014; Boari *et al*., 2015; Segura-Monroy *et al.*, 2015 AbdAllah, 2017). Additionally, some studies have been demonstrated its effect in solar radiation reflection (Rosati *et al*., 2006, 2007; Shellie and King, 2013), being described an increase of 86% in PAR reflection from KL-sprayed (5% w/v) olive trees (Nanos, 2015). Moreover, as the formed film not only reflects PAR and UV, but also IR (Brillante *et al*., 2016), the reduction of leaf-to-air vapor pressure deficit ($VPD_{leaf\text{-}air}$) is also a regular effect (Jifon and Syvertsen, 2003; Glenn and Puterka, 2005; Rosati *et al*., 2006).

KL film also have repercussions on water relations and leaf structural characteristics. The improvement of plant water status is a regular effect of KL application in stressed plants (Glenn *et al*., 2010; Denaxa *et al*., 2012; Boari *et al*., 2015; Nanos, 2015; AbdAllah, 2017; Dinis *et al.*, 2018). Some reports argued that KL may contrary the effects of stress on leaf structure (discussed in section 1.3). For instance, KL application increased succulence and decreased density (Denaxa *et al.*, 2012) and leaf mass area (LMA) of olive leaves (Nanos, 2015), and increased stomatal density and decreased the thickness and trichomes density of cape glossberry leaves (Segura-Monroy *et al*., 2015). This is easily explained, as the continuous white coat formed over the leaf causes a shade effect and artificially increase leaf thickness, increasing the path length of radiation to target cells within leaf (Glenn and Puterka, 2005); second, by reducing the heat load and the excessive water losses contributes to keep cells hydration; third,

stomatal density increases under moderate drought followed by a decrease with the rising of drought severity (Xu and Zhou, 2008), and decreases with temperature increase (Beerling and Chaloner, 1993).

KL was also found to largely influence gas exchange and photochemistry responses. Much studies support the theory that KL benefits on photosynthetic activity mainly occur under harsh conditions, when plants cannot use all the radiation that reach the leaves, while can decrease when the conditions for $A_n$ were optimal, due to the reduction light availability by increasing reflection. In fact, under water-limited environments, high temperatures, high PAR and/or large vapor pressure deficit (VPD), $g_s$ and $A_n$ were positively influenced in several species (Jifon and Syvertsen, 2003; Glenn, 2009; Denaxa *et al*., 2012; Correia *et al*., 2014; Boari *et al*., 2015; Nanos, 2015; Dinis *et al*., 2018). Moreover, the improvement in photosynthetic activity was also associated with the preservation of photosynthetic machinery integrity, as confirmed by the chlorophyll *a* fluorescence traits (Jifon and Syvertsen, 2003; Correia *et al*., 2014; Segura-Monroy *et al*., 2015; Dinis *et al*., 2016a, 2018). Nevertheless, the absence of influence with stress prevalence and/or high stress intensity had also been described (Shellie and Glenn, 2008; Nanos, 2015).

Despite KL function as an antitranspirant, the few studies that accessed leaf transpiration rate (E), revealed that KL may induce an increase on $g_s$ at a higher extent than on E (Correia *et al*., 2014; Boari *et al*., 2015). A possible explanation may be the reduced $VPD_{leaf\text{-}air}$ (Jifon and Syvertsen, 2003; Rosati *et al*., 2006), what can reduce the driving force for transpiration, while possibly promote an increase in $g_s$ (Zhang *et al.*, 2017). Due to the different variations in A and $g_s$ intrinsic water use efficiency ($WUE_i$) is differently affected by KL application, being usually higher with KL spray under stressful conditions (Jifon and Syvertsen, 2003; Correia *et al*., 2014; Boari *et al*., 2015; Brillante *et al*., 2016; Dinis *et al*., 2018;).

Interestingly, in some cases, although KL may affect negatively $A_n$ at leaf scale, this is not reflected at whole canopy scale and/or in plant biomass accumulation. For instance, concomitant with a decrease in photosynthesis at leaf scale, Roussos *et al*. (2010) described an increase in canopy dry weight of young olive trees and Rosati *et al*. (2007) estimate an increase in whole canopy $A_n$ of almond and walnut trees. This happens because KL alters light distribution within the canopy, increasing the incident radiation on inner-canopy leaves, that was previously shaded or partially-shaded (Glenn, 2009; Rosati *et al*., 2007).

The influence of KL on leaf biochemistry has been little studied, remaining basically on the photosynthetic pigments composition. Generally, KL prevents chlorophyll degradation

(Glenn *et al*., 2003; Roussos *et al*., 2010; Correia *et al*., 2014; Nanos, 2015; Segura-Monroy *et al*., 2015) and improves carotenoids concentrations (Shellie and King, 2013; Correia *et al*., 2014; Dinis *et al*., 2016b). KL also reduces clorophyll a/chlorophyll b ratio ($Chl_a/Chl_b$) (Shellie and King, 2013; Nanos, 2015) and increases total chlorophylls ($Chl_{(a+b)}$)/ carotenoids (Car) ratio (Shellie and King, 2013; Correia *et al*., 2014), typical features of low-light adapted leaves (Lichtenthaler *et al*., 2007). Meanwhile, Dinis *et al*. (2016b) and Bernardo *et al*. (2017) demonstrated that KL boosts the antioxidant defense systems and reduce the oxidative damages of grapevine leaves. Dinis *et al*. (2018) also reported a positive influence on total soluble proteins concentrations and changes in hormonal dynamics, with a slightly decrease in ABA and an increase in indoleacetic acid (IAA). Regarding mineral composition, Al-Absi and Archbold (2016) found no significant influence of KL application in apple trees under different irrigation regimes.

Although KL generally exerts a positive influence on plant growth and/or biomass accumulation (Roussos *et al*., 2010; Javan *et al*., 2013; Segura-Monroy *et al*., 2015), the positive influence on yield is more recognized (Glenn *et al*., 2003; Saour and Makee, 2003; Cantore *et al*., 2009; Javan *et al*., 2013; Correia *et al*., 2014; Segura-Monroy *et al*., 2015; AbdAllah, 2017). Moreover, in general KL also increases harvests quality. For instance, in olive trees were reported increase in fruit dry matter, oil content, oxidative stability and shelf life of the extracted olive oil and a reduction in free acidity (Saour and Makee, 2003; Khaleghi *et al*., 2015; Nanos, 2015). In apple trees, KL increased the postharvest fruit quality, reducing the loss of weight, soluble solids and titratable acidity after 70 days of storage (Ergun, 2012). In grapevines, KL increased the phenolics concentrations on berries, as well anthocyanins, vitamin C, and the antioxidant capacity (Shellie and King, 2013; Brillante *et al*., 2016; Conde *et al*., 2016; Dinis *et al*., 2016b). KL also increased lycopene in tomato (Cantore *et al*., 2009) and changed pepper mineral composition, increasing Ca, Na and B and decreasing Al concentration (Makus, 2005).

### 2.2.2. Salicylic acid

#### 2.2.2.1. Characterization and action on abiotic stress tolerance

Salicylic acid ($C_7H_6O_3$) is a naturally occurring phenolic compound and endogenously synthetized signaling molecule in plants (Khan *et al*., 2015). This molecule is known to regulate several physiological mechanisms in plants, as flowering induction, hormonal status,

photosynthesis, respiration, solutes transport and metabolism, antioxidant defense system, plant water relations, minerals uptake and defense responses against infections and pathogen attacks (Abd El-Razek *et al*., 2013; Kumar, 2014; Miura and Tada, 2014; Khan *et al*., 2015; Nazar *et al*., 2015). Moreover, increasing evidences suggest a key role of SA in response to multiple abiotic stresses, both the natural accumulation in plants subjected to stressful conditions (Bandurska, and Cieślak, 2013; Choudhary and Agrawal, 2014), and the induction of protective mechanisms by its exogenous application in plants subjected to major abiotic stresses, as drought (Kang *et al*., 2012; Nazar *et al*., 2015), salinity (Fayez and Bazaid, 2014; Khan *et al*., 2014), heat (Wang and Li, 2006; Wang *et al*., 2010), and high light (Zhao *et al*., 2011; Wang *et al*., 2014).

Nevertheless, the precise mechanisms by which SA induces plant tolerance against abiotic stresses remain least discussed and more comprehensive investigations are needed in this direction (Khan *et al*., 2015). It is believed that abiotic stress tolerance induction is related with the dual redox effect of SA, a first oxidative phase, characterized by a transient increase in ROS levels, that is followed by an increase in reducing power (Herrera-Vásquez *et al*., 2015). Some studies reported that SA pre-treatment lead to an initial increase in $H_2O_2$ levels, possibly by the inhibition of some antioxidant enzymes, such as catalase (Gunes *et al*., 2007; Belkadhi *et al*., 2014; Hao *et al*., 2014). Then, the slight increase in ROS levels triggers defense responses, mediated by the increase in the antioxidant enzymatic system (*e. g.* catalase, ascorbate peroxidase, superoxide dismutase) or by the synthesis of antioxidant metabolites and protective compounds (*e. g*. glutathione, ascorbic acid, phenolic compounds, heat shock proteins (HSPs), osmolites) (Horváth *et al*., 2007; Wang *et al*., 2010; Misra and Misra, 2012; Li *et al*., 2013; Khan *et al*., 2014; Herrera-Vásquez *et al*., 2015). SA might also induce genes responsible for protective mechanisms (Jumali *et al*., 2011; Li *et al*., 2013) and the production of signal transducers (e. g. ROS, protein kinases and ABA) (Sakhabutdinova *et al*., 2003; Horváth *et al*., 2007; Jesus *et al.*, 2015) (Figure 2). Nonetheless, lower concentrations of SA are generally more efficient than higher ones (Kang *et al*., 2012; Miura and Tada, 2014). High concentrations can cause a high level of ROS and then oxidative stress that plants are unable to overcome (Horváth *et al*., 2007; Hara *et al*., 2012; Miura and Tada, 2014).

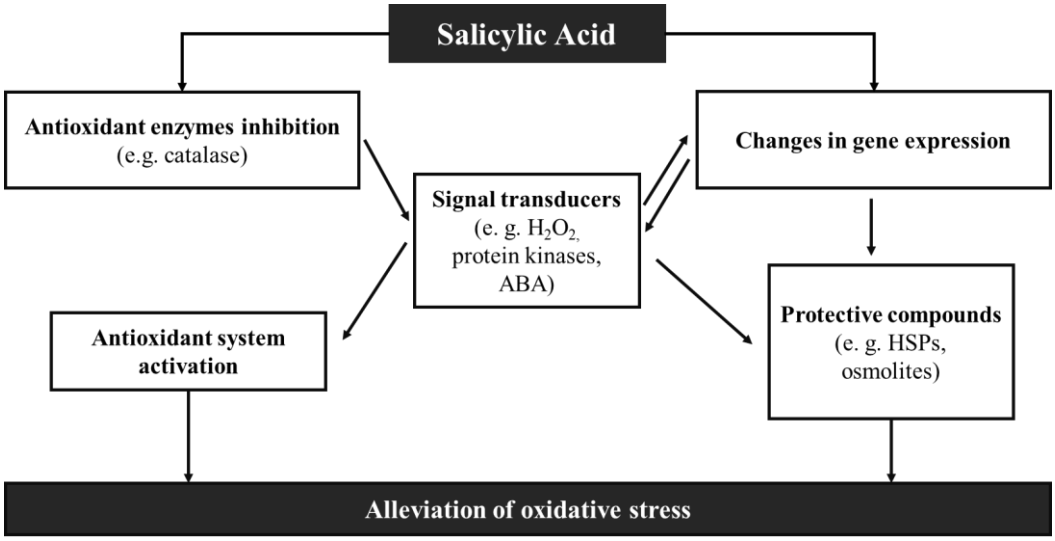


**Figure 2.** Schematization of salicylic acid action on the alleviation of oxidative stress (Adapted from Horváth *et al*., 2007).

#### 2.2.2.2. Effects on crops

It is frequent that exogenous SA effects on plant resistance to abiotic stress depend on a set of factors: (i) applied SA concentration (Kang *et al*., 2012; Agami *et al*., 2013; Jesus *et al*., 2015), as normally, for most plant species, the optimal levels for the maximum stress tolerance range between 0.1 mM to 0.5 mM (Hara *et al*., 2012); (ii) method of SA administration, as pre-soaking, addition to the growth medium or foliar spray (Singh and Usha, 2003; Kang *et al*., 2012); (iii) plant species and cultivars (Umebese *et al*., 2009; Khalil *et al*., 2012); (iv) plant developmental stage (Umebese *et al*., 2009; Ahmad *et al*., 2014); (v) kinds of stress (Sakhabutdinova *et al*., 2003; Fayez and Bazaid, 2014); (vi) stress level (El-Tayeb, 2005; Alam *et al*., 2013; Hashempour *et al*., 2014); (vii) system were the study was carried out, from cell suspensions to the whole plant (Pál *et al*., 2013); and (viii) frequency of application (Shaaban *et al*., 2011).

It is generalized that an accurate SA application improves abiotic stress tolerance in plants, by modulation of several important aspects of plants function and structure, with relevant consequences on growth, yield and harvests quality. Indeed, in a study conducted by Kang *et al.* (2012), the SA pre-treatment of droughted wheat seedlings regulates several proteins associated with signal transduction, stress defense, photosynthesis, carbohydrate metabolism, protein metabolism and energy production. Moreover, it is recurrent to find a positive influence of SA application on several physiological responses in stressed plants, including on water

relations and photosynthetic variables. A great number of studies reported that SA application improves water status of different plant species (Kang *et al*., 2012; Alam *et al*., 2013; Ahmad *et al*., 2014; Jesus *et al*., 2015; Nazar *et al*., 2015). Moreover, it is quite frequent to observe a positive effect on net photosynthesis, due to increase of stomatal conductance, Rubisco activity, chlorophyll concentration, quantum efficiency of PSII and electron transport rate or, at least, increase in some of the reported variables (Wang *et al*., 2010; Zhao *et al*., 2011; Ahmad *et al*., 2014; Khan *et al*., 2014; Wang *et al*., 2014; Jesus *et al*., 2015; Nazar *et al*., 2015). Interestingly, it was also reported a faster recovery of these variables after the end of heat and high light stress imposition (Wang *et al*., 2010, 2014; Zhao *et al*., 2011).

There are also evidences that SA affect leaf and chloroplast structure of stressed plants. SA increased leaf constituents thickness and the number and diameter of vessels of droughted plants (Agami, 2013). Similarly, SA increased leaf thickness of salt stressed plants, and the distance between vessels, reduced stomatal number and maintained the chloroplasts centrifugal organization (Cárcamo *et al*., 2012).

Some of the negative effects of stresses on plant function are mediated by the associated phenomenon of oxidative stress, thus ROS homeostasis is a convergence point to evaluate plant stress status. The available evidence supports that SA pre-treatment is related with reduced ROS accumulation under stressful conditions (Alam *et al*., 2013; Khan *et al*., 2014; Wang *et al*., 2014; Nazar *et al*., 2015). Moreover, SA also improved cell membrane integrity of stressed plants, a signal of lower oxidative stress (Agami *et al*., 2013; Alam *et al*., 2013; Hashempour *et al*., 2014; Fayez and Bazaid, 2014; Khan *et al*., 2014; Jesus *et al*., 2015; Nazar *et al*., 2015). Additionally, there are also evidences that SA prevents photosynthetic pigments stress-induced degradation (Agami *et al*., 2013; Alam *et al*., 2013; Ahmad *et al*., 2014; Fayez and Bazaid, 2014).

To alleviate oxidative stress, plants activate both enzymatic and non-enzymatic antioxidant mechanisms and increase the production of osmolites, responses that were found to be highly regulated by SA application (Khan *et al*., 2015). In general, strong evidences suggest that SA induces the enzymatic antioxidant system, including catalase, superoxide dismutase, ascorbate peroxidase, peroxidase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase, polyphenol oxidase, monodehydroascorbate reductase and dehydroascorbate reductase activities (Singh and Usha, 2003; Wang and Li, 2006; Alam *et al*., 2013; Hashempour *et al*., 2014; Khan *et al*., 2014; Wang *et al*., 2014). SA was also found to increase the transcription of certain ascorbate- glutathione cycle-related genes, allowing the maintenance of

higher contents of ascorbate and glutathione (Kang *et al*., 2013; Alam *et al*., 2013; Li *et al*., 2013; Khan *et al*., 2014), and to improve reduced-to-oxidized ascorbate and reduced-to-oxidized glutathione ratios (Wang and Li, 2006; Alam *et al*., 2013). By other side, while some studies reported increases in phenolic compounds (Hashempour *et al*., 2014; Jesus *et al*., 2015) and proteins (Agami *et al*., 2013; Hashempour *et al*., 2014) in response to SA application, others showed no influence and/or opposite responses for both phenolics (Agami *et al*., 2013; Fayez and Bazaid, 2014) and in soluble proteins (Singh and Usha, 2003; El-Tayeb, 2005; Fayez and Bazaid, 2014).The induction of osmolytes accumulation by SA application is frequent (Khan *et al*., 2015), including increases of soluble carbohydrates (Ahmad *et al*., 2014; Jesus *et al*., 2015), proline (Misra and Misra, 2012; Ahmad *et al*., 2014; Hashempour *et al*., 2014; Nazar *et al*., 2015) and glycinebetaine (Misra and Misra, 2012; Khan *et al*., 2014). However, in some studies SA was also associated with a reduced accumulation of soluble carbohydrates and proline (Agami *et al*., 2013; Fayez and Bazaid, 2014).

Moreover, SA also modulates mineral nutrients uptake and metabolism. Generally, SA improves its accumulation and changing its allocation among different plant organs (El-Tayeb 2005; Gunes *et al*. 2007; Khan *et al*. 2015; Nazar *et al*., 2015). SA also regulate several plant responses through signaling cross-talks with other phytohormones. For instance, the pretreatment with SA prevented salinity and drought-induced decline in indole-3-acetic-acid (IAA) (Sakhabutdinova *et al*., 2003; Fahad and Bano, 2012), promoted ABA accumulation (Sakhabutdinova *et al*., 2003; Jesus *et al*., 2015) and restricted ethylene formation (Khan *et al*., 2014; Nazar *et al*., 2015).

In line with the main SA-induced physiological and biochemical effects, plant growth and productivity are also modulated by SA. Indeed, several studies reported the increase of plant growth and/or biomass accumulation (Kang *et al*., 2012; Agami *et al*., 2013; Ahmad *et al*., 2014; Fayez and Bazaid, 2014; Khan *et al*., 2014; Nazar *et al*., 2015; Aliniaeifard *et al*., 2016) and crops yield (Javaheri *et al*., 2012; Khalil *et al*., 2012; Abd El-Razek *et al*., 2013; Kazemi *et al*., 2013). Furthermore, the harvests quality is also determined by the application of SA. Although negative effects were already reported in peaches (El-Shazly *et al*., 2013), the positive influence seems to prevail, as in tomatoes where SA increase fruit skin diameter, the amount of vitamin C and lycopene and Brix index (Javaheri *et al*., 2012), in strwberry where SA increase total soluble solids, titratable acidity, phenolics and vitamin C (Kazemi *et al*., 2013), and in olives where SA increase fruit and pulp weight and oil contents Khalil *et al*., 2012; Abd El-Razek *et al*., 2013).

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# CHAPTER 3

## Role of nighttime water balance under contrasting water regimes

## Briefing note

This chapter covers the nighttime water balance process of olive trees subjected to contrasting water regimes. Although usually neglected, nighttime water balance can have serious implications in plant water use efficiency, displaying nighttime stomatal open and the consequent water losses a key role in this process. But, if there is no opportunity for carbon gain, why do plants maintain a nighttime stomatal conductance? Some clues have been pointing to answer this question, but no argument is fully accepted yet. In addition, the occurrence and significance of this phenomenon in olive tree species and under drought conditions is even less explored. Considering the forecasted climate scenarios of drought and nighttime temperatures rise, it is mandatory to look deeper into this plant process.

In this context, this chapter is an adaptation of a research article entitled "The role of nighttime water balance on *Olea europaea* plants subjected to contrasting water regimes" published in *Journal of Plant Physiology* (226, 56-63). This article aimed to respond to the specific objective 1 of this thesis, "to get a more comprehensive information about the olive tree response to drought, specifically the role of nighttime water balance", having contributed to improve the scientific advance in the theme. This work revealed a different plant behaviour between different water regimes and exposed some clues regarding the mechanisms underlying this process. This work showed that nighttime water losses should be considered in simulation models and revealed the importance of selection of cultivars and the adoption of agronomic practices that enable a lower nighttime transpiration.

The authors contribution for the article converted in the present chapter was: Cátia Brito was responsible for establish and maintain the experiment, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and in the critical review of the article. Helena Ferreira collaborated in data collection on the field and assisted in the laboratory analysis. Carlos Correia was responsible for design the experiments, data collection on the field and critical review of the article. All the authors reviewed and approved the final manuscript.

## 3.1. The role of nighttime water balance on *Olea europaea* plants subjected to contrasting water regimes

## The role of nighttime water balance on Olea europaea plants subjected to contrasting water regimes

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### Abstract

The climate change scenarios besides foreseeing a severe drought imposition also emphasize the temperature rising in the Mediterranean region, with special prominence at nighttime. Despite the high olive tree tolerance to severe environmental conditions, stomatal nighttime water loss can change plant water relations, and the related consequences and opportunities, especially under water scarcity, must be clarified. A set of 3-year-old potted olive trees were subjected to three cycles of drought, imposed by withholding irrigation, while another group were continuously irrigated. At the end of the latter and more severe drought cycle, daytime gas exchange parameters, water status and membrane integrity were negatively affected by drought imposition. Moreover, the nighttime transpiration rate was far above cuticular water loss, suggesting sustained stomatal aperture during nighttime, leading to substantial water losses, which was higher under drought in the first hours of darkness. The higher nighttime stomatal conductance of droughted plants were related with higher starch concentration in their leaves, a thicker trichome layer and a lower intercellular $CO_2$ concentration, in a closely association with an inferior nighttime respiration. Still, whole-plant transpiration on droughted plants were much lower than leaf transpiration-based estimates, which is interpreted as compensation by water inputs due to dew deposition on leaves. Although unexpected, the increased of stomatal conductance in the first hours of the night, until a certain level of water deficit intensity, could be linked with potential benefits to the plants.

**Key-words:** olive tree; stomatal opening; transpiration; drought; nocturnal warming.

## 1. Introduction

Drought and high temperature stresses impair several physiological processes, like photosynthesis and water status (Fernández, 2014), and seriously affect membrane stability, increasing the permeability and leakage of ions (Elbasyoni *et al*., 2017), that may lead to decrease vegetative growth and yield. Moreover, those negative effects can be exacerbated by the projections of climate change in the Mediterranean, which anticipate a general reduction in rainfall and an increase in temperature, the last one more markedly at nighttime (IPCC, 2013). Since nocturnal warming could affect plants in several ways, the nighttime transpiration and respiration are worth considering. However, as far as we know these aspects are been poorly investigated.

Although nighttime stomatal opening is unexpected because there is no opportunity for carbon gain and the need to cool leaves is reduced or absent (Caird *et al*., 2007), a substantial leaf nighttime stomatal conductance ($g_{night}$) and transpiration ($E_{night}$) was observed in a wide range of species from different functional groups and ecosystems (Snyder *et al*., 2003; Dawson *et al*., 2007; Ogle *et al*., 2012; Resco de Dios *et al*., 2015). However, there are several factors that can determine those responses, such as soil water (Caird *et al*., 2007; Dawson *et al*., 2007; Howard and Donovan, 2007; Escalona *et al*., 2013; Zeppel *et al*., 2014) and nutrient availability (Caird *et al*., 2007), vapor pressure deficit (VPD) (Daley and Phillips, 2006; Caird *et al*., 2007; Dawson *et al*., 2007; Zeppel *et al*., 2014), wind speed (Daley and Phillips, 2006; Dawson *et al*., 2007), $CO_2$ concentration (Caird *et al*., 2007; Zeppel *et al*., 2014), dusts, aerosols and dew and/or fog (Burkhardt, 2010), previous day environmental conditions (Caird *et al*., 2007; Easlon and Richards, 2009), net photosynthesis ($A_n$) (Easlon and Richards, 2009) and stomatal conductance during the day ($g_{day}$) (Snyder *et al*., 2003), carbohydrate metabolism (Easlon and Richards, 2009; Resco de Dios *et al*., 2015), circadian rhythms (Caird *et al*., 2007; Resco de Dios *et al*., 2015) and leaf age (Caird *et al*., 2007; Zeppel *et al*., 2014). The occurrence and magnitude of gnight and Enight display conflicting patterns and the generalization about the factors that affect those traits is still not possible. Additionally, there are strong evidences that the responses are both species and cultivar dependent (Snyder *et al*., 2003; Daley and Phillips, 2006; Flexas *et al*., 2010; Ogle *et al*., 2012; Escalona *et al*., 2013).

Although recently we have noticed a growing awareness about $g_{night}$ and $E_{night}$, their implication in physiological processes remain unclear (Ogle *et al*., 2012; Escalona *et al*., 2013; Coupel-Ledru *et al*., 2016). The $g_{night}$ and, consequently, $E_{night}$ affect plant water balance, water use efficiency (WUE) (Escalona *et al*., 2013) and hydraulic redistribution (Howard *et al*., 2009).

In fact, substantial water losses have been reported to occur overnight at leaf and plant scales, strongly impacting global evapotranspiration (Forster, 2014; Resco de Dios *et al*., 2015). Nevertheless, it has also been postulated several benefits related with the continue water loss during the night that may outweighs those costs, including the improvement of nutrient uptake (Snyder *et al*., 2003; Scholz *et al*., 2007; Snyder *et al*., 2008;), preventing excess cell turgor at night when water availability increases and leaves still contain substantial contents of osmoticants (Donovan *et al*., 1999, 2001), supplying $O_2$, enhancing the capacitance of the trunk and stem (Daley and Phillips, 2006) and preventing $CO_2$ build-up in leaves from nighttime dark respiration ($R_{night}$) (Marks and Lechowicz, 2007). The prevalence of $g_{night}$ and $E_{night}$ in some conditions and the potential influence in plant growth and physiology can indicate a widespread behavior and an adaptive process that must be clarified. Furthermore, those outcomes have implications in plant water relations theory and in the studies, that utilize plant water use data at larger scales (Dawson *et al*., 2007).

Olive tree (*Olea europaea* L.) is a common species of Mediterranean region that displays important morphological and physiological adaptive mechanisms to withstand the environmental constraints that characterizes Mediterranean climate. This capacity, includes the ability to control leaf transpiration by a high thickness and density of the leaves, associated to a dense peltate trichomes layer (Bacelar *et al*., 2004). Additionally, also involves an efficient capacity to regulate stomatal aperture, and the effective ability to extract water from soils with very low water potential and/or to sustain very low internal water deficits (Connor and Fereres, 2005). However, there is a lack of information and understanding about $g_{night}$ and $E_{night}$ mechanisms in *Olea europaea*. Arquero *et al*. (2006) mentioned a very low stomatal conductance during the night, becoming to rise 3 h before dawn in olive cuttings of cv. Chemali de Sfax. In addition, the study of $g_{night}$ and $E_{night}$ by leaf-level gas exchange includes the loss across both cuticular and stomatal components (Caird *et al*., 2007). This limitation can be avoided by determining the cuticular transpiration ($E_{cuticular}$).

Nocturnal warming is likely to have a significant effect on respiration rate (R) (Turnbull *et al*., 2002; Catoni *et al*., 2013), especially on the maintenance component that, in opposite to growth respiration, increases exponentially with temperature (Peraudeau *et al*., 2015). As plant biomass production depends on the balance between $A_n$ and R (Pérez-Priego *et al*., 2014), R is a determining factor to maintain growth and productivity, particularly in conditions in which $A_n$ is negatively affected, such as under drought conditions (Flexas *et al*., 2005; Ribas-Carbo *et al*., 2005; Galmes *et al*., 2007). Although R usually presents an order of magnitude lower than

$A_n$, photosynthesis is limited temporally, while R occurs continuously in different plant organs (Flexas *et al*., 2005; Galmes *et al*., 2007). Contrarily to the $A_n$ response to drought, that is well investigated, the respiratory process has been commonly marginalized and the few information available tend to be contradictory. Apart to the trend, also the mechanisms underlying R regulation under drought are still largely unknown and most of the studies assessed R on darkened leaves during the day. However, these responses can differ at night, once may change substrate supply and sink demand compared with normal nocturnal conditions (Ribas-Carbo *et al*., 2005) and, because daytime and nighttime temperatures differ substantially. Improve the knowledge of the importance of R to plant carbon balance during water stress is the first step to enable prediction and management of crop growth and yields in water-stress-prone areas (Flexas *et al*., 2005). Moreover, may help to hypothesize more accurately species behavior under new climatic conditions (Varone and Gratani, 2015). Therefore, we address the following questions: (1) It is $E_{night}$ substantially higher than $E_{cuticular}$?; (2) If so, can different water regimes determine the occurrence and magnitude of $g_{night}$ and $E_{night}$?; (3) Can leaf anatomical structure influence nighttime water balance?; (4) How $R_{night}$ responds to different water regimes? (5) What are the most probable causes of $g_{night}$ and $E_{night}$?; (6) What are the implications and opportunities of $g_{night}$ and $E_{night}$ to olive tree?

## 2. Material and Methods

### 2.1. Plant material and growth conditions

The experiment was carried out with own-rooted 3 years-old olive trees (*Olea europaea* cv. Cobrançosa), between June and September 2014 at the University of Trás-os-Montes and Alto Douro, Vila Real, Northeast Portugal (41°17′17.83″N, 7°44′12.81″W, 448 m a.s.l.). Plants were grown outdoors in 16 l pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). The surfaces of containers were covered with a thin layer of perlite and sealed with plastic film and aluminum foil. This measure aimed to avoid the evaporation from soil surface and the rain water entering to the pots, and to minimize the temperature increase inside the containers. Pots were randomly arranged and periodically rotated to the neighboring position to minimize the effects of environmental heterogeneity. When applicable, plants were watered to field capacity, determined gravimetrically. Care was taken to ensure negligible leaching through the bottoms of the containers during irrigation. All the plants were manually defruited immediately after fruit set to avoid yield influences on the measured variables.

The climate of the study site is typically Mediterranean-like, a warm-temperate climate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which falls in the autumn-winter with negligible rainfall during the summer months, although 2014 was an atypical summer with some rainfall events. The warmest months are July/ August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 °C and 6.8/6.3 °C, respectively (IPMA, 2017). The climatic conditions during the field measurements are presented in Figure 1.

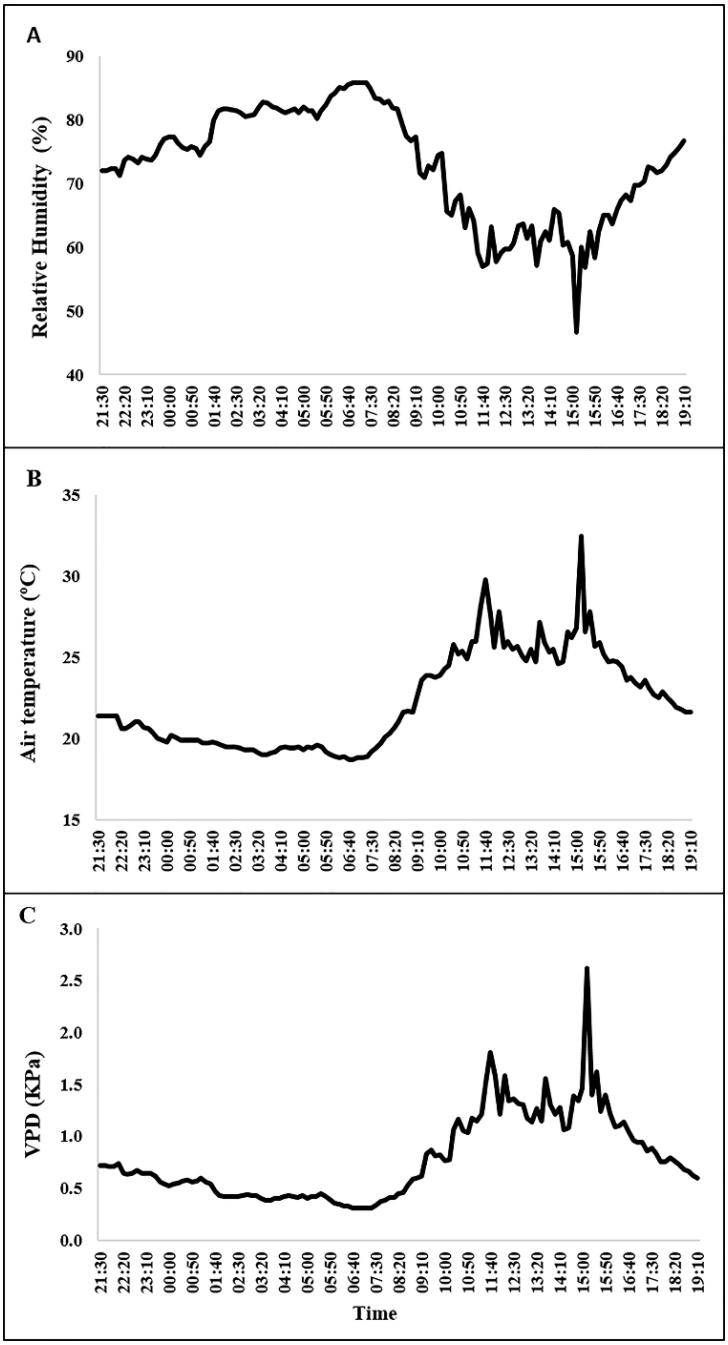


**Figure 1.** Relative humidity (A), air temperature (B) and ambient vapor-pressure deficit (VPD) (C) corresponding to the whole-day of physiological measurements.

### 2.2. Experimental plan

Forty uniform selected plants, based on height, leaf number and leaf area were submitted to an acclimatization period of 30 days, being watered every other day to field capacity, determined gravimetrically. At the beginning of the experiment, 6$^{th}$ July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining thirty-two plants were divided in two groups, each one comprising sixteen plants. One group was kept under well-watered conditions (WW, control plants) throughout the entire experimental period, in which plants were watered every day. The other group was subjected to three "drought-rewatering cycles" (WS, stressed plants) by withholding water until the occurrence of precipitation (1$^{st}$ and 2$^{nd}$ cycles), or until the stomatal conductance for water vapor ($g_{day}$) during mid-morning (peak of photosynthetic activity) dropped around 50 $mmolm^{-2}$ $s^{-1}$ (reached at 3$^{rd}$ cycle), a threshold value indicating a situation of severe drought stress experienced by the plants, at that value photosynthetic activity becomes predominantly inhibited by metabolic processes, besides stomatal limitations (Flexas and Medrano, 2002). When occurred precipitation, or when olive trees reached the desired drought intensity, they were re-watered to field capacity in the evening and also during the following days until $A_n$ was almost restored to control values (recovery). The 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ "drought-rewatering cycles" had the duration of 12-6 days, 9–3 days and 21–16 days, respectively.

Each group of sixteen plants was divided in two subgroups, each one with eight plants arranged in a completely randomized design with four replications (two plants per treatment). Plants from one subgroup were used for physiological destructive measurements, and plants from the other subgroup were used for night-time non-destructive measurements and final biomass assessment. A schematic representation of the experiment is presented in Figure 2.

All the measurements detailed bellow were performed 8 times per treatment (n=8), one per plant. Physiological, structural and biochemical measurements at leaf level, measured in healthy, full expanded mature leaves, and whole-plant transpiration records, were performed 21 days after starting the 3$^{rd}$ drought cycle, at the peak of stress. Final biomass accumulation was evaluated at the end of the experiment, 16 days after starting the 3$^{rd}$ rewatering period.

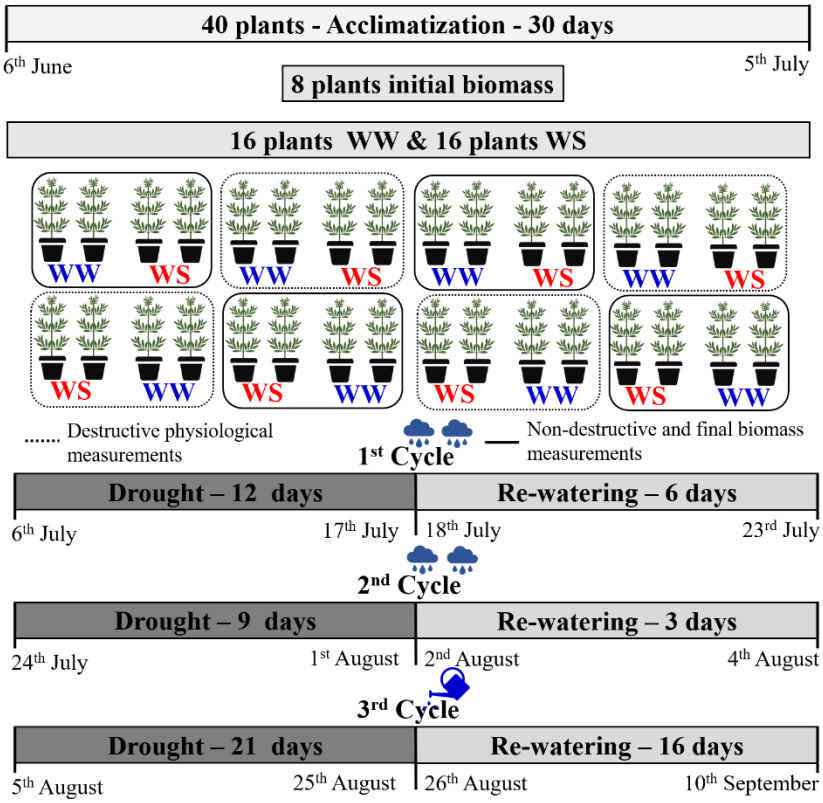


**Figure 2.** Schematization of the experiment. Abbreviations: WW, control plants; WS, stressed plants.

### 2.3. Leaf gas exchange, cuticular transpiration and total plant water balance

During daylight, leaf gas exchange measurements were performed in two periods, morning (mo, 10:00 local time) and midday (mid, 13:30 local time) of a summer cloudless day using a portable IRGA (LCpro+ ADC, Hoddesdon, UK), operating in the open mode. At night, the measurements were performed in the first hours of the night (22:30–23:30 local time). Just before gas exchange records were taken, dew water was removed with an absorbent paper to avoid interferences in gas exchange measurements. The measurements were made at photosynthetic photon flux density of 1618 ± 80, 1783 ± 75 and 0 μmol$m^{-2}$ $s^{-1}$, air temperature in the chamber of 25.0 ± 0.4, 30.7 ± 0.9 and 23.1 ± 0.3 °C, and $CO_2$ concentration of 391 ± 7, 380 ± 10 and 387 ± 6 μmol $CO_2$ $mol^{-1}$, at morning, midday and night, respectively. The air flow rate in all periods was set at 250 ml air $min^{-1}$. Net photosynthetic rate ($A_n$, μmol $CO_2$ $m^{-2}$ $s^{-1}$), stomatal conductance during the daylight ($g_{day}$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) and at night ($g_{night}$, mmol $H_2O$ $m^{-2}$ $s^{-1}$), transpiration rate during the daylight ($E_{day}$, g $H_2O$ $m^{-2}$ $h^{-1}$) and at night ($E_{night}$, g $H_2O$ $m^{-2}$ $h^{-1}$), ratio of intercellular to atmospheric $CO_2$ concentration during the daylight ($C_i/C_a$), respiration rate at night ($R_{night}$, μmol $CO_2$ $m^{-2}$ $s^{-1}$), and the concentration of $CO_2$ in intercellular spaces at night ($C_{i\text{-}night}$) were estimated using the equations developed by von Caemmerer and

Farquhar (1981). Whole-nighttime transpiration ($E_{W\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$) was extrapolated from the gas exchange measurements, assuming constant transpiration during the night, to compare it with the whole-plant water balance estimated by mass losses. Intrinsic water use efficiency was calculated as the ratio of $A_n/g_{day}$ (μmol $mol^{-1}$).

In order to discern whether measured values of $g_{night}$ and $E_{night}$ with IRGA were mostly cuticular or stomatal, cuticular water loss ($E_{cuticular}$, g $H_2O$ $m^{-2}$ $h^{-1}$) was estimated by the weight loss method, as described by Howard and Donovan (2007). Total plant water balance was accessed based on the gravimetric method. Pot mass changes were monitored during a period of 21 h, at early night (22:30 local time), at sunrise (5:30 local time) and at 19:30 (local time) of the following day (balance capacity of 30 kg at 1 g of precision, KERN FKB30K1A, KERN & Sohn Gmbh, Balingen, Germany). Plant leaf area was measured at the end of the experiment using the WinDias image analysis system (Delta- T Devices Ltd., Cambridge, UK). From pot mass changes and leaf area data were estimated the whole-nighttime transpiration ($PE_{W\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$) and the whole-daytime transpiration ($PE_{W\text{-}daytime}$, g $H_2O$ $m^{-2}$ $daytime^{-1}$).

### 2.4. Leaf water status, structural traits and chemical composition

After the midday gas exchange measurements, leaves were detached and immediately placed into air-tight containers and then the following parameters were examined: fresh weight (FW, g); weight at full turgor (TW, g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at 4 °C; leaf area (LA, $cm^2$), measured using the WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK); and dry weight (DW, g), measured after drying at 70 °C to a constant weight. Further, was calculated the relative water content (RWC) as RWC (%)=(FW−DW)/(TW−DW)×100, to characterize leaf water status, and the sclerophylly index, leaf mass area (LMA) as LMA ($gm^{-2}$)=DW/LA.

Leaves with the same characteristics, as those used for leaf gas exchange, were collected at the end of the experiment for anatomical tissue measurements. Leaf sections were taken from the middle of the leaves, to avoid differential thickness along the leaf. Cut sections were dehydrated, cleared and embedded in paraffin. Cross-sections of 4 μm were obtained using a rotary microtome, placed on slides and stained with toluidine blue. The thickness of total lamina, palisade and spongy parenchyma, upper and lower epidermis, cuticle and trichome layer were measured in the leaf cross-sections using an inverted optical microscope (Olympus IX51 with the image analysis software Cell^A).

The leaves adjacent to those used for RWC and LMA evaluation, with the same characteristics, were sampled for quantification of nonstructural carbohydrates by spectrophotometry. Total soluble sugars (SS) were extracted according to Irigoyen *et al*. (1992), by heating foliar discs in 80% ethanol during 1 h, at 80 °C. SS were quantified, at 625 nm, after the reaction of the alcoholic extract with fresh anthrone in a boiling water bath for 10 min. Thereafter, starch (St) was extracted from the same solid fraction by heating leaf discs in 30% perchloric acid during 1 h, at 60 °C, according to Osaki *et al*. (1991). The St concentration was determined by the anthrone method, as described above. Glucose was used as a standard for both SS and St quantification.

### 2.5. Electrolyte leakage

Leaf electrolyte leakage was measured as an indicator of cell membrane permeability, following a procedure described by Mena Petite *et al*. (2001) with some modifications. Leaves were washed three times in deionized water to remove surface ions, and then foliar discs (5) of 0.8 cm diameter were punched out of each leaf per plant, and placed in 10 ml deionized water within capped test tubes and incubated for 24 h, at 25 °C, on a rotary shaker. Electrical conductivity of the solution was measured after 24 h. Finally, samples were killed by autoclaving at 120 °C for 20 min, and the total conductivity reading was obtained upon equilibration at 25 °C. The 24 h conductivity was expressed as the percentage of the total conductivity value, having first subtracted the known conductivity value of the deionized water from both values.

### 2.6. Biomass accumulation and whole-plant water use efficiency

At the end of the experiment, the plants of each treatment were harvested and the dry weight of aboveground and belowground organs, after drying in a force-draft oven at 70 °C to a constant weight, were determined.

Water use efficiency of biomass production ($WUE_{WP}$) was determined for each plant by dividing total dry matter production by the cumulative amount of water used throughout the growing season, as previously described. Total dry matter included the oven-dried leaves, stems and roots.

### 2.7. Statistical analysis

All statistical calculations were performed using the statistical software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test ($P < 0.05$). The relationships $R_{night}$ and SS, $C_{i\text{-}night}$ and $R_{night}$, $R_{night}$ and $E_{night}$, $g_{night}$ and $C_{i\text{-}night}$, $g_{night}$ and St, $E_{night}$ and $g_{night}$, $g_{night}$ and $g_{mo}$, $g_{night}$ and $g_{mid}$, $g_{night}$ and $A_{n\text{-}mo}$ and $g_{night}$ and $A_{n\text{-}mid}$ was analyzed by the Pearson correlation test and significance was set at $P < 0.05$.

## 3. Results and Discussion

### 3.1. Influence of $E_{cuticular}$ on $E_{night}$, nighttime water balance and responses to water availability

The present study revealed that $E_{night}$ and $g_{night}$ are important factors that affect whole-plant water balance and water use efficiency in olive tree. The instantaneous $E_{night}$ were 2.7–5.9 times higher than the cuticular water losses ($E_{cuticular}$) in WW and WS plants, respectively (Table 1). The very strong positive correlation ($r=0.987$; $P < 0.001$) between $E_{night}$ (Table 1) and $g_{night}$ (Table 2) suggests that most nighttime water losses can be regulated (Howard and Donovan, 2007).

**Table 1.** Water losses of olive control plants (WW) and olive stressed plants (WS), based on cuticular losses ($E_{cuticular}$, g $H_2O m^{-2} h^{-1}$), gas exchange measurements (nighttime transpiration, $E_{night}$, g $H_2O$ $m^{-2} h^{-1}$; whole-nighttime transpiration, $E_{W\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$; transpiration at morning period, $E_{day\text{-}mo}$, g$H_2O m^{-2} h^{-1}$; transpiration at midday period, $E_{day\text{-}mid}$, g $H_2O$ $m^{-2}$ $h^{-1}$), and mass changes measurements (whole-nighttime transpiration, $PE_{W\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$, and whole-daytime transpiration, $PE_{W\text{-}day}$, g $H_2O$ $m^{-2}$ $daytime^{-1}$).

| | $E_{cuticular}$ | $E_{night}$ | $E_{W\text{-}night}$ | $E_{day\text{-}mo}$ | $E_{day\text{-}mid}$ | $PE_{W\text{-}night}$ | $PE_{W\text{-}day}$ |
|---|---|---|---|---|---|---|---|
| **WW** | 4.22±0.13 | 11.2±2.0 | 84.4±6.6 | 372.2±29.5 | 296.7±12.5 | 177.2±8.9 | 1042.0±72.6 |
| **WS** | 2.82±0.22 | 16.5±1.4 | 123.9±6.1 | 109.5±9.7 | 66.8±6.7 | 70.6±13.0 | 278.9±36.8 |
| **Sig.** | ** | ** | ** | *** | *** | *** | *** |

Values are means ± SE (n=8). Significant differences: ** – significant at $p < 0.01$; *** – significant at $p < 0.001$

**Table 2.** Leaf physiological parameters of olive control plants (WW) and olive stressed plants (WS), $A_{n\text{-}mo}$, $A_{n\text{-}mid}$ represent leaf morning and midday net photosynthetic rates, respectively; $g_{day\text{-}mo}$, $g_{day\text{-}mid}$, $g_{night}$ represent leaf morning, midday and night stomatal conductance's, respectively; $A_{n\text{-}mo}/g_{day\text{-}mo}$, $A_{n\text{-}mid}/g_{day\text{-}mid}$ represent leaf morning and midday intrinsic water use efficiency, respectively; $R_{night}$, represent respiration during the night; $C_{i\text{-}night}$ represent night intercellular $CO_2$ concentration; and $C_i/C_{a\text{-}mo}$, $C_i/C_{a\text{-}mid}$ represent the ratio of intracellular/ atmospheric $CO_2$ in morning and midday, respectively.

| **Leaf physiological parameters** | **WW** | **WS** | **Sig.** |
|---|---|---|---|
| **$A_{n\text{-}mo}$ (µmol $m^{-2}$ $s^{-1}$)** | 18.0±1.5 | 6.89±0.72 | ** |
| **$A_{n\text{-}mid}$ (µmol $m^{-2}$ $s^{-1}$)** | 16.2±0.96 | 2.93±0.65 | *** |
| **$g_{day\text{-}mo}$ (mmol $m^{-2}$ $s^{-1}$)** | 172.7±15.7 | 53.4±4.6 | *** |
| **$g_{day\text{-}mid}$ (mmol $m^{-2}$ $s^{-1}$)** | 222.1±17.4 | 26.9±2.4 | *** |
| **$g_{night}$ (mmol $m^{-2}$ $s^{-1}$)** | 12.2±0.98 | 16.5±1.1 | * |
| **$A_n/g_{day\text{-}mo}$ (µmol $mol^{-1}$)** | 102.5±2.5 | 131.9±25.2 | n.s. |
| **$A_n/g_{day\text{-}mid}$ (µmol $mol^{-1}$)** | 72.5±3.5 | 104.0±15.2 | * |
| **$R_{night}$ (µmol $m^{-2}$ $s^{-1}$)** | 2.53±0.33 | 1.12±0.33 | * |
| **$C_{i\text{-}night}$ (µmol $mol^{-1}$)** | 698.9±35.6 | 481.3±24.3 | ** |
| **$C_i/C_{a\text{-}mo}$** | 0.466±0.012 | 0.399±0.102 | n.s. |
| **$C_i/C_{a\text{-}mid}$** | 0.608±0.014 | 0.490±0.067 | n.s. |

Values are means ± SE (n=8). Significant differences: * – significant at $p < 0.05$; ** – significant at $p < 0.01$; *** – significant at $p < 0.001$; n.s.– not significant at $p > 0.05$.

Similar evidences of high values of $g_{night}$, which were far above the measured cuticular transpiration, were reported by Howard and Donovan (2007) in Helianthus species and by Escalona *et al*. (2013) in *Vitis vinifera*. In addition, $E_{cuticular}$ showed a significant response to water availability, being lower in WS plants, in a strictly association with the significant decrease of RWC from 94 to 64.7% (Table 3).

**Table 3.** Leaf relative water content (RWC), leaf mass area (LMA), leaf soluble sugars (SS) and starch (St) concentrations, leaf electrolyte leakage (EL) and plant biomass increase (BI) of olive control plants (WW) and olive stressed plants (WS).

| | **WW** | **WS** | **Sig.** |
|---|---|---|---|
| **RWC (%)** | 94.0±0.8 | 64.7±1.2 | *** |
| **LMA (g $m^{-2}$)** | 209.0±3.0 | 215.4±10.6 | n.s. |
| **SS (mg $g^{-1}$FW)** | 104.6±6.0 | 112.7±4.1 | n.s. |
| **St (mg $g^{-1}$FW)** | 21.8±3.6 | 33.7±3.0 | * |
| **EL (%)** | 18.0±1.3 | 25.7±0.5 | ** |
| **BI (%)** | 62.9±6.0 | 24.9±5.5 | ** |

Values are means ± SE (n=8). Significant differences: * – significant at $p < 0.05$; ** – significant at $p < 0.01$; *** – significant at $p < 0.001$; n.s. – not significant at $p > 0.05$

The low cuticular water permeability is one of the main factors supporting the survival and viability of plants under scarce water supply, particularly in species exposed to pronounced levels and rapid changes in temperature, as olive tree under the Mediterranean region, where the thermal stability of the cuticular transpiration barrier is decisive, maybe even to a higher degree than the baseline cuticular permeability (Schuster *et al*., 2016). In our study, the lower $E_{cuticular}$ of water stressed plants could be related with differences in cuticle structure and composition, or both, since no differences were observed in cuticle layer thickness (Table 4).

**Table 4.** Leaf tissues thickness (μm) (total section, TS; upper cuticle, UC; upper epidermis, UE; upper palisade parenchyma, UPP; spongy parenchyma, SP; lower palisade parenchyma, LPP; lower epidermis, LE; trichome layer, TL) of olive control plants (WW) and olive stressed plants (WS).

| | **WW** | **WS** | **Sig.** |
|---|---|---|---|
| **TS** | 485.7±11.2 | 478.3±8.7 | n.s. |
| **UC** | 7.03±0.26 | 7.03±0.23 | n.s |
| **UE** | 16.4±0.8 | 15.7±0.5 | n.s |
| **UPP** | 163.4±6.1 | 161.1±9.1 | n.s |
| **SP** | 216.5±5.3 | 206.9±3.6 | n.s |
| **LPP** | 28.6±1.7 | 27.6±1.0 | n.s. |
| **LE** | 15.6±0.4 | 15.3±0.3 | n.s |
| **TL** | 38.3±1.5 | 44.7±2.0 | * |

Values are means ± SE (n=8). Significant differences: * - significant at $p < 0.05$ and n.s. - not significant at $p > 0.05$.

Curiously, WS plants had higher $g_{night}$ and, thus, $E_{night}$, during the first hours of darkness (Tables 1 and 2), against the typical evidences that $g_{night}$ are most likely to occur when water availability is high (Dawson *et al*., 2007; Howard and Donovan, 2007; Flexas *et al*., 2010; Escalona *et al*., 2013; Fuentes *et al*., 2014). Nonetheless, the occurrence of $g_{night}$ in water-limited habitats was already reported in other species (Snyder *et al*., 2003; Ogle *et al*., 2012), as well the higher nocturnal sap flow during the dry season over the wet season (Forster, 2014), strongly suggesting that this behavior is species dependent, and that may provide an ecological advantage to this sclerophyllous species under most stressful conditions. Conversely, when nighttime transpiration was estimated based on the gravimetric method (PE), we observed an opposite trend relatively to transpiration extrapolated from $E_{night}$ (Table 1). In WW plants, $E_{W\text{-}night}$ was much lower than $PE_{W\text{-}night}$, suggesting a shift on the $g_{night}$ throughout the nighttime

and/or that gas exchange measurements may underestimate the water losses. Progressive increases of $g_{night}$ from early night hours to dawn were reported previously by Escalona *et al*. (2013), mainly due to a decrease of VPD, and also by some internal regulation of $g_{night}$ by sub-stomatal $CO_2$ concentration. Our environmental data support these hypothesis, as VPD and temperature decreased toward the dawn (Figure 1), and the latter may contribute to reduce respiration rate and $C_i$ values. On the other hand, errors when extrapolating $E_{night}$ to the whole-plant are likely to contribute also to such discrepancy, since different leaves, namely the younger leaves, may display significantly different $E_{night}$ values and the conditions inside the gas exchange cuvette might not fully reflect the actual conditions of the leaves, leading to biased estimates of $E_{night}$ (Escalona *et al*., 2013). Noteworthy, in opposite to WW plants, $E_{W\text{-}night}$ of WS plants was 75% higher than $PE_{W\text{-}night}$ (Table 1). Similar trend was already described in *Vitis vinifera* by Escalona *et al*. (2013). Plant water losses can be partially or fully compensated by dew deposition on leaves. It is remarkable the thicker trichome layer of WS plants (Table 4) and the typical (confirmed visually) paraheliotropism response of this species under drought conditions (Bacelar *et al*., 2009), that could largely increase this dew deposition over the leaves and contribute to the referred discrepancy. In fact, during the night of water balance measurements the dew deposition was confirmed visually, favored by the high relative humidity (72–86%) and the low and decreasing $VPD_{ambient}$ over the night (0.72-0.31 kPa). Additionally, the condensation can start at lower values of relative humidity if the plant disposes of pubescent leaf surfaces that promotes capillary condensation and higher accumulation of hygroscopic particles, as aerosols. This phenomenon takes special importance in arid climates, where is quite difficult to reach high humidity during the drought season (Burkhardt and Hunsche, 2013; Konrad *et al*., 2015). Meanwhile, dew in a dense trichome layer is able to improve WUE indirectly, even without being absorbed by the plant (Konrad *et al*., 2015), although there are evidences that trichomes play a key role in dew absorption by leaves (Munné-Bosch *et al*., 1999; Savé *et al*., 2000). Interestingly, although in WS plants the relative nighttime water losses were slightly more significant than in WW plants, reaching 20.2% against 14.5%, respectively, of the whole-day transpiration (Table 1), the difference was lower than we expected, possibly related with the dew deposition. These results strengthen the need, when is possible, to incorporate both gas exchange and gravimetric measurements in the study of plant nighttime water balance, once extrapolating transpiration from leaf gas exchange measurements to whole-plant transpiration or vice-versa could be erratic.

### 3.2. Photosynthesis and respiration responses to water availability

Net $CO_2$ assimilation rate of olive trees generally declines in response to severe water deficit (Bacelar *et al*., 2007a; Ben Abdallah *et al*., 2018) as in the present study, due to both stomatal and non-stomatal limitations, being the latter more evident at midday period, judging by the changes in $g_{day}$, $A_n/g_{day}$ and $C_i/C_a$ data (Table 2). On the other hand, the trend of R is still not so clear (Varone and Gratani, 2015). It was suggested that R response varies generally from inhibition, with low to moderate stress, to stimulation, with severe stress, being the response closely linked to the species drought tolerance (Flexas *et al*., 2005; Atkin and Macherel, 2009; Varone and Gratani, 2015). Moreover, it has been assumed that this different response is strongly related with RWC (Flexas *et al*., 2005; Varone and Gratani, 2015), with 50% being indicated as a threshold value to induce an increase in R (Flexas *et al*., 2005). This assumption is consistent with our results, since WS plants, with lower $R_{night}$ (Table 2), presented RWC of 64.7% (Table 3). Respiration and net photosynthesis are strongly coupled and intrinsically interdependent because $A_n$ provides photosynthetic substrates to R, and R supplies ATP and carbon skeletons to sustain plant energy requiring processes (Cannell and Thornley, 2000). Although $A_n$ declined as a consequence of water deficit, the decrease in $R_{night}$ was not related to a decay in the concentration of SS (Table 3).

To reinforce this idea, Pearson's correlation between $R_{night}$ and SS ($r=-0.168$; $P=.534$) shows that the probability of these variables being correlated is highly unlikely. Similar results were found by Rodríguez-Calcerrada *et al*. (2011), who suggested that, apart the effect of drought on respiratory capacity, drought-induced reduction of plant growth could translate into a reduction of R in fully developed leaves, via reduced sucrose loading into the phloem and ATP demand. Meanwhile, WS plants accumulate more St in leaves than WW plants (Table 3). Under drought conditions, is expected an increase in SS in spite of St, once drought is known to induce St degradation, resulting in an increase in SS (Chaves, 1991). However, an increase in leaf St content in drought stressed olive trees was already documented (Bacelar *et al*., 2006), possibly because carbon was not translocated out of the leaves, as these plants were sink-limited as confirmed by lower accumulation of biomass (Table 3), and due to a reduced necessity to increment the use of reserves to increase the maintenance component of R to support extra repair costs (Atkin and Macherel, 2009; Varone and Gratani, 2015). Some studies associate R to other leaf traits, such as leaf tissues thickness and leaf mass per unit of leaf area (LMA) (Wright *et al*., 2006; Lewis *et al*., 2011; Pérez-Priego *et al*., 2014). However, we did not observe this trend on the present study (Tables 2 and 3), probably because sclerophylly does not change

substantially through time in mature leaves (Bacelar *et al*., 2004), especially in the present growth conditions, with 2 periods of recovery before the 3rd cycle of drought stress. Moreover, Varone and Gratani (2015) argued that higher membrane injury requires an energetic input to the maintenance of the ion gradient across membranes, resulting in higher R. However, although WS plants exhibited higher electrolyte leakage (EL) (Table 3), indicating a drought-induced membrane impairment, it was not recorded an increase in $R_{night}$. These data confirm that olive tree is highly drought tolerant, since after several days of water shortage the plants are not yet under extreme conditions, being able to reduce the metabolism and conserve the photosynthates. The capacity to maintain low $R_{night}$ rates during stressful conditions, associated with a decline in $A_n$, allows this species to allocate more assimilates for biomass accumulation and, consequently, for growth (Varone and Gratani, 2015). This could be an important advantage from an evolutionary point of view for plants inhabiting drought-prone and climatic change susceptible habitats.

### 3.3. $g_{night}$ and $E_{night}$ drivers, implications and opportunities

Understand the mechanisms underlying the responses of $g_{night}$ and $E_{night}$ to water availability and the characterization of costs–benefits relationship can help to determine its causes and whether the occurrence and magnitude of those traits have or not acclimation value. In a previous study (Snyder *et al*., 2003), the magnitude of $g_{night}$ was positively correlated with the magnitude of $g_{day}$, but in the present investigation, we observed an opposite pattern (Table 2), as $g_{night}$ presented a significant negative correlation with $g_{day\text{-}mo}$ ($r=-0.941$; $P < 0.001$) and $g_{day\text{-}mid}$ ($r=-0.926$; $P < 0.001$), in line with the evidences of a separate genetic control of $g_{night}$ and $g_{day}$ (Caird *et al*., 2007; Christman *et al*., 2009). Similarly, some reports stated that daytime conditions favoring $A_n$ resulted in higher $g_{night}$ (Easlon and Richards, 2009), while in the present study (Table 2), we observed a negative correlation between $g_{night}$ and $A_{n\text{-}mo}$ ($r=-0.913$; $P < 0.001$) and $g_{night}$ and $A_{n\text{-}mid}$ ($r=-0.911$; $P < 0.001$) as in the study of Christman *et al*. (2009). Resco de Dios *et al*. (2015) also suggested that $g_{night}$ is independent of $A_n$, since different levels of radiation did not affect $g_{night}$. Together, these data demonstrated that in olive tree the previous $g_{day}$ and $A_n$ do not determine following $g_{night}$.

It was proposed that a by-product of starch-metabolism (osmoticant) may affect guard cells osmoregulation (Easlon and Richards, 2009), causing a great stomatal opening when St levels are high (Caird *et al*., 2007). Likewise, a positive correlation between $g_{night}$ and St concentrations were found in the present study ($r=0.974$; $P < 0.001$) (Tables 2 and 3).

Meanwhile, higher $E_{night}$ of WS plants can play a crucial role, once may lower leaf temperature by evaporative cooling, thereby decreasing carbon losses through R (Coupel-Ledru *et al*., 2016), an hypothesis supported by the significant negative correlation between $E_{night}$ and $R_{night}$ ($r=-0.622$; $P < 0.05$). This response takes especially importance in the first hours of darkness, once temperatures are usually higher. In addition, a reduced accumulation of $C_{i\text{-}night}$ (Table 2), due to the reduced $R_{night}$ ($r=0.949$; $P < 0.001$), may have contributed to stomatal open (Escalona *et al*., 2013), as attested by the negative correlation between $C_{i\text{-}night}$ and $g_{night}$ ($r=-0.734$; $P < 0.001$). Furthermore, the possible dew absorption by trichomes (Munné-Bosch *et al*., 1999; Savé *et al*., 2000), may immediately increase foliar hydration and guard cells turgidity, what in turns could induce the higher $g_{night}$ observed in this study. Moreover, this mechanism engineered by some plants to withstand drought stress could be suppressed under water availability, since is no longer needed (Munné-Bosch, 2010).

The substantial water cost of $g_{night}$ could represent a major problem for agronomic development (Resco de Dios *et al*., 2015), reducing daily WUE, particularly under water deficit conditions (Escalona *et al*., 2013; Fuentes *et al*., 2014; Medrano *et al*., 2015). Nonetheless, in the present experiment the whole-plant WUE (Figure 3), as well the $A_{n\text{-}mid}/g_{day\text{-}mid}$ (Table 2) were higher in WS than in WW plants. Likewise, higher $WUE_{WP}$ in drought stressed plants was reported previously in this olive tree genotype (Bacelar *et al*., 2007b).

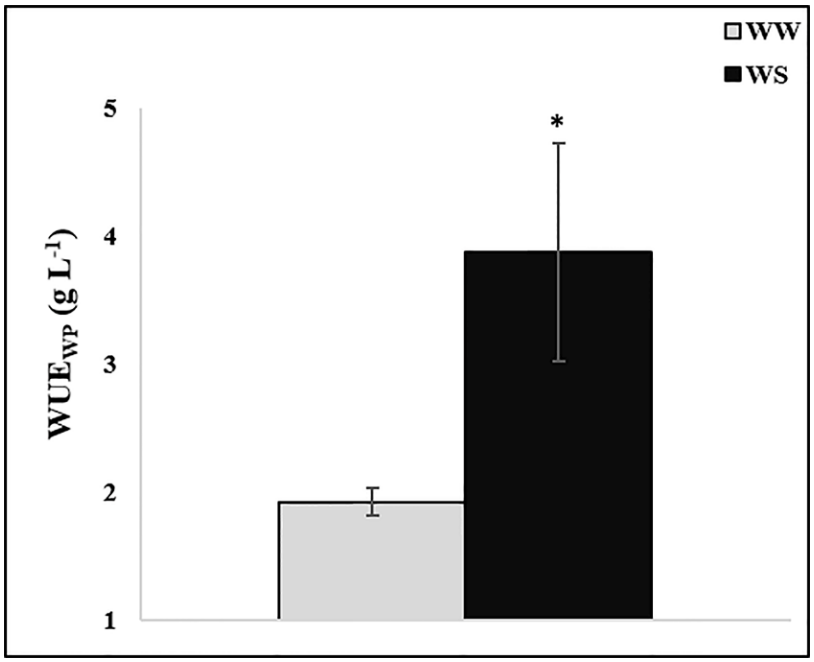


**Figure 3.** Whole-plant water use efficiency ($WUE_{WP}$) of olive control plants (WW) and olive stressed plants (WS). Bars are means ± SE (n=8). Significant differences: * – significant at $p < 0.05$.

In addition to evaporative cooling (Coupel-Ledru *et al*., 2016), the nutrient acquisition at root level and distribution within the plant has been proposed as a potential benefit of $E_{night}$ (Snyder *et al*., 2003, 2008; Scholz *et al*., 2007). Drought is known to reduce both nutrient uptake capacity and availability (Kreuzwieser and Gessler, 2010). In the present study, WS plants presented a lower production of biomass and substantial changes in concentrations, root uptake efficiency and physiological use efficiency of some mineral elements, being particularly more relevant the significant decrease of root uptake efficiency of phosphorus, potassium, sulfur and copper and the significant increase of phosphorus use efficiency, joining with slight increases of the physiological use efficiency of the other three elements (data not shown). Thus, altogether, these responses suggest that WS plants could increase nighttime transpiration in order to compensate the reduced mineral absorption capacity induced by drought stress. Another potential benefit of $E_{night}$ relies on the nocturnal foliar uptake of nutrients (Burkhardt and Hunsche, 2013), as the moisture accumulated by hygroscopic particles on dense trichome layers can favor the "hydraulic activation of stomata", forming a continuous thin liquid water films on stomatal walls from external leaf surface to the apoplast, allowing the flow of water and solutes driven by concentration differences (Burkhardt, 2010; Burkhardt *et al*., 2012). Nevertheless, if too many stomata are activated transpiration can highly increase and reduce drought tolerance of plants (Burkhardt, 2010; Pariyar *et al*., 2013).

## 4. Conclusions

We were able to establish some correlations between some leaf traits and the nighttime transpiration response in the first hours of the night, as summarized in Figure 4. However, in some cases when it starts the causes or the consequences of this response, as well as the costs and benefits, are still not clear. The present study revealed the increase of $g_{night}$ in the first hours of the night, until certain level of water deficit could be coupled with some potential benefits to the plant. Nevertheless, the maintenance of $E_{night}$ for longer periods of drought could have devastating consequences, leading us to believe that the potential benefits conferred by $E_{night}$ might be dependent on stress severity, suggesting a threshold drought stress intensity for $g_{night}$ that may be species dependent.

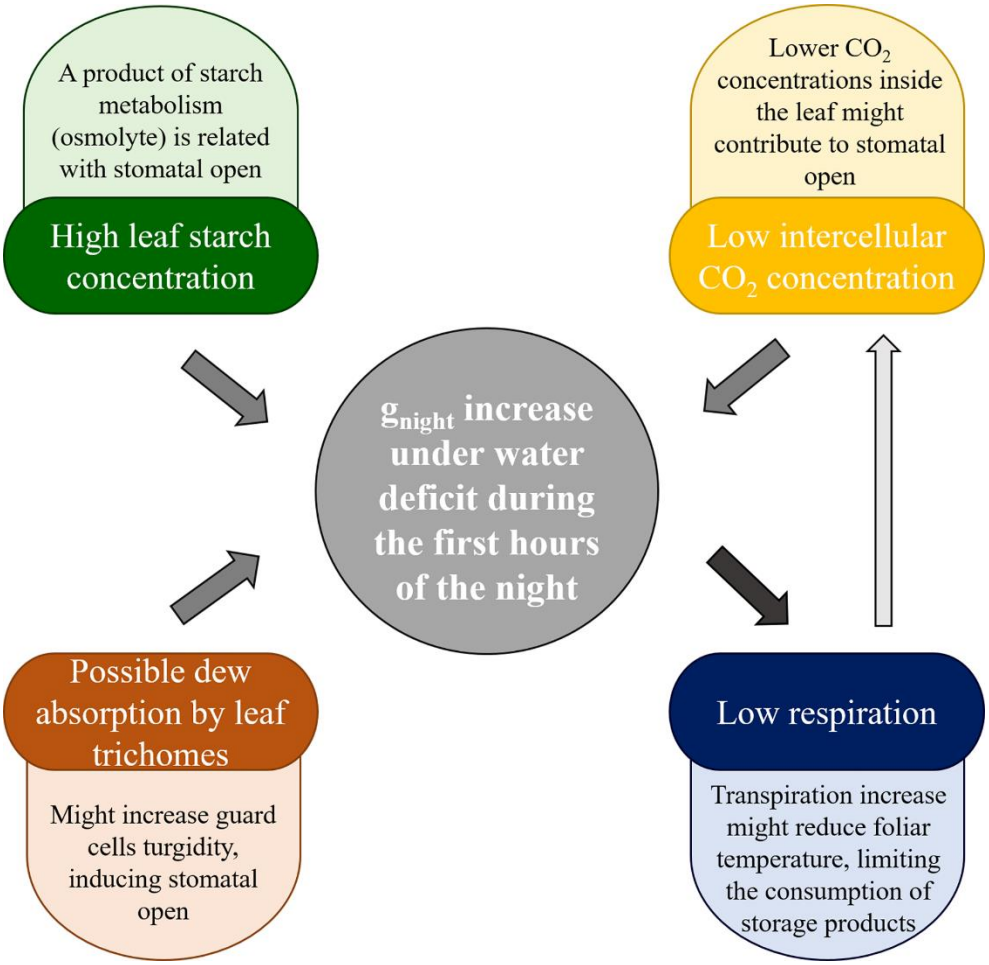


**Figure 4.** Schematic representation relating leaf nighttime stomatal conductance ($g_{night}$) with starch concentrations, intercellular $CO_2$, trichome layer and respiration rate.

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# CHAPTER 4

## Kaolin as a short-term mitigation strategy under drought and rewatering

## Briefing note

This chapter covers the olive tree mechanisms modulated by kaolin particle film under both drought and recovery events. Although this reflective clay has been studied in fruit tree species, including in olive tree, the plant induced changes are still not fully understood, namely under the pivotal drought recovery events. The importance of evaluation of KL influence in olive tree responses lays on the necessity to improve their performance under the typical Mediterranean ecosystems, frequently exposed to episodic droughts and highly susceptible to climate change.

Following, this chapter is an adaptation of a research article published in *Plant Physiology and Biochemistry* entitled "Kaolin particle film modulates morphological, physiological and biochemical olive tree responses to drought and rewatering". The manuscript aimed to respond to the specific objective 2 of this thesis, "to evaluate the influence of exogenously applied KL on olive tree responses to drought and rewatering events". This work revealed a more comprehensive information about how KL modulates olive tree responses to drought, exposing new processes influenced by this technology. Additionally, the study divulged how KL can influence the olive tree responses under rewatering.

The authors contribution for the article converted in the present chapter was: Cátia Brito was responsible for establish and maintain the experiment, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and in the critical review of the article. Helena Ferreira, Luis Rocha and Ivo Pavia collaborated in data collection on the field. Helena Ferreira also assisted in the laboratory analyses. Carlos Correia was responsible for design the experiments, data collection on the field and critical review of the article. All the authors reviewed and approved the final manuscript.

## 4.1. Kaolin particle film modulates morphological, physiological and biochemical olive tree responses to drought and rewatering

**Kaolin particle film modulates morphological, physiological and biochemical olive tree responses to drought and rewatering**

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### Abstract

Regarding the foreseeing climate change is reasonable to expect harmful consequences to olive tree (*Olea europaea* L.), an iconic species of Mediterranean region. Thus, the selection of practices that allow a better drought resistance and recovery capacity needs the immediate attention of scientific community. This study evaluates the strategies adopted by young potted olive trees, subjected to three cycles of drought and rewatering, in the presence of a reflective clay, kaolin (KL). The results demonstrated that KL induced shade-related leaf structural changes and was effective in keeping leaf water status during the most stressful periods. In general, photosynthetic activity of sprayed plants was improved by the alleviation of drought-induced stomatal and non-stomatal limitations. Moreover, during stress imposition sprayed leaves showed reduced oxidative damages, allowing lower investment in antioxidant defences. Furthermore, sprayed plants also had lower nighttime water losses due to inferior nighttime stomatal conductance, and are able to maintain higher respiration rates. Upon rewatering, the shaded effect conferred by KL limited gas exchange restauration but improved the plants' capacity to restore the metabolic functions. In spite of the induced physiological and biochemical changes, no significant differences were found in whole-plant water use efficiency and plant biomass accumulation, possibly by the attenuation of photosynthesis restauration during the recovery events. In conclusion, the changes induced by KL might be beneficial under severe conditions, as on realistic Mediterranean field environments.

**Keywords:** drought, recovery, photosynthesis, mitigation strategies, oxidative stress, antioxidants.

## 1. Introduction

In the current settings, olive tree growing under the typical Mediterranean semi-arid conditions are already affected by multiple environmental constraints factors, as drought stress is commonly associated with high temperature and irradiance. Moreover, severe summer conditions and extreme climatic events are predicted to increase in frequency in most future climate scenarios (IPCC, 2013). Although olive is a crop well-adapted to harsh conditions, water deficit has negative repercussions on water relations, carbon assimilation, oxidative pathways, nutrient uptake and biomass accumulation (Bacelar *et al*., 2006, 2007a, b). In addition, the presence of simultaneous abiotic stresses, as heat and high irradiance levels, can exacerbate drought effects, affecting plant growth and yield and consequently the economic viability of the olive sector. Therefore, is important to increase trees ability to use the scarce available water and the low and unexpected rainfall during the summer.

Undeniably, the recovery of plant functions when water shortage is relieved is crucial to restart growth under drought and rewatering events. Recovery after stress is a very complex process involving the rearrangement of many metabolic pathways to repair drought-induced damages and restore plant growth and productivity (Chen *et al*., 2016). In olive tree, it has been identified a conservative bearing after rewatering, as trees restoring rapidly the water status along with a slow recovery of stomatal conductance (Perez-Martin *et al*., 2014). Although have been suggested that drought recovery may play a more important role than drought resistance (escape, avoidance and tolerance) in water deficit response (Chen *et al*., 2016), the capacity for recovery after successive drought and rewatering cycles have poorly been studied. Those evidences highlight the importance to adopt agronomic practices that allow a better drought adaptability, i.e. the capacity to integrate both drought resistance and recovery capacity (Chen *et al*., 2016) of rainfed olive orchards. Kaolin, which main constituent is kaolinite, is a white mineral chemically inert (Glenn and Puterka, 2005) that have been proved to be efficient in summer stress alleviation. Once sprayed on the leaf surface, water evaporates leaving a protective particle film that increases the reflection of excess radiation (photosynthetically active, PAR, ultraviolet, UV and infrared, IR), avoiding the accumulation of heat load and reducing the risk of leaf and fruit damage from high temperatures and solar injury (Glenn and Puterka, 2005). In general, positive effects were recorded in plant water status, photosynthetic responses and yield, but the results appear to be largely influenced by a set of factors, such as species and cultivars, environmental conditions, plant age and structure (Jifon and Syvertsen, 2003; Rosati *et al*., 2006; Glenn, 2009; Roussos *et al*., 2010; Denaxa *et al*., 2012; Nanos, 2015).

Hence, with this study we aimed to evaluate if kaolin application alleviates the negative effects associated to cyclic water deficit events. For this propose, we studied the effects of kaolin application on (i) physiological and biochemical variables under drought and rewatering; (ii) on leaf anatomical traits; and (iii) on growth responses.

## 2. Material and Methods

### 2.1. Plant material and experimental set-up

The study was carried out in Vila Real, Northeast Portugal (41°17'17.83"N, 7°44'12.81"W, 448 m a.s.l.) with own-rooted 3-years-old olive trees (*Olea europaea* cv. Cobrançosa). Plants were grown outdoors in 16 L pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). The surfaces of containers were covered with a thin layer of perlite and sealed with plastic film and aluminum foil. This measure aimed to avoid the evaporation from soil surface and the rain water entering to the pots, and to minimize the temperature increase inside the containers. Pots were randomly arranged and periodically rotated to the neighboring position to minimize the effects of environmental heterogeneity. The climate of the study site is typically Mediterranean-like, a warm-temperate climate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which falls in the autumn-winter with negligible rainfall during the summer months, although 2014 was an atypical summer with some rainfall events (13.7, 11.8 and 13.0 mm during the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ recovery periods, respectively. The warmest months are July/August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 ºC and 6.8/6.3 °C, respectively. The maximum, minimum and average air temperature recorded during the experimental period are shown in supplemental Figure 1 (IPMA, 2017).

Prior to the experiment, forty uniform plants, selected based on height, leaf number and leaf area were left for 30 days in the study site for acclimatization, being watered every other day to field capacity, determined gravimetrically. Then, at the beginning of the experiment, 6$^{th}$ July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining thirty-two plants were subjected to three "drought-rewatering cycles" by withholding water until the occurrence of precipitation (1$^{st}$ and 2$^{nd}$ cycles), or until the stomatal conductance for water vapour ($g_{day}$) during mid-morning (peak of photosynthetic activity) dropped around 50 mmol m$^{-2}$ s$^{-1}$ (reached at 3$^{rd}$ cycle), a threshold value indicating a situation of severe drought stress experienced by the plants, a value where photosynthetic

activity becomes predominantly inhibited by metabolic processes, besides stomatal limitations (Flexas and Medrano, 2002). When occurred precipitation, or when olive trees reached the desired drought intensity, they were re-watered to field capacity, determined gravimetrically, in the evening and also during the following days until $A_n$ was almost restored to well-watered every day control values, as described in a parallel study (Brito *et al*., 2018). The 1st, 2nd and 3rd "drought-rewatering cycles" had the duration of 12-6 days, 9-3 days and 21-16 days, respectively. Consecutive drought-rewatering cycles simulate what usually occurs under Mediterranean-type ecosystems (Munné-Bosch and Peñuelas, 2003).

Plants were divided in two groups, the first group (C, control plants) was sprayed with distilled water and the second group (KL) was sprayed with an aqueous solution of kaolin (Surround® WP, Engelhard Corporation, Iselin, NJ), at the manufacturer recommended dosage 5% (w/v). Each plant was treated with a mean volume of 150 mL of spraying solution. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. Care was taken during the application of foliar sprays to avoid overspraying non-target trees, covering them with a plastic sheet. The treatment was made in the absence of wind in the morning and kaolin were applied when it was necessary due to the rain events. A second application in the same day was done for KL trees, in order to ensure the adhesion uniformity of kaolin clay particles. The applications were made in the beginning of the experiment, 6th July, and after the rain events, 18th July and 2nd August.

Each group of sixteen plants was divided in two subgroups, each one with eight plants arranged in a completely randomized design with four replications (two plants per treatment). Plants from one subgroup were used for physiological and biochemical destructive measurements, and plants from the other subgroup were used for non-destructive and final biomass assessment. A schematic representation of the experiment is presented in Figure 1.

All physiological and biochemical measurements at leaf level were measured in healthy, full expanded mature leaves. The daytime leaf gas exchange and leaf relative water content measurements (n=8) were taken periodically during the three drought-recovery cycles. Nighttime leaf gas exchange, cuticular transpiration and total plant water balance (n=8) were done at the peak of severest drought period (DP) (3rd cycle). Leaf samples for biochemical analysis (n=8) were taken at the peak of severest DP (3rd cycle) and eight days after the respective recovery period (RP). Leaves for anatomical tissue measurements (n=8) were

collected at the end of the experiment. For growth, biomass accumulation and whole-plant water use efficiency (n=8), plants were harvested at the end of the experiment.

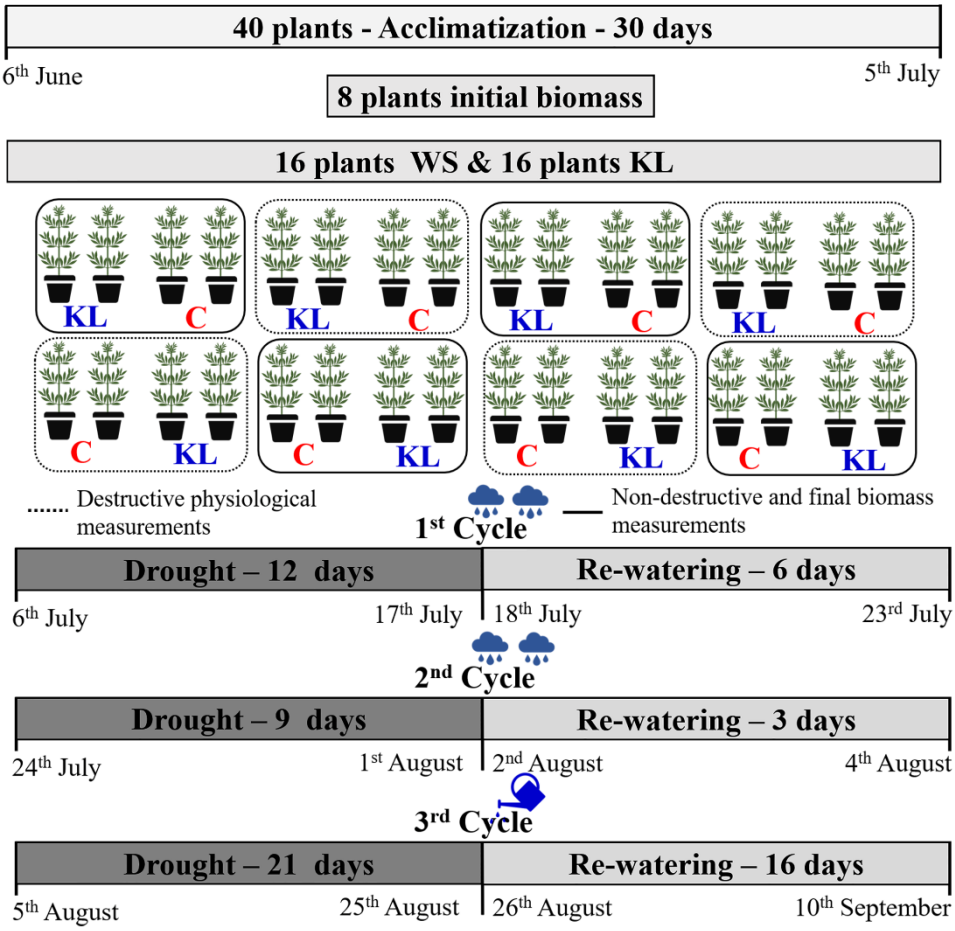


**Figure 1.** Schematization of the experiment. Abbreviations: C, control; KL, kaolin treatment.

### 2.2. Leaf water status, sclerophylly and structural traits

Leaves detached were immediately placed into air-tight containers and then the following parameters were examined: fresh weight (FW, g); weight at full turgor (TW, g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at 4 ºC; leaf area (LA, $cm^2$), measured using the WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK); and dry weight (DW, g), measured after drying at 70 ºC to a constant weight. Further, was calculated the relative water content, RWC (%) = (FW – DW)/(TW – DW) x 100, and succulence (mg $H_2O$ $cm^{-2}$) = (FW – DW)/LA, to characterize leaf water status, and the sclerophylly index, density of foliar tissue (g $kg^{-1}$) = DW/FW.

For structural analysis, leaves were prepared and analysed as described in Brito *et al.* (2018). The following variables were determined, thickness of total section (TS), spongy (SP),

upper (UPP) and lower (LPP) palisade parenchyma, upper (UE) and lower (LE) epidermis, upper cuticle (UC) and trichome layer (TL).

### 2.3. Leaf gas exchange and chlorophyll *a* fluorescence

Leaf gas exchange measurements were performed using a portable IRGA (LCpro+, ADC, Hoddesdon, UK), operating in the open mode. During daylight were performed in the morning (10:00 local time) of summer cloudless days under natural irradiance, and at night in the first hours of the night (22:30-23:30 local time). At night, just before gas exchange quantifications were taken, dew water was removed with an absorbent paper to avoid interferences in gas exchange measurements. The photosynthetic photon flux density (PPFD) and air temperature are provided as supplementary material (supplemental Table 1). Net photosynthetic rate ($A_n$, μmol $CO_2$ $m^{-2}$ $s^{-1}$), daytime ($g_{day}$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) and nighttime ($g_{night}$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) stomatal conductance, daytime ($E_{day}$, mmol $H_2O$ $m^{-2}$ $h^{-1}$) and nighttime ($E_{night}$, mmol $H_2O$ $m^{-2}$ $h^{-1}$) transpiration rate, daytime ratio of intercellular to atmospheric $CO_2$ concentration ($C_i/C_a$), nighttime respiration rate ($R_{night}$, μmol $CO_2$ $m^{-2}$ $s^{-1}$), and nighttime concentration of $CO_2$ in intercellular spaces ($C_{i\text{-}night}$, μmol $mol^{-1}$) were estimated using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency was calculated as the ratio of $A_n/g_{day}$ (μmol $mol^{-1}$).

Chlorophyll *a* fluorescence parameters were measured in the same leaves and environmental conditions used for gas exchange measurements, with a pulse-amplitude-modulated fluorometer (FMS 2, Hansatech Instruments, Norfolk, England). Prior to the measurements, a small part of the leaves was dark-adapted for 30 min using dark-adapting leaf-clips. After this period, the minimal fluorescence ($F_0$) was measured when all photosystem II (PSII) reaction centers are open using a low intensity pulsed measuring light source. The maximal fluorescence ($F_m$) was measured when all PSII reactions centers are closed during a pulse saturating light (0.7 s pulse of 15000 μmol photons $m^{-2}$ $s^{-1}$ of white light). The difference between these two levels ($F_m$-$F_0$) is called variable fluorescence ($F_v$). Maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m\text{-}F_0)/F_m$ (Krause and Weis, 1991). Following $F_v/F_m$ estimation, after a 20 s exposure to actinic light (1500 μmol $m^{-2}s^{-1}$), light-adapted steady-state fluorescence yield ($F_s$) was averaged over 2.5 s, followed by exposure to saturating light (15000 μmol $m^{-2}s^{-1}$) for 0.7 s to establish $F'_m$. The sample was then shaded for 5 s with a far-red light source to determine $F'_0$. From these measurements, several fluorescence attributes were calculated according to Bilger and Schreiber (1986) and Genty *et al*. (1989): photochemical quenching ($qP = (F'_m\text{-}F_s)/(F'_m\text{-}F'_0)$), capture efficiency of excitation energy by

open PSII reaction centers ($F´_v/F´_m = (F'_m-F'_0)/F'_m$) and effective quantum efficiency of PSII ($\Phi PSII = \Delta F/F'_m = (F'_m-F_s)/F'_m$). Due to a problem in the fluorometer, it was not possible to assess the chlorophyll *a* fluorescence responses in the last monitored date (16(R3)).

### 2.4. Leaf cuticular transpiration and total plant water balance

In order to discern whether measured values of $g_{night}$ and $E_{night}$ with IRGA were mostly cuticular or stomatal, cuticular water loss ($E_{cuticular}$, mmol $m^{-2}$ $s^{-1}$) was estimated by the weight loss method, as described by Howard and Donovan (2007). Total plant water balance was accessed based on the gravimetric method. Pot mass changes was monitored during a period of 21 hours as reported elsewhere (Brito *et al*., 2018). From these measurements were estimated the whole-nighttime transpiration ($PE_{W\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$) and the whole-daytime transpiration ($PE_{W\text{-}daytime}$, g $H_2O$ $m^{-2}$ $daytime^{-1}$).

### 2.5. Foliar metabolic assays and oxidative stress markers

For leaf biochemical analysis, the harvested leaves were immediately frozen in liquid nitrogen and stored at -80ºC until be analysed. To express the metabolites by dry mass, a representative sample of each analyzed leaf was evaluated in fresh and after drying at 60ºC until constant weight. Chlorophylls and carotenoids were extracted with acetone/water (80/20, v/v). Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$), total chlorophyll ($Chl_{(a+b)}$) and $Chl_a/Chl_b$ ratio were determined according to Arnon (1949) and Sesták *et al*. (1971) and total carotenoids according to Lichtenthaler (1987) and expressed as mg $g^{-1}$DW. Lycopene and β-carotene were extracted with acetone/hexane (4/6, v/v), determined according to Barros *et al*. (2011), and expressed as mg $g^{-1}$DW.

Soluble sugars were extracted according to Irigoyen *et al*. (1992), by heating the samples in ethanol/water (80/20, v/v) during 1 h, at 80 ºC. Then, the soluble fractions were separated from the solid fraction. Starch was extracted by heating the same solid fraction in 30% perchloric acid during 1 h, at 60 ºC, according to Osaki *et al*. (1991). Both SS and St concentration was determined by the anthrone method and expressed as mg $g^{-1}$ DW, using glucose as a standard.

Total soluble proteins were quantified using the method of Bradford (1976), using bovine serum albumin as a standard, and expressed as mg $g^{-1}$DW. Then, total thiols in soluble proteins extract were assessed according to Ellman (1959), using an extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$, and being expressed as nM $mg^{-1}$DW.

Total phenolics in leaf extracts were quantified following the Folin–Ciocalteu procedure (Singleton and Rossi, 1965) and expressed as mg $g^{-1}$DW, using gallic acid as a standard. Flavonoids were determined according to Zhishen *et al*. (1999), using (+)-catechin as a standard, and expressed as mg $g^{-1}$DW.

Ascorbate was quantified using a method adapted from Klein and Perry (1982), using L-ascorbic acid as a standard, and expressed as mg $g^{-1}$DW.

Total antioxidant capacity (TAC) based on DPPH-free radical scavenging capacity of leaf extracts was evaluated according to a method adapted from Xu and Chang (2007). Leaf methanolic extracts, and methanol for negative control, were mixed with DPPH methanolic solution (0.1 mM) and left to stand for 30 min in dark at room temperature. The absorbance for the sample ($A_{sample}$) and negative control ($A_{control}$) was measured at 517 nm against methanol blank. The percent of DPPH radical reduction was calculated as follows = 100 x ($A_{control}$ – $A_{sample}$) / $A_{control}$. The free radical scavenging activity was expressed as µM of Trolox equivalents (TE) per $g^{-1}$DW (TE = (% DPPH radical reduction / a)), where *a* is the slope of the standard curve (y = *a*x).

Total reactive oxygen species (ROS) were determined with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma–Aldrich,Germany) (Kong *et al*., 2013). A 25 mM solution was prepared in dimethyl sulphoxide for pending use. Twenty microliters of each sample were loaded into a small well ELISA plate containing 0.2 ml of PBS buffer (pH 7.4) and 12 µM of DCFH-DA and incubated for 20 min at 25º◦C. Fluorescence was measured at 485 nm and 530 nm (excitation and emission wavelength, respectively), in a CARY 50 Bio (Eclipse, Australia) every 15 min until 60 min after the incubation. 2',7',-dichlorofluorescein was used to obtained a calibration curve. Results were expressed as nM DCF $g^{-1}$DW.

$H_2O_2$ concentration were determined using a method described by Junglee *et al*. (2014), with some modifications. The absorbance was measured at 350 nm and $H_2O_2$ was used to obtain a calibration curve. Results were expressed in µM $g^{-1}$DW.

Leaf electrolyte leakage was measured as an indicator of cell membrane permeability, following a procedure adapted from Mena-Petite *et al*. (2001) and described in Brito *et al*. (2018).

### 2.6. Biomass accumulation and whole-plant water use efficiency

Plants were harvested and total leaf area (WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK) and the dry weight of aboveground and belowground organs, after drying

in a force-draft oven at 70º C to a constant weight, were determined. Based on these data were determined the mean net assimilation rate (NAR, rate of biomass gain per leaf area), using the equation proposed by Hunt (1978), and the relative alleviation index (RAI), estimated according to Gupta *et al*. (1995).

Water use efficiency of biomass production ($WUE_{WP}$, g $kg^{-1}$) was determined, for each plant, by dividing total dry matter production by the cumulative amount of water used throughout the growing season. Total dry matter included the oven-dried leaves, stems and roots.

### 2.7. Statistical analysis

All statistical calculations were performed using the software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test ($P < 0.05$). For statistical analysis of RWC, EL and BI arcsine transformation was performed in percentage data. The relationships between $R_{night}$ and $E_{night}$, $R_{night}$ and $C_{i\text{-}night,}$ and $g_{night}$ and $C_{i\text{-}night}$ was analyzed by the Pearson correlation test and significance was set at $P < 0.05$.

## 3. Results and Discussion

### 3.1. Physiological and structural traits modulated by kaolin

Kaolin has been used as a tool for saving water and to improve crop performance, leading to lower temperature of sprayed organs (Segura-Monroy *et al*., 2015; Dinis *et al*., 2018), as the particle film reflects PAR, UV and IR radiation. The reduction of leaf-to-air vapor pressure deficit ($VPDl_{eaf\text{-}air}$) is also a regular effect (Jifon and Syvertsen, 2003; Rosati *et al*., 2006). Consequently, KL contributed to attenuate the drought-induced decline of RWC during the 3$^{rd}$ DP (Figure 2) and to increase leaves succulence (Table 1). Hence, our data demonstrate the effectiveness of this treatment in water-saving under severe conditions, as observed in other studies (Denaxa *et al*., 2012; Boari *et al*., 2015; Nanos, 2015). This ability to maintain turgid leaves in drought environments have several physiological advantages, allowing the maintenance of turgor dependent processes, such as growth, stomatal activity and photosynthesis (Mullan and Pietragalla, 2011).

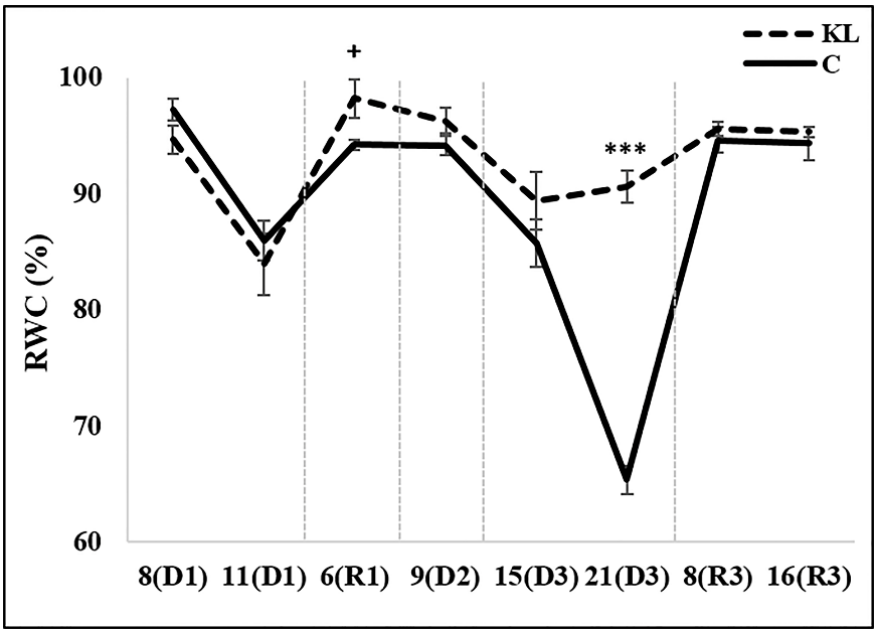


**Figure 2.** Changes of leaf relative water content (RWC) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n=8). Significance: +0.1>P>0.05, ***P<0.001.

In a closely association with plant water status (Figure 2), kaolin film had implications on leaf sclerophylly and structural traits, as KL leaves displayed lower density and thickness (Table 1 and Figure 3). KL plants exhibited thinner leaves, due to lower UC and UPP, that in turns contributed to reduce PP/SP ratio, which indicates a less compact arrangement of mesophyll cells (Bacelar *et al*., 2004). Interestingly, all these changes are typical plant responses to shade. Similar results for leaf density (Denaxa *et al*., 2012), leaf thickness (Segura-Monroy *et al*., 2015), and other shade-related characteristics (Nanos, 2015) were reported previously in response to kaolin application. On the other hand, no significant differences were observed for both epidermis, SP, LPP and TL (Table 1).

**Table 1.** Leaf sclerophylly and structural traits of control (C) and kaolin (KL) plants. Succulence (mg $H_2O$ $cm^{-2}$), density (g $kg^{-1}$), and leaf tissues thickness (µm; total section, LT, upper cuticle, UC, upper epidermis, UE, upper palisade parenchyma, UPP, spongy parenchyma, SP, lower palisade parenchyma, LPP, lower epidermis, LE, palisade/ spongy parenchyma ratio, PP/SP and trichome layer, TL).

| | C | KL | Sig. |
|---|---|---|---|
| **Succulence** | 18.97±1.19 | 27.9±0.88 | ** |
| **Density** | 576.7±6.5 | 495.7±7.2 | * |
| **LT** | 512.5±9.3 | 473.8±12.1 | * |
| **UC** | 7.53±0.24 | 6.61±0.24 | * |
| **UE** | 16.84±0.53 | 18.03±0.64 | n.s. |
| **UPP** | 172.6±9.77 | 138.9±4.09 | * |
| **SP** | 221.7±3.8 | 214.7±6.09 | n.s. |
| **LPP** | 29.54±1.09 | 29.20±1.92 | n.s. |
| **LE** | 16.36±0.28 | 16.45±0.52 | n.s. |
| **PP/SP** | 0.960±0.042 | 0.825±0.029 | * |
| **TL** | 47.86±2.10 | 49.81±3.80 | n.s. |

Values are means±SE (n=8). Significance: n.s., not significant, *P < 0.05, **P < 0.01.

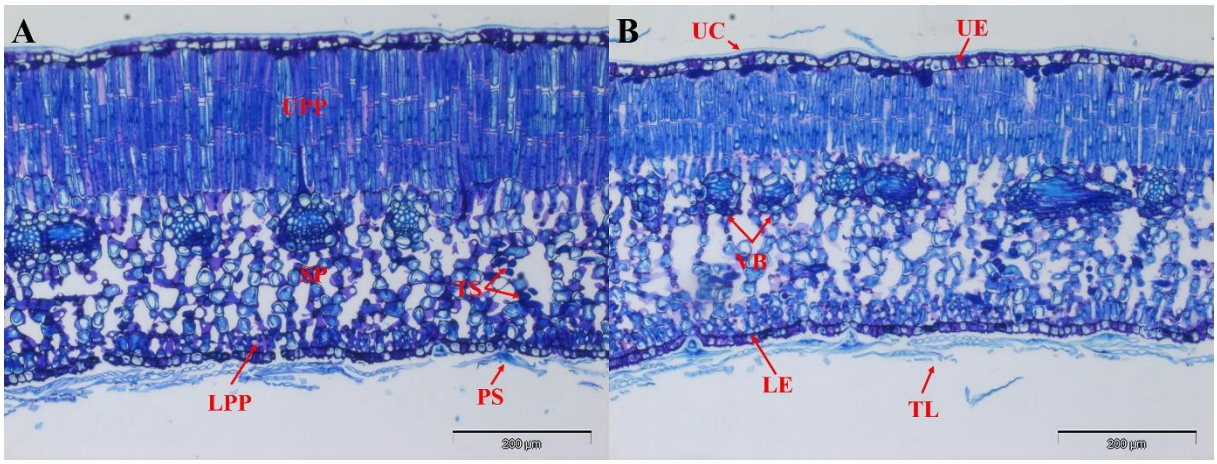


**Figure 3.** Light microscopy images of olive leaf transversal sections stained with toluidine blue. Control (A) and Kaolin (B) treatments. Abbreviations: UC = upper cuticle; UE = upper epidermis UPP = upper palisade parenchyma; SP = spongy parenchyma; LPP = lower palisade parenchyma; VB = vascular bundles; TS = trichosclereids; LE = lower epidermis; TL= trichome layer; PS = peltate scales.

Olive leaf stomata respond strongly to leaf RWC, as well to higher vapor pressure deficit (VPD). So, as KL may lower $VPD_{leaf\text{-}air}$, the sprayed plants were able to keep higher $g_{day}$ and, thus, $A_n$ and $E_{day}$ during the DPs (Figure 4 A, B, C). Similar effects of kaolin on leaf gas

exchange variables were reported previously in different plant species (Jifon and Syvertsen, 2003; Glenn, 2009; Boari *et al*., 2015), including olive tree (Denaxa *et al*., 2012; Nanos, 2015). Nonetheless, KL influence was higher on $g_{day}$ than on $A_{n,}$ contributing to lower $A_n/g_{day}$ during the 2nd and 3rd DPs (Figure 4D).

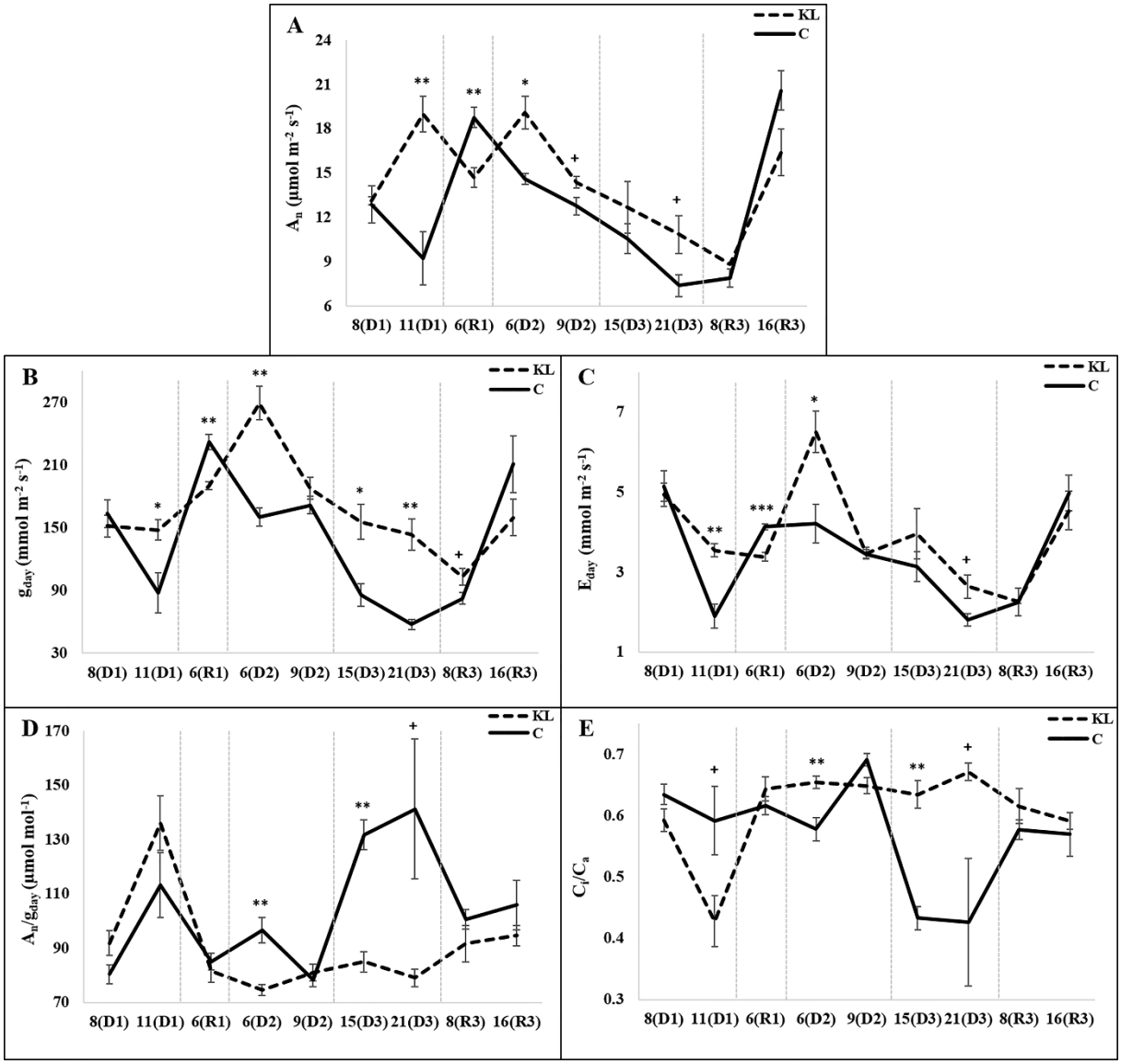


**Figure 4.** Changes of net photosynthetic rate ($A_n$, A), stomatal conductance ($g_{day,}$ B), transpiration rate ($E_{day}$, C), intrinsic water use efficiency ($A_n/g_{day}$, D) and ratio of intercellular to atmospheric $CO_2$ concentration ($C_i/C_a$, E) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n=8). Significance: +0.1>P>0.05, *P < 0.05, **P < 0.01, ***P<0.001.

Despite the predominance of stomatal effects, also corroborated by $C_i/C_a$ data (Figure 4D), KL influenced photosynthetic activity by the preservation of photosynthetic machinery, due to the reduction of heat load and irradiation levels, as demonstrated by Dinis *et al*. (2018). In this

study, KL spray was effective in the photochemistry processes protection (Figure 5), as demonstrated by the lower $F_0$ until the middle of the 3rd DP (Figure 5A), the higher $F_v/F_m$, until the 2nd DP (Figure 5B), and the general trend to superior ΦPSII, with the exception at the peak of the 3rd DP (Figure 5C). Nevertheless, KL did not interfere with the proportion of open PSII reaction centers during drought, as demonstrated by the absence of significant differences on qP (Figure 5D) (Baker, 2008). As qP had a slight influence on ΦPSII, the capacity of KL plants to keep higher ΦPSII was more related to $F'_v/F'_m$ response (Figure 5E), which indicates a reduced loss of excitation energy by thermal dissipation, which could compete with its transfer to PSII reaction centers (Baker, 2008). A positive kaolin influence on the light-dependent reactions of photosynthesis was also described in other studies, with higher effectiveness around midday period (Jifon and Syvertsen, 2003; Segura-Monroy *et al*., 2015).

Regarding recovery events, RWC values showed a prompt recovery (Figure 2). This is a typical response of olive tree that through a conservative behaviour rapidly restore water status after stress relief (Perez-Martin *et al*., 2014). On the other hand, although only significant values of $g_{day}$ were found at the 1st RP, $g_{day}$ showed a reverse pattern with respect to the DP (Figure 4B), demonstrating that KL trees had a delay on $g_{day}$ recovery after rewatering. Under optimal hydration, plants increase transpiration and the resulted evaporative cooling effect lower $VPD_{leaf\text{-}air}$. Under these conditions the low light intensity promoted by kaolin may prevail leading to a reduction of $g_{day}$ (Gregoriou *et al*., 2007). Similar findings were reported previously (Roussos *et al*., 2010; Denaxa *et al*., 2012; Shellie and King, 2013; Boari *et al*., 2015). As a consequence of lower $g_{day}$, KL also retarded the $A_n$ recovery (Figure 4A). In addition, both the partial shade-related characteristics developed by KL leaves and the reduction in solar irradiation limited the recovery of photosynthetic activity. It is important to highlight that the year of the study had an atypical summer season with enhanced cloudy days than usual, meaning that with low PPFD the shade effect is undesirable for KL effectiveness. Denaxa *et al*. (2012) also showed that during the time of the day when light intensity is lower, kaolin limited $A_n$ of irrigated olive trees, while no such effect was recorded at midday hours.

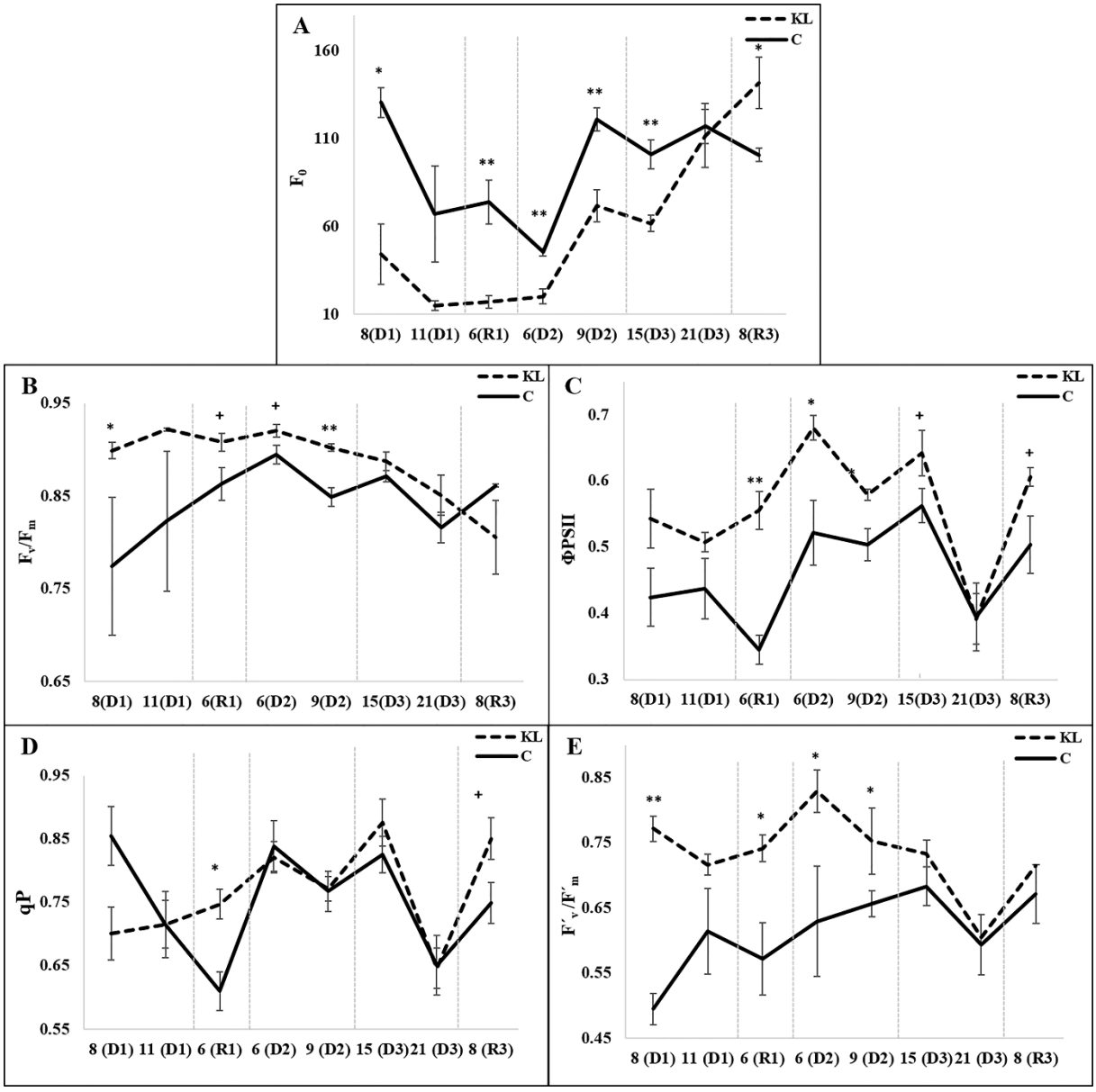


**Figure 5.** Changes in minimal florescence emitted from dark adapted leaves ($F_0$, A), maximum ($F_v/F_m$, B) and effective (ΦPSII, C) quantum efficiency of PSII, photochemical quenching (qP, D), and capture efficiency of excitation energy by open PSII reaction centers ($F'_v/F'_m$, E) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n=8). Significance: +0.1>P>0.05, *P < 0.05, **P < 0.01, ***P<0.001.

Regarding physiological responses during nighttime, this study demonstrated that $g_{night}$ and, consequently, $E_{night}$ respond to kaolin application, being the leaf water losses during the first hours of night repressed by KL (Table 2).

**Table 2.** Leaf nighttime stomatal conductance ($g_{night}$, mmol $m^{-2}$ $s^{-1}$), nighttime transpiration rate ($E_{night}$, mmol $m^{-2}$ $s^{-1}$), cuticular transpiration ($E_{cuticular}$, mmol $m^{-2}$ $s^{-1}$), nighttime respiration rate ($R_{night}$, µmol $m^{-2}$ $s^{-1}$) and nighttime intercellular $CO_2$ concentration ($C_{i\text{-}night}$, µmol $mol^{-1}$) and whole-plant transpiration during the nighttime ($PE_{w\text{-}night}$, g $H_2O$ $m^{-2}$ $n^{-1}$) and daytime ($PE_{w\text{-}daytime}$, g $H_2O$ $m^{-2}$ $daytime^{-1}$) of control (C) and kaolin (KL) plants.

| | C | KL | Sig. |
|---|---|---|---|
| $g_{night}$ | 17.65±1.15 | 5.84±0.75 | *** |
| $E_{night}$ | 0.273±0.014 | 0.098±0.003 | *** |
| $E_{cuticular}$ | 0.046±0.003 | 0.095±0.005 | *** |
| $R_{night}$ | 1.20±0.36 | 1.90±0.07 | * |
| $C_{i\text{-}night}$ | 515.6±26.0 | 981.6±84.9 | ** |
| $PE_{w\text{-}night}$ | 79.4±14.7 | 39.1±6.1 | * |
| $PE_{w\text{-}daytime}$ | 298.8±39.5 | 413.4±42.1 | + |

Values are means±SE (n=8). Significance: +0.1>P>0.05, *P < 0.05, **P < 0.01, ***P<0.001).

Meanwhile, in C leaves $E_{night}$ was highly far above $E_{cuticular}$, demonstrating that $E_{night}$ is mainly due to stomatal losses, while in KL leaves, the lower $E_{night}$ was more related with $E_{cuticular}$, as a negligible water loss by stomata was observed (Table 2). Moreover, the differences in $E_{cuticular}$ were related with the histological analysis, as KL leaves presented thinner cuticle layer (Table 1). The higher nighttime transpiration of C plants was also confirmed gravimetrically, while during the daytime period was observed a clear opposite tendency (Table 2), in a closely association with $g_{day}$ values (Figure 4B). It has been demonstrated that nighttime transpiration can account for around 10% of daily transpiration (Escalona *et al*., 2013; Medrano *et al*., 2015), reducing water use efficiency at whole-plant scale (Medrano *et al*., 2015). Our data indicate that kaolin contributed to reduce the relative nighttime transpiration, as in KL plants $PE_{W\text{-}night}$ account to 8.6 % of whole-day transpiration, against 21 % in C plants (Table 2). However, since nighttime transpiration can lead to a reduction in WUE raises the question, why control stressed-plants risk to lose water when there is no opportunity for carbon gain? There is no consistent knowledge about $g_{night}$ and much factors can act isolated or in combination. $E_{night}$ may lower leaf temperature by evaporative cooling, thereby decreasing carbon losses through $R_{night}$ (Coupel-Ledru *et al*., 2016), a hypothesis supported in our study by the significant negative correlation between $E_{night}$ and $R_{night}$ ($r=-0.723$; $P < 0.01$). This response takes especially importance in the first hours of darkness, once temperatures are usually higher. In addition, a reduced accumulation of $C_{i\text{-}night}$ (Table 2), due to the reduced $R_{night}$ ($r=0.789$; $P < 0.001$), may also have contributed to stomatal open (Escalona *et al*., 2013), as attested by the

negative correlation between $C_{i\text{-}night}$ and $g_{night}$ ($r=-0.945$; $P < 0.001$). Thus, this study confirms that the olive tree capacity to maintain low $R_{night}$ rates during more stressful conditions, allows this species to allocate more assimilates for biomass accumulation and, consequently, for growth (Varone and Gratani, 2015), despite the higher leaf density and thickness (Table 1) and decline in $A_n$ (Figure 4A).

### 3.2. Changes in non-structural carbohydrates and oxidative stress markers induced by kaolin

Environmental stresses perturb the equilibrium between the production and scavenging of ROS, inducing damage to several biomolecules (Sharma e*t al*., 2012). Nevertheless, only few studies reported data about cellular metabolic dynamics in relation to kaolin application, and those were mainly related with photosynthetic pigment analysis. Our data showed higher concentration of chlorophylls in KL plants (Table 3), a sign of lower oxidative stress, allowing that these plants can use light energy more efficiently. Chlorophylls preservation associated to kaolin application had also been reported in other studies (Nanos, 2015; Segura-Monroy e*t al*., 2015). In addition, KL leaves exhibited lower $Ch_a/Chl_b$ ratio during drought (Table 3), as in other works (Shellie and King, 2013; Nanos, 2015). Lower $Ch_a/Chl_b$ ratio is a typical feature of low-light adapted leaves, indicating that photosystems have larger antenna sizes at the expense of reaction centers pigment proteins, to enhance their ability to capture and utilize photon energy (Gregoriou *et al*., 2007; Lichtenthaler *et al*., 2007). Furthermore, as total carotenoids concentration was not significant different between treatments, $Chl_{(a+b)}$/Carotenoids ratio was significantly higher in KL leaves (Table 3), also a shade-related trait (Lichtenthaler *et al*., 2007). Since carotenoids play an important role in photoprotection, scavenging ROS and releasing the excess energy by thermal dissipation via xanthophyll cycle (Lisar *et al*., 2012; Sharma *et al*., 2012), the higher $Chl_{(a+b)}$/Carotenoids ratio observed in KL leaves indicates a lower need for photoprotection of chlorophylls. Meanwhile, the concentration of chlorophylls was restored immediately after rewatering (Table 3), probably as a result of *de novo* synthesis of chlorophylls, as in the study of Tognetti *et al*. (1995). The maintenance of high chlorophyll concentration during drought was noted to contribute to a rapid recovery of photosynthesis (Chen *et al*., 2016). However, we found no such influence on $A_n$ recovery of KL leaves, meaning that photosynthetic recovery of KL trees was mainly limited by stomata. Although the concentration of total carotenoids was not affected by rewatering, the relative proportion of individuals changed, as revealed by higher investment on lycopene and β-carotene by control

plants (Table 3). Lycopene is the starting compound of various end group modifications that produces a large variety of carotenoids, such as β-carotene, which display the ability to quench triplet chlorophyll and singlet oxygen (Domonkos *et al*., 2013), preventing photosynthetic apparatus damage.

**Table 3.** Foliar pigments concentrations (mg $g^{-1}$ DW) of control (C) and kaolin (KL) at the peak of severest drought period (21(D3)) and 8 days after rewatering (8(R3)). Total chlorophyll ($Chl_{(a+b)}$), chlorophyll a/b ratio ($Chl_a/Chl_b$), total carotenoids, $Chl_{(a+b)}$/Carotenoids ratio, lycopen and β-Carotene.

| | | C | KL | Sig. |
|---|---|---|---|---|
| **$Chl_{(a+b)}$** | **21(D3)** | 2.80±0.13 | 3.21±0.05 | * |
| | **8(R3)** | 3.36±0.06 | 3.31±0.07 | **n.s.** |
| **$Chl_a/Chl_b$** | **21(D3)** | 3.11±0.01 | 3.04±0.02 | + |
| | **8(R3)** | 2.97±0.015 | 3.12±0.02 | **n.s.** |
| **Carotenoids** | **21(D3)** | 0.656±0.032 | 0.707±0.012 | **n.s.** |
| | **8(R3)** | 0.720±0.023 | 0.717±0.018 | **n.s.** |
| **$Chl_{(a+b)}$/Carotenoids** | **21(D3)** | 4.48±0.032 | 4.77±0.042 | ** |
| | **8(R3)** | 4.91±0.25 | 4.85±0.05 | **n.s.** |
| **Lycopene** | **21(D3)** | 1.21±0.03 | 1.30±0.04 | **n.s.** |
| | **8(R3)** | 1.35±0.05 | 1.10±0.04 | * |
| **β-Carotene** | **21(D3)** | 0.554±0.012 | 0.534±0.010 | **n.s.** |
| | **8(R3)** | 0.637±0.013 | 0.564±0.026 | * |

Values are means±SE (n=8). Significance: n.s., not significant, +0.1>P>0.05, *P < 0.05, **P < 0.01.

A fraction of carbon acquired via photosynthesis is retained in the form of non-structural carbohydrates. At the peak of the 3rd DP, KL leaves presented higher and lower concentrations of soluble sugars and starch, respectively (Table 4). Upon rewatering, no significant differences were observed in soluble sugars levels, as a result of the higher increase of soluble sugars in C plants. On the other hand, St concentration remained higher in C plants, in spite of the higher decrease during the RP, suggesting a mobilization of these reserves. These results are consistent with a dual view of non-structural carbohydrates function. Soluble sugars are sources of carbon for maintenance and regrowth during recovery and may act as osmoprotectants (Chaves *et al*., 2002). Since the primary function of compatible solutes is to prevent water loss, by maintaining cell turgor and the gradient for water uptake into cells (Lisar *et al*., 2012) might contributed to

the higher RWC and stomatal opening exhibited by KL leaves (Figures 2 and 4B). Moreover, compatible solutes are also involved in detoxification of ROS and stabilization of cellular macromolecules structures (Lisar *et al*., 2012).

**Table 4.** Foliar metabolite concentrations of control (C) and kaolin (KL) at the peak of severest drought period (21(D3)) and 8 days after rewatering (8(R3)). Soluble sugars (mg $g^{-1}$DW), starch (mg $g^{-1}$DW), soluble proteins (mg $g^{-1}$ DW), total thiols (nmol $mg^{-1}$ DW), total phenolics (mg $g^{-1}$ DW), flavonoids (mg $g^{-1}$ DW), ascorbate (mg $g^{-1}$ DW), reactive oxygen species (ROS, nmol $g^{-1}$ DW) and hydrogen peroxide ($H_2O_2$, µmol $g^{-1}$ DW) concentrations, total antioxidant capacity (TAC, µmol TE $g^{-1}$ DW), and electrolyte leakage (%).

| | | C | KL | Sig. |
|---|---|---|---|---|
| **Soluble Sugars** | **21(D3)** | 209.0±6.1 | 230.9±6.6 | ***** |
| | **8(R3)** | 242.5±14.7 | 254.0±14.8 | **n.s.** |
| **Starch** | **21(D3)** | 64.35±5.80 | 41.37±3.50 | ****** |
| | **8(R3)** | 53.29±6.64 | 35.31±2.42 | ***** |
| **Soluble proteins** | **21(D3)** | 2.74±0.14 | 4.59±0.30 | ****** |
| | **8(R3)** | 3.72±0.13 | 7.00±0.35 | ******* |
| **Total thiols** | **21(D3)** | 1.04±0.07 | 1.27±0.05 | ***** |
| | **8(R3)** | 1.15±0.04 | 1.28±0.07 | **n.s.** |
| **Total phenolics** | **21(D3)** | 44.54±0.30 | 42.07±0.39 | ** |
| | **8(R3)** | 44.00±0.39 | 47.13±0.30 | *** |
| **Flavonoids** | **21(D3)** | 23.73±0.25 | 18.64±0.28 | *** |
| | **8(R3)** | 18.47±0.40 | 19.53±0.22 | * |
| **Ascorbate** | **21(D3)** | 1.68±0.05 | 1.36±0.02 | ****** |
| | **8(R3)** | 2.27±0.04 | 2.62±0.03 | ******* |
| **TAC** | **21(D3)** | 163.4±1.2 | 152.6±3.4 | ***** |
| | **8(R3)** | 199.1±4.2 | 195.1±3.9 | **n.s.** |
| **ROS** | **21(D3)** | 0.443±0.020 | 0.554±0.043 | ****** |
| | **8(R3)** | 0.588±0.041 | 0.511±0.053 | **n.s.** |
| **$H_2O_2$** | **21(D3)** | 12.48±0.15 | 19.53±0.23 | ******* |
| | **8(R3)** | 18.74±0.59 | 24.18±0.35 | ******* |
| **Electrolyte leakage** | **21(D3)** | 27.56±0.56 | 18.54±3.41 | ***** |
| | **8(R3)** | 24.05±1.78 | 19.87±0.89 | + |

Values are means±SE (n=8). Significance: n.s., not significant, +0.1>P>0.05, *P < 0.05, **P < 0.01, ***P<0.001).

On the other hand, starch acts mostly as a reservoir of carbon for future use, depending on the source-sink dynamics concept. The higher starch contents in C leaves, also verified earlier in olive tree under severe drought conditions (Bacelar *et al*., 2006), in spite of lower $A_n$, suggest that carbon was not translocated out of the leaves, reflecting an excess supply relative to demand. Conversely, the lower starch concentrations in KL leaves may be linked to tissue osmotic adjustment, as one of the main sources of osmolytes are the starch reserves, which supply soluble sugars. Although it is usual an increased demand of non-structural carbohydrates to rapid recovery of physiological activities and growth after rewatering (Da costa and Huang, 20006), this is not the general picture of our data (Table 4). To illustrate, was observed an increase of soluble sugars during rehydration, namely in C plants, in line with a tendency to lower starch content. These results showed that, following rehydration, olive trees divert a higher proportion of the assimilated carbon into soluble sugar export for plant growth, and less to temporary storage, as starch, a common response of fast growing species (Liu *et al*., 2017). Thus, we may assume that growth of trees under drought and rewatering cycles are positively associated with higher soluble sugars/starch ratio, particularly clear in KL plants, and not to higher non-structural carbohydrates concentration.

Kaolin application contributed to keep higher total soluble proteins concentrations (Table 4), possibly due to the better water status and the plausible reduced leaf temperature. Indeed, a reduction in leaf soluble proteins concentration was already reported in plants subjected to drought (*Bacelar et al*., 2007) and heat (Gulen and Eris, 2004). The accumulation of ROS under stressful conditions induces oxidative damage in proteins (Farooq *et al*., 2009), and high temperatures also causes protein denaturation and aggregation (Hasanuzzaman *et al*., 2013). One of the more susceptible targets in proteins are thiol groups, with relevant role in signalling in a range of physiological processes, but when suffer from irreversible oxidation can seriously damage proteins (Chouchani *et al*., 2011). Thiol groups are usually affected by water deficit, as already reported in olive trees (Bacelar *et al*., 2006, 2007), but kaolin contributed to keep higher total thiols levels during the DP (Table 4). After stress relief, both treatments increased soluble proteins contents (Table 4), suggesting that proteins were suppressed due to water deficit in both treatments, as in Flexas *et al*. (2006), although in a different extent. In addition to the higher soluble proteins concentration upon rewatering, KL leaves also presented higher increase from the peak of drought stress, with an enhancement of 52% against 35% exhibited by C leaves. Moreover, only the last leaves increased total thiols levels (Table 4) in response to

rewatering, suggesting, at some extent, a reversible oxidation during drought and possible effects in signalling pathways.

As a result of the worst physiological status, reported before, C plants needed to invest more resources in antioxidant defences during drought, as demonstrated by ascorbate and total phenolic concentrations and by total antioxidant activity data (Table 4). Ascorbate, besides to directly scavenge ROS, is also a substrate to ascorbate peroxidase that use it as specific electron donor to reduce $H_2O_2$ to $H_2O$ (Mattos and Moretti, 2015; Sharma *et al*., 2012). Moreover, in chloroplasts, ascorbate acts as a cofactor of violaxantin de-epoxidase, thus sustaining dissipation of excess excitation energy (Mattos and Moretti, 2015). Phenolic compounds, to which flavonoids belong, possess the ideal chemistry for free radical scavenging because of their strong capacity to donate electrons or hydrogen atoms, acting actively as plant antioxidants (Mattos and Moretti, 2015). It is noteworthy that total phenolics increase was largely associated with the rise of flavonoids (Table 4), which, in addiction to be efficient scavengers, serve multiple functions in photoprotection under high sunlight conditions and act as UV-B screening (Agati *et al*., 2013). 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). Interestingly, the concentrations of total phenolics, flavonoids and ascorbate increased with rewatering, namely in KL leaves, being reversed the previous pattern, as those concentrations were now higher in KL trees (Table 4). This suggest a rearrangement of many metabolic pathways, in order to a better balance between repair of drought-induced damages, activation of a battery of plant defences and stimulation of plant growth processes. Furthermore, kaolin contributed to maintain lower cellular membrane dysfunction, as showed by the inferior electrolyte leakage during drought (Table 4). Upon rewatering, C leaves had less leakage of ions, but still presented a tendency to higher electrolyte leakage than KL leaves (Table 4).

Despite KL leaves had lower signals of oxidative damage, they exhibited higher concentrations of both total ROS and $H_2O_2$ during drought (Table 4). Conversely, Dinis *et al*. (2016) observed inferior accumulation of ROS in response to kaolin application in grapevines leaves. ROS are an inevitable by-product of aerobic metabolism, damaging biomolecules if were excessively produced (Sharma *et al*., 2012). However, at low/moderate levels they act as second messengers in a variety of cellular processes, including conferment of tolerance to environmental stresses (Sharma *et al*., 2012), specially $H_2O_2$ due to its long half-life and the ability to cross cellular membranes (Petrov and Van Breusegem, 2012). Because of the

multifunctional roles of ROS, it is necessary for the cells to control their levels tightly to avoid any oxidative injury, but not eliminating them completely (Sharma *et al*., 2012). Taken together, these results suggest that ROS levels in KL leaves may still not be excessively harmful. In addition, in C plants, the oxidative damages already caused might triggered the antioxidative responses, that effectively reduced the ROS levels, to avoid extra oxidative damages, showing that C plants are not severely stressed. Moreover, the increase in total ROS in C leaves after stress relief, linked with the increase of $H_2O_2$ in both treatments (Table 4) suggest that these levels might occur due to normal plant metabolism.

### 3.3. Growth biomass accumulation and water use efficiency under kaolin application

In general, is recurrent to find a positive effect of kaolin application on plant growth and/or yield under drought stress conditions (Roussos *et al*., 2010; Segura-Monroy *et al*., 2015). However, in the present study, in spite of the lower oxidative injury and the best physiological performance observed during the DPs in KL plants, leaf area, net assimilation rate, total biomass increase, and water use efficiency for biomass production were not significant different between treatments (Table 5), which were associated with the negative $A_n$ responses after rewatering (Figure 4A). Moreover, the differences were also mitigated by the high number of days (25 out of 66) of the rehydration periods, given the unusual climatic conditions. Nonetheless, there was a tendency to all those variables being higher in KL plants, contributing to an increase of 8% on RAI.

**Table 5.** Total leaf area (TLA, $cm^2\ plant^{-1}$), total biomass increase (BI, %), whole-plant water use efficiency ($WUE_{WP,}$ $g\ kg^{-1}$), relative alleviation index (RAI) and net assimilation rate (NAR, $g\ m^2\ day^{-1}$) of control (C) and kaolin (KL) plants at the end of the experiment.

| | C | KL | Sig. |
|---|---|---|---|
| **TLA** | 1078.1±44.8 | 1208.2±76.3 | **n.s.** |
| **BI** | 26.67±5.84 | 37.22±9.60 | **n.s.** |
| **$WUE_{WP}$** | 4.15±0.91 | 5.78±1.49 | **n.s.** |
| **RAI** | 100.0±4.4 | 108.0±7.3 | **n.s.** |
| **NAR** | 3.19±0.67 | 4.24±1.08 | **n.s.** |

Values are means±SE (n=8). Significance: n.s., not significant.

The negative influence of kaolin in leaf $A_n/g_{day}$ during some drought periods of the experiment (Figure 4D) was not traduced in $WUE_{WP}$, that presented a slight tendency to be higher in KL trees (Table 5). In fact, $A_n/g_{day}$ and $WUE_{WP}$ cannot be always strictly related, due to spatial and temporal variations, since the first denotes responses of individual leaves at specific environmental conditions, while the second represents the whole-plant carbon and biomass acquisition per unit of water used along all the growth season, integrating other physiological processes like respiration and night transpiration processes (Medrano *et al*., 2015). A positive influence of kaolin application on $WUE_{WP}$ was described in cape gooseberry by Segura-Monroy *et al*. (2015).

## 4. Conclusions

Kaolin is suitable for improving water status and preserving cellular function during the most stressful periods, reducing investment costs in extra repair damages during recovery periods. Kaolin is appropriate to alleviate stomatal and non-stomatal limitations to photosynthesis under water shortage conditions, allowing a better photosynthetic activity during the more severe drought events. Nevertheless, the shaded effect conferred by kaolin interfere with gas exchange restauration after stress relief and also with photosynthetic activity under low light conditions. Kaolin mitigate oxidative stress during drought episodes, reducing the investment in antioxidant defences, and allow a better capacity to restore metabolic functions upon rewatering. Under the present conditions, kaolin did not influence whole-plant water use efficiency and plant biomass accumulation. Taken together, the present results clearly demonstrate the complexity of plant responses to kaolin-induced microclimate changes, which vary depending on the respective biological and environmental conditions. Nevertheless, kaolin may have beneficial effects on realistic field conditions under the prevalence of sunny days, in trees with higher dense canopies, benefiting from the light redistribution within the canopy, and in semi-arid areas, where is recurrent to find groves under rainfed conditions where the incident PAR usually exceeds the capacity of plant use.

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## Supplementary material

**Figure 1.** Maximum, minimum and average air temperature (ºC) during the experimental period. Days of drought (D1, D2, D3) and rewatering (R1, R3) in each cycle.


**Table 1.** Photosynthetic photon flux density (PPFD) and air temperature during the leaf gas exchange measurements in days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle.

| Analysis days | | PPFD ($\mu molm^{-2}\ s^{-1}$) | Temperature (ºC) |
|---|---|---|---|
| **8(D1)** | **morning** | 1744±86 | 27.9±0.1 |
| **11(D1)** | **morning** | 1652±65 | 28.5±0.1 |
| **6(R1)** | **morning** | 651±30 | 25.1±0.5 |
| **6(D2)** | **morning** | 1737±143 | 27.0±1.7 |
| **9(D2)** | **morning** | 1010±55 | 25.3±0.1 |
| **15(D3)** | **morning** | 1408±221 | 26.2±1.7 |
| **21(D3)** | **morning** | 1609±91 | 25.5±2.0 |
| | **night** | 0.0±0.0 | 21.1±0.5 |
| **8(R3)** | **morning** | 1573±202 | 25.5±1.3 |
| **16(R3)** | **morning** | 1424±156 | 27.3±0.7 |

Values are means±SD.

# CHAPTER 5

## Salicylic acid as a short-term mitigation strategy under drought and rewatering

## Briefing note

This chapter covers the olive tree mechanisms modulated by SA application, under both drought and recovery events. Although the use of this phytohormone in drought tolerance has been largely studied, the available information is essentially under herbaceous species and, as far as we know, none study was performed in olive tree under drought stress. Moreover, the influence in drought recovery capacity is also usually neglected. The importance of evaluation of SA influence in olive tree responses lays on the necessity to improve their performance under the typical Mediterranean ecosystems, frequently exposed to episodic droughts and highly susceptible to climate change.

The chapter is an adaptation of two research articles. One published in *Journal of Plant Physiology* (230, 21-32) entitled "Salicylic acid modulates olive tree physiological and growth responses to drought and rewatering events in a dose dependent manner", related to the point 5.1, while the other corresponds to a submitted manuscript entitled "Salicylic acid increases drought adaptability of young olive trees by changes on redox status and ionome", related to the point 5.2. These articles aimed to respond to the specific objectives 3 and 4 of this thesis, "to evaluate the influence of exogenously applied SA on olive tree responses to drought and rewatering events" and "to evaluate how different SA concentrations determine olive tree responses to drought and rewatering events". These studies revealed how SA modulates olive tree responses to drought and rewatering. Moreover, allows to select a suitable SA concentration to improve olive tree responses under drought stress.

The authors contribution for the article converted in the point 5.1. was: Cátia Brito was responsible for establish and maintain the experiment, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and in the critical review of the article. Helena Ferreira collaborated in data collection on the field and assisted in the laboratory analyses. Lia-Tânia Dinis, Glória Pinto and Mónica Meijón gave the support for the immunohistochemical studies. Carlos Correia was responsible for design the experiments, data collection on the field and critical review of the article. All the authors reviewed and approved the final manuscript.

The authors contribution for the article converted in the point 5.2. was: Cátia Brito was responsible for establish and maintain the experiment, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and in the critical

review of the article. Helena Ferreira collaborated in data collection on the field and assisted in the laboratory analyses. João Coutinho carried out the minerals analysis. Carlos Correia was responsible for design the experiments, data collection on the field, critical review and final approval of the manuscript. All the authors reviewed and approved the final manuscript.

## 5.1. Salicylic acid modulates olive tree physiological and growth responses to drought and rewatering events in a dose dependent manner

# Salicylic acid modulates olive tree physiological and growth responses to drought and rewatering events in a dose dependent manner

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## Abstract

The predicted accentuation of drought events highlights the importance of optimize plants capacity to tolerate drought, but also the capacity to recovery from it, especially in species, as olive tree (*Olea europaea* L.), that grows in particularly susceptible regions. Three different concentrations (10, 100 and 1000 μM) of salicylic acid (SA), a stress signaling phytohormone, was sprayed on 3-year-old potted olive trees subjected to three successive drought and rewatering events. Trees responses to SA application are concentration dependent, being 100 μM the most effective concentration to improve drought tolerance and recovery capacity. During drought events, this effectiveness was achieved by osmolytes accumulation, leaf water status maintenance, reduced photosynthetic systems drought-associated damages, and by optimizing shoot/root ratio. The better plant fitness during drought allowed a fast recovery of the physiological functions upon rewatering and reduced the necessity to invest in extra repair damages, allowing the regrowth. The intense abscisic acid (ABA) signal close to upper epidermis in stressed controls suggests a "memory" of the worst water status displayed by those plants. SA attenuated the limitation of total biomass accumulation imposed by drought, mainly in root system, increased water use efficiency and lead to a higher intense signal of indoleacetic acid (IAA) in leaves during recovery period. In summary, in a suitable concentration, SA demonstrate to be a promising tool to increase drought adaptability of olive trees.

**Keywords:** drought; recovery; water status; photosynthesis; hormones; plant biomass.

## 1. Introduction

The Mediterranean region is well known by the widespread area of olive orchards, which has been growing due to the recognition of the benefits of olive fruits and oil to human health, as are rich sources of valuable nutrients, bioactives and monounsaturated fatty acids (Ghanbari *et al*., 2012). However, the accentuation of extreme events in this area, with decreased water availability and increased air temperature and evaporative demand, will became an acute problem (IPCC, 2013). Among the abiotic stresses, drought is perhaps the most responsible for decreased agricultural production worldwide (Wani *et al*., 2016), being highly exacerbated in the presence of other stressors. Although olive tree (*Olea europaea* L.) is a sclerophyllous species well adapted to the Mediterranean region (Fernández, 2014), the investment in defense strategies against drought (Bacelar *et al*., 2006, 2007a,b, 2009) seriously compromise growth and yield.

In response to drought, tissue water content declines, mesophyll compactness increases (Bacelar *et al*., 2007a) and stomatal ($g_s$) and mesophyll ($g_m$) conductance decrease, affecting photosynthetic rate ($A_n$) (Perez-Martin *et al*., 2014). As drought severity increases, the biochemical component of photosynthesis might also be inhibited (Bacelar *et al*., 2009; Boughalleb and Hajlaoui, 2011), along with photosynthetic pigments degradation and perturbations of photochemical processes (Bacelar *et al*., 2006; Boughalleb and Hajlaoui, 2011). Consequently, carbohydrate production and availability (Farooq *et al*., 2009) may be compromised. Still, plant biomass production depends on the balance between An and respiration rate (R), and thus, particularly in conditions in which An is negatively affected, R is a determining factor (Galmés *et al*., 2007). However, the respiratory process under drought conditions has been commonly marginalized, and the few information available about its pattern and regulation is usually contradictory (Flexas *et al*., 2005). Moreover, plants modify their physiology and biochemistry in response to stress conditions by the cross-talk of several hormones (Munné-Bosch and Müller, 2013), changing their production, distribution or signal transduction (De Diego *et al*., 2012, 2013; Escandón *et al*., 2016).

Although drought response is detrimental to plants growth and development, the recovery capacity has a key role in drought adaptability (drought resistance and recovery capacity) (Chen *et al*., 2016). This take special importance in the Mediterranean-type ecosystems, where plants are continuously exposed to repeated cycles of drought-rewatering during their life, depending they survival on the ability to use the scarce and unexpected rainfall water during summer (Munné- Bosch and Peñuelas, 2003). However, compared to the developing drought, studies of

recovery have been neglected. The recovery degree is species-dependent and it is also influenced by the duration and intensity of previous drought, depending on compatible solutes accumulation, phytohormones dynamics and carbon allocation between plant organs (Chaves *et al*., 2009; De Diego *et al*., 2012, 2013).

To cope with the negative impacts of drought in olive trees, short-term mitigation strategies that improve both drought resistance and recovery capacity must be evaluated. Although the precise mechanisms remain largely unknown, salicylic acid (SA) appears to be a key molecule in drought resistance in some species (Khan *et al*., 2015a,b; Wani *et al*., 2016). SA can regulate various plant metabolic processes, activate the antioxidant system, modulate the production of diverse osmolytes and secondary metabolites, and maintain nutrient status, resulting in stress tolerance (Khan *et al*., 2015a,b). However, the effect might depend on the combination of several factors, including genotype, kind and level of stress, and the applied concentration (Khan *et al*., 2003; Umebese *et al*., 2009; Kang *et al*., 2012; Fayez and Bazaid, 2014; Kabiri *et al*., 2014; Jesus *et al*., 2015). To our knowledge, research on SA application and drought adaptability in olive tree species remains completely absent. The existing studies focus on the freezing (Hashempour *et al*., 2014) and salt stress (Aliniaeifard *et al*., 2016), revealed that suitable SA concentrations were effective to cope with these stresses. Still, in other species SA is known to be effective in improving tolerance to drought (Khan *et al*., 2015a,b), heat (Khan *et al*., 2013) and salt stress (Khan *et al*., 2014). Hence, we hypothesize that the exogenous application of SA mitigates the drought-induced negative effects under both drought developing and recovery, upon rewatering. To test this hypothesis will be: (i) evaluated physiological and biochemical responses to SA application under drought episodes; (ii) evaluated physiological and biochemical responses to SA application under rewatering; (iii) evaluated the SA influence in plant biomass accumulation, and (iv) selected the most effective SA concentration to intermittent events of drought.

## 2. Material and methods

### 2.1.Plant material and experimental set-up

The study was carried out in Vila Real, Northeast Portugal (41°17'17.83"N, 7°44'12.81"W, 448 m a.s.l.), with own-rooted 3-years-old olive trees (*Olea europaea* cv. Cobrançosa) between June and September 2014. Plants were grown outdoors in 16 l pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). The surfaces of containers were covered with a thin layer of perlite and sealed with plastic film

and aluminum foil. This measure aimed to avoid the evaporation from soil surface and the rain water entering to the pots, and to minimize the temperature increase inside the containers. Pots were randomly arranged and periodically rotated to the neighboring position to minimize the effects of environmental heterogeneity. The climate of the study site is typically Mediterranean-like, a warm-temperate climate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which falls in the autumn-winter with negligible rainfall during the summer months, although 2014 was an atypical summer with some rainfall events. The warmest months are July/August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 °C and 6.8/6.3 °C, respectively (IPMA, 2017). Prior to the experiment, eighty-eight uniform plants, selected based on height, leaf number and leaf area were left for 30 days in the study site for acclimatization, being watered every other day to field capacity, determined gravimetrically. Then, at the beginning of the experiment, 6th July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining eighty plants were divided in five groups, each one comprising sixteen plants. One group was sprayed with distilled water and kept under well-watered conditions (WW, control plants) throughout the entire experimental period, in which plants were watered every day. The other four groups were subjected to three drought-rewatering cycles", as reported elsewhere (Brito *et al*., 2018). One group was sprayed with distilled water (D0), while the other three groups were sprayed with different salicylic acid (SA) concentrations (10 μM, D10; 100 μM, D100; and 1000 μM, D1000). Each plant was treated with a mean volume of 150 ml of spraying solution. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. Care was taken during the application of foliar sprays to avoid overspraying non-target trees, covering them with a plastic sheet.

Each group of sixteen plants was divided in two subgroups, each one with eight plants arranged in a completely randomized design with four replications (two plants per treatment). Plants from one subgroup were used for physiological and biochemical destructive measurements, and plants from the other subgroup were used for final biomass assessment. A schematic representation of the experiment is presented in Figure 1

All physiological and biochemical measurements at leaf level were measured in healthy, full expanded mature leaves. The daytime leaf gas exchange and leaf relative water content measurements (n=8) were taken periodically during the three drought-recovery cycles and

respiration rate (n=8) was done at the peak of severest drought period (DP) (3rd cycle). Leaf samples for biochemical analysis (n=8) were taken at the peak of severest DP (3rd cycle) and eight days after the respective recovery period (RP). Leaves for abscisic acid (ABA) and indoleacetic acid (IAA) immunolocalization (n=3) were sampled at the end of the experiment. For growth, biomass accumulation and whole-plant water use efficiency (n=8) plants were harvested at the end of the experiment.

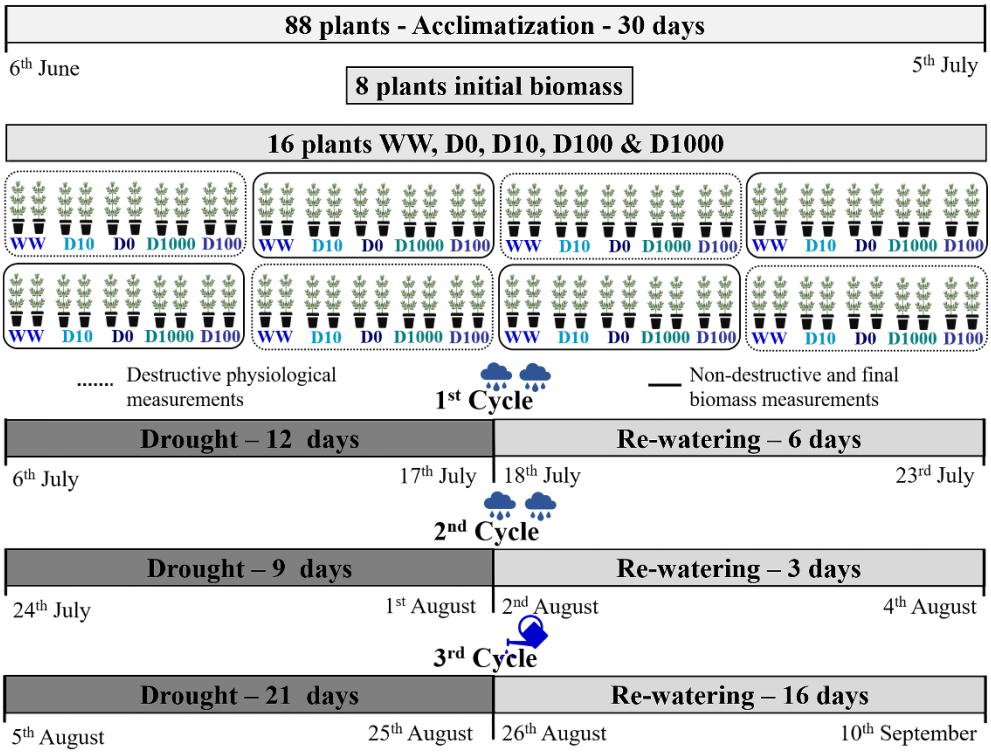


**Figure 1.** Schematization of the experiment. Well-watered controls (WW) and droughted plus salicylic acid plants, 0µM (D0), 10 µM (D10), 100 µM (D100) and 1000 µM (D1000).

### 2.2. Leaf gas exchange and water status

During daylight, leaf gas exchange measurements were taken periodically on summer cloudless days using a portable IRGA (LCpro+, ADC, Hoddesdon, UK), operating in the open mode. At night, the respiration rate measurements were performed once, at the peak of the 3rd drought cycle, in the first hours of the night (22:30‑23:30 local time). Net photosynthetic rate ($A_n$, µmol $CO_2$ $m^{-2}$ $s^{-1}$), stomatal conductance ($g_s$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) and respiration rate (R, µmol $CO_2$ $m^{-2}$ $s^{-1}$), were estimated using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency was calculated as the ratio of $A_n/g_s$ (µmol $mol^{-1}$).

For water status analysis detached leaves were immediately placed into air-tight containers and then the following parameters were examined: fresh weight (FW, g); weight at full turgor (TW, g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at 4 °C; and dry weight (DW, g), measured after drying at 70 °C to a constant weight. Further, was calculated the relative water content (RWC) as RWC (%)=(FW – DW)/(TW – DW)×100

### 2.3. Carbohydrates and photosynthetic pigments

For quantification of non-structural carbohydrates and photosynthetic pigments the leaves adjacent to those used for RWC were sampled. Total soluble sugars (TSS) were extracted according to Irigoyen *et al*. (1992), by heating foliar discs in 80% ethanol during 1 h, at 80 ºC. TSS were quantified by spectrophotometry reading absorbance at 625 nm, after the reaction of the alcoholic extract with fresh anthrone in a boiling water bath for 10 min. Thereafter, starch (St) was extracted from the same solid fraction by heating leaf discs in 30% perchloric acid during 1 h, at 60 ºC, according to Osaki *et al*. (1991). The St concentration was determined by the anthrone method, as described above for TSS. Glucose was used as a standard for both SS and St quantification. Chlorophylls and carotenoids were extracted with 80% (v/v) acetone. Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$) and total chlorophyll ($Chl_{(a+b)}$) were determined according to Arnon (1949) and Sesták *et al*. (1971), and total carotenoids (Car) according to Lichtenthaler (1987).

### 2.4. Immunolocalization of indole-3-acetic acid (IAA) and abscisic acid (ABA)

To immunolocalization of IAA and ABA, leaves of each treatment were sampled at the end of the experiment and immediately fixed in 3 % (w/v) paraformaldehyde containing 0.1 % (v/v) Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA) and 4 % (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich Co., St Louis, MO, USA) according with Escandón *et al*. (2016). IAA and ABA immunolocalization was performed following the protocol of Escandón *et al*. (2016); and finally, fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation. Images were processed using Fiji Software (Schindelin *et al*. 2012).

### 2.5. Growth, biomass accumulation and whole-plant water use efficiency

Plants were and total leaf area (WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK) and the dry weight of aboveground and belowground organs, after drying in

a force-draft oven at 70ºC to a constant weight, were determined. Based on the data of leaf area and/or dry weight, the mean net assimilation rate (NAR, rate of biomass gain per leaf area), leaf area ratio (LAR, leaf area per total plant biomass) and specific leaf area (SLA, leaf area per leaf biomass) were calculated using the equations proposed by Hunt (1978). Moreover, based on the dry matter accumulation, the relative alleviation index (RAI) and the relative tolerance index (RTI) were estimated according to Gupta *et al*. (1995). Shoot to root ratio, relative aboveground biomass increase (RAB) and relative belowground biomass increase (RBB) were also calculated.

Water use efficiency of biomass production ($WUE_{WP}$, g $kg^{-1}$) was determined for each plant by dividing total dry matter production by the cumulative amount of water used throughout the growing season, as previously described. Total dry matter included the oven-dried leaves, stems and roots.

### 2.6. Statistical analysis

The statistical analysis was performed using the statistical software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test ($P < 0.05$).

## 3. Results

### 3.1. Leaf gas exchange and water status

Keeping WW plants as reference, in general, droughted plants presented lower $A_n$ and $g_s$ (Figure 2). The $g_s$ of D0 and D100 plants decreased over the course of the 1st DP, while $g_s$ of D10 showed a decrease at midday, contrary to D1000 that remains statistically equivalent to WW plants in both periods of the day. During the 6 days of the correspondent RP none of the stressed plants recovered completely $g_s$, being D0 and D100 the most conservative plants. During the 2nd DP, the progressive gs decrease was delayed by the SA application. The delay of $g_s$ fall, promoted by SA, was even more relevant during the 3rd DP, particularly in the end of the DP in D100 and D1000 plants that presented higher $g_s$ than D0 plants, averaging 69.8% and 112.6% in the morning and midday periods, respectively. Thereafter, during the 3rd RP, none of the stressed plants recovered gs completely, although D10 and D100 exhibited a faster recovery after 8 days of irrigation (Figure 2B). Although $A_n$ changes were closely related with

gs responses, some exceptions were recorded (Figure 2). During the 1st DP, in opposite to gs response, D100 plants showed, in the morning, $A_n$ values similar to WW, D10 and D1000 treatments. The alleviation of $A_n$ drought-induced decrease, promoted by SA, was also recorded during the 2nd DP, and at midday at the end of the 3rd DP, where SA-treated plants presented higher $A_n$ than D0 plants, averaging 110%. During the 3rd RP, in a closely association with $g_s$, the recovery of $A_n$ is minimal after 8 days of irrigation, presenting D100 plants higher An than D0 and D1000 trees, reaching 41.2% and 33.1%, respectively. After 16 days of irrigation, although the incomplete recovery of $g_s$, all plants presented similar $A_n$ (Figure 2A and B).

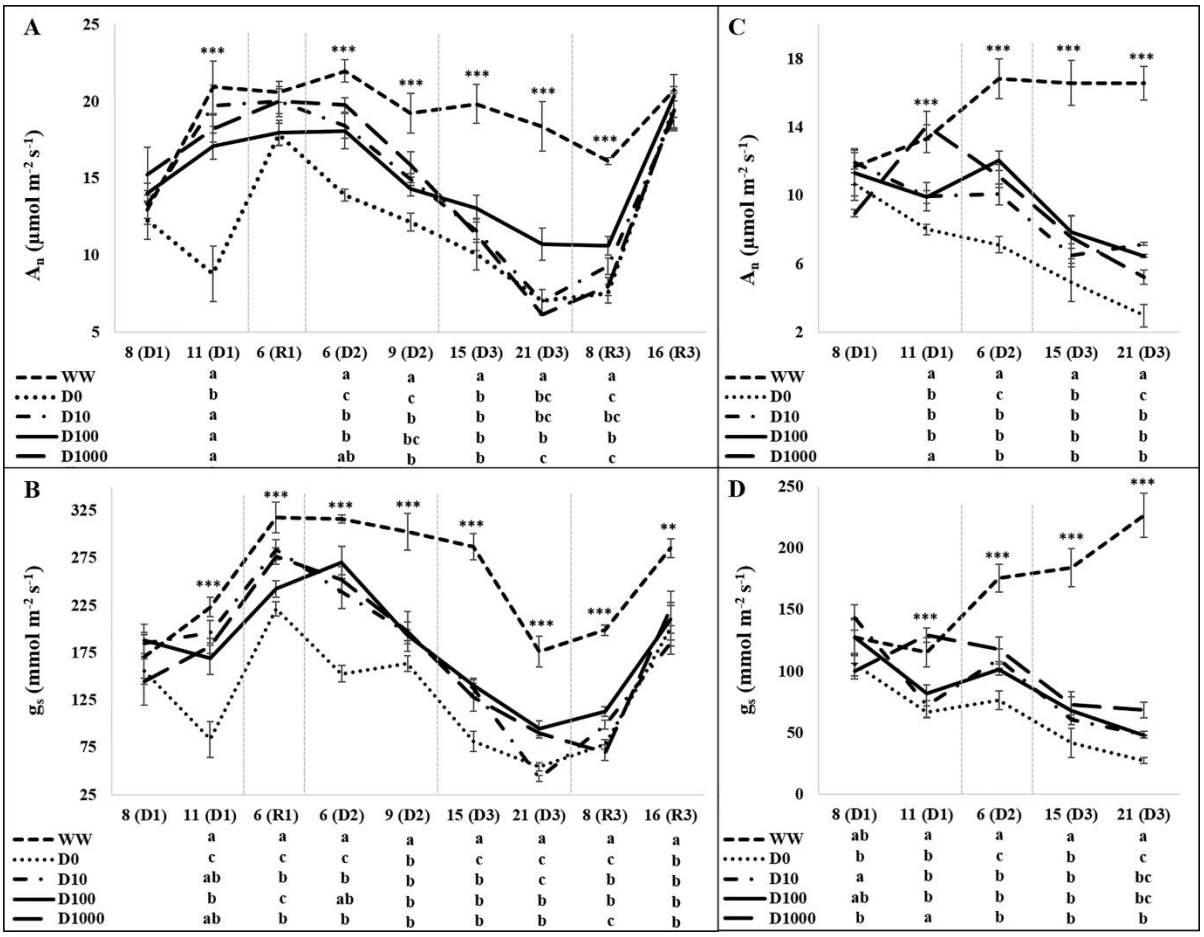


**Figure 2.** Changes of net photosynthetic rate ($A_n$) and stomatal conductance ($g_s$), at morning (A-B) and midday (C-D) of days of drought (D1-D3) and recovery (R1, R3) in each cycle, in leaves of well-watered (WW) and droughted plus salicylic acid (D) plants. Each point is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments within each date (**P < 0.01, ***P<0.001).

In general, droughted plants had higher $A_n/g_s$ and lower $C_i/C_a$ than WW plants throughout the experiment, especially D0 plants in the morning period, while D10 and D100 presented higher $A_n/g_s$ than D0, D1000 and WW plants, averaging 33.6%, 84.3% and 91.7%, respectively, and lower $C_i/C_a$ at midday of the severest DP, averaging 31.9%, 46.4% and 45.1%, respectively (Figure 3). Conversely, during this DP, D1000 plants presented comparable $A_n/g_s$ as WW, and

the higher $C_i/C_a$ during the morning, whereas they had the higher $A_n/g_s$ and the lower $C_i/C_a$ 8 days after rewatering (Figure 3).

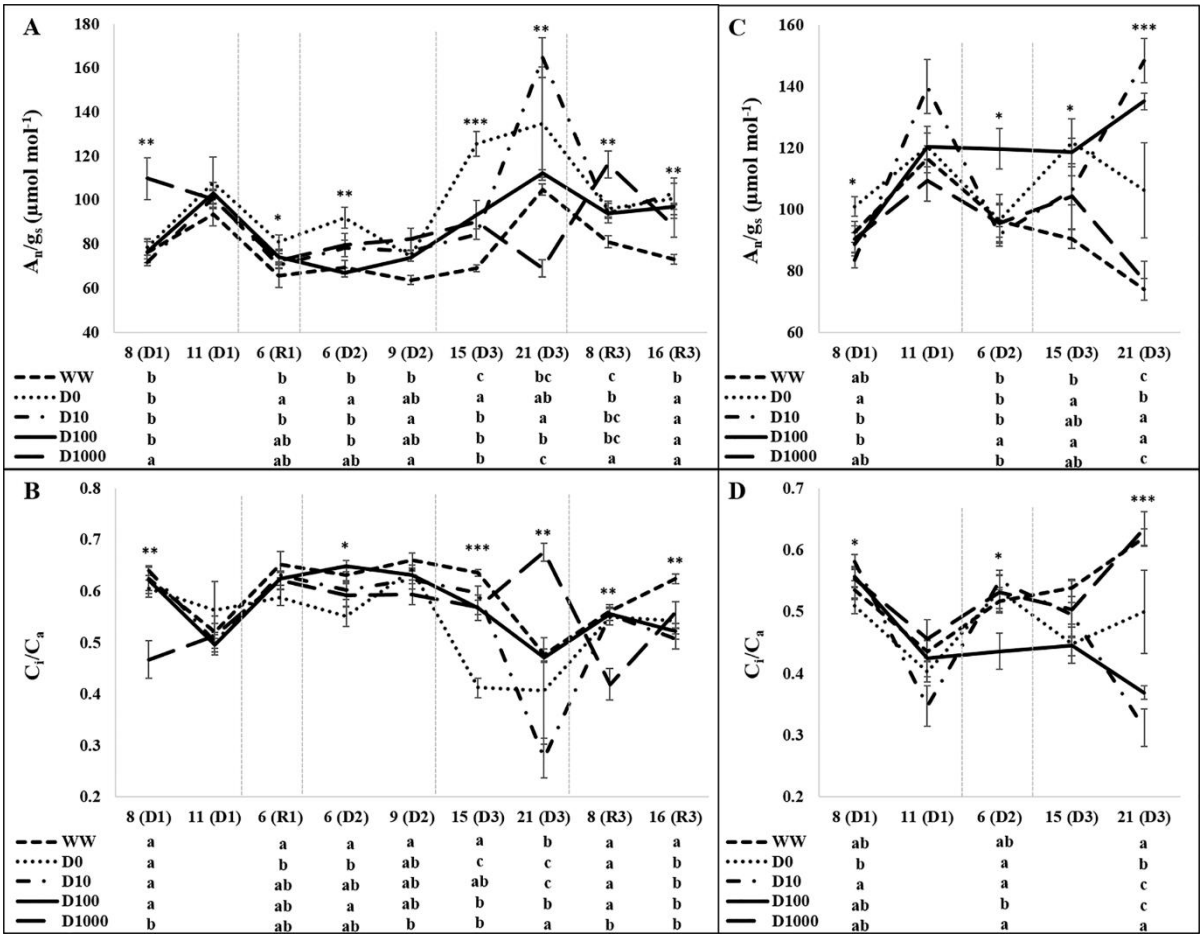


**Figure 3.** Changes of the ratio of intercellular to atmospheric $CO_2$ concentration ($C_i/C_a$) and intrinsic water use efficiency ($A_n/g_s$), at morning (A-B) and midday (C-D) of days of drought (D1-D3) and recovery (R1, R3) in each cycle, in leaves of well-watered (WW) and droughted plus salicylic acid (D) plants. Each point is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments within each date (*P < 0.05, **P < 0.01, ***P<0.001).

Regarding respiration rate, D0 plants had lower values than WW, D10 and D100 trees, averaging 55.8%, 49.6% and 52.5%, respectively (Figure 4).

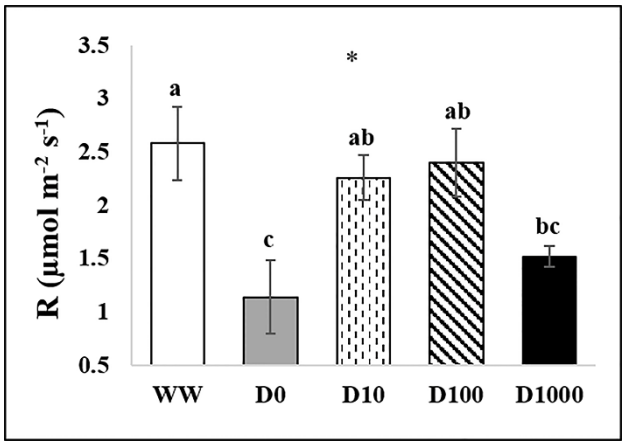


**Figure 4.** Night-time respiration rate (R) at the peak of the third drought period (21 days of drought) in leaves of well-watered (WW) and droughted plus salicylic acid (D) plants. Each column is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments within each date (*P < 0.05).

RWC drop slightly during the 1st DP in all stressed plants, showing D1000 an earlier decrease (Figure 5). At the 2nd DP, D100 plants stand out slightly from the other stressed plants, and during the 3rd and more severe DP, both D10 and D100 plants presented higher RWC than D0, averaging 8.4% and 18.9%, respectively. In contrast, D1000 plants had the lowest RWC at that DP, reaching 59%, against the maximum and stable value of WW plants, around 94%. Stressed plants showed a full RWC recovery during the 6 days of the 1st RP, and a largely uncompleted recovery during the 3rd RP, as only D100 plants exhibited full recovery at 8 days after resuming irrigation (Figure 5).

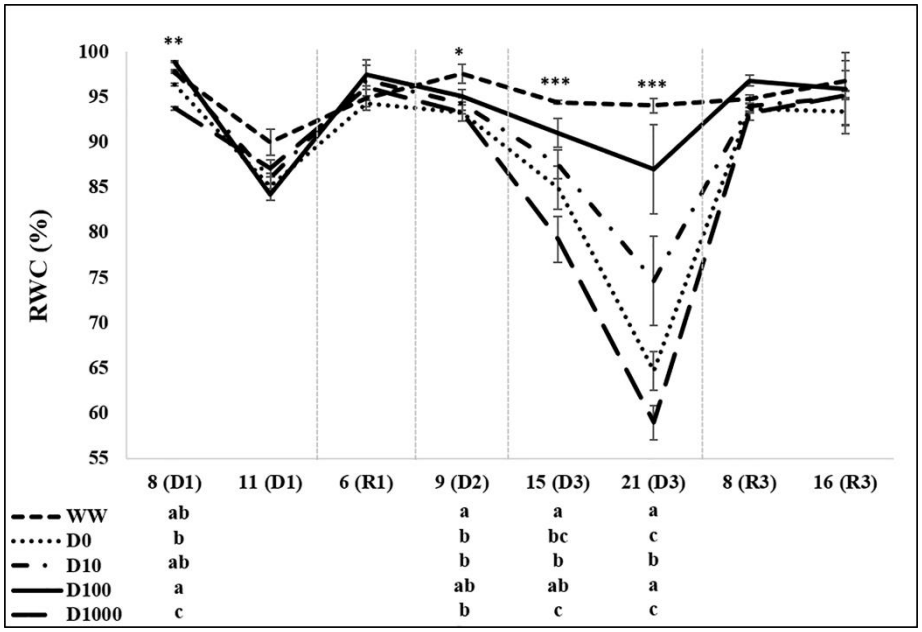


**Figure 5.** Changes of relative water content (RWC), of days of drought (D1-D3) and recovery (R1, R3) in each cycle, in leaves of well-watered (WW) and droughted plus salicylic acid (D) plants. Each point is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments within each date (*P < 0.05, **P < 0.01, ***P<0.001).

### 3.2. Photosynthetic pigments and carbohydrates

Total soluble sugars and St concentrations were significantly different among treatments, both at the peak of drought and after stress relief (Table 1). During drought, D100 and WW exhibited higher TSS concentration than D0, D10 and D1000 treatments, averaging 15.5%, 13.7% and 14.6%, respectively. The application of SA lead to lower St accumulation in leaves, presenting D0 plants the highest concentration. The TSS and St dynamics after the RP were quite similar, although the higher rise of TSS and St on D1000 and D10 plants, respectively (Table 1). Watering regime, SA concentration and sampling point significantly affected the concentration of chlorophylls dynamics. At the end of the 3$^{rd}$ DP, D0 plants presented lower concentration of $Chl_{(a+b)}$ than WW (15%) and D100 (10.4%) plants, with a similar trend in $Chl_a/Chl_b$ ratio. Following 8 days of rehydration, D0 plants recovered completely to control values. Meanwhile, the Car concentration was not affected by the applied treatments, neither the $Chl_{(a+b)}$/Car ratio (Table 1).

**Table 1.** Concentration (mg $g^{-1}$ DW) of soluble sugars (TSS), starch (St), total chlorophylls ($Chl_{(a+b)}$) and total carotenoids (Car), and the ratio of chlorophyll a/ chlorophyll b ($Chl_a/Chl_b$) and chlorophylls/carotenoids ($Chl_{(a+b)}$/Car) in leaves of well-watered (WW) and droughted plus salicylic acid (D) plants, at the peak of the third drought period (21(D3)) and after resuming irrigation (8(R3)).

| | | WW | D0 | D10 | D100 | D1000 | Sig. |
|---|---|---|---|---|---|---|---|
| **TSS** | **21(D3)** | 228.3±13.0$^{a}$ | 199.1±5.8$^{b}$ | 202.3±2.6$^{b}$ | 231.7±9.1$^{a}$ | 200.7±4.4$^{b}$ | * |
| | **8(R3)** | 257.8±6.0$^{a}$ | 231.0±14.0$^{b}$ | 220.7±7.1$^{b}$ | 272.2±3.0$^{a}$ | 255.1±5.2$^{a}$ | ** |
| **St** | **21(D3)** | 47.5±5.6$^{b}$ | 61.3±5.5$^{a}$ | 33.6±3.7$^{c}$ | 32.4±2.9$^{c}$ | 28.1±2.5$^{c}$ | *** |
| | **8(R3)** | 55.3±6.4$^{ab}$ | 50.7±6.3$^{ab}$ | 58.7±4.5$^{a}$ | 40.3±5.8$^{bc}$ | 29.9±2.3$^{c}$ | ** |
| **$Chl_{(a+b)}$** | **21(D3)** | 3.14±0.09$^{a}$ | 2.67±0.12$^{b}$ | 2.87±0.08$^{ab}$ | 2.98±0.05$^{a}$ | 2.93±0.10$^{ab}$ | * |
| | **8(R3)** | 3.32±0.05 | 3.20±0.06 | 2.87±0.06 | 2.97±0.17 | 3.17±0.18 | n.s. |
| **$Chl_a/Chl_b$** | **21(D3)** | 3.11±0.03$^{a}$ | 2.96±0.01$^{c}$ | 3.01±0.02$^{bc}$ | 3.07±0.05$^{ab}$ | 3.01±0.01$^{bc}$ | ** |
| | **8(R3)** | 3.04±0.07 | 2.83±0.14 | 2.97±0.04 | 3.08±0.03 | 3.00±0.05 | n.s. |
| **Car** | **21(D3)** | 0.710±0.016 | 0.625±0.03 | 0.683±0.01 | 0.698±0.013 | 0.669±0.023 | n.s. |
| | **8(R3)** | 0.692±0.032 | 0.686±0.022 | 0.612±0.008 | 0.698±0.036 | 0.709±0.039 | n.s. |
| **$Chl_{(a+b)}$/Car** | **21(D3)** | 4.42±0.05 | 4.31±0.03 | 4.2±0.007 | 4.28±0.10 | 4.37±0.02 | n.s. |
| | **8(R3)** | 4.81±0.15 | 4.6±0.24 | 4.69±0.04 | 4.25±0.10 | 4.47±0.02 | n.s. |

Values are means±SE. Different letters within a line demonstrate significant differences between treatments (n.s., not significant, *P < 0.05, **P < 0.01, ***P<0.001).

### 3.3. Immunolocalization of ABA and IAA

The analysis of ABA and IAA immunolocalization showed differences in signal intensity and distribution through the leaf tissues in response to post-drought recovery and SA application, although each phytohormone exhibited a specific dynamic (Figure 6).

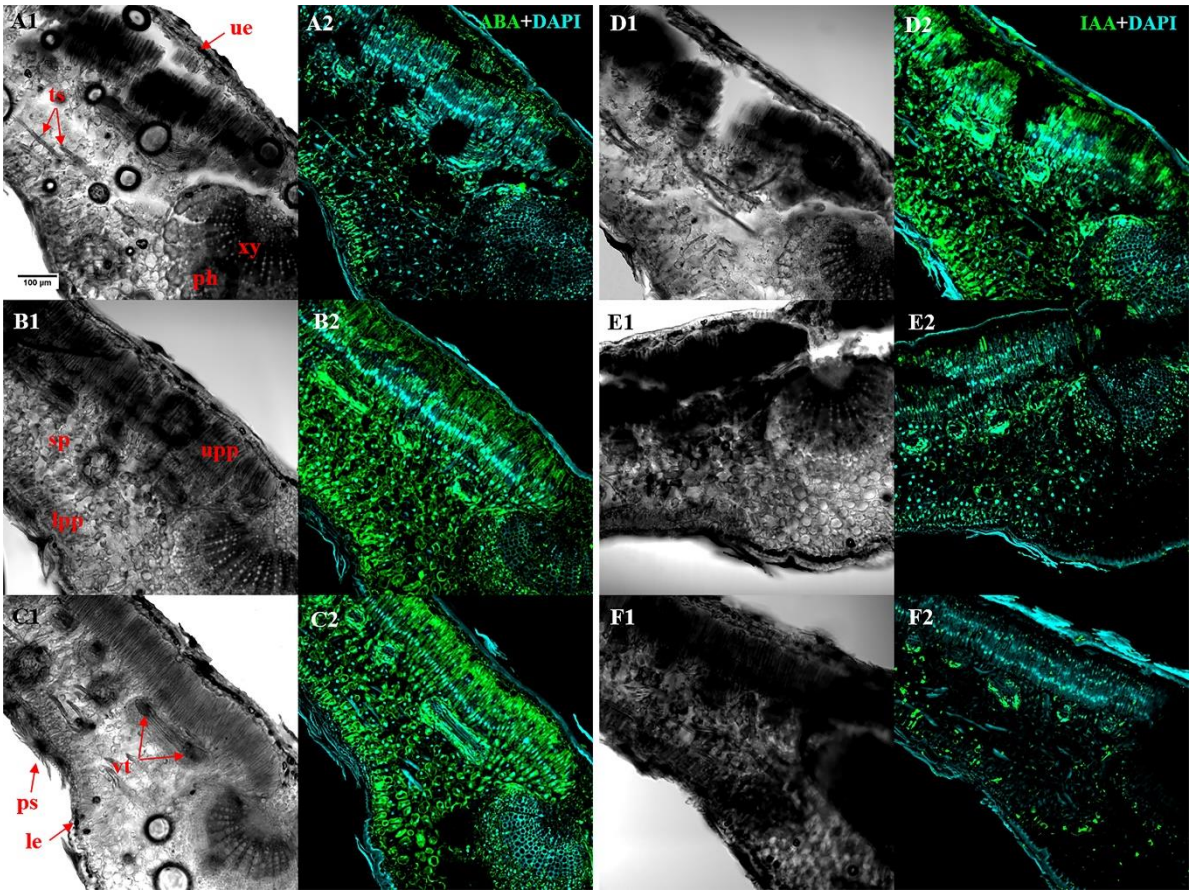


**Figure 6.** Immunodetection of ABA (A, B, C) and IAA (D, E, F) in sections of olive leaves using confocal microscope (n=3). Differential interference contrast (A1, B1, C1, D1, E1, F1); DAPI nucleic acid stain (blue signals) plus ABA (green signals) (A2, B2, C2); and DAPI nucleic acid stain (blue signals) plus IAA (green signals) (D2, E2, F2). A1, A2, D1 and D2 are from WW plants, B1, B2, E1and E2 from D100 plants and C1, C2, F1 and F2 are from D0 plants. Abbreviations: ue=upper epidermis upp=upper palisade parenchyma; sp=spongy parenchyma; lpp=lower palisade parenchyma; xy=xylem; ph=phloem; vt=vascular tissue; ts=trichosclereids; le=lower epidermis; ps=peltate scales. A negative control was performed (bars = 100 µm).

ABA signal was more intense in both D0 and D100 plants (Figure 6A,B,C). Furthermore, in WW plants, it can be noted an almost absence of signal in xylem and phloem and a slight accumulation close to lower epidermis (Figure 6A). In D100 (Figure 6B) plants the ABA signal was homogeneously distributed throughout the leaf tissues and in D0 plants (Figure 6C) it is evident an intense signal close to the upper epidermis. On the other hand, the IAA signal revealed a crescent intensity from D0 (Figure 6F), to D100 (Figure 6E), and to WW plants

(Figure 6D). Moreover, in WW plants was identified an intense IAA signal close to the lower epidermis and in vascular tissues (Figure 6D).

### 3.4. Growth, biomass accumulation and whole-plant water use efficiency

Water availability had a significant effect on total biomass production and biomass allocation patterns, with plants under lower water availability producing less biomass, particularly in the aboveground organs (Table 2). The extent of these changes was dependent on SA concentration, as D100 plants had higher biomass than D0 (+X%) and higher RBB than all other treatments, that contribute to the lowest shoot/root ratio (Table 2).

**Table 2.** Plant biomass (g plant$^{-1}$), relative aboveground biomass (RAB, % increase), relative belowground biomass (RBB, % increase), shoot/root ratio, leaf area (cm$^2$ plant$^{-1}$), net assimilation rate (NAR, g m$^{-2}$ day$^{-1}$), leaf area per total plant biomass (LAR, m$^2$ Kg$^{-1}$), leaf area per leaf biomass (SLA, m$^2$ Kg$^{-1}$), relative tolerance index (RTI, %), relative alleviation index (RAI, %) and the and whole-plant water use efficiency ($WUE_{WP}$, g kg$^{-1}$) of well-watered (WW) and droughted plus salicylic acid (D) plants.

| | WW | D0 | D10 | D100 | D1000 | Sig. |
|---|---|---|---|---|---|---|
| **Plant biomass** | 135.4±5.1$^{a}$ | 103.4±4.6$^{c}$ | 111.8±6.1$^{bc}$ | 125.2±7.8$^{ab}$ | 115.6±7.1$^{bc}$ | * |
| **RAB** | 92.5±6.0$^{a}$ | 37.1±7.7$^{b}$ | 51.3±8.7$^{b}$ | 59.2±11.3$^{b}$ | 57.7±11.6$^{b}$ | ** |
| **RBB** | 80.5±9.9$^{b}$ | 59.92±5.86$^{b}$ | 66.1±8.7$^{b}$ | 108.9±10.2$^{a}$ | 69.1±8.1$^{b}$ | ** |
| **Shoot/Root** | 2.37±0.07$^{a}$ | 1.90±0.09$^{bc}$ | 2.02±0.06$^{b}$ | 1.68±0.05$^{c}$ | 2.06±0.11$^{b}$ | *** |
| **Leaf area** | 1472.5±60.2$^{a}$ | 1026.8±42.7$^{b}$ | 1162.6±81.7$^{b}$ | 1210.1±105.7$^{b}$ | 1123.0±119.2$^{b}$ | * |
| **NAR** | 6.43±0.48$^{a}$ | 3.04±0.64$^{c}$ | 3.97±0.69$^{bc}$ | 5.72±0.85$^{ab}$ | 4.58±0.81$^{abc}$ | * |
| **LAR** | 1.09±0.02 | 0.994±0.018 | 1.04±0.04 | 0.965±0.061 | 0.966±0.058 | n.s. |
| **SLA** | 4.28±0.07 | 4.25±0.09 | 4.19±0.08 | 4.29±0.15 | 4.13±0.10 | n.s. |
| **RTI** | 100.0±3.8$^{a}$ | 76.4±3.4$^{c}$ | 82.6±4.5$^{bc}$ | 92.5±5.7$^{ab}$ | 85.4±5.2$^{bc}$ | * |
| **RAI** | 130.9±4.9$^{a}$ | 100.0±4.4$^{c}$ | 108.1±5.9$^{bc}$ | 121.1±7.5$^{ab}$ | 111.8±6.9$^{bc}$ | * |
| **$WUE_{WP}$** | 1.96±0.11$^{c}$ | 3.95±0.87$^{bc}$ | 5.54±1.14$^{ab}$ | 8.07±1.46$^{a}$ | 6.26±1.34$^{ab}$ | ** |

Values are means±SE. Different letters within a line demonstrate significant differences between treatments (n.s., not significant, *P < 0.05, **P < 0.01, ***P<0.001).

Total biomass production was positively correlated with leaf area, although some differences exist among treatments. In D0 (biomass = 3,461 + 0.097 x leaf area) each unit decrease (1 cm$^2$) in total leaf area resulted in a reduction of 97 mg in total biomass production, whereas in WW (biomass = 21.745 + 0.077 x leaf area), in D10 (biomass = 29.872+ 0.070 x leaf area), in D100 (biomass = 58.812+ 0.055 x leaf area) and in D1000 (biomass = 54.968+ 0.049 x leaf area) the decreases were lower (76, 70, 55 and 49 mg, respectively). Nonetheless,

the biomass decreases under drought depended more on NAR reductions, especially evident in D0 plants. On the other hand, LAR and SLA were not affected by treatments (Table 2). D100 plants displayed higher RTI (92.5%) among the drought treatments, against 85.4 %, 82.6% and 76.4% of D1000, D10 and D0 plants respectively. Moreover, RAI demonstrate that D100 exert a higher alleviating efficacy, with RAI of 121.1 %, closely to the 130.9 % of WW plants. All SA concentrations, namely D100, contributed to increase $WUE_{WP}$ relatively to WW (Table 2).

## 4. Discussion

### 4.1. Drought phase

The significantly decrease of $g_s$ and $A_n$ in all droughted plants is a typical acclimation mechanism in order to save water. Still, SA alleviates the adverse effects of drought on $g_s$ and $A_n$, in line with the findings of Hayat *et al*. (2008) and Nazar *et al*. (2015). These results suggest that SA plays a role in retarding the drought-induced stomatal closure, possibly because SA reverses the stomatal closure induced by ABA, as described by Rai *et al*. (1986). Indeed, there are also evidences that SA prevents salinity and drought-induced decline in IAA (Sakhabutdinova *et al*., 2003; Fahad and Bano, 2012), that in turns can stimulates stomatal opening (Peleg and Blumwald, 2011) by impairing ABA-inhibition response (Tanaka *et al*., 2006). As a result of higher $g_s$ of SA-treated plants, D0 exhibited, in general, a higher $A_n/g_s$ throughout the entire experience. Nonetheless, the applied SA concentrations induced distinct $g_s$ and $A_n$ responses during the experiment, in a closely association with the higher RWC of D100 and D10 plants at the most stressful period of the experiment. Likewise, the positive SA influence on water status was earlier reported in other species (Jesus *et al*., 2015; Nazar *et al*., 2015).

Against the typical response, where $g_s$ is usually the first variable affected by drought (Perez-Martin *et al*., 2014), in this study RWC was the first physiological trait that discriminate the treatments effects. While D1000 plants already exhibited a lower RWC 8 days after the start of the experiment, leaf gas exchange variables responded later, when D1000 plants kept higher $g_s$ at midday than the other droughted plants. That RWC might be already a response to a slightly accumulated larger $g_s$, which became evident later on. Furthermore, the tendency to maintain higher $g_s$ throughout the experiment than the other stressed trees, especially at midday, suggests that D1000 concentration might induced an anisohydric-like behavior, where $g_s$ is kept and water status is allowed to decline as soil dries (Landsberg *et al*., 2017). Under optimal conditions and mild-to-moderate drought conditions, anisohydric plants can maintain higher $g_s$

and $A_n$ than isohydric plants, leading to greater productivity (Sade *et al*., 2012), but it will be a valuable agronomic trait under the severe conditions of Mediterranean region? In the present study, the gain in $CO_2$ assimilation arising from the higher $g_s$ was more evident in the beginning of the experiment. In the long-term, the extreme low RWC (59%) at the stress peak was associated with photosynthetic performance impairment. Indeed, while under moderate stressful conditions $g_s$ and $g_m$ are the main limitations to $A_n$, at severe stress levels the biochemical component of photosynthesis can be severely inhibited (Boughalleb and Hajlaoui, 2011; Fernández, 2014). In fact, the lower $g_s$ and $A_n/g_s$ and the higher $C_i/C_a$ ratio of D1000 plants at the peak of the drought indicate that non-stomatal factors are involved in $A_n$ limitation, what is commonly reported under severe drought conditions (Bacelar *et al*., 2009). Although species-dependent, it was already assumed that high concentrations of SA can impose negative effects on the photosynthetic machinery (Gururani *et al*., 2015), though in the this work the effect was mediated, indirectly, by the induced anisohydric-like behavior.

The initial reduction of $g_s$, larger in D100 plants than in D10 and D1000, apparently contributed to keep RWC of D100 plants relatively more constant throughout the experiment. This fine control of plant water status at the initial stage of drought contributed to maintain higher $g_s$ in the long term, comparatively to D0 plants. A decrease in stomatal resistance after an initial increase during the early days of stress may be an adaptive mechanism to drought in olive trees (Saei *et al*., 2006). Thus, in contrast to D1000 concentration, D100 seems to maintain the isohydric-like behavior, as these plants reduce their $g_s$ with drought imposition (Landsberg *et al*., 2017), and regulate strictly RWC (Sade *et al*., 2012). Such behavior allows a return in photosynthetic terms, as these plants showed the highest $A_n$ among stressed plants in the most severe DP. The higher efficiency in photosynthetic terms was also evident with the relatively higher values of $A_n/g_s$ and lower values of $C_i/C_a$ recorded in the two last DPs, at midday. Our results are in agreement with Khan *et al*. (2003), who reported that $A_n$ SA-induced increase was closely correlated with low concentration of $C_i$, in line with other studies that showed a positive effect of SA on Rubisco activity under drought (Wang *et al*., 2010; Nazar *et al*., 2015). These evidences suggest that, unlike what happens in other SA treatments, the $A_n$ increases in D100 plants was also the result of higher carboxylation efficiency, rather than to simple increases in stomatal opening (Khan *et al*., 2003). Regarding D10 plants, the change to a more $g_s$ conservative response after the 1$^{st}$ DP contributed to maintain higher RWC than D1000 and D0. However, did not contribute to extra photosynthetic returns, compared with both D100 and D1000 plants.

Under drought, in spite of lower $A_n$, plants usually increase TSS concentration in leaves (Chaves *et al*., 2003). Thus, our results contradict our expectations, since none of the stressed plants overaccumulate TSS in relation to the WW controls. However, carbohydrates accumulation in leaves is largely dependent on drought duration and intensity (Liu *et al*., 2015). Moreover, different SA concentrations also induced different carbohydrates responses. The higher acumulation of these compatible solutes in D100 plants, as in WW controls, suggest some degree of osmotic adjustment (OA) activity. This allows plants to maintain, to some extent, tissue turgor under drought conditions, and thus increase in $g_s$ (Bosabalidis and Kofidis, 2002). Hence, this might explain the decrease in stomatal resistance in the peak of drought and the relatively constant RWC throughout the experiment. In agreement, the SA role on osmoregulation ability was already described (Jesus *et al*., 2015; Nazar *et al*., 2015; Umebese *et al*., 2009). On the other hand, St accumulation on leaves had a different pattern from TSS. The higher St concentration in D0 leaves in association with the lowest $A_n$, as already verified in olive tree (Bacelar *et al*., 2006), suggest that carbon was not translocated out of the leaves because these plants were sink-limited, acting as a short-term reserve or buffer against changes in environmental conditions. Conversely, the lower St concentrations in SA-treated plants are more related with $A_n$, although might be also linked to tissue OA, as one of the main sources of osmolytes are the starch reserves, which supply soluble sugars.

Respiration and photosynthetic rates are strongly coupled and intrinsically interdependent because $A_n$ provides photosynthetic substrates to R, and R supplies ATP and carbon skeletons to sustain plant energy requiring processes (Cannell and Thornley, 2000). Although $A_n$ declined in response to drought, the decline in R was not so clear, being only significant in D0 and D1000 plants. Moreover, was not related to a decay in carbohydrates concentration. Similar results were found by Rodríguez-Calcerrada *et al*. (2011), who suggested that drought-induced reduction of plant growth could translate into a R reduction in fully developed leaves, via reduced sucrose loading into the phloem and ATP demand. Furthermore, this work confirms that olive tree can reduce the metabolism and conserve photosynthates. The capacity to maintain low R rates during stressful conditions, associated with a decline in $A_n$, allows this species to allocate more assimilates for reserve accumulation (*e.g*. starch) which could be used later on, under favorable conditions, in growth (Varone and Gratani, 2015). This could be an important advantage from an evolutionary point of view for plants inhabiting drought and climatic change susceptible habitats. Interestingly, the maintenance of higher R in D10 and D100 plants might suggest the consumption of carbohydrates to growth and/or defense mechanisms.

The drought-induced reduction in photosynthetic pigments concentration observed in D0 plants is a typical symptom of oxidative stress (Bacelar *et al*., 2007b; Kabiri *et al*., 2014). Furthermore, the lower $Chl_a/Chl_b$ ratio in D0 leaves reflects fewer numbers of photosystem II reaction center complexes and greater chlorophyll b-containing light-harvesting complexes, resulting in lower value for nitrogen to chlorophyll ratio of pigment-protein complexes and, concomitantly, in a decrease in electron transport capacity (Evans and Poorter, 2001). Similar decreases of $Chl_{(a+b)}$ and $Chl_a/Chl_b$ ratio were reported previously in olive tree (Bacelar *et al*., 2007b). Notably, in general, all SA concentrations contributed to alleviate the negative effect of drought on $Chl_{(a+b)}$ and $Chl_a/Chl_b$ ratio, but D100 clearly stood out. The ability of SA-treated plants, especially D100, to maintain higher chlorophyll concentrations under drought conditions demonstrate that can use light energy more efficiently (Fang and Xiong, 2015). In this sense, the higher $A_n$ of D100 plants in the peak of drought could be, in part, a consequence of this response. Likewise, the positive influence of SA in preventing chlorophyll degradation under different types of abiotic stresses was already described (Fayez and Bazaid, 2014; Kabiri *et al*., 2014).

In summary, D100 demonstrate to be the most effective concentration in improving stress resistance. The fine control of $g_s$ allowed to improve $CO_2$ assimilation without excessive water losses. This response, associated with the soluble sugars accumulation, contributed to improve plant water status, avoiding relevant oxidative damages. In line, D100-treated plants exhibited better photosynthetic performance and the preservation of photosynthetic pigments that contributed to use light energy more efficiently. On the other hand, D10 and D1000 concentrations exhibited a behavior close to D0 plants. While D10 might be an extremely low concentration to clearly observe the benefits influence of SA, D1000 might be excessively high, exacerbating the plant responses. To illustrate, D1000 promoted an excessive $g_s$ during the initial stage of the stress period, leading to excessive water losses, what is evident latter on in RWC values. Moreover, the reduced R rates demonstrate that D0 and D1000 plants experienced higher stress, reducing the metabolism to conserve carbon reserves. A possible reason for the detrimental effect of the higher concentration, D1000, lays on its proposed action mode. It is believed that abiotic stress tolerance induction is related with the dual redox effect of SA. A first oxidative phase, characterized by a transient increase in reactive oxygen species (ROS) levels, is followed by an increase in antioxidant responses (Herrera-Vásquez *et al*., 2015). Thus, high concentrations of SA can cause high levels of ROS and then oxidative stress that plants are unable to overcome (Miura and Tada, 2014).

### 4.2. Recovering Phase

Olive tree is known to rapidly uptake water immediately after soil water is newly available. However, the rate of water status recovery depends largely on the severity of previous drought (Fernández, 2014), as severe conditions lead to a lack of response in absorption when full irrigation is restarted (Moriana *et al*., 2007). Although RWC of all stressed plants returned to control values 6 and 8 days after rewatering, during the 1st and 3rd RP, respectively, a tendency to higher RWC exists on D100 plants in the last sampling point. Thus, treatment history had a significant effect on the final values of RWC at the end of the RP. Furthermore, RWC recovered earlier than $g_s$ in both RP evaluated, as none of the stressed plants recovered $g_s$ at 6 (1st RP) and 16 days (3rd RP) after rehydration. The partially stomatal opening after rewatering can be interpreted as a physiological "stress memory", facilitating water conservation for subsequent dehydration stress events (Virlouvet and Fromm, 2015). This conservative behavior after rewatering is a typical olive tree response, as confirmed by other studies (Moriana *et al*., 2007; Perez-Martin *et al*., 2014). On the other hand, even with the similar dependence from previous drought intensity and duration, $A_n$ recovery had a different pattern. In the 1st RP, the complete recovery was reached at 6 days, whereas in the last RP was delayed to 16 days, despite the incomplete recovery of $g_s$, confirming higher damage to photosynthetic processes during the last DP. In agreement, such differential $g_s$ and $A_n$ responses were reported earlier (Galle *et al*., 2011).

The quick restoration of chlorophyll concentrations in stressed plants, as in Chen *et al*. (2016), might have contributed to the rapid $A_n$ recovery after rewatering. Meanwhile, with the RWC amelioration during the 8 days of RP, $A_n/g_s$ decreased in four treatments, a common response under better water status (Galle *et al*., 2011). The exception was the great $A_n/g_s$ increase in D1000 due to the absence of $g_s$ recovery.

Usually, the availability and utilization of carbohydrates change during recovery from stress (DaCosta and Huang, 2006). Although the most common response is the reduction of their concentration, due to the increased demand to rapid recovery of physiological activities and growth (DaCosta and Huang, 2006; Liu *et al*., 2015), this is not the general picture of our data. In fact, we observed a significant TSS increase in all treatments during rehydration, namely in D1000, a slight rise of St concentration in SA treatments, although only significant in D10, and a tendency to lower St in D0. These results showed that, following rehydration, olive trees divert a higher proportion of the newly assimilated carbon into soluble sugar export for plant growth, and less to temporary storage, as starch, a common response of fast growing

species (Liu *et al*., 2017). Thus, we may assume that trees growth under drought and rewatering cycles are positively associated with higher soluble sugars/starch ratio, particularly clear in D100 and D1000 plants, and not to higher concentration of non-structural carbohydrates. The knowledge about SA influence on recovery capacity is quite limited. However, there are evidences that SA, although concentration-dependent, exert a positive effect on the recovery of photosynthetic related variables and chlorophyll concentrations of heat and high light stressed grapevines and wheat (Wang *et al*., 2010). Altogether, the results showed that the influence of different SA concentrations in recovery responses is less distinctive. Still, in response to the better performance during the DPs, D100 plants slightly stood out. Meanwhile, the St decrease in D0 plants suggests the use of these reserves to possible repairing damages or re-growth after stress relief.

### 4.3. ABA and IAA immunohistochemistry response after recovery

It is known that SA can crosstalk with other phytohormones regulating various aspects of plant responses (Khan *et al*., 2015), although some contradiction can be found. For instance, SA was shown to induce ABA accumulation under stress conditions (Sakhabutdinova *et al*., 2003; Bandurska and Stroinski 2005; Szepesi *et al*., 2009; Fahad and Bano, 2012), while no significant effects of SA on ABA accumulation was recorded under normal conditions (Fahad and Bano, 2012). By other side, was shown a repression of auxin signaling (Iglesias *et al*., 2011), the induction of its accumulation (Fahad and Bano, 2012) or the prevention of its degradation in stressed plants (Sakhabutdinova *et al*., 2003), and also no significant effects of SA on IAA concentration was observed under normal conditions (Fahad and Bano, 2012). Although the hormonal dynamics has been receiving more attention under stress conditions, some studies already suggested that hormones play important roles in the recovery process (De Diego *et al*., 2012; Correia *et al.*, 2014). In addition to hormones accumulation, the distribution along the different leaf tissues could also be determinant in an accurate response to stress and recovery.

As illustrated in Figure 6, even after 16 days of rewatering ABA signal was more intense in both D0 and D100 than in WW plants, what is consistent with the retarded recovery capacity of $g_s$ exhibited by those plants. These results confirm the persistence of ABA, after a long-period of rewatering, as a signal of "stress memory", as also reported by Virlouvet and Fromm (2015). Still, contrary to the commonly described under dehydration (De Diego *et al*., 2013; Jesus *et al*., 2015), no special accumulation of ABA was found at guard cells. This finding

suggests that during recovery ABA might start to be mobilized and that, in addition to a local effect of ABA on stomatal guard cells, exerts an indirect effect regulating stomatal opening via leaf hydraulic conductance, which decreased by ABA by inactivating bundle sheath aquaporins (Pantin *et al*., 2013). Meanwhile, it is important to highlight the intense immunohistochemical signal close to the upper epidermis of D0 leaves, where are located the higher amount of chloroplasts. Considering that: (1) ABA biosynthesis is initiated on plastids and ends in cytoplasm where is inactivated, being glucose-conjugated ABA (ABA-GE) the major storage and transport of ABA (Schroeder and Nambara, 2006); (2) ABA-GE accumulates in vacuoles and in apoplast, but is activated and relocalized to the endoplasmic reticulum in response to dehydration (Finkelstein, 2013); (3) ABA-GE increased in response to repetitive cycles of dehydration/rehydration (Zeevaart, 1983) and the cleavage of this pre-existing pool is proposed as an alternative route for rapid ABA synthesis in response to changes in environmental conditions (Schroeder and Nambara, 2006); (4) for ABA immunolocalization there was no discrimination between free ABA and ABA-GE (Escandón *et al*., 2016), we propose that the intense signal present in D0 plants and absent in D100 could reflect a "memory" of the worst water status exhibited during the experiment.

Concerning the IAA function in growth (Wani *et al*., 2016), the crescent signal intensity with the order D0<D100<WW is well correlated with the rate of biomass accumulation recorded at the end of the experiment. In addition, it was already reported that SA application prevents the drought-induced IAA degradation (Sakhabutdinova *et al*., 2003; Fahad and Bano, 2012), revealing an advantage under recovery conditions. Furthermore, the intense IAA signal close to the lower epidermis of WW plants highlight their function in stomatal opening promotion (Peleg and Blumwald, 2011). An unequally distribution of both ABA ad IAA in response to different stresses and recovery and to exogenous SA application were already described, though species dependent (De Diego *et al*., 2013; Jesus *et al*., 2015; Escandón *et al*., 2016).

### 4.4. Growth, biomass accumulation and whole-plant water use efficiency

Despite the three RPs during the experiment, drought affected negatively biomass accumulation, confirming earlier studies (Bacelar *et al*., 2007a; Di Vaio *et al*., 2013). Noteworthy, SA alleviates, to some extent, the inhibitory effect of drought on biomass production, with statistically significance with D100. Similar SA effects were found in other studies (Fayez and Bazaid, 2014; Kang *et al*., 2012; Nazar *et al*., 2015). Moreover, the RTI and

RAI, calculated based on biomass accumulation, indicate that D100 was the most appropriate concentration to help olive to offset drought.

The drought-induced biomass decline was associated with the decrease of NAR and total plant leaf area, whereas LAR was not significantly affected, mainly due to the absence of effects on SLA. Similar SLA and LAR results were obtained by Erice *et al*. (2010), though the more common responses are reductions in both traits (Anyia and Herzog, 2004; Bacelar *et al*., 2007a). Decreases in NAR are closely related with reduced $A_n$ due to stomatal and non-stomatal limitations, and changed carbohydrate metabolism and TSS levels in leaves that spills over to a decreased export rate, as in the study of Farooq *et al*. (2009).

The carbon balance of plants during periods of drought and recovery depend on the velocity and degree of photosynthetic recovery, as it depends on the degree and velocity of photosynthesis decline during water depletion (Chaves *et al*., 2009). Thus, the enhanced photosynthetic capacity of SA-treated plants along the DP and in the 8 days of the 3$^{rd}$ RP, highlighting D100, certainly contributed to maintain some degree of growth and biomass accumulation during these phases. In addition, the levels of TSS in D100 plants during drought might contributed to OA, maintaining turgor and meristem viability, and serve as the primary source of carbohydrates, enhancing rapid regrowth of plants after rewatering (Chen *et al*., 2016; DaCosta and Huang, 2006). Moreover, some studies suggest that growth-stimulating effect of SA could be related to changes in hormonal status (Shakirova *et al*., 2003; Abreu and Munné-Bosch, 2008), in line with the strong IAA signal of D100 plants during the last RP.

A differential allocation pattern of dry matter between below and aboveground organs, as well the leaf area reduction are common plant mechanisms to tolerate repeated cycles of drought (Toscano *et al*., 2014). Indeed, all droughted plants exhibited higher investment in roots, as evidenced by the lower shoot/root ratio, a known mechanism to improve water uptake (Toscano *et al*., 2014). Interestingly, D100 were the plants that invest more in root biomass. By optimizing shoot/root ratio, these plants improve and maintain the equilibrium between water supply and demand and improve the partitioning of assimilates to recovery, after mobilization of store reserves in roots. Moreover, as roots are also important organs for water storage in trees (Scholz *et al*. 2011), as described in neotropical savanna woody species (Dommec *et al*., 2006), D100 trees can store more water, allowing higher $g_s$ and $A_n$ during drought. In agreement, the root development stimulation by SA application was already described in olive trees, tomato and wheat (Umebese *et al*., 2009; Kang *et al*., 2012; Aliniaeifard *et al*., 2016).

Furthermore, it is well established that plant biomass production depends on the amount of water used for growth, as well on water use efficiency (Anyia and Herzog, 2004). For production on relatively dry sites, plants that display high $WUE_{WP}$ appear to be the most promising (Bacelar *et al*., 2007a). Interestingly, $WUE_{WP}$ was significantly higher in SA-treated plants, highlighting D100. Meanwhile, the absence of a significant association between $A_n/g_s$ and $WUE_{WP}$, in some experimental periods, reflects the difference in time scale of both processes and non-accounted energy expenses in growth and maintenance respiration rates. Similar results were reported previously in olive tree (Bacelar *et al*., 2007a).

## 5. Conclusions

Our results confirm that both drought resistance and drought recovery are key determinants of plant drought adaptation. The accumulation of osmolytes, leaf water status maintenance and reduced photosynthetic systems drought-associated damage induced a rapid recovery after rewatering. Recovery capacity might influence largely growth and biomass accumulation, and are intimately associated with hormonal dynamics.

Drought adaptability trough the implementation of short-term measures, such as SA exogenous application, is fundamental to respond rapidly to the current and predicted adverse conditions. Moreover, the results of the present study suggest that the determination of an optimum SA concentration for each species and environmental condition is a prerequisite, once SA applied beyond a certain range might be detrimental. Taken together, the presented evidences indicate that, among the SA tested concentrations, 100 µM contributed to higher drought adaptability by inducing the highest drought resistance and recovery capacities. This SA concentration improved adaptative responses of olive tree to drought by the accumulation of osmolytes that are determinant to maintain turgor, more favorable water status and higher photosynthetic capacity during drought and recovery, by optimizing shoot/root ratio, investing largely in root biomass accumulation, and allowing higher accumulation of IAA under recovery. Thus, all these SA-induced changes contribute to attenuate the limitation of total biomass accumulation imposed by drought.

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## 5.2. Salicylic acid increases drought adaptability of young olive trees by changes on redox status and ionome

### Salicylic acid increases drought adaptability of young olive trees by changes on redox status and ionome

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### Abstract

Current and predicted environmental conditions claim the necessity to improve Mediterranean crops drought adaptability (drought resistance and recovery capacity). Salicylic acid (SA) has been recognized as a stress tolerance promoter when applied at suitable concentration. Here, different concentrations of SA (10, 100 and 1000 µM) were applied in young olive trees (*Olea europaea* L.) subjected to drought and rewatering. Plants treated with 10 µM SA exhibited a close behavior to SA-starved plants. Although both 100 and 1000 µM concentrations contributed to improve the balance between reactive oxygen species (ROS) production and scavenging, 100 µM SA was more efficient. During drought, 100 µM SA induced a higher detoxification and scavenging of ROS by the maintenance or overaccumulation of total soluble proteins in detriment of phenolics, ascorbate and β-carotene. Conversely, during recovery this picture was reversed, as soluble proteins return to well-watered control values and the investment in that group of antioxidant compounds was increased. Concomitant with the improved redox status, 100 µM SA concentration was the most effective in plant ionome regulation, by the improvement of the uptake of important macro and micronutrients, namely P, Fe, Mn and Zn, and by changes on mineral allocation patterns. As a consequence, 100 µM SA also countered the drought induced decline in total plant biomass accumulation, mainly due to the promotion of root development. Thus, this study demonstrated that the application of a suitable SA concentration is an efficient tool to improve the cellular homeostasis and growth of plants subjected to recurrent drought episodes.

**Keywords:** antioxidants, growth minerals, recovery, ROS, water deficit.

## 1. Introduction

The major factors affecting crop production in Mediterranean agro-ecosystems are water and minerals (Porras-Soriano *et al*., 2009). Olive tree (*Olea europaea* L.) is an iconic crop in this Region being traditionally grown under poor soils and water limited environments (Therios, 2009). Concomitant with a rainfall decrease during summer, climate models predict a stronger inter- and intra-annual weather variability (IPCC, 2013). These scenarios give even more prominence to the concept of drought adaptability, that integrates much more than drought resistance, playing recovery capacity also a fundamental role in plants growth and survival (Chen *et al*., 2016).

Drought stress results in increased generation of reactive oxygen species (ROS) due to energy accumulation, which increases the photooxidative effect (Waraich *et al*., 2011). Prevention of cellular oxidative damage has been suggested as one of the mechanisms of stress tolerance (Yildirim *et al*., 2008). To fight against the resulting oxidative stress, olive trees invest in several enzymatic and non-enzymatic antioxidant mechanisms (Sofo *et al*., 2004, 2008; Bacelar *et al*., 2006, 2007; Petridis *et al*., 2012). However, high stress levels can create an imbalance between ROS production and scavenging (Farooq *et al*., 2012), damaging lipids, proteins, carbohydrates, pigments and DNA (Sofo *et al*., 2004; Bacelar *et al*., 2006, 2007; Farooq *et al*., 2009; Petridis *et al*., 2012). Those limitations may also influence the biochemical metabolism and the signal cascade in recovery by rewatering events (Xu *et al*., 2010). In addition, drought stress affects uptake, transport, and subsequent distribution of nutrients within the plant (Farooq *et al*., 2009), causing an imbalance in plant nutrition. The disequilibrium in plant nutrition is associated with several secondary effects, since mineral nutrients serve numerous functions in plants, as structural components in macromolecules, co-factors in enzymatic reactions, osmotic solutes and maintenance of charge balance in cellular compartments (Grusak, 2001). Therefore, the occurrence of oxidative damage in droughted plants can be even more notorious when plants also suffer nutrient deficiencies (Cakmak, 2008). Consequently, biomass accumulation, allocation patterns and productivity are eventually affected (Farooq *et al*., 2009, 2012).

Salicylic acid (SA) is a phytohormone increasingly recognized as abiotic stress-tolerance enhancer, via SA-mediated control of major plant-metabolic processes (Khan *et al*., 2015). Nevertheless, the basic mechanisms supporting this tolerance remain less discussed (Khan *et al*., 2015). SA regulates several proteins associated with signal transduction, stress defense and protein metabolism (Kang *et al*., 2012). and modulates mineral nutrients uptake and

metabolism, affecting growth and development under stressful conditions (El-Tayeb, 2005; Gunes *et al*., 2007; Yildirim *et al*., 2008; Khan *et al*., 2015). However, the effect of SA on ROS concentration, cell membrane stability, osmoprotectants and proteins accumulation and antioxidant defense system is highly dependent on several factors, as applied concentration, kind and level of stress and the species in study (El-Tayeb, 2005; Chen *et al*., 2007; Harfouche *et al*., 2008; Hayat *et al*., 2008; Yildirim *et al*., 2008; Kang *et al*., 2012; Fayez and Bazaid, 2014; Kabiri *et al*., 2014). As far as we know, only two studies (Hashempour *et al*., 2014; Alineaeifard *et al*., 2016) reported the use of SA in abiotic stress mitigation in olive tree. These works showed that SA in a suitable concentration (1 mM) improved the biochemical responses under freeze conditions (Hashempour *et al*., 2014) and at 0.25 mM SA promoted growth under salt stress conditions (Aliniaeifard *et al*., 2016).

We hypothesized that a suitable SA concentration can improve olive trees drought adaptability. Accordingly, we aimed: 1) to appraise the SA influence on oxidative damage and antioxidant defense system during drought and recovery events; 2) to assess the SA influence on plant mineral status and growth after drought and rewatering events; 3) and, to achieve a most profit SA concentration to improve olive tree drought tolerance and recovery capacity.

## 2. Material and Methods

### 2.1. Growth conditions, plant material and experimental set-up

The experiment was carried out between June and September 2014 at the University of Trás-os-Montes and Alto Douro, Vila Real, Northeast Portugal (41°17'17.83"N, 7°44'12.81"W, 448 m a.s.l.). Climate is Mediterranean-like, warm-temperate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which in the autumn-winter and negligible during the summer. However, 2014 had an atypical summer with some rainfall events (13.7, 11.8 and 13.0 mm during the 1st, 2nd and 3rd recovery periods, respectively). The warmest months are July/August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 ºC and 6.8/6.3 °C, respectively (IPMA, 2017).

Own-rooted 3 years-old olive trees (*Olea europaea* cv. Cobrançosa), were grown outdoors in 16 L pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). Pots surface was covered with a thin layer of perlite and then were sealed with plastic film and aluminum foil to avoid temperature raise, evaporation and rain water entering. Pots were randomly arranged and periodically rotated to

the neighboring position to minimize the effects of environmental heterogeneity. When applicable, plants were watered to field capacity, determined gravimetrically. Care was taken to ensure negligible leaching through the pots bottoms during irrigation.

Prior to the experiment, eighty-eight uniform plants, selected based on height, leaf number and leaf area were left for 30 days in the study site for acclimatization, being watered every other day to field capacity, determined gravimetrically. Then, at the beginning of the experiment, 6$^{th}$ July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining eighty plants were divided in five groups, each one comprising sixteen plants. One group was sprayed with distilled water and kept under well-watered conditions (WW, control plants) throughout the entire experimental period, in which plants were watered every day. The four other groups were subjected to three "drought-re-watering cycles" by withholding water until the occurrence of precipitation (1$^{st}$ and 2$^{nd}$ cycles), or until the stomatal conductance for water vapour during mid-morning (peak of photosynthetic activity) dropped around 50 mmol $m^{-2}$ $s^{-1}$, reached at 3$^{rd}$ cycle, a threshold value indicating a situation of severe drought stress experienced by the plants, a value where photosynthetic activity becomes predominantly inhibited by metabolic processes, besides stomatal limitations (Flexas and Medrano, 2002). When occurred precipitation, or when olive trees reached the desired drought intensity, they were rewatered to field capacity in the evening and during the following days until net photosynthesis was almost restored to control values (recovery). From the four droughted groups (D), one was sprayed with distilled water (D0), while the other three groups were sprayed with different salicylic acid (SA) concentrations, namely 10 µM (D10), 100 µM (D100) and 1000 µM (D1000). Each plant was treated with a mean volume of 150 mL of spraying solution. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. Care was taken during the application of foliar sprays to avoid overspraying non-target trees, for this, non-target trees were covered by a plastic sheet. The 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ "drought-re-watering cycles" had the duration of 12-6 days, 9-3 days and 21-16 days, respectively.

Each group of sixteen plants was divided in two subgroups, each one with eight plants. Plants from one subgroup were used for biochemical destructive measurements, and plants from the other subgroup were used for final biomass and plant minerals assessment. A schematic representation of the experiment is presented in Figure 1. All the measurements detailed bellow were performed 8 times per treatment (n=8), one per plant. Biochemical measurements at leaf

level, were measured in healthy, full expanded mature leaves 21 days after starting the 3rd drought period (DP), at the peak of stress and 8 days after starting the respective recovery period (RP). Final biomass accumulation and plant organs ionome was evaluated at the end of the experiment, 16 days after starting the 3rd RP.

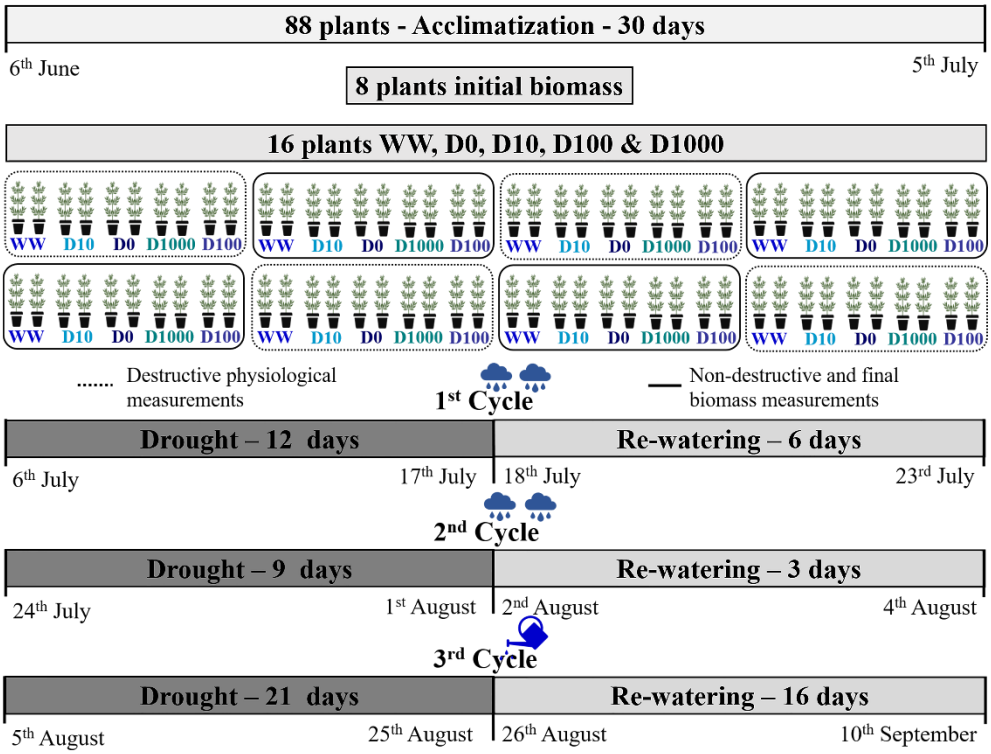


**Figure 1.** Schematization of the experiment. Well-watered controls (WW) and droughted plus salicylic acid plants, 0µM (D0), 10 µM (D10), 100 µM (D100) and 1000 µM (D1000).

### 2.2. Foliar metabolic assays

Total reactive oxygen species (ROS) were determined with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma–Aldrich,Germany) (Kong *et al*., 2013). A 25 mM solution was prepared in dimethyl sulphoxide for pending use. Twenty microliter of each sample were loaded into a small well ELISA plate containing 0.2 ml of PBS buffer (pH 7.4) and 12 µM of DCFH-DA and incubated for 20 min at 25 ºC. Fluorescence was measured at 485 nm and 530 nm (excitation and emission wavelength, respectively), in a CARY 50 Bio (Eclipse, Australia) every 15 min until 60 min after the incubation. 2',7' -dichlorofluorescein was used to obtain a calibration curve. Results were expressed as nM DCF $g^{-1}$ DW. Hydrogen peroxide ($H_2O_2$) concentration were determined using a method described by Junglee *et al*. (2014), with some modifications. The absorbance was measured at 350 nm and $H_2O_2$ was used to obtain a calibration curve.

β-carotene was extracted with acetone–hexane mixture (4:6) and determined according to Barros *et al*. (2011). Total soluble proteins (TSP) were quantified using the method of Bradford (1976), using bovine serum albumin as a standard. Total thiols (–SH) in TSP extract were assessed according to Ellman (1959), using an extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$. Total phenolic compounds (TPC) in leaf methanolic extracts were quantified following the Folin–Ciocalteu procedure (Singleton and Rossi, 1965), using gallic acid as a standard. Flavonoids were determined according to Zhishen *et al*. (1999) in the same leaf extracts of TPC, using (+)-catechin as a standard. Ascorbate was quantified using a method adapted from Klein and Perry (1982), using L-ascorbic acid as a standard.

Total antioxidant capacity (TAC), based on DPPH-free radical scavenging, was evaluated according to a method adapted from Xu and Chang (2007). Leaf methanolic extracts, and methanol for negative control, were mixed with DPPH methanolic solution (0.1 mM) and left to stand for 30 min in dark at room temperature. The absorbance for the sample ($A_{sample}$) and negative control ($A_{control}$) was measured at 517 nm against methanol blank. The percent of DPPH radical reduction was calculated as follows = 100 x ($A_{control} - A_{sample}$) / $A_{control}$. The free radical scavenging activity was expressed as µM of Trolox equivalents, TE = (% DPPH radical reduction / *a*), where *a* is the slope of the standard curve (y = *a*x).

### 2.3. Plant biomass accumulation and mineral analysis

Dry weight of plant samples (leaves, stems and roots) were oven-dried at 70 ºC to a constant weight. Based on these data, the total biomass increase (TBI) and the fraction of biomass allocation among plant organs were calculated. Following ground, N and P concentrations were determined by molecular absorption spectrophotometry (SanPlus, Skalar, The Netherlands), after digestion with $H_2SO_4$ and $H_2O_2$ (Mills and Jones, 1996). The concentrations of other elements (Ca, Mg, Fe, Cu, Zn, and Mn) were determined by atomic absorption spectrophotometry (3100, Perkin Elmer, USA), and K was determined by flame emission photometry (PFP7, Jenway, UK), after digestion with $HNO_3$ and $HClO_4$ (Mills and Jones, 1996).

The quantity of nutrients accumulated per plant was obtained by multiplying the concentration (dry weight basis) in each plant organ by the respective biomass. Physiological nutrient-use efficiency (UE) has been calculated as total plant biomass per unit of mineral element acquired, while minerals root uptake efficiency has been calculated as the total amount of minerals in each plant per unit of root biomass.

### 2.4. Statistical analysis

The statistical analysis was performed using the statistical software program SPSS for Windows (v. 22). All data sets satisfied the assumptions of ANOVA based on homogeneity of variances and normality. In all parameters, data were analyzed one-way factorial ANOVA and the post hoc Tukey's test. Significant differences were considered for ($P < 0.05$). For statistical analysis of TBI, arcsine transformation was performed in percentage data.

## 3. Results

### 3.1. Oxidative stress and defense systems

Oxidative stress, at leaf level, can be accessed based on total ROS and $H_2O_2$ concentrations (Table 1). The results showed that water regime and SA concentrations modulate total ROS and $H_2O_2$ accumulation in both sampling dates. Treatments influenced total ROS concentration in the following order: D10≥D0≥D1000≥D100=WW and D0≥D10≥D1000≥D100=WW during the drought and recovery periods, respectively. Meanwhile, $H_2O_2$ concentrations varied in the following way: WW=D1000>D100>D10>D0 and D100>WW=D10=D0>D1000 during drought and after recovery, respectively.

**Table 1.** Oxidative stress indicators and foliar metabolites concentrations of well-watered (WW) and droughted plus salicylic acid (D) plants during drought (DP) and recovery (RP) periods. Total reactive oxygen species (ROS, nmol.g$^{-1}$ DW), $H_2O_2$ (µmol.g$^{-1}$ DW), total soluble proteins (TSP, mg.g$^{-1}$ DW ), total thiols (-SH, nmol.mg$^{-1}$ DW), β-Carotene (mg.g$^{-1}$ DW), total phenolic compounds (TPC, mg.g$^{-1}$ DW), flavonoids (mg.g$^{-1}$ DW ) and ascorbate (mg.g$^{-1}$ DW).

| | | WW | D0 | D10 | D100 | D1000 | Sig. |
|---|---|---|---|---|---|---|---|
| **ROS** | **DP** | $0.276\pm0.020^{c}$ | $0.422\pm0.019^{ab}$ | $0.470\pm0.040^{a}$ | $0.297\pm0.048^{c}$ | $0.356\pm0.024^{bc}$ | ** |
| | **RP** | $0.391\pm0.026^{c}$ | $0.560\pm0.039^{a}$ | $0.490\pm0.022^{ab}$ | $0.380\pm0.029^{c}$ | $0.437\pm0.012^{bc}$ | ** |
| **$H_2O_2$** | **DP** | $22.7\pm0.1^{a}$ | $11.9\pm0.1^{d}$ | $19.9\pm0.2^{c}$ | $21.1\pm0.2^{b}$ | $22.6\pm0.1^{a}$ | *** |
| | **RP** | $18.0\pm0.5^{b}$ | $17.8\pm0.6^{b}$ | $17.8\pm0.9^{b}$ | $25.6\pm0.5^{a}$ | $14.4\pm0.6^{c}$ | *** |
| **TSP** | **DP** | $7.04\pm0.36^{b}$ | $2.65\pm0.13^{c}$ | $9.92\pm0.60^{a}$ | $8.78\pm0.38^{ab}$ | $7.57\pm0.85^{b}$ | *** |
| | **RP** | $7.87\pm0.48^{a}$ | $3.54\pm0.12^{c}$ | $6.02\pm0.26^{b}$ | $6.78\pm0.38^{ab}$ | $7.37\pm0.88^{ab}$ | *** |
| **-SH** | **DP** | $1.96\pm0.07^{a}$ | $0.99\pm0.07^{b}$ | $1.77\pm0.16^{a}$ | $1.95\pm0.18^{a}$ | $1.60\pm0.11^{a}$ | *** |
| | **RP** | $3.28\pm0.51^{a}$ | $1.10\pm0.04^{b}$ | $1.06\pm0.04^{b}$ | $1.36\pm0.13^{b}$ | $1.20\pm0.02^{b}$ | *** |
| **β-Carotene** | **DP** | $0.562\pm0.010^{a}$ | $0.528\pm0.011^{b}$ | $0.519\pm0.004^{b}$ | $0.512\pm0.004^{bc}$ | $0.492\pm0.005^{c}$ | *** |
| | **RP** | $0.536\pm0.010^{bc}$ | $0.607\pm0.012^{a}$ | $0.527\pm0.014^{c}$ | $0.582\pm0.013^{ab}$ | $0.572\pm0.021^{abc}$ | ** |
| **TPC** | **DP** | $38.6\pm0.5^{b}$ | $42.4\pm0.3^{a}$ | $39.2\pm0.3^{b}$ | $35.5\pm0.3^{c}$ | $34.2\pm0.4^{d}$ | *** |
| | **RP** | $34.2\pm0.5^{c}$ | $41.9\pm0.4^{b}$ | $42.9\pm0.5^{b}$ | $46.8\pm0.4^{a}$ | $47.3\pm0.3^{a}$ | *** |
| **Flavonoids** | **DP** | $12.0\pm0.6^{d}$ | $22.6\pm0.2^{a}$ | $15.9\pm0.4^{b}$ | $14.5\pm0.1^{c}$ | $16.6\pm0.1^{b}$ | *** |
| | **RP** | $15.4\pm0.3^{e}$ | $17.6\pm0.4^{d}$ | $21.9\pm0.5^{b}$ | $20.1\pm0.3^{c}$ | $24.6\pm0.4^{a}$ | *** |
| **Ascorbate** | **DP** | $1.36\pm0.44^{b}$ | $1.60\pm0.05^{a}$ | $0.485\pm0.01^{c}$ | $0.227\pm0.009^{d}$ | $1.41\pm0.02^{b}$ | *** |
| | **RP** | $1.15\pm0.02^{d}$ | $2.16\pm0.04^{a}$ | $1.56\pm0.05^{b}$ | $1.28\pm0.04^{cd}$ | $1.38\pm0.06^{c}$ | *** |

Values are means±SE. Different letters indicate significant differences among treatments within each date (**P < 0.01, ***P<0.001).

Water regime and SA also modulated leaf non-enzymatic antioxidant defenses, which usually change upon rewatering (Table 1, Figure 2). Total soluble proteins concentration was higher in D10 and D100 than in D0 plants during drought, whereas WW and D100 leaves showed higher TSP levels than in D0 plants after rewatering. Drought induced decline in total thiols concentration was annulated by the application of SA during the drought period, whereas their concentration was lower in all stressed plants upon rewatering (Table1). Regarding carotenoids, only β-carotene was significantly affected by the applied treatments, presenting control plants higher values during the drought phase, whereas D0 leaves had higher values than WW and D10 treatments, upon rewatering (Table 1). Ascorbate concentration was modulated by water regime and SA treatments. During drought, D0 and D100 plants presented the highest and lowest values, respectively, whereas upon rewatering D0 continued to present the highest concentrations and D100 and control plants showed the lowest levels (Table 1). The concentrations of TPC and flavonoids were affected during the drought period following the order D0>WW=D10>D100>D1000 and D0>D10=D1000>D100>WW, respectively, while after rewatering the orders were D1000=D100>D10=D0>WW and D1000>D10>D100>D0>WW, respectively (Table 1).

The influence of treatments on antioxidant system is somehow reflected in the TAC estimated by the DPPH assay, being TAC higher in D0 under drought, namely relatively to D1000 plants, whereas upon rewatering D100 and WW plants presented lower values than the other treatments (Figure 2).

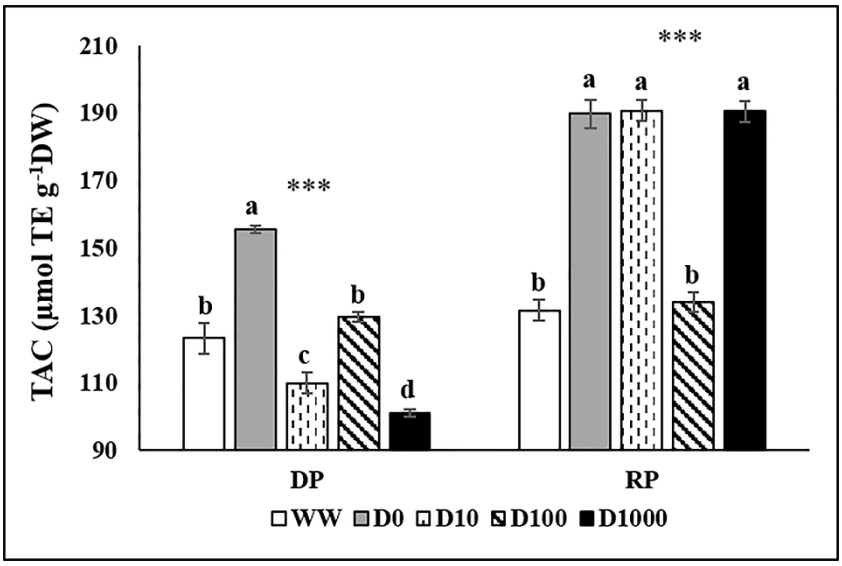


**Figure 2.** Total antioxidant capacity (TAC) of well-watered (WW) and droughted plus salicylic acid (D) plants during drought (DP) and recovery (RP) periods. Each column is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments within each date (***P<0.001).

### 3.2. Plant biomass accumulation and mineral dynamics

Total biomass increase through the experiment was reduced by drought imposition, being this decline attenuated by SA in a dose dependent manner, as D100 plants presented higher TBI than D0 plants (Figure 3A). The fraction of biomass allocation among plant organs was also distinctly affected by water regime and SA concentration. All stressed plants increased the investment in roots, highlighting D100 plants, that conversely reduced the investment in stems and leaves fractions (Figure 3B).

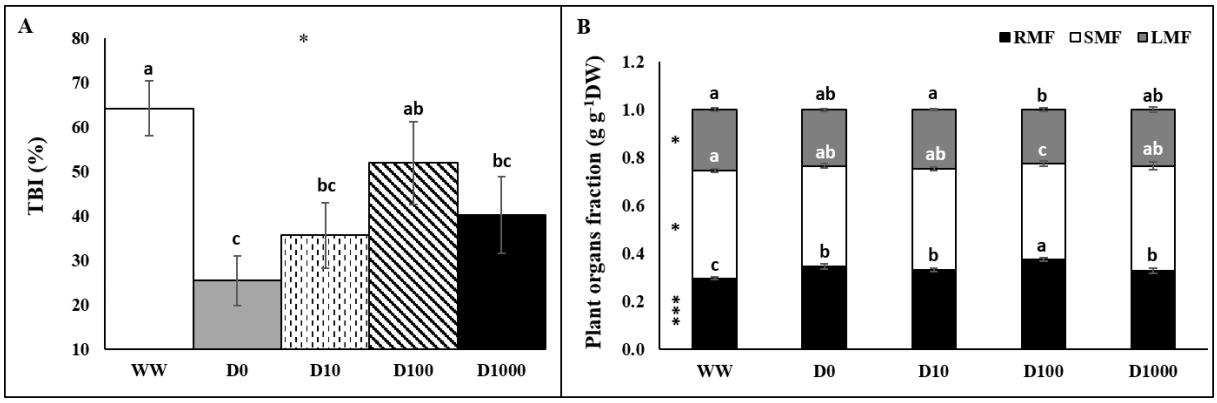


**Figure 3.** Total biomass increase (TBI) (A) and plant organs fraction (RMF, root mass fraction; SMF, stem mass fraction; LMF, leaf mass fraction) (B) of well-watered (WW) and droughted plus salicylic acid (D) plants at the end of the experiment. Each column is average and vertical bars represent the S.E. (n=8). Different letters indicate significant differences among treatments (*P < 0.05, ***P<0.001)

The concentration minerals, except for nitrogen and calcium, was affected by water availability and exogenous SA supply, although dependent on the plant organ (Table 2). The application of SA decreased leaf P, while drought reduced stem P, mainly in the absence of SA. On the other hand, drought dropped K concentration on leaves and roots, mainly in SA-treated and D0 and D10 plants, respectively. Furthermore, substantial changes were observed in magnesium concentrations. Leaf Mg was higher in D10 than in SA-starved drought plants, stem Mg was superior in D0, whereas root Mg was higher in D100 than in D0 and D10 plants. Meanwhile, the leaf sulfur concentration was higher in D0 plants, at the expenses of stem S concentration. In addition, interesting changes were observed in micronutrients concentrations, namely under the application of SA. The concentration of boron increased in roots of droughted plants, namely in D100 trees, whereas the concentrations of iron and manganesium increased in leaves and roots with SA application, highlighting D10 on leaves and D100 on both organs for Fe, and D10 and D100 on leaves and D1000 on roots for Mn. On the other hand, the concentration of Zn only changed on roots, being higher in D100 than in D0 and D10 plants,

while the concentration of Cu on leaves was higher in D10 than on SA-starved plants, while the concentration on roots decreased under drought.

**Table 2.** Concentration of nutrients (g.kg$^{-1}$DW for macronutrients, and mg.kg$^{-1}$DW for micronutrients) in the different plant organs of well-watered (WW) and droughted (D) plus salicylic acid plants at the end of the experiment.

| | Nutrients concentration | | | | | Sig. |
|---|---|---|---|---|---|---|
| | WW | D0 | D10 | D100 | D1000 | |
| $N_{Leaf}$ | 20.0±0.8 | 20.0±0.9 | 19.6±0.5 | 19.6±0.8 | 19.8±0.8 | n.s. |
| $N_{Stem}$ | 5.02±0.23 | 5.80±0.21 | 5.54±0.20 | 5.54±0.14 | 5.79±0.33 | n.s. |
| $N_{Root}$ | 13.8±0.4 | 15.0±0.6 | 13.2±0.8 | 14.3±0.5 | 15.5±0.5 | n.s. |
| $P_{Leaf}$ | 3.52±0.21$^{a}$ | 2.93±0.11$^{a}$ | 2.41±0.15$^{b}$ | 2.66±0.11$^{b}$ | 2.62±0.12$^{b}$ | *** |
| $P_{Stem}$ | 2.93±0.20$^{a}$ | 1.57±0.34$^{c}$ | 2.22±0.05$^{b}$ | 2.59±0.13$^{ab}$ | 2.42±0.26$^{ab}$ | ** |
| $P_{Root}$ | 1.97±0.29 | 2.11±0.40 | 1.72±0.58 | 1.28±0.15 | 1.71±0.31 | n.s. |
| $K_{Leaf}$ | 12.6±0.7$^{a}$ | 10.4±0.2$^{b}$ | 8.30±0.98$^{c}$ | 7.16±0.31$^{c}$ | 8.59±0.56$^{c}$ | *** |
| $K_{Stem}$ | 6.61±1.33 | 5.14±0.17 | 2.67±0.97 | 5.21±0.94 | 5.63±1.02 | n.s. |
| $K_{Root}$ | 7.87±0.44$^{a}$ | 3.40±0.77$^{c}$ | 3.44±1.03$^{c}$ | 5.98±0.33$^{ab}$ | 5.56±0.45$^{b}$ | *** |
| $Ca_{Leaf}$ | 3.37±0.26 | 3.80±0.96 | 4.93±0.39 | 4.62±0.65 | 3.84±0.97 | n.s. |
| $Ca_{Stem}$ | 1.74±0.40 | 2.345±0.43 | 0.93±0.42 | 1.80±0.60 | 1.32±0.23 | n.s. |
| $Ca_{Root}$ | 1.99±0.523 | 2.51±0.93 | 2.75±0.57 | 2.23±0.17 | 3.25±0.38 | n.s. |
| $Mg_{Leaf}$ | 0.462±0.094$^{b}$ | 0.402±0.086$^{b}$ | 0.808±0.034$^{a}$ | 0.694±0.099$^{ab}$ | 0.586±0.12$^{ab}$ | * |
| $Mg_{Stem}$ | 0.217±0.076$^{b}$ | 0.492±0.080$^{a}$ | 0.288±0.067$^{b}$ | 0.267±0.060$^{b}$ | 0.189±0.01$^{b}$ | * |
| $Mg_{Root}$ | 1.78±0.13$^{ab}$ | 1.27±0.21$^{b}$ | 1.34±0.26$^{b}$ | 1.59±0.08$^{ab}$ | 1.97±0.12$^{a}$ | * |
| $S_{Leaf}$ | 2.95±0.17$^{ab}$ | 3.29±0.26$^{a}$ | 2.25±0.19$^{b}$ | 2.35±0.06$^{b}$ | 2.35±0.38 $^{b}$ | * |
| $S_{Stem}$ | 0.328±0.047$^{a}$ | 0.135±0.042$^{b}$ | 0.152±0.04$^{b}$ | 0.324±0.006$^{a}$ | 0.284±0.03$^{a}$ | ** |
| $S_{Root}$ | 0.667±0.095 | 0.759±0.264 | 0.627±0.302 | 0.683±0.079 | 0.646±0.110 | n.s. |
| $B_{Leaf}$ | 28.4±0.8 | 25.8±1.8 | 26.6±1.9 | 27.8±2.2 | 27.9±2.0 | n.s. |
| $B_{Stem}$ | 22.5±1.1 | 29.0±2.7 | 28.6±3.0 | 33.3±3.4 | 32.8±4.4 | n.s. |
| $B_{Root}$ | 29.4±1.7$^{c}$ | 39.2±3.3$^{b}$ | 38.1±2.7$^{b}$ | 50.1±2.7$^{a}$ | 40.9±2.9$^{b}$ | ** |
| $Fe_{Leaf}$ | 14.4±1.9$^{bc}$ | 9.9±0.9$^{c}$ | 30.8±3.9$^{a}$ | 25.2±4.5$^{ab}$ | 17.8±5.0$^{bc}$ | ** |
| $Fe_{Stem}$ | 10.6±2.1 | 11.3±2.7 | 5.5±1.5 | 12.7±3.9 | 9.78±1.17 | n.s. |
| $Fe_{Root}$ | 246.1±22.7$^{bc}$ | 262.3±49.8$^{bc}$ | 179.2±33.3$^{c}$ | 499.4±107.0$^{a}$ | 377.5±38.1$^{ab}$ | ** |
| $Zn_{Leaf}$ | 17.4±2.2 | 17.0±2.0 | 21.7±1.0 | 18.7±2.3 | 18.4±4.9 | n.s. |
| $Zn_{Stem}$ | 9.8±1.7 | 14.9±1.6 | 7.6±2.9 | 14.6±2.8 | 16.7±2.6 | n.s. |
| $Zn_{Root}$ | 54.0±3.9$^{ab}$ | 34.0±9.7$^{bc}$ | 30.1±6.3$^{c}$ | 64.6±5.3$^{a}$ | 54.7±2.3$^{ab}$ | ** |
| $Mn_{Leaf}$ | 27.3±5.6$^{c}$ | 21.8±6.7$^{c}$ | 59.2±2.6$^{ab}$ | 62.1±11.0$^{a}$ | 40.2±4.9$^{bc}$ | ** |
| $Mn_{Stem}$ | 4.94±0.15 | 9.65±1.90 | 6.64±1.73 | 13.09±5.89 | 5.96±1.60 | n.s. |
| $Mn_{Root}$ | 35.9±9.9$^{b}$ | 54.1±17.4$^{b}$ | 50.3±11.4$^{b}$ | 71.7±12.3$^{ab}$ | 92.9±7.7$^{a}$ | * |
| $Cu_{Leaf}$ | 14.0±1.0$^{b}$ | 12.9±1.6$^{b}$ | 22.8±1.3$^{a}$ | 18.1±2.5$^{ab}$ | 17.3±3.9$^{ab}$ | * |
| $Cu_{Stem}$ | 9.17±1.37 | 10.6±2.5 | 7.95±1.35 | 10.6±0.9 | 10.5±0.9 | n.s. |
| $Cu_{Root}$ | 25.5±2.6$^{a}$ | 10.1±2.4$^{bc}$ | 9.74±1.49$^{bc}$ | 7.14±1.88$^{c}$ | 14.3±2.3$^{b}$ | *** |

Values are means±SE. Different letters indicate significant differences among treatments (n.s.- not significant, *P < 0.05, **P < 0.01, ***P<0.001).

The minerals allocation patterns, with the exception of calcium, were affected by the applied treatments, although differently among SA concentrations and water regime, being leaves and roots more influenced than stems (Table 3).

**Table 3.** Nutrients allocation (%) by the different plant organs of well-watered (WW) and droughted (D) plus salicylic acid plants at the end of the experiment.

| | Nutrients allocation | | | | | Sig. |
|---|---|---|---|---|---|---|
| | WW | D0 | D10 | D100 | D1000 | |
| $N_{Leaf}$ | 48.1±0.4$^{a}$ | 40.2±1.6$^{b}$ | 45.1±0.4$^{a}$ | 39.5±0.5$^{b}$ | 40.3±1.9$^{b}$ | *** |
| $N_{Stem}$ | 26.2±0.7 | 24.7±1.31 | 26.8±0.8 | 25.1±0.8 | 26.2±2.1 | n.s. |
| $N_{Root}$ | 25.7±1.0$^{b}$ | 35.1±2.9$^{a}$ | 28.1±0.7$^{b}$ | 35.4±0.9$^{a}$ | 33.5±2.3$^{a}$ | ** |
| $P_{Leaf}$ | 31.1±1.7$^{b}$ | 38.6±1.5$^{a}$ | 28.0±2.5$^{b}$ | 26.9±2.2$^{b}$ | 26.8±3.0$^{b}$ | ** |
| $P_{Stem}$ | 55.6±0.5$^{a}$ | 34.7±5.5$^{b}$ | 54.1±3.6$^{a}$ | 57.6±2.0$^{a}$ | 54.1±6.4$^{a}$ | ** |
| $P_{Root}$ | 13.3±1.8 | 26.7±4.6 | 17.9±5.6 | 15.5±1.7 | 19.1±4.6 | n.s. |
| $K_{Leaf}$ | 40.2±6.4$^{ab}$ | 41.1±1.8$^{ab}$ | 51.5±5.8$^{a}$ | 28.3±1.8$^{b}$ | 33.9±5.6$^{b}$ | * |
| $K_{Stem}$ | 41.2±7.2 | 42.8±2.6 | 31.1±6.9 | 42.3±4.7 | 43.7±5.6 | n.s. |
| $K_{Root}$ | 18.6±1.3 | 16.1±4.3 | 17.4±4.37 | 29.4±3.2 | 22.4±2.2 | n.s. |
| $Ca_{Leaf}$ | 39.9±3.0 | 34.9±8.1 | 52.8±3.8 | 41.9±7.0 | 36.8±5.1 | n.s. |
| $Ca_{Stem}$ | 40.5±7.5 | 42.1±4.1 | 18.7±7.2 | 33.0±10.5 | 28.0±4.1 | n.s. |
| $Ca_{Root}$ | 19.6±5.3 | 23.0±7.7 | 28.5±6.6 | 25.1±3.6 | 35.2±5.0 | n.s. |
| $Mg_{Leaf}$ | 20.6±4.9$^{b}$ | 13.2±1.8$^{b}$ | 31.4±3.1$^{a}$ | 21.2±2.6$^{b}$ | 18.6±2.9$^{b}$ | * |
| $Mg_{Stem}$ | 19.6±6.0$^{b}$ | 37.3±6.6$^{a}$ | 23.1±5.9$^{ab}$ | 18.5±4.6$^{b}$ | 13.6±1.3$^{b}$ | * |
| $Mg_{Root}$ | 59.8±3.6$^{ab}$ | 49.5±6.0$^{b}$ | 45.5±7.0$^{b}$ | 60.3±2.0$^{ab}$ | 67.8±3.7$^{a}$ | * |
| $S_{Leaf}$ | 70.9±2.1$^{ab}$ | 76.2±3.91$^{a}$ | 72.3±6.6$^{ab}$ | 60.1±1.80$^{b}$ | 62.8±3.9$^{b}$ | * |
| $S_{Stem}$ | 16.7±1.9$^{a}$ | 6.3±1.63 $^{b}$ | 11.4±4.5$^{ab}$ | 18.6±1.21$^{a}$ | 18.5±4.4$^{a}$ | * |
| $S_{Root}$ | 12.4±2.0 | 17.5±4.66 | 16.3±7.4 | 21.3±2.30 | 18.7±2.5 | n.s. |
| $B_{Leaf}$ | 28.6±0.4$^{a}$ | 19.6±0.55$^{c}$ | 21.9±0.8$^{b}$ | 17.0±0.7$^{d}$ | 19.6±0.9$^{c}$ | *** |
| $B_{Stem}$ | 48.7±0.5 | 46.1±1.85 | 48.8±1.7 | 45.1±1.5 | 49.8±2.7 | n.s. |
| $B_{Root}$ | 22.7±0.5$^{c}$ | 34.3±1.97$^{ab}$ | 29.3±1.5$^{b}$ | 37.9±1.6$^{a}$ | 30.6±2.8$^{b}$ | *** |
| $Fe_{Leaf}$ | 6.6±0.9$^{b}$ | 3.3±0.7$^{b}$ | 16.7±4.7$^{a}$ | 4.2±0.8$^{b}$ | 4.3±1.3$^{b}$ | ** |
| $Fe_{Stem}$ | 9.9±1.3 | 10.3±4.4 | 6.8±2.7 | 5.5±2.4 | 5.2±1.0 | n.s. |
| $Fe_{Root}$ | 83.5±1.0 | 86.4±2.0 | 76.5±7.3 | 90.3±3.0 | 90.5±2.2 | n.s. |
| $Zn_{Leaf}$ | 21.6±1.8$^{b}$ | 19.9±3.4$^{b}$ | 35.3±3.8$^{a}$ | 14.5±1.9$^{b}$ | 16.0±3.2$^{b}$ | *** |
| $Zn_{Stem}$ | 25.9±3.8 | 36.9±6.0 | 23.1±6.1 | 24.6±3.9 | 31.8±3.1 | n.s. |
| $Zn_{Root}$ | 52.5±4.4 | 43.2±8.5 | 41.6±6.6 | 60.9±2.6 | 52.2±3.6 | n.s. |
| $Mn_{Leaf}$ | 41.3±4.7$^{ab}$ | 23.3±6.3$^{c}$ | 52.2±6.4$^{a}$ | 34.8±5.3$^{bc}$ | 26.8±3.7$^{bc}$ | ** |
| $Mn_{Stem}$ | 18.3±2.8 | 20.8±3.2 | 11.0±2.3 | 16.9±7.6 | 8.4±1.9 | n.s. |
| $Mn_{Root}$ | 40.4±5.0$^{bc}$ | 55.9±6.3$^{ab}$ | 36.8±4.5$^{c}$ | 48.3±5.1$^{bc}$ | 64.8±4.2$^{a}$ | ** |
| $Cu_{Leaf}$ | 26.7±2.1$^{b}$ | 30.5±5.2$^{b}$ | 47.4±3.2$^{a}$ | 35.5±3.46$^{b}$ | 30.1±4.3$^{b}$ | ** |
| $Cu_{Stem}$ | 36.2±4.0 | 45.1±3.5 | 33.9±4.6 | 47.7±5.01 | 42.0±2.7 | n.s. |
| $Cu_{Root}$ | 37.1±2.6$^{a}$ | 24.4±3.9$^{b}$ | 18.7±2.5$^{b}$ | 16.8±3.26$^{b}$ | 27.9±4.8$^{ab}$ | ** |

Values are means±SE. Different letters indicate significant differences among treatments (n.s.- not significant, *P < 0.05, **P < 0.01, ***P<0.001).

D10 plants maintained the allocation of N into leaves and roots, as in WW plants, whereas the other stressed plants decreased their allocation of N into leaves, in order to invest more N in roots. SA-treated plants presented similar patterns of P allocation, as well-irrigated plants, while drought-starved SA increased allocation into leaves, at the expenses of stems. Meanwhile, minor changes were observed in K allocation, as only D10 plants presented more investment of K on leaves than D100 and D1000 counterparts. More evident were the effects of the application of SA and drought on the allocation of magnesium, as D10 plants presented higher distribution of Mg to leaves, D0 exhibited superior Mg allocation to stems than WW, D100 and D1000 trees, and D1000 had larger Mg investment in roots than D0 and D10 plants. Relatively to sulfur allocation, D0 plants showed higher allocation to leaves than D100 and D1000, while these treatments, joining with well-watered, had higher S allocation to stems than drought-starved SA plants. On the other hand, the allocation of B to leaves decreased in plants submitted to drought, whereas the opposite trend was observed in roots, namely in D100 treatment. Iron, zinc and copper allocation patterns were almost similar, being registered higher allocations to leaves of D10 plants. Nonetheless, WW plants presented higher Cu allocation to roots than droughted plants. Relatively to Mn, D10 plants presented again the highest distribution to leaves, whereas D1000 trees stand out in allocation of Mn to roots.

The total quantity of minerals acquired by plants showed in Table 4 reflects treatment effects on both tissue concentrations and biomass production and are, in general, strictly associated with the root uptake efficiency (RUE). Droughted plants presented lower P, K, S and Cu root uptake efficiency and, thus, inferior minerals yield than well-watered trees, whereas Ca, Mg and B were not significantly affected. In addition, WW plants had larger $N_{RUE}$ than D0 and D100, while D100 presented superior uptake of P than D0 plants. Moreover, in general, D100 and D1000 plants absorbed more Fe, Zn and Mn than the other treatments.

**Table 4.** Minerals acquired (A, g.plant$^{-1}$for macronutrients and mg.plant$^{-1}$ for micronutrients) and root uptake efficiency (RUE) (macronutrients, mg.g$^{-1}$ root, and micronutrients, µg.g$^{-1}$ root) of well-watered (WW) and droughted (D) plus salicylic acid plants at the end of the experiment.

| | WW | D0 | D10 | D100 | D1000 | Sig. |
|---|---|---|---|---|---|---|
| $N_A$ | 1.43±0.08 | 1.20±0.06 | 1.21±0.10 | 1.38±0.07 | 1.33±0.12 | n.s. |
| $P_A$ | 0.393±0.031$^{a}$ | 0.216±0.004$^{c}$ | 0.240±0.011$^{bc}$ | 0.284±0.028$^{b}$ | 0.267±0.011$^{bc}$ | *** |
| $K_A$ | 1.142±0.111$^{a}$ | 0.612±0.017$^{b}$ | 0.478±0.078$^{b}$ | 0.743±0.111$^{b}$ | 0.728±0.093$^{b}$ | *** |
| $Ca_A$ | 0.301±0.037 | 0.285±0.059 | 0.259±0.019 | 0.318±0.029 | 0.273±0.035 | n.s. |
| $Mg_A$ | 0.079±0.003 | 0.071±0.010 | 0.074±0.009 | 0.091±0.005 | 0.083±0.006 | n.s. |
| $S_A$ | 0.14±0.01$^{a}$ | 0.105±0.008$^{b}$ | 0.087±0.007$^{b}$ | 0.110±0.008$^{b}$ | 0.098±0.011$^{b}$ | ** |
| $B_A$ | 3.43±0.22 | 3.18±0.26 | 3.42±0.40 | 4.55±0.29 | 3.89±0.44 | n.s. |
| $Fe_A$ | 7.78±0.76$^{b}$ | 8.35±1.51$^{b}$ | 5.83±0.87$^{b}$ | 18.3±3.1$^{a}$ | 11.8±1.3$^{ab}$ | *** |
| $Zn_A$ | 2.78±0.33$^{ab}$ | 2.22±0.37$^{bc}$ | 1.82±0.29$^{c}$ | 3.60±0.18$^{a}$ | 3.04±0.30$^{ab}$ | ** |
| $Mn_A$ | 2.25±0.40$^{c}$ | 2.56±0.76$^{c}$ | 3.32±0.40$^{bc}$ | 4.96±0.39$^{a}$ | 4.09±0.34$^{ab}$ | ** |
| $Cu_A$ | 1.86±0.21$^{a}$ | 1.12±0.16$^{b}$ | 1.33±0.02$^{b}$ | 1.42±0.16$^{ab}$ | 1.49±0.13$^{ab}$ | * |
| $N_{RUE}$ | 54±1.5$^{a}$ | 43.9±3.9$^{b}$ | 46.8±2.7$^{ab}$ | 40.3±0.6$^{b}$ | 46.9±3.0$^{ab}$ | * |
| $P_{RUE}$ | 14.8±0.8$^{a}$ | 7.83±0.36$^{b}$ | 9.32±0.33$^{b}$ | 8.21±0.48$^{b}$ | 9.47±0.57$^{b}$ | *** |
| $K_{RUE}$ | 43.1±3.6$^{a}$ | 22.3±1.5$^{b}$ | 18.8±3.7$^{b}$ | 21.3±2.5$^{b}$ | 25.4±2.6$^{b}$ | *** |
| $Ca_{RUE}$ | 11.3±1.3 | 10.1±1.8 | 10.3±1.4 | 9.25±0.72 | 9.61±1.20 | n.s. |
| $Mg_{RUE}$ | 2.99±0.17 | 2.54±0.31 | 2.86±0.32 | 2.64±0.11 | 2.93±0.20 | n.s. |
| $S_{RUE}$ | 5.43±0.29$^{a}$ | 3.86±0.50$^{b}$ | 3.38±0.30$^{b}$ | 3.20±0.12$^{b}$ | 3.45±0.40$^{b}$ | *** |
| $B_{RUE}$ | 129.6±4.6 | 114.7±8.6 | 130.1±6.6 | 133.0±8.1 | 137.8±15.7 | n.s. |
| $Fe_{RUE}$ | 294.4±26.0$^{b}$ | 295.1±44.8$^{b}$ | 225.2±32.4$^{b}$ | 541.7±103.1$^{a}$ | 415.2±34.7$^{ab}$ | ** |
| $Zn_{RUE}$ | 104.5±8.6$^{a}$ | 78.7±10.0$^{ab}$ | 72.1±14.4$^{b}$ | 106.0±7.8$^{a}$ | 106.58±7.9$^{a}$ | * |
| $Mn_{RUE}$ | 85.0±15.1$^{b}$ | 90.4±24.9$^{b}$ | 129.0±16.9$^{ab}$ | 146.2±14.2$^{a}$ | 144.4±10.3$^{a}$ | * |
| $Cu_{RUE}$ | 69.7±5.6$^{a}$ | 39.4±4.1$^{b}$ | 52.4±4.5$^{b}$ | 40.9±2.7$^{b}$ | 52.9±5.0$^{b}$ | *** |

Values are means±SE. Different letters indicate significant differences among treatments (n.s.- not significant, *P < 0.05, **P < 0.01, ***P<0.001).

In terms of physiological nutrient use efficiency (UE), our data demonstrated that $P_{UE}$ increased in water-stressed trees, $K_{UE}$ was highest in D10, WW had larger $B_{UE}$ than D100 and D1000 and higher $Mn_{UE}$ than all exogenous-SA treatments, and D10 presented superior $Fe_{UE}$ and $Zn_{UE}$ than D100 and D1000 plants. Meanwhile, no significant effects of treatments were reported on nitrogen, calcium, magnesium, sulfur and copper physiological use efficiency (Table 5).

**Table 5.** Physiological nutrient use efficiency (UE) (g biomass g $^{-1}$ mineral for macronutrients and mg biomass g$^{-1}$ mineral for micronutrients) of well-watered (WW) and droughted (D) plus salicylic acid plants at the end of the experiment.

| | Nutrients Use Efficiency | | | | | Sig. |
|---|---|---|---|---|---|---|
| | WW | D0 | D10 | D100 | D1000 | |
| $N_{UE}$ | 95.3±2.7 | 86.4±3.4 | 93.2±3.3 | 90.4±3.0 | 87.6±3.7 | n.s. |
| $P_{UE}$ | 351.3±23.4$^{b}$ | 480.0±26.6$^{a}$ | 467.3±22.2$^{a}$ | 448.4±23.8$^{a}$ | 432.2±11.5$^{a}$ | ** |
| $K_{UE}$ | 123.2±12.8$^{b}$ | 168.7±5.8$^{b}$ | 257.1±36.2$^{a}$ | 182.8±27.4$^{b}$ | 167.8±21.9$^{b}$ | * |
| $Ca_{UE}$ | 484.7±72.8 | 422.4±82.6 | 440.2±38.3 | 402.6±30.8 | 444.7±49.7 | n.s. |
| $Mg_{UE}$ | 1729.6±66.0 | 1581.4±246.1 | 1572.7±136.2 | 1388.9±66.0 | 1404.5±67.8 | n.s. |
| $S_{UE}$ | 956.3±60.7 | 1010.8±87.9 | 1332.6±147.0 | 1142.1±40.1 | 1265.7±192.7 | n.s. |
| $B_{UE}$ | 39.8±1.7$^{a}$ | 33.2±2.7$^{ab}$ | 33.8±2.7$^{ab}$ | 27.8±1.9$^{b}$ | 30.9±3.0$^{b}$ | * |
| $Fe_{UE}$ | 19.2±1.6$^{ab}$ | 15.5±3.9$^{ab}$ | 26.5±6.5$^{a}$ | 8.02±1.39$^{b}$ | 10.7±1.3$^{b}$ | * |
| $Zn_{UE}$ | 50.5±4.5$^{ab}$ | 51.6±8.3$^{ab}$ | 67.0±9.0$^{a}$ | 35.0±2.5$^{b}$ | 38.9±3.0$^{b}$ | * |
| $Mn_{UE}$ | 67.7±10.8$^{a}$ | 57.1±17.5$^{ab}$ | 35.7±4.4$^{bc}$ | 25.8±2.2$^{c}$ | 28.4±0.6$^{bc}$ | * |
| $Cu_{UE}$ | 75.9±7.4 | 102.6±17.8 | 84.7±6.0 | 90.3±5.3 | 79.7±8.3 | n.s. |

Values are means±SE. Different letters indicate significant differences among treatments (n.s.- not significant, *P < 0.05, **P < 0.01).

## 4. Discussion

### 4.1. Oxidative stress and defense systems

Whether ROS would act as signaling molecules or might cause oxidative stress to the tissues depend on the refined balance between ROS production and scavenging (Mattos and Moretti, 2015). In this study, the sharp increase in total ROS observed in D10 and D0 plants, both under drought and after rewatering, suggest that D100 and D1000 treatments contributed to maintain reduced values of ROS, just as in WW plants. ROS react with structural and functional proteins, lipids and nucleic acids, causing oxidative damage and impairing cellular functioning (Farooq *et al*., 2009). Yet, each type of ROS molecule has its own distinct reactivity, being singlet oxygen ($^{1}O_2$) and hydroxyl radical ($OH^{\bullet}$) the most reactive, while $H_2O_2$ is less reactive (Gill and Tuteja, 2010; Pinto-Marijuan and Munne-Bosch, 2014). Nonetheless, when accumulated at high level, $H_2O_2$ becomes toxic because it can be converted to $OH^{\bullet}$ (Belkadhi *et al*., 2014). Still, none of the droughted plants overaccumulate $H_2O_2$ in relation to the WW controls. Furthermore, $H_2O_2$ being the product of other ROS detoxification (Gill and Tuteja, 2010; Pinto-Marijuan and Munne-Bosch, 2014), is also a potent signaling molecule, due to its long half-life and the ability to cross cellular membranes (Petrov and Van Breusegem, 2012). Interestingly, the distinct pattern of $H_2O_2$ and total ROS accumulation during drought, with higher $H_2O_2$ accumulation in WW, D100 and D1000 treatments might be a reflex of the balance between each type of ROS. Thus, D0 and D10 plants seems to display a reduced

capacity to scavenge and detoxify highly reactive species. In agreement, there are evidences that SA pretreatment induces $H_2O_2$ production (Chen *et al*., 2007; Harfouche *et al*., 2008; Belkadhi *et al*., 2014), which in turns might induce antioxidant enzymes activity, decreasing cellular ROS levels (Arfan, 2009).

General responses to stress involve the signaling stress detection and, consequently, the increase in antioxidant responses (Farooq *et al*., 2009). The synthesis of stress proteins plays crucial role in drought tolerance development (Farooq *et al*., 2009). However, although in some studies total soluble proteins increased in response to drought (Bacelar *et al*., 2006, 2007), we found a markedly decline of TSP in D0 plants, in agreement with other experiments (El-Tayeb, 2005; Jalal *et al*., 2012; Kabiri *et al*., 2014). In fact, depending on drought severity (Jalal *et al*., 2012), the generation of ROS causes the oxidation of amino acids and burst proteins structure (Kabiri *et al*., 2014). Notably, SA prevented the TSP decline and/or induced its overaccumulation in droughted leaves, starting to stabilize during the recovery period. The positive effect of SA on proteins accumulation is commonly observed in stressed plants, either through developing mechanisms that avoids their degradation or by inducing specific stress related proteins (Jalal *et al*., 2012; Kang *et al*., 2012; Belkadhi *et al*., 2014; Hashempour *et al*., 2014; Kabiri *et al*., 2014). In fact, the application of SA changed the protein patterns of drought stressed plants (Jalal *et al*., 2012; Kang *et al*., 2012), inducing the expression of proteins associated with signal transduction, stress defense, photosynthesis, carbohydrate metabolism, protein metabolism, and energy (Kang *et al*., 2012). Again, the lower -SH concentrations of D0 plants during drought stress must be the result of the oxidation by ROS (Zagorchev *et al*., 2013). In agreement, similar results were reported in droughted olive trees by Bacelar *et al*. (2006, 2007). The maintenance of high -SH levels during water scarcity is an advantage to SA-treated plants, since -SH groups are involved in the antioxidant defense system and, thus, they are crucial to plants stress tolerance (Zagorchev *et al*., 2013).

The restauration and/or overaccumulation of drought-declined β-carotene concentration after stress relief suggests an important function of this carotenoid in recovery processes. β-carotene allows the physical quenching of $^3$Chl* and $^1O_2$ (Pinto-Marijuan and Munne-Bosch, 2014), helping in the detoxification processes. Many abiotic stresses, and the resulted oxidative stress, induce the phenylpropanoid metabolism in plants (Grace, 2007), leading to an accumulation of phenolic compounds in olive leaves in response to drought (Bacelar *et al*., 2006; Petridis *et al*., 2012), as observed in our study. Noteworthy, the increase of TPC in D0 plants during drought was largely associated with the increase in flavonoids, important

bioactives plant secondary metabolites (Gill and Tuteja, 2010). This association suggests an increased necessity of protection against photooxidative damage, as flavonoids protects photosynthetic apparatus against photoinhibition under excessive light (Zhou *et al*., 2016) and absorbs the damaging UV-B radiation (Grace, 2007). The lower accumulation of TPC in SA-treated plants during drought, mainly in D100 and D1000 is in conformity with a previous study in salt and drought stressed barley plants (Fayez and Bazaid, 2014). Such data suggests a reduced oxidative stress in D100 and D1000 trees, which was confirmed by the lower accumulation of ROS in these plants, and/or the investment in other defense mechanisms, such as the maintenance of high TSP concentrations. Meanwhile, ascorbate, besides to directly scavenge ROS is also substrate to ascorbate peroxidase, that use it as specific electron donor to reduce $H_2O_2$ to $H_2O$ (Pinto-Marijuan and Munné-Bosch, 2014; Mattos and Moretti, 2015). Thus, the overaccumulation of ascorbate and the lowest $H_2O_2$ concentration in D0 suggest an improvement of ascorbate peroxidase activity. Moreover, in chloroplasts ascorbate acts as a cofactor of violaxantin de-epoxidase, sustaining the dissipation of excess excitation energy (Mattos and Moretti, 2015), suggesting again higher necessity of photoprotection.

The higher TAC exhibited by D0 plants at the peak of drought claim the role of phenolic compounds as potent free radical scavengers, since the measured TAC depends on the reduction of DPPH radical, where phenolics are known to overcome other antioxidants (Xu and Chang, 2007; Mattos and Moretti, 2015). In agreement, the lower accumulation of phenolic compounds in SA-treated plants than in D0 plants was also evident on TAC data. Still, the higher TAC of D100 plants, relatively to D10 and D1000 plants suggests that other antioxidant substances, rather phenolic compounds, were involved in this response, that were further repressed during rewatering.

In short, D10 plants although invested largely in TSP accumulation, exhibited a close behavior to D0 plants. D0 plants, exhibited higher accumulation of phenolic compounds and ROS, and invested in β-carotene and ascorbate pool restauration upon rewatering. The responses induced by D100 and D1000 treatments were more similar. D100 plants had a slightly higher and lower accumulation of TSP and ROS, respectively, and increased the investment in TPC and β-carotene concentrations. Upon rewatering, both D100 and D1000 plants increased largely the investment in phenolic compounds. However, although D100 plants reduced TSP to D1000 and WW levels, continued to display the lower and higher concentrations of total ROS and $H_2O_2$, respectively. This response might be the result of a higher detoxification of

stronger ROS. By this, the activation or restoration of other mechanisms and/or metabolites, like ascorbate, may be involved in this response.

### 4.2. Plant ionome dynamics

Olive trees in many areas of the world are among the least fertilized trees, and because of such inadequate nutrition, among other factors, biennial bearing is quite frequent (Therios, 2009). Moreover, limited nutrient uptake and reduced minerals concentrations is a regular response to lower water availability (Farooq *et al*., 2009), although in our study we only observed relevant decreases in some elements (P, K, S and Cu), in a closely association with root uptake efficiency. Several reasons can be given for the reduction of nutrient uptake under drought stress, including the lower transpiration rate, due to inferior stomatal conductance and total leaf area, as demonstrated previously (Brito *et al*., 2018a), the inhibition of ATP synthesis, the reduction of nutrient supply through mineralization (Sanaullah *et al*., 2012), the reducing nutrient diffusion and mass flow in the soil (Chapin, 1991) and the decrease in the concentration of root nutrient-uptake proteins (Bista *et al*., 2018). It is noteworthy that the decline in the concentrations of those minerals in some plant organs was evident even with the concentration effect due to the lower production of biomass. In opposition, drought-starved SA plants presented higher concentrations of Mg and B on stems and roots, respectively, in association with higher allocation of minerals to these organs. In the same way, these plants showed higher allocation of N to roots, at the expenses of leaves, and of P to leaves, at the expenses of stems, suggesting that the change on the allocation of minerals is an adaptive mechanism for growth under water limitation, confirming that the 'functional equilibrium' or 'resource balancing' theory might apply for the allocation of mineral nutrients within plants, as proposed by Weih *et al*. (2011). Meanwhile, as drought stress did not affect the uptake and transport of all minerals to the same extent, changes in the element stoichiometry may cause nutrient imbalances, conducting to perturbations of physiological functions, as reported earlier (Brito *et al*., 2018a,b), and to a reduction of biomass accumulation. In addition, nutrient imbalances can also lead to ROS formation, as demonstrated above, which can result in oxidative stress. Thus, for all these reasons, we believe that the optimization of fertilizer practices in a drought environment would offer a considerable challenge.

Notably, this study clearly demonstrated that plant ionome of droughted plants was modulated by the application of SA. Although some reductions in element concentrations were observed, as P, K and S in leaves, and Mg in stems, mainly associated to changes in allocation

patterns among organs, in general organs of SA-treated plants presented higher concentrations of mineral elements, as P and S on stems, Fe and Mn on leaves, and K, Mg, B, Fe, Mn and Zn on roots, highlighting D1000 and, mainly, D100 plants. The concentrations on roots are particularly interesting, as the higher values were obtained in spite of the higher root biomass accumulation in D100 plants, meaning that the responses were more related with enhanced nutrient root uptake efficiency, namely for Fe, Zn and Mn. Overall, the drought-induced decline in minerals uptake and concentrations were attenuated, and in some cases even overcompensated by SA application in relation to WW controls. Despite the fact that few studies have been done, particularly that they include all plant organs, these results were consistent with other works who reported nutrient status improvement by SA application in drought and salt stressed plants (El-Tayeb 2005; Gunes *et al*., 2007; Yildirim *et al*., 2008; Nazar *et al*., 2015). In the present study it is also notorious that exogenous SA affected micronutrients at a higher extent than macronutrients. Although micronutrients are presented in plant tissues in much lower concentration than macronutrients, they are largely required to activate several physiological, biochemical and metabolic processes, being essential to help macronutrients in growth and drought alleviation (Waraich *et al*. 2011; Duman, 2012).

The higher concentration of K on roots of D100 and D1000, relatively to D0 plants, has a significant relevance as K is a principal cation in vacuoles, contributing to osmotic adjustment and thus to increased expansion of cells via high cell turgor pressure, while the superior root Mg, more evident in D1000 plants, is important due to the involvement in protein synthesis and phosphorylation reactions, and as enzymatic cofactor (Grusak 2001; Waraich *et al*., 2011). Meanwhile, B has major functions on the structure of cell walls and on regulation of carbohydrates metabolism, promoting root cell elongation and, thus, root growth (Li *et al*., 2016), whereas zinc is a micronutrient required in many plant processes, as various enzymatic and oxidation-reduction reactions, membrane integrity, energy transfer and protein synthesis (Babar *et al*. 2013). Zn is also involved in tryptophan synthesis, a precursor of an essential growth hormone, IAA (Waraich *et al*. 2011; Babar *et al*. 2013), that enhance root growth, which in turn improves drought tolerance. Furthermore, the general higher Fe and Mn concentrations, both on leaves and roots of superior exogenous SA treatments, have major effects on drought adaptation as Fe is required for chlorophyll synthesis, respiration, photosynthesis and is an enzymatic cofactor (Rout and Sahoo, 2015). In addition, iron promote cell division in the meristematic cells of adventitious root primordial, as well the lateral root elongation, mediated by auxins signals (Hilo *et al*., 2017). On the other hand, Mn assists iron in chlorophyll

formation, is fundamental on the splitting of water and on electron transport to the chlorophyll reaction centers, being also and enzymatic cofactor, including in an important antioxidant enzyme, superoxide dismutase (Ciríaco da Silva *et al*., 2011). Thus, for all these reasons we may assume that SA regulates the responses of olive tree to drought stress and could be used as plant growth regulator to rebalancing mineral nutrients stoichiometry in plant tissues and to increase nutrient reserves that can promote plant's capacity to recover from drought stress events.

Nutrient allocation reflects the balance between the capacity to obtain, transport and store nutrients (He *et al*., 2015), being also dependent on where and how nutrients are used by the plant, and whether this pattern is changed under atypical conditions (McGrath and Lobell, 2013). In this way, the different allocation patterns verified in SA-treated plants suggest a selective behavior according to the plant needs, for instance of elements that acts as antioxidant-enzyme cofactors (Ciríaco da Silva *et al*., 2011; Waraich *et al*. 2011), which is also supported by the higher concentration of TSP in these plants. Likewise, the influence of SA in plant minerals allocation was also confirmed by other studies (El-Tayeb, 2005; Yildirim *et al*., 2008). Meanwhile, minor changes in physiological nutrients use efficiency were induced by water availability. In fact, only PUE was affected, being higher under drought stress, which suggests a better distribution of phosphorus resources among the different metabolic processes involved in biomass production and, thus, we may conclude that P use efficiency is an important trait to improve growth under drought conditions. On the contrary, salicylic acid provoked higher changes on nutrients use efficiency, depending from dosage, as D10 plants presented enhanced KUE, FeUE and ZnUE values, mainly relatively to the D100 plants, in a strictly association with the higher allocation of these minerals into leaves and to a lesser investment of minerals in protection mechanisms.

### 4.3. Biomass accumulation and allocation

The reduced growth and dry matter accumulation under drought is well established (Bacelar *et al*., 2007; Farooq *et al*., 2012), primarily due to lower leaf area dimension and to stomatal closure and then due to the inhibition of C assimilation by photosynthetic impairment (Medrano *et al*. 2002; Farooq *et al*. 2009). In fact, none of the stressed plants reached the TBI exhibited by WW controls, although SA attenuated this decline in a dose dependent manner, highlighting D100 treatment. Likewise, this positive effect of SA has been described on the literature (Umebese *et al*., 2009; Habibi, 2012; Kang *et al*., 2012; Fayez and Bazaid, 2014;

Nazar *et al*., 2015). This response was found to be related with the higher capacity to maintain and recover the photosynthetic performance, during and after drought relief (Brito *et al*., 2018b). Moreover, the maintenance of a better balance between oxidants and antioxidants, as discussed above, certainly contributed to the improved photosynthetic capacity and consequent biomass accumulation. In the same way, Gunes *et al*. (2007) argued that the dry matter increases in SA-treated salt stressed plants might been related to the induction of antioxidant responses.

A differential partitioning of dry matter between root and shoot is commonly observed in Mediterranean species to improve tolerance to repeated cycles of drought (Toscano *et al*., 2014; Brito *et al*., 2018b). As observed in our study, with special emphasis in D100 plants, instead of investing in photosynthetic tissues, olive tree enhances the allocation of dry matter into roots, as in other studies (Umebese *et al*., 2009; Kang *et al*., 2012; Aliniaeifard *et al*., 2016), in order to improve water and minerals uptake from soil. Moreover, roots also give mechanical support to plants and supply hormones that affect many physiological and biochemical processes associated with growth and development (Fageria and Moreira, 2011).

## 5. Conclusions

Overall, the results successfully confirmed our previous hypothesis, as a suitable concentration of SA (100 µM SA) improves olive trees drought adaptability. This effectiveness is achieved by the root biomass accumulation, the higher uptake of essential minerals, mainly micronutrients, and the improvement of the equilibrium between ROS production and scavenging. Notably, our findings give new insights about how SA regulate drought and recovery responses in olive tree. This understanding is of great importance as olive tree growing areas are typically subjected to oscillating drought-rewatering events. Moreover, in this study SA-treated plants were exposed to a realistic combination of abiotic stresses, making the present results very promising for established olive groves. Hence, the use of an appropriate SA concentration could be a cost-effective tool under the current and predicted extreme and variable environmental conditions, being a procedure of major interest in the commercial production of olive trees and others fruit crops.

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# CHAPTER 6

## Kaolin and salicylic acid effectiveness on summer stress mitigation in rainfed olive orchards

## Briefing note

This chapter covers the rainfed olive orchards response to KL and SA, in terms of plant performance and fruit and olive oil quality. Following the promising results obtained in pots experiments, it was important to confirm the efficacy of these products under realistic field conditions, where harvests can also be taken into account.

Following, this chapter is an adaptation of two research articles. The first, published in *Scientia Horticulturae* (246, 201-211) was entitled "Kaolin and salicylic acid alleviate summer stress in rainfed olive orchards by modulation of distinct physiological and biochemical responses", corresponding to the point 6.1, and the other published in *Scientia Horticulturae* (237, 176-183), entitled "Kaolin and salicylic acid foliar application modulate yield, quality and phytochemical composition of olive pulp and oil from rainfed trees", corresponding to the point 6.2. These articles aimed to respond to the specific objectives 5, 6 and 7 of this thesis, "to gain a greater understanding of the KL and SA effects on rainfed olive orchards responses", "to test how long KL and SA effects are prolonged in rainfed conditions" and "to evaluate the influence of KL and SA on olive fruit and oil quality". These studies contributed to confirm the efficacy of the applied products in increasing drought tolerance during the summer season and showed that also improve the plants responses during the winter months. Moreover, revealed how KL and SA determines the olives and olive oil quality parameters.

The authors contribution for the article converted in the point 6.1 was: Cátia Brito was involved in the establishment of the field trial, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and critical review of the article. Ana Luzio, Ermelinda Silva and Alexandre Gonçalves collected data on the field. Mónica Meijón and Monica Escandón gave the support for the immunohistochemical studies. Manuel Ângelo Rodrigues was involved in field trial establishment and maintenance. Margarida Arrobas carried out the minerals analysis. Carlos Correia was responsible for design the experiments, data collection on the field and critical review of the article. All the authors reviewed and approved the final manuscript.

The authors contribution for the article converted in the point 6.2 was: Cátia Brito was involved in the establishment of the field trial, collected data on the field, performed the laboratory analyses and was responsible for data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection on the field and critical review of the article. Ermelinda Silva and Alexandre Gonçalves collected data on the field. Manuel

Ângelo Rodrigues was involved in field trial establishment, maintenance and data collection on the field. Ana Barros gave the support for the laboratory analyses. Carlos Matos assisted in the olive oil quality parameters evaluation. Carlos Correia was responsible for design the experiments, data collection on the field and critical review of the article. All the authors reviewed and approved the final manuscript.

## 6.1. Kaolin and salicylic acid alleviate summer stress in rainfed olive orchards by modulation of distinct physiological and biochemical responses

### Kaolin and salicylic acid alleviate summer stress in rainfed olive orchards by modulation of distinct physiological and biochemical responses

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### Abstract

In a changing world, the search for new agronomic practices that help crops to maintain and/or increase yields and quality is a continuous challenge. We aim to evaluate kaolin (KL) and salicylic acid (SA) effectiveness as summer stress alleviating agents through physiological, biochemical and immunohistochemical analysis. Olive trees (*Olea europaea* L. cv. Cobrançosa) grown under rainfed conditions were sprayed with 5% KL and 100 µM SA, at the beginning of summer, during two consecutive years. KL enhanced relative water content (RWC), stomatal conductance ($g_s$) net photosynthesis (A) and leaf indole-3-acetic acid (IAA) signal, and decreased leaf sclerophylly, secondary metabolites and non-structural carbohydrates accumulation and abscisic acid (ABA). The trees treated with SA showed changes on IAA and ABA dynamics, and an enhancement in RWC, $g_s$, A, soluble proteins, and leaf P and Mg concentrations during the summer. Notably, KL and SA also allowed a faster restauration of the physiological functions during stress relief. In sum, KL and SA foliar sprays alleviated the negative effects induced by summer stress in olive trees performance, by modulation of distinct physiological and biochemical responses.

**Key-words:** Adaptation strategies, mineral nutrition, photosynthesis, phytohormones, secondary metabolism.

## 1. Introduction

In the current settings, olive trees (*Olea europaea* L.) growing under the typical Mediterranean semi-arid conditions are affected by multiple environmental constraint factors, and since the region is particularly vulnerable to climate change (IPCC, 2013), we may expect more severe summer stress. Although olive tree is well-adapted to harsh conditions, a considerable expense of energy resources is used in defense mechanisms, compromising plant growth and productivity (Fernández, 2014). Water deficit, commonly associated to heat and high irradiance stresses, impairs plant water status, drives stomatal closure, mesophyll compactness and photoinhibition, compromising photosynthetic capacity (Bacelar *et al*., 2004, 2006, 2007; Petridis *et al*., 2012). The continuous stress imposition induces oxidative damages and improves the investment in secondary metabolism, leading to reserves depletion (Bacelar *et al*., 2006, 2007; Petridis *et al*., 2012; Mattos and Moretti 2015). Furthermore, the cross-talk between different phytohormones mediate a wide range of adaptative responses, as growth, development, nutrient allocation, and source/sink transitions (Peleg and Blumwald 2011). Although abscisic acid (ABA) is the most studied stress-responsive hormone, the role of indole-3-acetic acid (IAA) during environmental stress is emerging (Peleg and Blumwald 2011).

Global climate change might compromise the economic viability of the olive rainfed sector, leading to the abandonment of traditional groves, with devastating environmental consequences. In this sense, it is required the implementation of agronomic strategies in order to alleviate the adverse effects of summer stress. Accordingly, the foliar application of kaolin (KL) and salicylic acid (SA) has been considered short-term adaptations for that purpose. KL, once sprayed on leaf surface, leaves a white protective particle film after water evaporation, increasing the reflection of excess radiation (ultraviolet, visible and infrared radiations), reducing the risk of leaf and fruit damage from heat load accumulation and solar injury (Glenn *et al*., 2005). The KL use to mitigate the negative influence of summer stress in olive trees was already appraised by some studies that report positive effects in plant water status, photosynthetic responses and yield (Roussos *et al*., 2010; Denaxa *et al*., 2012; Nanos, 2015), although this effectiveness was dependent from stress level and genotype (Roussos *et al*., 2010; Nanos, 2015). Thus, it is important to fill the lack in the understanding of KL action mode by studying other induced plant responses. Meanwhile, SA is a signaling phytohormone with diverse regulatory roles in plant metabolism, such as the antioxidant defense system activation, secondary metabolites production, osmolytes synthesis modulation and optimization of mineral nutrients status (Khan *et al*., 2015). Moreover, SA appears to be a key molecule to maintain a

proper balance between photosynthesis and growth (Rivas-San Vicente and Plasencia 2011). However, the precise mechanisms by which SA induces plant tolerance against abiotic stresses remain unknown (Rivas-San Vicente and Plasencia 2011; Khan *et al*., 2015). As far as we know, SA application to improve stress tolerance in olive trees was only described under freezing (Hashempour *et al*., 2014) and salinity (Aliniaeifard *et al*., 2016) conditions, where suitable concentrations of SA revealed to be effective. In sum, the influence of KL (Shellie and Glenn 2008; Nanos, 2015; Brillante *et al*. 2016) and SA (Kang *et al*. 2012; Fayez and Bazaid 2014; Wang *et al*., 2014Nazar *et al*., 2015) on stress mitigation is not consensual, since it depends on several factors that act in isolation or in combination, including genotypes, growth stage, concentration, administration mode and environmental conditions. Therefore, we aim to test the effectiveness of KL and SA as summer stress alleviating products in rainfed olive orchards. For this, a deep analysis was accomplished, evaluating specifically KL and SA influence on leaf structure, plant water status, photosynthetic performance, primary and secondary metabolites fluctuations, foliar phytohormones distribution and plant nutritional status.

## 2. Material and Methods

### 2.1. Site description, cultural practices and plant material

The experimental trial took place in Bragança, Northest Portugal, at Pinheiro Manso farm (41º 48´ N, 6º 44´ W), during two consecutive growing seasons (2015 and 2016), on a 5-years-old rainfed olive orchard (cv. 'Cobrançosa') planted at 7×6 m. The orchard was planted late in 2010 and produced the first fruits in 2013. Year 2014 was the first year that olive yield was recorded. The climate is typically Mediterranean with some Atlantic influence. Under the Koppen-Geiger climate classification, Bragança is classified as Csb, a temperate climate with hot and dry summers and rainy winters (IPMA, 2017). Annual precipitation in 2015 was 419.4 mm and 707.1 mm in 2016, 34% and 83% of it between January and May, respectively. The average air temperature and monthly precipitation recorded during the experimental period are shown in supplementary Figure 1. At the beginning of the experiment, soil total organic carbon (C) was 25.6 g $kg^{-1}$ (Incineration method), pH (soil:water, 1:2.5) was 5.8, extractable phosphorus (P) (Egner-Rhiem method) was 87.9 mg $kg^{-1}$, extractable potassium (K) (Egner-Rhiem method) was 102 mg $kg^{-1}$, extractable boron (B) (Azomethine method) was 0.5 mg $kg^{-1}$, exchangeable calcium (Ca) (ammonium acetate method, pH 7) was 7.2 cmolc $kg^{-1}$ and exchangeable magnesium (Mg) (ammonium acetate method, pH 7) was 2.2 cmolc $kg^{-1}$. Soil

fertilization consisted in the annual application (in the last week of March) of a compound NPK (10% N, 10% $P_2O_5$, 10% $K_2O$) fertilizer and borax (11% B). The fertilizers were localized in squares of 16 (4 x 4) $m^2$ (2 m distance from the trunk) per tree. In these areas, the compound fertilizer was applied at a rate corresponding to 50 kg $ha^{-1}$ of N, $P_2O_5$ and $K_2O$ and borax at a rate of 2 kg B $ha^{-1}$. The ground was managed with a non-selective herbicide (glyphosate, isopropylammonium salt, 360 g $L^{-1}$ active ingredient) applied beneath the trees in the areas corresponding to the application of fertilizers and at a dose of 3 L $ha^{-1}$. The fertilizers were left on the ground without incorporation. The inter-rows were tilled once a year late in April. The trees were yearly subjected to light interventions of pruning and training in February.

### 2.2. Treatments applied and monitoring

The experiment comprises three treatments: control (C) trees, sprayed with distilled water; kaolin (KL), sprayed with an aqueous solution of KL (Surround® WP, Engelhard Corporation, Iselin, NJ), at the manufacturer recommended dosage of 5% (w/v); and salicylic acid (SA), sprayed with an aqueous solution of 100 µM SA (Sigma-Aldrich, St. Louis, USA), selected based on results of preliminary research. Each plant was treated with a mean volume of 500 mL of spraying solution. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. The treatments were applied in the absence of wind in the morning of 30$^{th}$ June 2015 and 23$^{th}$ June 2016. A second application in the same days was done for KL trees to ensure the adhesion uniformity of kaolin clay particles to form the required film. The KL treatment was repeated in 27$^{th}$ August 2016 after a heavy rain event. Each treatment included three replicates, completely randomized, with three trees of similar canopy size per plot, separated by a buffer line of trees.

All the physiological, structural, biochemical and immunohistochemical measurements done at leaf level were taken in healthy, full expanded and mature leaves. The leaf gas exchange, chlorophyll *a* fluorescence and leaf relative water content measurements were taken periodically during the two years of the study (n=9), while the samples for leaf histological analysis (n=9), leaf and stem biochemical analyses (n=9) and leaf IAA and ABA immunolocalization (n=3) were collected only in 2016, at the peak of stress, 22$^{th}$ August. To determine the nutritional status of olive trees, a pool of leaf samples per plot was taken in July 2016, during summer, at endocarp sclerification, and in January 2017, during the winter resting period (n=3). A schematic representation of the experiment procedure is presented in Figure 1.

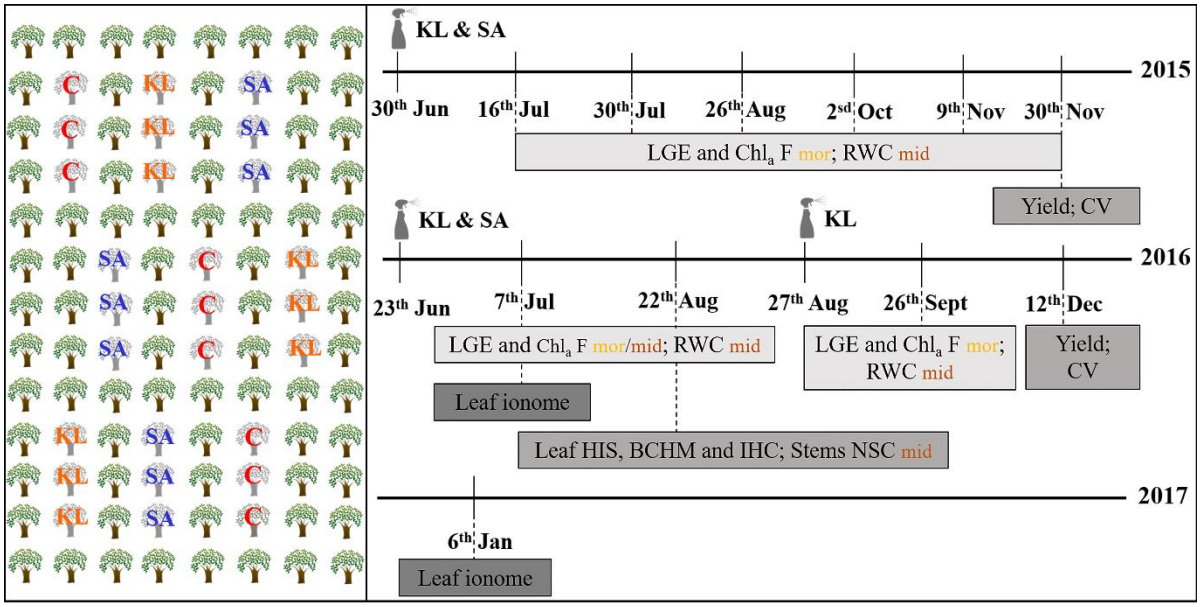


**Figure 1.** Schematization of field trial and monitoring analysis performed during 2015, 2016 and 2017. Abbreviations: KL – kaolin; SA – salicylic acid; C – control; LGE – leaf gas exchange; $Chl_a$ F – chlorophyll *a* fluorescence; RWC – relative water content; CV – canopy volume; HIS – histology; BCHM – biochemistry; IHC – immunohistochemistry; NSC – non-structural carbohydrates; mor – morning; mid -midday.

### 2.3. Leaf water status and structural analysis

Leaf samples, detached in a similar position, were immediately placed into air-tight containers and the following parameters were examined: fresh weigh (FW; g); fresh weigh at full turgor (TW; g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at 4 ºC; and dry weigh (DW; g), measured after drying in a force-draft oven at 60 ºC to a constant weight. Further, was calculated the relative water content (RWC = (FW – DW)/(TW – DW) x 100; %).

For histological analysis, leaf sections were taken from the middle of the leaves, to avoid differential thickness along the leaf. Cut sections were dehydrated, cleared and embedded in paraffin. Four µm cross-sections were obtained using a rotary microtome (Leica RM 2135, Germany) placed on slides and stained with toluidine blue. Leaf tissues thickness were measured in the leaf cross-sections using an inverted optical microscope (Olympus IX51 with the image analysis software Cell^A). To make stomatal impressions, one or two coats of polish (colodium) were applied to the abaxial surface of each leaf, after peltate hairs were removed. The polish was then carefully peeled off and placed on a microscope slide (Bacelar *et al*., 2004).

### 2.4. Leaf gas exchange and chlorophyll *a* fluorescence

Leaf gas exchange measurements were performed using a portable IRGA (LCpro+, ADC, Hoddesdon, UK), operating in the open mode. Measurements were performed on cloudless days

under natural irradiance and environmental conditions on sun exposed leaves. Net photosynthetic rate (A, μmol $CO_2$ $m^{-2}$ $s^{-1}$), stomatal conductance ($g_s$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) and the ratio of intercellular to atmospheric $CO_2$ concentration ($C_i/C_a$) were estimated using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency was calculated as the ratio of $A/g_s$ (μmol $mol^{-1}$).

Chlorophyll *a* fluorescence parameters were measured *in vivo* with a pulse-amplitude-modulated fluorometer (FMS 2, Hansatech Instruments, Norfolk, UK) on the same leaves and environmental conditions used for gas exchange measurements. Prior to the measurements, a small part of the leaves was dark-adapted for 30 min using dark-adapting leaf-clips. After this period, the minimal fluorescence ($F_0$) was measured when all photosystem II (PSII) reaction centers are open using a low intensity pulsed measuring light source. The maximal fluorescence ($F_m$) was measured when all PSII reactions centers are closed during a pulse saturating light (0.7 s pulse of 15000 μmol photons $m^{-2}$ $s^{-1}$ of white light). The difference between these two levels ($F_m$-$F_0$) is called variable fluorescence ($F_v$). Maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m-F_0)/F_m$ (Krause and Weis, 1991). Following $F_v/F_m$ estimation, after a 20 s exposure to actinic light (1500 μmol $m^{-2}s^{-1}$), light-adapted steady-state fluorescence yield ($F_s$) was averaged over 2.5 s, followed by exposure to saturating light (15000 μmol $m^{-2}s^{-1}$) for 0.7 s to establish $F'_m$. The sample was then shaded for 5 s with a far-red light source to determine $F'_o$. From these measurements, several fluorescence attributes were calculated (Bilger, 1986; Genty, 1989): photochemical quenching ($qP= (F'_m-F_s)/(F'_m-F'_o)$), non-photochemical quenching ($NPQ= (F_m-F'_m)/F'_m$) and efficiency of electron transport as a measure of the quantum effective efficiency of PSII ($\Phi PSII = \Delta F/F'_m = (F'_m-F_s)/F'_m$). The apparent electron transport rate was estimated as ETR (μmol $e^-$ $m^{-2}$ $s^{-1}$) = $(\Delta F/F'_m)$ x PPFD x 0.5 x 0.84, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is the factor that assumes equal distribution of energy between the two photosystems, and the leaf absorbance used was 0.84, the most common value for $C_3$ plants (Bilger, 1986).

### 2.5. Biochemical assays

Chlorophylls and carotenoids were extracted with 80% (v/v) acetone. Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$) and total chlorophyll ($Chl_{(a+b)}$) were determined according to Arnon (1949) and Sesták (1971) and total carotenoids (Car) according to Lichtenthaler (1987). Lycopene and β-carotene were extracted with acetone–hexane mixture (4:6) and determined according to Barros *et al*. (2011). Total soluble proteins (TSP) were quantified using the method of Bradford

(1976), using bovine serum albumin (Sigma-Aldrich, St. Louis, USA) as a standard. Total phenolic compounds (TPC) were quantified following the Folin–Ciocalteu procedure (Singleton and Rossi 1965), using gallic acid (Sigma-Aldrich, St. Louis, USA) as a standard. Flavonoids were determined according to Zhishen *et al*. (1999), using (+)-catechin (Sigma-Aldrich, St. Louis, USA**)** as a standard. Ascorbate was quantified using a method adapted from Klein and Perry (1982), using L-ascorbic acid (Fisher Chemical, UK) as a standard. Total antioxidant capacity (TAC) based on DPPH (2,2-Diphenyl-1-picrylhydrazyl)-free radical scavenging capacity was evaluated according to a method adapted from Xu and Chang (2007). Leaf methanolic extracts, and methanol for negative control, were mixed with DPPH methanolic solution (0.1 mM) and left to stand for 30 min in dark at room temperature. The absorbance for the sample ($A_{sample}$) and negative control ($A_{control}$) was measured at 517 nm against methanol blank. The percent of DPPH radical reduction was calculated as follows = 100 x ($A_{control} - A_{sample}$) / $A_{control}$. The free radical scavenging activity was expressed as µM of Trolox equivalents (Sigma-Aldrich, St. Louis, USA), TE = (% DPPH radical reduction / *a*), where a is the slope of the standard curve (y = ax). Total soluble sugars (SS) were extracted according to Irigoyen *et al*. (1992), by heating the samples in 80% ethanol during 1 h, at 80 ºC. Then, the soluble fractions were separated from the solid fraction. Starch (St) was extracted by heating the same solid fraction in 30% perchloric acid during 1 h, at 60 ºC according, to Osaki *et al*. (1991). Both SS and St concentrations were determined by the anthrone method, using glucose (Merck, Germany) as a standard.

### 2.6. Immunodetection of ABA and IAA

For the immunodetection of indole-3-acetic acid (IAA) and abscisic acid (ABA), mature leaves of each treatment were fixed and processed according with Escandón *et al*. (2016). Propidium iodide was used as counterstain. Fluorescence, in both immunochemical essays, was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation and the images were processed with Fiji Software (Schindelin *et al*. 2012). Negative controls in both immunochemical essays were obtained replacing the primary antibody by PBS (See supplementary Figure 2).

### 2.7. Leaf mineral analyses

Leaves were collected from the middle of current season shoots of the four quadrants around the tree canopy. The samples were then oven-dried at 70 ºC and ground. Tissue analyses

were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn, and Mn) methods (Walinga *et al*. 1989).

### 2.8. Statistical analysis

The statistical analysis was performed using the statistical software program SPSS for Windows (v. 22). All data sets satisfied the assumptions of ANOVA based on homogeneity of variances and normality. In all parameters, data were analyzed one-way factorial ANOVA and the post hoc Tukey's test. Significant differences were considered for $P < 0.05$. For statistical analysis of RWC and CVI arcsine transformation was performed in percentage data.

## 3. Results

### 3.1. Leaf water status and leaf structure

The influence of KL and SA on RWC was dependent on the seasonal period of the experiment and the analyzed year (Figure 2a, b). In the first year (Figure 2a), KL contributed to increase RWC in the first two analyzed dates, losing this capacity at the end of summer, while SA stands out in the middle of the summer season. On the second year (Figure 2b), in August, both products contributed to increase RWC.

Leaf histological analysis revealed that KL induced thinner leaves, due to the reduced thickness of upper palisade parenquyma (UPP), lower epidermis (LE) and trichome layer (TL) (Table 1). As a result, a reduced palisade/spongy parenchyma (PP/SP) ratio was observed in KL leaves. Regarding SA plants, the leaves presented lower TL thickness than C plants. Both KL and SA contributed to increase the stomatal density (Table 1).

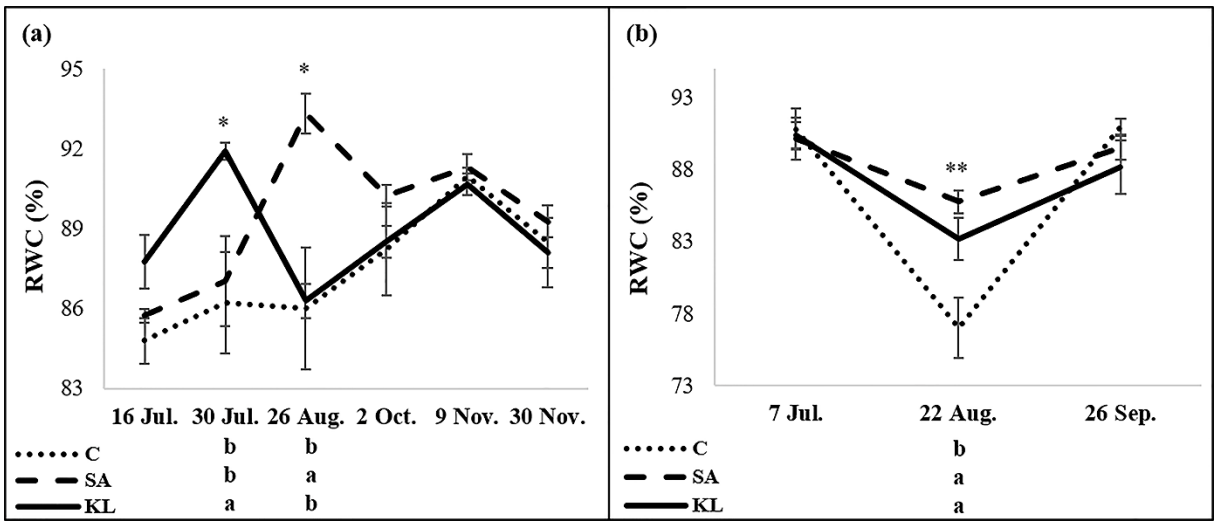


**Figure 2.** Evolution of leaf relative water content (RWC) in control (C), salicylic acid (SA) and kaolin (KL) plants throughout the experiment in 2015 (a) and 2016 (b). Values are means±SE. Different letters demonstrate significant differences between treatments in each analyzed date (*P < 0.05, **P < 0.01).

**Table 1.** Leaf tissues thickness (µm) and stomatal density (stomatal number $mm^{-2}$) of control (C), salicylic acid (SA) and kaolin (KL) plants. Total section (LT), upper cuticle (UC), upper epidermis (UE), upper palisade parenchyma (UPP), spongy parenchyma (SP), lower palisade parenchyma (LPP), lower epidermis (LE), palisade/ spongy parenchyma ratio (PP/SP) and trichome layer (TL).

| | C | SA | KL | P-value |
|---|---|---|---|---|
| **LT** | 548.8±3.9$^{a}$ | 543.8±4.9$^{a}$ | 501.8±8.7$^{b}$ | *** |
| **UC** | 5.73±0.17 | 6.41±0.33 | 5.90±0.18 | n.s. |
| **UE** | 18.00±0.61$^{ab}$ | 17.11±0.52$^{b}$ | 19.29±0.53$^{a}$ | * |
| **UPP** | 224.5±5.0$^{a}$ | 231.4±3.4$^{a}$ | 193.8±5.3$^{b}$ | *** |
| **SP** | 220.1±4.8 | 218.2±4.8 | 214.5±4.2 | n.s. |
| **LPP** | 29.51±0.85 | 27.67±0.68 | 27.20±0.65 | n.s. |
| **LE** | 15.53±0.36$^{a}$ | 15.01±0.45$^{ab}$ | 13.96±0.36$^{b}$ | * |
| **PP/SP** | 1.17±0.04$^{a}$ | 1.20±0.03$^{a}$ | 1.03±0.02$^{b}$ | ** |
| **TL** | 36.65±1.43$^{a}$ | 28.40±1.92$^{b}$ | 26.51±1.11$^{b}$ | *** |
| **Stomatal density** | 565.5±13.9$^{b}$ | 744.6±27.4$^{a}$ | 683.7±16.6$^{a}$ | *** |

Values are means±SE. Different letters within a line demonstrate significant differences between treatments (n.s., not significant, *P < 0.05, **P < 0.01, ***P<0.001).

### 3.2. Leaf gas exchange and chlorophyll *a* fluorescence

In general, both KL and SA foliar sprays contributed to keep higher A and $g_s$ in relation to C plants (Figure 3). In 2015, an exception was observed in the first sampling date, as KL plants exhibited the lower A, while SA plants already exhibited higher A than C plants. Stomatal conductance followed a similar pattern than A, although no statistical differences were found among treatments on July 30$^{th}$ and November 30$^{th}$ (Figure 3b). The $A/g_s$ was not statistically affected by treatments (Figure 3c), while regarding $C_i/C_a$, it was only observed a reduction with KL application on July 30$^{th}$ (Figure 3d). In 2016, it was evident the typical midday depression of A and $g_s$ in all treatments, particularly in August (Figure 3e, f). Concerning the treatment effect, on July 7$^{th}$ and September 26$^{th}$, both KL and SA plants presented higher A than C plants, whereas on August 22$^{th}$ only SA trees had superior A at midday (Figure 3e). Stomatal conductance follows generally a pattern like A (Figure 3f). $A/g_s$ was significantly affected by treatments only on July 7$^{th}$. At morning, KL plants exhibited the lower $A/g_s$, while at midday period SA plants presented higher values than the other treatments (Figure 3g). In the same way, $C_i/C_a$ ratio was only affected by the treatments on July 7$^{th}$. At morning, KL plants exhibited the highest ratios, while at midday the lower ratio was observed in SA plants (Figure 3h).

Regarding chlorophyll *a* fluorescence analysis, in 2015, only on 30$^{th}$ July was observed a significant influence of the applied products. At that date, SA leaves exhibited higher ΦPSII and ETR and lower NPQ than C plants (Figure 4), whereas both KL and SA-sprayed leaves showed higher qP (Figure 4g). Furthermore, in general, all determined variables decreased progressively until October and, drastically in November (Figure 4). In 2016, at midday period of July 7$^{th}$, KL plants had higher $F_v/F_m$, ΦPSII, $F'_v/F'_m$ and ETR and lower NPQ than the other treatments (Figure 4), whereas on August 22$^{th}$, SA presented higher ΦPSII and ETR (Figure 4e, k). Finally, on September 26$^{th}$, KL leaves showed higher $F_v/F_m$ (Figure 4g).

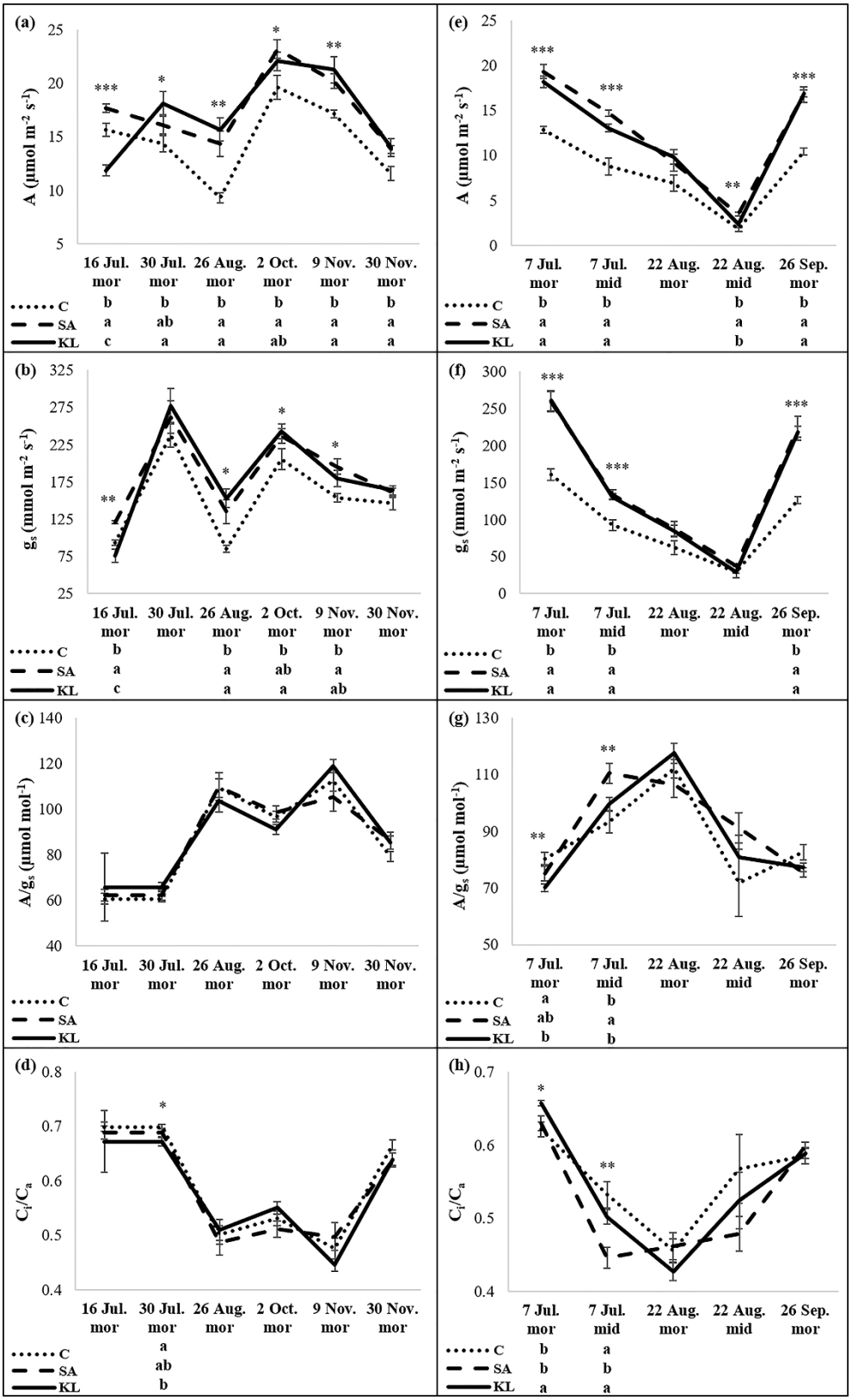


**Figure 3.** Evolution of leaf gas exchange parameters in control (C), salicylic acid (SA) and kaolin (KL) plants throughout the experiment during the morning (mor) period of 2015 (A-D) and both morning (mor) and midday (mid) periods of 2016 (E-H). Net photosynthetic rate (A, a, e), stomatal conductance ($g_s$, b, f), intrinsic water use efficiency ($A/g_s$, c, g) and ratio of intercellular to atmospheric $CO_2$ concentration ($C_i/C_a$, d, h). Values are means±SE. Different letters demonstrate significant differences between treatments in each analyzed date (*P < 0.05, **P < 0.01, ***P<0.001).

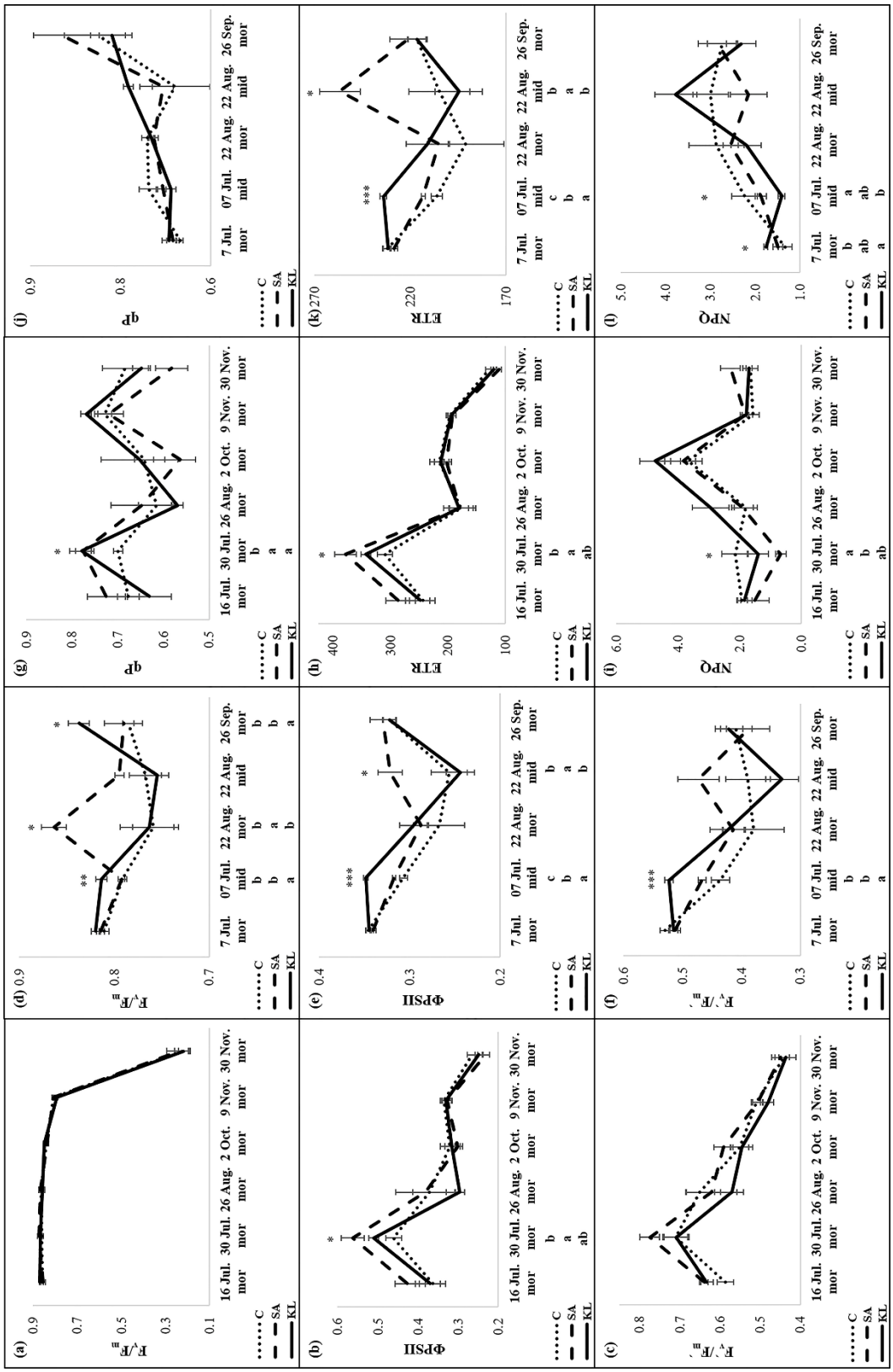


**Figure 4.** Evolution of chlorophyll fluorescence variables in control (C), salicylic acid (SA) and kaolin (KL) plants throughout the experiment during the morning (mor) period of 2015 (A-C and G-I) and both morning (mor) and midday (mid) periods of 2016 (D-F and J-L). Maximum ($F_v/F_m$, a, d) and effective (ΦPSII, b, e) quantum efficiency of PSII, capture efficiency of excitation energy by open PSII reaction centers ($F'_v/F'_m$, c, f), photochemical quenching (qP, g, j), electron transport rate (ETR, μmol $e^-$ $m^{-2}$ $s^{-1}$, h, k) and non-photochemical quenching (NPQ, i, l). Values are means±SE. Different letters demonstrate significant differences between treatments in each analyzed date (*P < 0.05, **P < 0.01, ***P<0.001).

### 3.3. Biochemical components

Leaf biochemical analyses are summarized in Table 2. None of the applied products induced changes on $Chl_{(a+b)}$ and Car concentrations, neither on $Chl_a/Chl_b$ and $Chl_{(a+b)}$/Car ratios, on flavonoids and TPC, although a tendency for higher concentration of TPC in C plants. KL and SA lead to higher levels of lycopene and β-carotene, whereas TSP were enhanced only by SA. The ascorbate concentration was higher in C and SA than in KL plants. Regarding carbohydrates concentration, SS and St values followed the order KL<C and SA, and SA<KL<C, respectively. The TAC based on DPPH radical scavenging was reduced in both KL and SA treatments. The accumulation of SS and St in stems followed the order C≤SA≤KL and C<SA<KL, respectively.

**Table 2.** Leaf and stem biochemical analyses of control (C), salicylic acid (SA) and kaolin (KL) plants. In leaves: total chlorophylls ($Chl_{(a+b)}$, mg $g^{-1}$ DW), chlorophyll a/b ratio ($Chl_a/Chl_b$), total carotenoids (Car, mg $g^{-1}$ DW), $Chl_{(a+b)}$/Car ratio, lycopene (mg $g^{-1}$ DW), β-carotene (mg $g^{-1}$ DW), total soluble proteins (TSP, mg $g^{-1}$ DW), total phenolic compounds (TPC, mg $g^{-1}$ DW), flavonoids (mg $g^{-1}$ DW), ascorbate (mg $g^{-1}$ DW), total antioxidant activity (TAC, µmol $g^{-1}$ DW), soluble sugars ($SS_{leaf}$, mg $g^{-1}$ DW) and starch ($St_{leaf}$, mg $g^{-1}$ DW) concentrations. In stems: soluble sugars ($SS_{Stems}$, mg $g^{-1}$ DW) and starch ($St_{Stems}$, mg $g^{-1}$ DW) concentrations.

| | C | SA | KL | P-value |
|---|---|---|---|---|
| **$Chl_{(a+b)}$** | 2.80±0.05 | 2.79±0.10 | 2.65±0.05 | n.s. |
| **$Chl_a/Chl_b$** | 3.05±0.03 | 3.13±0.02 | 3.13±0.04 | n.s. |
| **Car** | 0.605±0.008 | 0.609±0.015 | 0.579±0.008 | n.s. |
| **$Chl_{(a+b)}$/Car** | 4.63±0.036 | 4.57±0.057 | 4.58±0.045 | n.s. |
| **Licopene** | 0.294±0.011$^{b}$ | 0.352±0.008$^{a}$ | 0.337±0.005$^{a}$ | ** |
| **β-Carotene** | 0.151±0.007$^{b}$ | 0.179±0.006$^{a}$ | 0.173±0.003$^{a}$ | * |
| **TSP** | 5.39±0.40$^{b}$ | 8.18±0.21$^{a}$ | 5.94±0.10$^{b}$ | *** |
| **TPC** | 46.05±1.24 | 43.24±0.59 | 42.21±1.36 | n.s. |
| **Flavonoids** | 24.22±0.46 | 24.04±1.09 | 24.20±0.23 | n.s. |
| **Ascorbate** | 0.885±0.022$^{a}$ | 0.920±0.027$^{a}$ | 0.537±0.043$^{b}$ | *** |
| **TAC** | 133.9±1.2$^{a}$ | 126.5±0.7$^{b}$ | 128.6±0.9$^{b}$ | *** |
| **$SS_{leaf}$** | 134.0±4.6$^{a}$ | 121.3±2.9$^{a}$ | 94.3±4.9$^{b}$ | *** |
| **$St_{leaf}$** | 67.20±3.20$^{a}$ | 49.14±1.96$^{c}$ | 58.05±3.10$^{b}$ | ** |
| **$SS_{Stems}$** | 63.37±2.00$^{b}$ | 66.80±1.31$^{ab}$ | 71.02±2.62$^{a}$ | * |
| **$St_{Stems}$** | 40.80±2.00$^{c}$ | 59.32±1.31$^{b}$ | 71.26±2.81$^{a}$ | *** |

Values are means±SE. Different letters within a line demonstrate significant differences between treatments (n.s., not significant, *P < 0.05, **P < 0.01, ***P<0.001).

### 3.4. Immunodetection of ABA and IAA

The immunodetection of ABA and IAA revealed that both KL and SA induced differences in the signal intensity and distribution through the leaf tissues (Figures 5and 6). In general, ABA signal showed a uniform distribution throughout the leaf in all treatments. However, the intensity was higher in C and SA than in KL leaves (Figure 5a, b, c). SA leaves showed an increased signal intensity in the main vascular tissues (Figure 5b), compared to C (Figure 5a). The IAA signal was substantially less evident than the ABA signal in all the analyzed leaves (Figure 5d, e, f). In C leaves, it was possible to observe a uniform distribution of IAA signal across the leaf limb and an almost absence of signal in the main vascular tissues, especially in xylem (Figure 5d). Both KL and SA leaves showed an increase in signal intensity in the main vascular tissues, especially in phloem (Figure 5e, f), and KL plants also exhibited an increase in signal intensity in the UPP (Figure 5f).

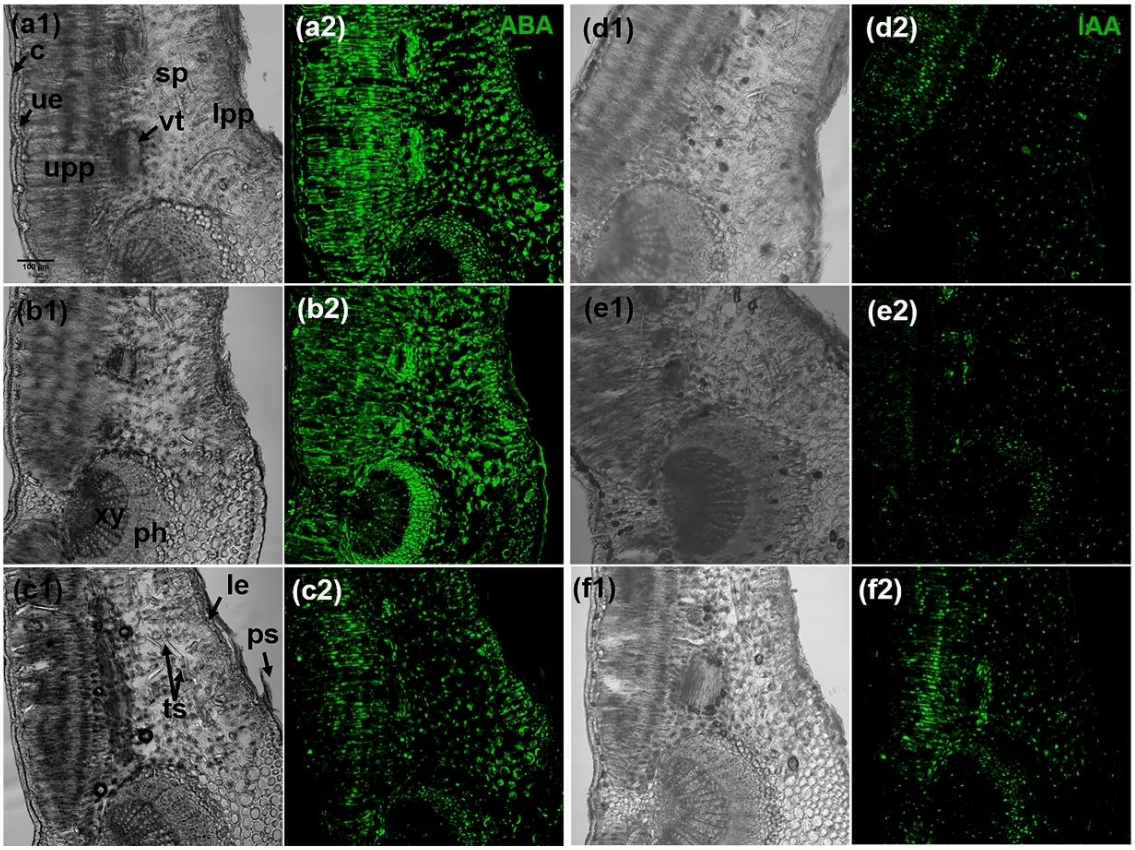


**Figure 5.** Immunolocalization of ABA (a, b, c) and IAA (d, e, f) in sections of olive leaves using confocal microscope (20×). Differential interference contrast (a1, b1, c1, d1, e1, f1) and ABA (a2, b2, c2) and IAA (d2, e2, f2) signal. Control (C) plants (a, d), salicylic acid (SA) plants (b, e) and kaolin (KL) plants (c, f). Abbreviations: c=cuticle; ue=upper epidermis upp=upper palisade parenchyma; sp=spongy parenchyma; lpp=lower palisade parenchyma; xy=xylem; ph=phloem; vt=vascular tissue; ts=trichosclereids; le=lower epidermis; ps=peltate scales. A negative control was performed (bars = 100 μm).

### 3.5. Leaf mineral analyses

The indicators of tree nutrient status are presented in Table 3. A great part of the evaluated minerals, namely N, P, K, B, Cu, Fe and Zn, were found in higher concentration in July than in January. At the same time, the applied products induced changes in the amounts of some minerals. In summer, N concentration followed the order KL<SA=C, P the order KL=C<SA, K the order KL≤C≤SA, Mg the order C=KL<SA and Cu the order KL≤SA≤C. Meanwhile, in the winter, K concentration followed the order SA<KL=C and Cu the order C<SA<KL.

**Table 3.** Leaf macronutrients (N, P, K, Ca, Mg, g $kg^{-1}$ DW) and micronutrients (B, Cu, Fe, Zn, Mn, mg $kg^{-1}$ DW) of control (C), salicylic acid (SA) and kaolin (KL) plants in summer 2016 and winter 2017.

| | | C | SA | KL | P-value |
|---|---|---|---|---|---|
| Summer | N | 22.53±0.43$^{a}$ | 21.93±0.03$^{a}$ | 20.80±0.06$^{b}$ | ** |
| | P | 1.27±0.14$^{b}$ | 1.85±0.09$^{a}$ | 1.23±0.03$^{b}$ | ** |
| | K | 13.30±2.2$^{ab}$ | 16.33±1.34$^{a}$ | 8.60±0.40$^{b}$ | * |
| | Ca | 5.93±0.45 | 7.12±0.48 | 7.01±0.22 | n.s. |
| | Mg | 0.99±0.06$^{b}$ | 1.59±0.01$^{a}$ | 1.08±0.01$^{b}$ | *** |
| | B | 27.38±0.61 | 25.92±0.32 | 25.34±1.46 | n.s. |
| | Cu | 10.56±0.41$^{a}$ | 8.60±0.42$^{ab}$ | 7.67±0.65$^{b}$ | * |
| | Fe | 111.62±18.7 | 93.29±1.16 | 86.24±7.52 | n.s. |
| | Zn | 36.25±1.59 | 35.97±1.46 | 38.44±8.41 | n.s. |
| | Mn | 65.08±1.19 | 56.81±5.04 | 67.56±3.32 | n.s. |
| Winter | N | 18.50±0.35 | 19.90±0.91 | 19.83±0.59 | n.s. |
| | P | 0.95±0.06 | 0.86±0.04 | 0.98±0.02 | n.s. |
| | K | 5.650.15$^{a}$ | 4.44±0.15$^{b}$ | 5.49±0.27$^{a}$ | ** |
| | Ca | 7.61±0.08 | 9.99±1.47 | 9.09±0.61 | n.s. |
| | Mg | 1.03±0.04 | 1.26±0.18 | 1.12±0.05 | n.s. |
| | B | 13.01±0.53 | 13.11±0.55 | 12.40±0.42 | n.s. |
| | Cu | 4.08±0.01$^{c}$ | 4.12±0.01$^{b}$ | 4.17±0.01$^{a}$ | ** |
| | Fe | 85.22±7.03 | 83.49±7.98 | 62.13±2.50 | n.s. |
| | Zn | 23.04±1.88 | 21.90±1.32 | 18.92±0.28 | n.s. |
| | Mn | 54.53±4.72 | 59.23±5.32 | 54.47±3.59 | n.s. |

Values are means±SE. Different letters within a line demonstrate significant differences between treatments (n.s., not significant, *P < 0.05, **P < 0.01, ***P<0.001).

## 4. Discussion

### 4.1. KL and SA modulate leaf water status and structure

The general improvement of leaf water status during the most stressful periods was corroborated by other studies with KL (Denaxa *et al*., 2012; Nanos, 2015) and SA (Kang *et al*. 2012; Nazar *et al*., 2015). The lower thickness of KL leaves, associated with the lower PP/SP ratio, indicate a less compact arrangement of mesophyll cells (Bacelar *et al*., 2004), reflecting a reduced necessity to restrict water loss. Similar KL-induced leaf structural shade adaptations were described previously (Nanos, 2015; Segura-Monroy *et al*., 2015). The reduced TL in both KL and SA leaves also revealed a reduced necessity of protection, since trichomes are more abundant in leaves subjected to severe drought and high irradiance conditions (Savé *et al*., 2000; Bacelar *et al*., 2009).

The higher stomatal density in KL and SA leaves verified in the present study was also reported in response to KL in drought-stressed plants (Segura-Monroy *et al*., 2015) and in response to SA in salt-stressed plants (Ma *et al*., 2017). This response is of great ecophysiological relevance because higher stomatal densities improve stomatal regulation capacity, increasing the ability to balance water loss with photosynthetic performance (Casson and Gray, 2008).

### 4.2. KL and SA application boosts photosynthetic activity

The general positive influence of KL and SA on A and $g_s$ comes in agreement with previous studies in stressed plants treated with KL (Denaxa *et al*., 2012; Nanos 2015) and SA (Wang *et al*., 2014; Nazar *et al*., 2015). Regarding KL, in 2015, the lowest A and $g_s$ recorded in recently sprayed plants, followed by a larger recovery, demonstrated that leaves need a period of acclimation to benefit from KL application. Moreover, it is possible to infer that the period depends on the environmental conditions, being shorter when they are severe, as in 2016. This happens because KL leaves reflect a significant part of the incident radiation, as demonstrated by Nanos (2015). In 2015, the influence of KL on A was mainly due to $g_s$ stimulation, but also to lower non-stomatal limitations, not related with photochemistry processes, judging by the relative variation of $g_s$, $A/g_s$, $C_i/C_a$ and chlorophyll fluorescence variables. By reducing the heat load, this may reduce the leaf-to-air vapor pressure deficit ($VPDl_{eaf-air}$) (Jifon and Syvertsen, 2003; Rosati *et al*., 2006), and consequently the driving force for transpiration, promoting an increase of $g_s$ (Zhang *et al*., 2017). In 2016, the lower A of C plants on July 7$^{th}$ was due to stomatal, but mainly to non-stomatal limitations considering the higher $C_i/C_a$ ratio recorded at

midday period, in spite of the reduced $g_s$. In terms of photochemical processes, the higher $F_v/F_m$ and ETR of KL leaves indicate lower photoinhibitory damage (Maxwell and Johnson, 2000) and greater electron transport rate through PSII. Besides, although KL did not interfere with the proportion of open PSII reaction centers (e.g. similar qP), the higher ΦPSII values indicate that the open PSII reaction centers captured the light absorbed by PSII antenna more efficiently (Baker, 2008). This response was certainly due to a reduced loss of excitation energy by thermal dissipation, which could compete with its transfer to PSII reaction centers, as evidenced by the higher and lower values of $F'_v/F'_m$ and NPQ, respectively (Baker, 2008). The positive effects of kaolin on the preservation of the photochemical processes was already described in other species (Jifon and Syvertsen, 2003; Dinis *et al*., 2016) Nonetheless, in the severest drought period of 2016, KL plants lose the effectiveness to maintain higher $g_s$ and A values, being this change related with the worsening of summer environmental conditions from 2015 to 2016. In agreement, the higher efficiency of photochemical processes recorded on July 7$^{th}$ was also lost. Likewise, the loss of KL effectiveness in keeping $g_s$ and A with higher stress severity was documented previously (Shellie and Glenn, 2008; Nanos, 2015).

Regarding SA, the positive influence on A in 2015 was due to $g_s$ stimulation, but also to lower non-stomatal limitations, as previously pointed out to KL responses. In 2016, SA was the most effective product to decrease the midday depression of A, a typical response of Mediterranean species (Bacelar *et al*., 2007). At midday period of August 22$^{th}$, the absence of significant differences in $g_s$ and the higher A recorded in SA plants indicate reduced non-stomatal limitations in relation to KL and C plants, which include a better performance of photochemical endpoints (ETR, ΦPSII and $F'_v/F'_m$). The SA-induced protection of photosynthetic machinery under stress was also reported in other studies (Wang *et al*., 2014; Nazar *et al*., 2015).

Interestingly, the protection conferred by KL and SA during the summer period might allowed a faster recovery of $g_s$ and A at the end of the summer and a better response in the autumn cold days of 2015.

### 4.3. KL and SA influence positively the foliar metabolites fluctuations

In contradiction to our results, it is recurrent to find that both KL (Nanos, 2015; Segura-Monroy *et al*., 2015) and SA (Fayez and Bazaid, 2014; Wang *et al*., 2014) prevent chlorophylls degradation under stressful conditions. By other side, although the total carotenoids concentrations were not affected by the applied products, the relative composition was changed.

The higher amounts of lycopene and β-carotene in sprayed trees may be an added value to those plants, as lycopene is the starting compound of various end group modifications that produces a large variety of carotenoids, such as β-carotene which display the ability to quench triplet chlorophyll and singlet oxygen (Domonkos *et al*., 2013). Meanwhile, apart from preventing TSP degradation, SA might increase its synthesis. In agreement, Kang *et al*. (2012) reported that SA application in droughted plants induces the expression of several proteins and Jalal *et al*. (2012) reported that SA alleviates the negative effect of drought on proteins concentrations, increasing its values and changing its patterns. Thus, the higher concentration of TSP might support the improvement of photosynthetic performance in SA plants in the severest drought period of 2016.

The tendency to higher accumulation of TPC in C leaves is somehow reflected in the higher DPPH radical scavenging activity, since phenolic compounds are known to overcome other antioxidants in the scavenging of this radical (Xu and Chang, 2007; Mattos and Moretti, 2015). Such TPC response is common in stresses olive leaves (Bacelar *et al*., 2007; Petridis *et al*., 2012), and a similar reaction was found in drought stressed barley plants sprayed with SA (Fayez and Bazaid, 2014). On the other hand, the reduced accumulation of ascorbate in KL leaves suggests a reduced necessity of those plants to invest in secondary metabolism, while the high value in SA plants may be associated with its proposed action mode (Khan *et al*., 2015). Besides to directly scavenge ROS, it is also a substrate to ascorbate peroxidase that use it as specific electron donor to reduce $H_2O_2$ to $H_2O$ (Mattos and Moretti, 2015).

The higher accumulation of SS in C and SA leaves might be considered a protective mechanism to maintain cell homeostasis, a mechanism typically observed in droughted olive trees (Bacelar *et al*., 2006; Boussadia *et al*., 2013) and induced by SA application (El-Tayeb, 2005; Kang *et al*. 2012). On the other hand, the lower SS accumulation in KL leaves indicates that newly assimilated carbon was exported. In addition, the St depletion in both KL and SA plants also indicates the use of carbohydrates reserves, since St is an important storage carbohydrate that are usually mobilized in the form of SS (Rosa *et al*., 2009). As treated plants presented, in general, a better water status, we assume the preferential use of these carbon sources to growth and fruit development, instead of secondary metabolism investment. In fact, summer is a season of maximum carbohydrate demand for fruit growth and oil production (Bustan *et al*., 2011), and KL and SA plants exhibited the higher yields, averaging 97% and 72%, respectively (Brito *et al*., 2018). Moreover, KL plants exhibited the higher canopy volume increase between November 2015 and December 2016 (83.0% in KL plants against 59.5% in

C plants). Meanwhile, as SA might induce the development of antioxidant responses (Khan *et al*., 2015), the higher St depletion in SA than in KL plants may be related to the activation of antioxidant defense mechanisms, as the increase in TSP and ascorbate.

During autumn and winter, when carbon sink demands are small, St and SS tends to accumulate (Bustan *et al*., 2011). Thus, the lower SS and mainly St accumulation in C stems, at the end of the summer, demonstrates that these plants had a reduced capacity to allocate reserves and/or transport carbohydrates, in a strictly association with the lower photosynthetic activity. In addition, the lower St concentration in C stems could also be explained by the conversion of St to sugars and the transportation of sugars for regrowth, fruit development and extra repair damages. Consequently, the higher carbohydrates accumulation in KL and SA stems can have a profoundly positive effect on trees performance in the following year.

### 4.4. KL and SA induce changes in ABA and IAA dynamics

Plant hormones signaling, particularly IAA and ABA is crucial for regulating plants adaptation capacity to different environment conditions (Peleg and Blumwald, 2011). The reduction of ABA signal in KL leaves, reflects the better water status and higher $g_s$ of those plants. The higher ABA signal intensity in the main vascular tissues of SA leaves suggests its transport, highlighting SA signaling in ABA accumulation (Shakirova *et al*., 2003; Jesus *et al*., 2015). Furthermore, the intense signal in phloem may be related to the ABA involvement in assimilates flow and distribution regulation (Peng *et al*., 2003).

On the other hand, the reduced IAA signal detection, when compared with ABA signal, reflects the main function of this phytohormone as growth promoter (Wani *et al*., 2016). The higher signal detection in the main vascular tissues of KL and SA leaves suggests IAA transport. Indeed, some studies have shown that drought stress influence local auxin concentration and distribution (Shen *et al*., 2010; Shojaie *et al*., 2015), allowing to maintain a balance between vegetative growth and survival (Shojaie *et al*., 2015). The higher signal detected in KL leaves, especially in UPP, might be a response to the reduced irradiation incidence due to kaolin particle film. In fact, shaded cotyledons and leaves had higher IAA synthesis (Zheng *et al*., 2016), and in response to unilateral light IAA moves to the shaded site of shoots (Fankhauser and Christie, 2015). On the other hand, it has been reported that SA application prevents drought and salinity induced IAA degradation (Sakhabutdinova *et al*., 2003; Shakirova *et al*., 2003; Fahad and Bano, 2012), justifying the higher IAA signal detection in SA than in C leaves. Moreover, this higher detection can also be associated with IAA function as stress signaling hormone in an early stage

of stress (Jain and Khurana, 2009; Sharma *et al*., 2015). In fact, it was reported a markedly decrease of IAA with a transient increase during the initial stage of drought (Wang *et al*., 2008) and heat stress (Escandón *et al*., 2016).

### 4.5. KL and SA modulate leaf minerals

Despite the higher $g_s$ exhibited during summer, no positive effect of KL was recorded in leaf mineral status. In this case, a possible dilution effect may take place, since those plants exhibited the higher increase in canopy volume. Moreover, higher $g_s$ does not necessarily means an increase in water loss, since the expected reduction in $VPDl_{eaf\text{-}air}$ with KL application (Jifon and Syvertsen, 2003; Rosati *et al*., 2006) may decrease the driving force for water movement (Zhang *et al*., 2017). As leaf K concentration in July was lower in KL than in SA plants, K is involved in important biochemical and physiological processes, such as osmoregulation (Hu and Schmidhalter, 2005), and as KL plants had lower SS accumulation, it is possible to infer that KL plants had a reduced necessity to invest in osmotic adjustment.

The tendency of SA plants to have higher mineral concentrations in summer was corroborated by other studies with stressed plants (El-Tayeb, 2005; Yildirim *et al*., 2008; Nazar *et al*., 2015), what could be, in part, promoted by the water movement associated with higher $g_s$. However, the observed significant differences among some elements indicate that plant minerals responses are complex and might be related to changes in specific nutrient metabolic processes. Due to the higher yields of both KL and SA plants (Brito *et al*., 2018), it was expected higher translocation of N from leaves to fruits, since olive fruits are an important sink for N in the initial phase of growth (Rodrigues *et al*., 2012). However, this was not verified in SA leaves, probably due to the higher TSP concentration, since N is an important constituent of all amino acids and proteins. In winter, after the harvest period, the influence of the applied products was reduced, exhibiting SA leaves the lower concentration of K. Since olive fruits are important sinks of K, may reaching 40% of total K (Rodrigues *et al*., 2012), higher amounts of K may have been exported to fruits.

## 5. Conclusions

The results of the present study revealed that KL and SA were effective in preventing the adverse effects of summer stress, contributing to better olive tree physiological performance. After the summer period, the attenuated negative effects induced by summer stress on KL and SA plants allowed a faster restauration of the physiological functions during the stress relief.

Nevertheless, the effectiveness of each product was associated with distinct protective actions. KL contributed to keep a better water status possibly due to a specific microclimate created around the leaves, reducing water losses by transpiration, while it keeps high stomatal conductance. These effects better contributed to increase the photosynthetic activity and the IAA immunodetected signal, to decrease ABA and to reduce the necessity to invest in leaf sclerophylly and secondary metabolism traits. Meanwhile, the protective action of SA was associated with the induction of some stress tolerance responses and the improvement in specific mineral status. Specifically, the maintenance of a better water status, stomatal conductance and photosynthetic machinery integrity, the increase in soluble proteins concentrations, in phytohormones immunodetected signal and in some non-enzymatic antioxidants contributed to alleviate summer stress.

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## Supplementary material

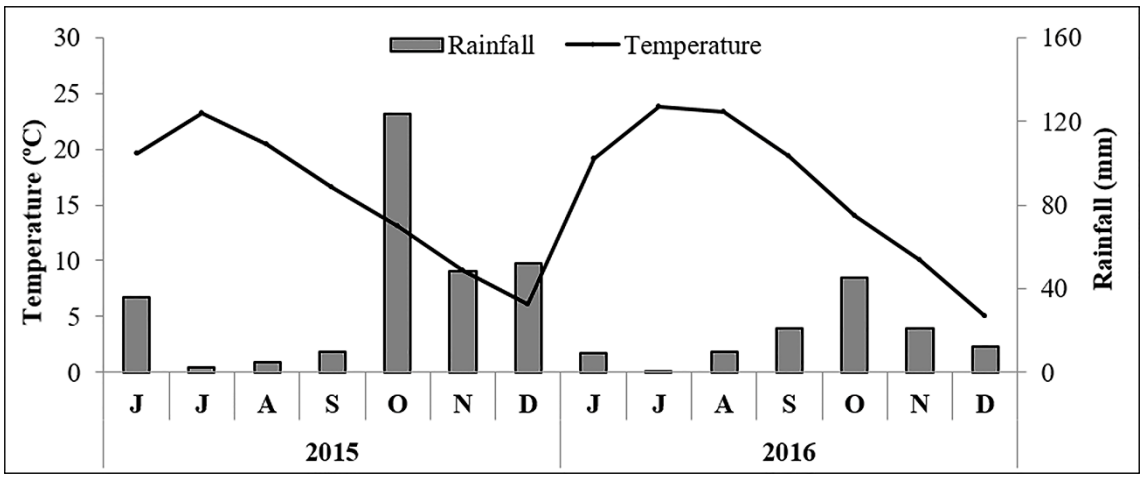


**Figure 1.** Climatic conditions (rainfall and average temperature) during the experimental period in 2015 and 2016. By order, the letters mean the months of the year monitored: June (J), July (J), August (A), September (S), October (O), November (N) and December (D).

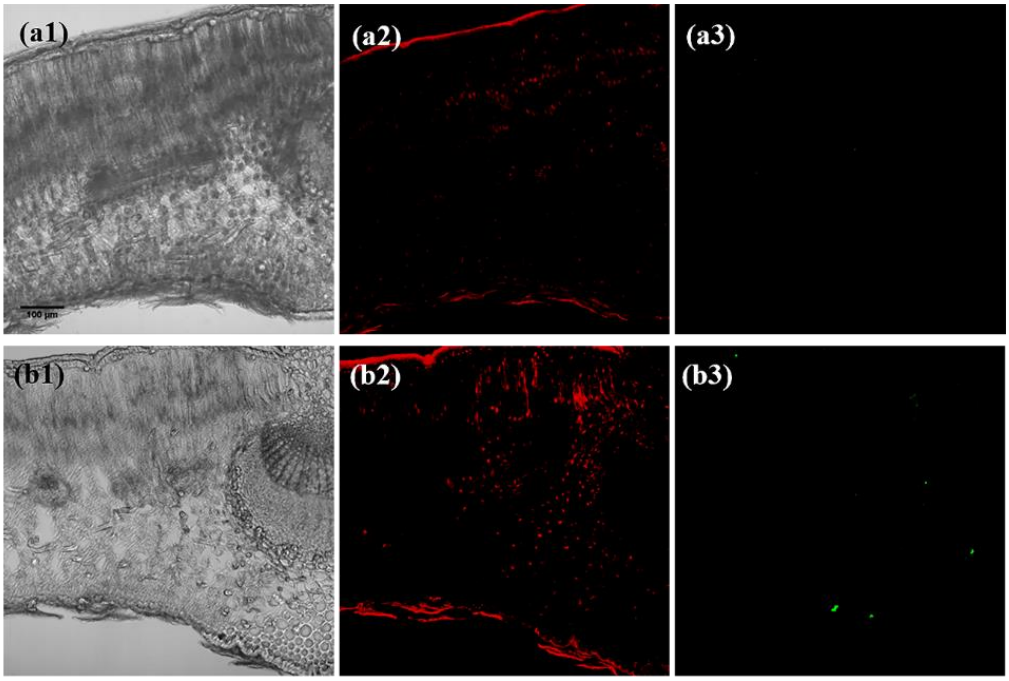


**Figure 2.** Negative controls of ABA (a) and IAA (b) analysis in sections of olive leaves using a confocal microscope (20×). Differential interference contrast (a1, b1), propidium iodide signal (a2, b2) and negative control of ABA (a2) and negative control of IAA (b2)

## 6.2. Kaolin and salicylic acid foliar application modulate yield, quality and phytochemical composition of olive pulp and oil from rainfed trees

### Kaolin and salicylic acid foliar application modulate yield, quality and phytochemical composition of olive pulp and oil from rainfed trees

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## Abstract

Olive orchards, rainfed managed, are threatened by the current and predicted adverse environmental conditions, which change the yield and quality of olive products, largely known for its benefits to human health. To mitigate these problems, it is highly recommended to perform some adjustments in agronomic practices, such as the use of foliar sprays that cloud help the trees to cope with climate change. During two consecutive years, olive trees were pre-harvest sprayed with kaolin (KL) and salicylic acid (SA) to attenuate the adverse effects of summer stress. Olive yield was increased by 97% and 72% with KL and SA, respectively. Phenolics and antioxidant capacity of both olives and olive oil increased and decreased in the first and second year, respectively, in a closely association with the prevailing climatic conditions. The foliar sprays did not significantly affect the oil quality indices, free acidity, peroxide value and K232 coefficient and decreased the K270 coefficient. This stud strongly suggests that the applied products might be effective in mitigating the adverse environmental conditions without substantial changes in fruit and olive oil quality.

**Keywords:** Olive tree, summer stress, frosts, phytochemicals, antioxidant capacity.

## 1. Introduction

Olive tree (*Olea europaea* L.) is one of the oldest and emblematic cash crops of the Mediterranean Basin, being cultivated mostly under rainfed production systems. Although the species can tolerate harsh conditions, the sector is under threat by the current adverse environmental circumstances and even more by the future scenarios of climate change (IPCC, 2013). Agricultural yield losses due to environmental stresses are well documented and many studies have shown that is crucial to increase the efforts in adapting measures to help the plants to cope with such adverse conditions (Wang and Frei, 2011). The limited water resources associated with the rugged topography of traditional olive growing areas hinders the implementation of irrigation systems, and/or make it economically unsustainable. Alternatively, the exogenous application of kaolin (KL) and salicylic acid (SA) can be adequate short-term solutions to attenuate the adverse effects of summer associated stresses. KL is a white mineral clay that avoid the accumulation of heat load through the reflection of sunlight, reducing the risk of leaf and fruit damage from high temperatures and solar injury (Glenn and Puterka, 2004) and SA is a signaling phytohormone with diverse regulatory roles in plant metabolism and abiotic stress tolerance (Khan *et al*., 2015). However, the influence of these substances on crop quality have received less attention than the influence on yield, possibly because they are more difficult to detect and sometimes are not consensual. The application of SA increased the yield of olive (Khalil *et al*., 2012; Abd El- Razek *et al*., 2013), peach (El-Shazly *et al*., 2013) and strawberry (Jamali *et al*., 2011; Kazemi, 2013) crops. In peach trees, the fruit quality was negatively affected, with lower soluble solids and anthocyanins accumulation and higher acidity (El-Shazly *et al*., 2013), whereas the quality of strawberry fruits was improved, with higher accumulation of total phenols, both flavonoids and non-flavonoids, soluble solids and vitamin C (Kazemi, 2013). With KL application, an increase in yield of olive (Saour and Makee, 2003), grapevine (Correia *et al*., 2014), mango (Chamchaiyaporn *et al*., 2013) and apple (Glenn *et al*., 2003) crops was reported. In olive trees, KL affected positively the olive oil quality and composition, in terms of total phenols, pigments, oil content, fatty acids, free acidity, peroxide value and ultraviolet (UV) absorption coefficients (Saour and Makee, 2003; Khaleghi *et al*., 2015). In grapevines, KL contributed to the increase of secondary metabolites in fruits, as total phenols, flavonoids, anthocyanins and vitamin C, as well as antioxidant capacity (Dinis *et al*., 2016).

The beneficial effects of olives and olive oil in health can be attributed to the antioxidant properties associated to the phenolic composition (Silva *et al*., 2006; Ghanbari *et al*., 2012;

Sousa *et al*., 2014a). Phenolic compounds in olives comprise 1‑3% of the fresh pulp weight, standing out the phenolic acids, phenolic alcohols, flavonoids, and the secoiridoids, which include the predominant phenolic compound found in fresh olive, oleuropein (Silva *et al*., 2006; Charoenprasert and Mitchell, 2012). In extra virgin olive oil, the concentration of phenols varies from 50 to 800 mg kg−1 oil (Charoenprasert and Mitchell, 2012). Reactive oxygen species (ROS), formed as a result of oxidative stress, are known to be responsible for the development of some diseases, targeting lipids, proteins and deoxyribonucleic acid (DNA) in living organisms (Ghanbari *et al*., 2012). Phenolic compounds in olives can restrict the deleterious effects of ROS, either by their free radical scavenging ability (by donating a hydrogen atom to the ROS, reducing and stabilizing it), or by chelating transition metals, suppressing the oxidative reaction in which them are involved (Charoenprasert and Mitchell, 2012). These phytochemicals also affect the sensorial and aromatic characteristics of both olive fruits and oil and the chemical stability of olive oil (Servili *et al*., 2009; Ghanbari *et al*., 2012). However, the composition and concentration of phenolic compounds is the result of a complex interaction of various pre-harvest factors, such as cultivars, environmental conditions, ripening stage and agronomic practices (Vinha *et al*., 2005; Gómez-Rico *et al*., 2006; Silva *et al*., 2006; Damak *et al*., 2008; Jemai *et al*., 2009; Ghanbari *et al*., 2012; Barros *et al*., 2013; Brahmi *et al*., 2013; Kazemi, 2013; Machado *et al*., 2013; Soufi *et al*., 2014; Sousa *et al*., 2014a, 2015; Sousa *et al*., 2014b; Talhaoui *et al*., 2015, 2016; Dinis *et al*., 2016).

We hypothesized that foliar sprays KL and SA might improve the olive trees yield without substantial changes in olives and olive oil quality. Hence, the aim of this work was to assess the influence of KL and SA in yield and in olive fruits and oil phenolics concentration and antioxidant capacity, as well in oil quality indices. The effects of harvest date and differences among years on those variables were also considered.

## 2. Material and methods

### 2.1.Field trial and sampling

The experiment took place in Bragança, Portugal, at Pinheiro Manso farm (41° 48´ N, 6° 44′ W), during two consecutive growing seasons (2015 and 2016), on a 5-years-old rainfed olive orchard (cv. “Cobrançosa”) planted at 7×6 m. The climate is of Mediterranean type with some Atlantic influence. Under the Koppen-Geiger climate classification, Bragança had a warm and temperate climate with dry and warm summer (Csb) and rainy winters (IPMA, 2017). The

average air temperature and monthly precipitation recorded during the experimental period are shown in Figure 1.

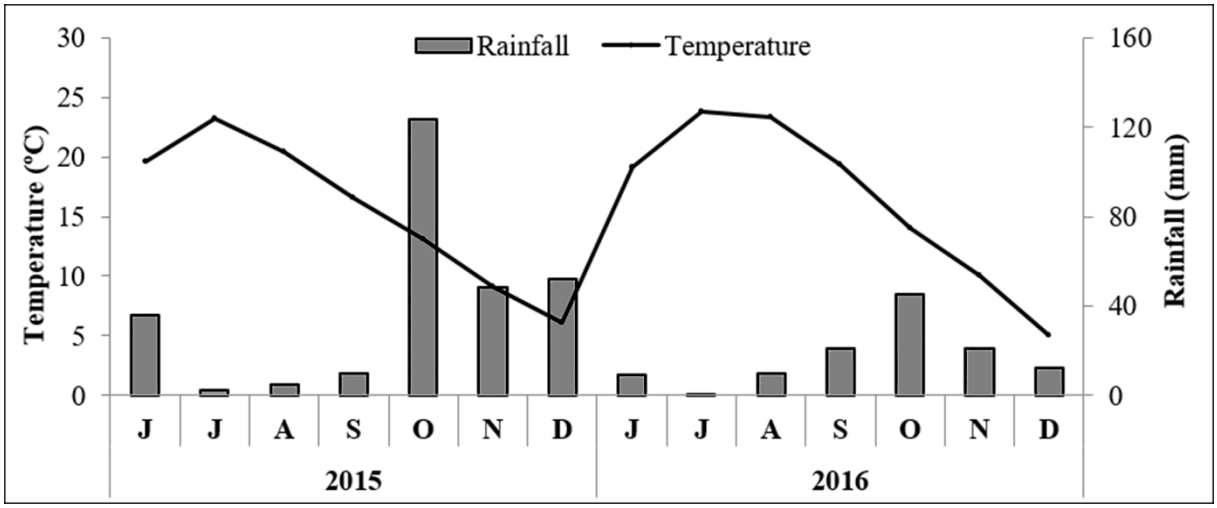


**Figure 1.** Climatic conditions (rainfall and average temperature) during the experimental period in 2015 and 2016. By order, the letters mean the months of the year monitored: June (J), July (J), August (A), September (S), October (O), November (N) and December (D).

Selected physico-chemical characteristics of the olive grove soil (0–20 cm depth) at the beginning of the experiment are presented in Table 1.

**Table 1.** Selected properties of the soil used in the field experiment sampled shortly before the trial started at a depth of 0-20 cm.

| **Soil properties** | | **Soil properties** | |
|---|---|---|---|
| **Clay (%)** | 14.5 | **Extractable P (mg $P_2O_5$ $kg^{-1}$)[d]** | 87.9 |
| **Silt (%)** | 27.7 | **Extractable K (mg $K_2O$ $kg^{-1}$)[d]** | 102.0 |
| **Sand (%)** | 57.8 | **Exchangeable bases[e]** | |
| **$pH_{H2O}$** | 5.8 | **Ca ($cmol_c$ $kg^{-1}$)** | 7.2 |
| **$pH_{KCl}$** | 4.6 | **Mg ($cmol_c$ $kg^{-1}$)** | 2.2 |
| **Oxidizable C (g $kg^{-1}$)[a]** | 8.5 | **K ($cmol_c$ $kg^{-1}$)** | 0.2 |
| **Total organic C (g $kg^{-1}$)[b]** | 25.6 | **Na ($cmol_c$ $kg^{-1}$)** | 0.4 |
| **Extractable B (mg $kg^{-1}$)[c]** | 0.5 | **Exchangeable acidity ($cmol_c$ $kg^{-1}$)** | 0.5 |

[a]Walkley-Black; [b]Incineration; [c]Azomethine H; [d]Ammonium-lactate; [e]Ammonium-acetate, pH 7

After drying (40 °C) and sieving (2mm mesh), soil samples were subjected to several analytical determinations: 1) clay, silt and sand, by the Robinson pipette method; 2) pH (H2O, KCl); 3) organic carbon (C), determined by the Walkley- Black method (easily oxidizable C), and by incineration (total organic C); 4) extractable B by the hot water extraction method and determined by azometine-H colorimetric procedure; 5) extractable P and K, by using ammonium lactate solution at pH 3.7; 6) exchangeable bases ($Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$),

determined by ammonium acetate, pH 7.0; and 7) exchangeable acidity extracted by 1 M KCl. Methods 1–3 and 6-7 are fully described in Van Reeuwijk (2002), 4 in Keren, 1996 and 5 in Houba *et al*. (1997).

The experiment comprises three treatments: control (C) trees, sprayed with distilled water; kaolin (KL) trees, sprayed with an aqueous solution of kaolin (Surround® WP, Engelhard Corporation, Iselin, NJ), at the manufacturer recommended dosage 5% (w/v); and salicylic acid (SA) trees, sprayed with an aqueous solution of 100 μM SA, selected based on results of preliminary research. The treatments were made in the absence of wind in the morning of 30th June 2015 and 23th June 2016. A second application in the same days was done for KL trees, in order to ensure the adhesion uniformity of kaolin clay particles. The kaolin treatment was repeated in 27th August 2016 after a heavy rain event. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. Each treatment included three replicates, completely randomized, with three trees of similar canopy size per plot. Each treatment was separated by a buffer line of trees and all trees were managed without irrigation and cared with the same fertilization, pruning, weed control and pest management practices, as applied by local commercial farmers.

Olive fruits were handpicked from the selected olive trees. In 2015 two harvests were performed in order to evaluate the maturation index and the evolution of the chemical composition of fruits, from an earlier harvest (9$^{th}$ November) (H1 2015) to the traditional harvest for olive oil extraction at the study site (30$^{th}$ November) (H2 2015). In 2016 only the last sampling (9$^{th}$ December) (H1 2016) was performed. Additionally, in both years, the overall production per tree was evaluated. In the H2 2015 and H1 2016 were also collected fruits for olive oil extraction.

### 2.2. Maturation index

The maturation index (MI) was determined according to the method proposed by Hermoso *et al*. (1991) and varied between 0 and 7. Olive fruits were classified into the following categories: 0 – olives with intense green epidermis; 1 – olives with yellowish green epidermis; 2 – olives with red spots or areas in less than half of the fruit; 3 – olives with red or light violet epidermis over more than half of the fruit; 4 – olives with black epidermis and totally white pulp; 5 – olives with black epidermis and less than half purple pulp; 6 – olives with black

epidermis and more than half purple pulp; 7 – olives with black epidermis and totally purple pulp. With a to h being the number of fruits in each category, the MI was calculated as follows:

$$MI= (a\times0+b\times1+c\times2+d\times3+e\times4+f\times5+g\times6+h\times7)/100$$

### 2.3. Olive oil extraction

Olive oil extraction was extracted within 24 h of the olive harvest. A bench hammer mill that reproduces industrial oil extraction was used for the extraction of olive oil from two kg of healthy fruits, without any kind of infection or physical damage. Then, the paste was slowly malaxed at about 25 °C for 40 min, centrifuged in a two-phase decanter at 3500 × for 10 min, and the oil collected was placed in dark glass bottles and kept at 4 °C for latter analysis.

### 2.4. Extraction of polyphenolic compounds from olive fruits and olive oil

The fruits and olive oil extraction was adapted from a procedure described by Sousa *et al*. (2014b). Freeze-dried olive pulp (300 mg) was grind and homogenized with 6 ml of MeOH/$H_2O$ (70:30, v/v) for 30 min at room temperature. Then, the samples were centrifuged at 2800g for 10 min and the supernatant was removed and reserved in a flask after filtration. This procedure was repeated three times. To remove the fat phase, the mixture was washed tree times with 6 mL of hexane and the organic phase was discarded. Extractions were performed in triplicate. Each extract was introduced into a 25 mL round bottom flask, which was filled up to the mark with MeOH/$H_2O$ (70:30, v/v). Four mL of olive oil was weighed in a tube and followed by the addition of 2.5 mL of hexane and 2.5 mL of MeOH/$H_2O$ (70:30, v/v). Then, the samples were centrifuged for 10 min at 2800g. The lower phase was carefully removed and reserved in a flask. This procedure was repeated three times. Extractions were performed in triplicate. Each extract was introduced into a 5 mL round bottom flask, which was filled up to the mark with MeOH/$H_2O$ (70:30, v/v).

### 2.5. Quantification of phenolic compounds

#### 2.5.1. Total phenolic compounds

The concentration of total phenolic compounds (TP) in methanolic extracts was determined using a Folin–Ciocalteu reagent according to the described by Barros *et al*. (2013) with some adaptations to microplates, using gallic acid as standard. This method is based on the reduction of a phosphowolframate–phosphomolybdate complex by phenolics to blue reaction products.

To 20 μL of properly diluted methanolic extracts was added 100 μL of the Folin–Ciocalteu reagent and 80 μL of 7.5% sodium carbonate solution. After 30 min of incubation at 40–45 °C the absorbance of both standard and samples, was measured at 750 nm. All measurements were performed in triplicate. The results were expressed as milligrams of gallic acid equivalents per gram of olive flesh (dry weight) (mg GAE $g^{-1}$) or kilogram of olive oil (mg GAE $kg^{-1}$).

### 2.5.2. Flavonoids

The concentration of flavonoids (Fl) was determined following the method proposed by Zhishen *et al*. (1999) with adaptations to microplates, using catechin as a standard. To 24 μL of properly diluted methanolic extracts was added 28 μL NaNO2 5%. After 5 min in the dark, was added 28 μL of AlCl3 10% and after 6 min in the dark, 120 μL of 1M NaOH was added. The mixture was shaken and the absorbance of the standard and samples was measured at 510 nm. All measurements were performed in triplicate. The results were expressed as milligrams of catechin equivalents per gram of olive flesh (dry weight) (mg CE $g^{-1}$) or kilogram of olive oil (mg CE $kg^{-1}$).

### 2.5.3. Ortho-diphenols

The concentration of ortho-diphenols (o-DP) was determined following the method proposed by Mateos *et al*. (2001) with adaptations to microplates, using gallic acid as a standard. To 160 μL of properly diluted methanolic extracts was added 40 μL of sodium molybdate 5% (w/v) in 50% methanol. After 15 min of incubation at room temperature the absorbance of the standard and samples was measured at 375 nm. All measurements were performed in triplicate. The results were expressed as milligrams of gallic acid equivalents per gram of olive flesh (dry weight) (mg GAE $g^{-1}$) or kilogram of olive oil (mg GAE $kg^{-1}$).

## 2.6. Quantification of the antioxidant capacity

### 2.6.1. $ABTS^{\bullet+}$ radical scavenging assay

The radical-scavenging activity determined by the 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS) radical cation decolorization assay was based on a method described by Ozgen *et al*. (2006) and Barros *et al*. (2011) with adaptations to microplates, using trolox as a standard. $ABTS^{+}$ radical was prepared by mixing an ABTS stock solution (7mM in water) with 2.45mM potassium persulfate. This mixture was allowed to stand for 12–16 h at room temperature in the dark until reaching a stable oxidative state. The ABTS solution was diluted

with 20mM sodium acetate buffer (pH 4.5) to an absorbance of 0.70 ± 0.02 at 734 nm. To a 12 μL of properly diluted methanolic extracts was added 188 μL of $ABTS^+$ solution. After 30 min of incubation in dark at room temperature the absorbance of the standard and samples was measured at 734 nm. All measurements were performed in triplicate. The antioxidant activity of the extract was calculated as Trolox Equivalent Antioxidant Capacity (TEAC) and was expressed as mmol of Trolox equivalents per kg of olive flesh (dry weight) and oil (mmol TEAC $kg^{-1}$). A standard curve of the percentage of $ABTS^+$ inhibition in function of Trolox concentration was used for the calculations.

### 2.6.2. DPPH radical scavenging assay

DPPH• radical scavenging assay was carried out as previously reported by Domínguez-Perles *et al*. (2014) with some modifications, using trolox as a standard. For this assay, it was prepared a solution of 8.87mM of DPPH and diluted in methanol/$H_2O$ (70:30, v/v) to achieve an absorbance closer to 1.000 at 520 nm. To 10 μL of properly diluted methanolic extracts was added 190 μL of DPPH solution. After 30 min of incubation in dark at room temperature the absorbance of the standard and samples was measured at 520 nm. All measurements were performed in triplicate. The antioxidant activity of the extract was calculated as Trolox Equivalent Antioxidant Capacity (TEAC) and was expressed as mmol of Trolox equivalents per kg of olive flesh (dry weight) and oil (mmol TEAC $kg^{-1}$). A standard curve of the percentage of $DPPH^+$ inhibition in function of Trolox concentration was used for the calculations.

### 2.6.3. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) was performed according to Bolanos de la Torre *et al*. (2015) with minor alterations, using trolox as a standard. The FRAP working solution was prepared daily by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of 2, 4, 6-tris(2-pyridyl)-S-triazine (TPTZ) (10mM dissolved with 40mM HCl) and 1 volume of ferric chloride (FeCl3) (20mM in water). The solution was warmed at 37 °C for 10 min before use. To 20 μL of properly diluted methanolic extracts was added 280 μL of FRAP working solution. After 30 min of incubation in dark at 37 °C the absorbance of the standard and samples was measured at 593 nm. All measurements were performed in triplicate. The antioxidant activity of the extract was calculated as Trolox Equivalent Antioxidant Capacity (TEAC) and was expressed as mmol of Trolox equivalents per kg of olive flesh (dry weight)

and oil (mmol TEAC $kg^{-1}$). A standard curve of the reading absorbance in function of Trolox concentration was used for the calculations.

### 2.7.Determination of oil quality parameters

The oil quality parameters were determined according to the European Community regulation EEC/2568/91(Regulation, 1991). Free acidity (FA) measures the hydrolytic breakdown of triglycerides to mono and di-glycerides, leading to fatty acid liberation. This variable is an indicator of the olives quality, the procedures of harvesting, handling, transportation and storage prior to olive milling (Khaleghi *et al*., 2015). FA was expressed as % oleic acid per 100 g oil. Peroxide value (PV) is a measure of the active oxygen bound by the oil, which reflects the hydroxyperoxide value (Khaleghi *et al*., 2015). PV was expressed as mgEqO2 kg−1 oil. The ultraviolet spectrophotometric analysis gives indications about the oxidation stage of the olive oil. The extinctions at specified wavelengths, 232 nm (K232) and 270 nm (K270), are related with the formation of conjugated diene and triene compounds, respectively, due to oxidation (Khaleghi *et al*., 2015). ΔK is the variation of the specific extinction.

### 2.8.Statistical analysis

All statistical calculations were performed using the statistical software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA)., followed by the post hoc Tukey's test (p values < 0.05). * - significant at $p < 0.05$; ** - significant at $p < 0.01$; *** - significant at $p < 0.001$; n.s. – not significant at $p > 0.05$. The relationship between the concentration of phenolic compounds and the antioxidant activity in olive fruits and oil was analyzed by the Pearson correlation test and significance was set at $p < 0.05$.

## 3. Results

### 3.1.Yield and ripening degree of fruits

Figure 2 shows that both applied substances increased the yield of young olive trees. In 2015, KL-treated trees presented higher increases (171%) in olive yield than in the SA-treated trees (65%). In 2016, both products presented similar performance, averaging 78% and 73%

for KL and SA treatments, respectively, which resulted in cumulative increases in the two harvests of 97% and 72%.

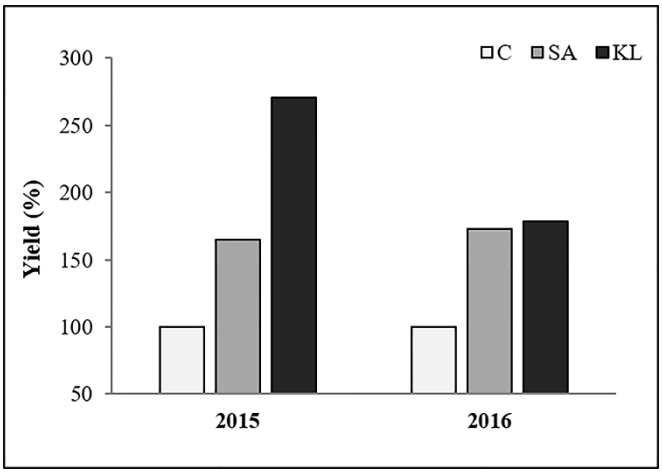


**Figure 2**. Influence of control (C), salicylic acid (SA) and kaolin (KL) treatments on yield in the two years of experiment (the values mean relative change against the minimum value, set out 100%) (n=9).

Both SA and KL treatments and harvest date influenced the MI of the fruits (Table 2). The tendency is constant in the three harvest dates, being the maturation stage slightly delayed by the application of SA and KL, namely in the 2016 season.

**Table 2.** Influence of control (C), salicylic acid (SA) and kaolin (KL) treatments, on maturation index of olives harvested in 2015 (November 9$^{th}$ (H1 2015) and 30$^{th}$ (H2 2015)) and in 2016 (December 9$^{th}$ (H1 2016)).

| | Date | C | SA | KL |
|---|---|---|---|---|
| **Maturation Index** | **H1 2015** | 3.12 | 3.04 | 2.95 |
| | **H2 2015** | 3.45 | 3.39 | 3.22 |
| | **H1 2016** | 3.90 | 3.36 | 3.26 |

### 3.2. Phenolic compounds and antioxidant capacity of olive fruits

The content of phenolic compounds, namely TP, o-DP and Fl and antioxidant capacity responded differently to the harvest date during the 2015 season (Table 3). While the fruits from KL and SA-treated trees exhibited a decrease in both TP and Fl over the fruit ripening, the fruits from C plants exhibited increases of 8.8% and 23.7% in TP and Fl, respectively. By another side, the concentration of o-DP decreased in all treatments, but with higher depletion in KL fruits (22.5%).

**Table 3.** Influence of control (C), salicylic acid (SA) and kaolin (KL) treatments, on olive pulp chemical parameters: total phenols, *ortho*-Diphenols and flavonoids content and antioxidant capacity (AC) based on DPPH, ABTS and FRAP assays. Olives were harvested in 2015 on November 9th (H1 2015) and 30th (H2 2015) and in 2016 on December 9th (H1 2016). Means (n=9) followed by the same letter within the same harvest season are not significantly different at $P < 0.05$.

| | Date | C | SA | KL | Sig. |
|---|---|---|---|---|---|
| **Total Phenols (mg GAE g$^{-1}$)** | **H1 2015** | 27.6$^{c}$ | 32.7$^{b}$ | 36.3$^{a}$ | *** |
| | **H2 2015** | 29.6$^{b}$ | 29.3$^{b}$ | 31.7$^{a}$ | ** |
| | **Sig.** | * | ** | *** | |
| | **H1 2016** | 41.2$^{a}$ | 38.4$^{b}$ | 39.0$^{b}$ | *** |
| ***ortho*-Diphenols (mg GAE g$^{-1}$)** | **H1 2015** | 40.5$^{b}$ | 43.1$^{ab}$ | 43.9$^{a}$ | * |
| | **H2 2015** | 34.9 | 35.6 | 34.0 | **n.s.** |
| | **Sig.** | *** | *** | *** | |
| | **H1 2016** | 40.8$^{a}$ | 38.0$^{b}$ | 38.5$^{b}$ | *** |
| **Flavonoids (mg CE g$^{-1}$)** | **H1 2015** | 26.9$^{b}$ | 42.7$^{a}$ | 43.4$^{a}$ | *** |
| | **H2 2015** | 33.3 | 32.1 | 32.8 | **n.s.** |
| | **Sig.** | *** | *** | *** | |
| | **H1 2016** | 47.4 | 49.4 | 48.5 | **n.s.** |
| **C- DPPH (mmol Trolox kg$^{-1}$)** | **H1 2015** | 81.7$^{b}$ | 123.8$^{a}$ | 131.7$^{a}$ | *** |
| | **H2 2015** | 110.0 | 105.2 | 107.9 | **n.s.** |
| | **Sig.** | *** | ** | *** | |
| | **H1 2016** | 130.6$^{a}$ | 130.5$^{a}$ | 121.8$^{b}$ | ** |
| **AC - ABTS (mmol Trolox kg$^{-1}$)** | **H1 2015** | 141.6$^{c}$ | 176.2$^{b}$ | 207.7$^{a}$ | *** |
| | **H2 2015** | 174.5$^{b}$ | 170.6$^{b}$ | 190.1$^{a}$ | ** |
| | **Sig.** | *** | **n.s.** | ** | |
| | **H1 2016** | 319.0 | 311.2 | 320.3 | **n.s.** |
| **AC - FRAP (mmol Trolox kg$^{-1}$)** | **H1 2016** | 214.3$^{a}$ | 190.2$^{b}$ | 185.1$^{b}$ | *** |

* - significant at p<0.05; ** - significant at p<0.01; *** - significant at p<0.001; n.s. – not significant at p>0.05.

The antioxidant capacity based on ABTS and DPPH assays followed a similar trend to TP and Fl (Table 3). During the first harvest of 2015, both substances contributed to increase the levels of all phenolic compounds in fruits, with the order C < SA < KL for TP, C≤SA≤KL for o-DP and C < SA and KL for Fl (Table 3). However, the higher decline in SA and KL fruits during ripening attenuate the differences in the second harvest, where only the concentrations of TP were still influenced by the treatments, being higher in KL fruits (Table 3). Relatively to

2015, in 2016 was noticed an increase in phenolics accumulation in all the analyzed treatments highlighting the C fruits (Table 3). However, in 2016 harvest the response to the treatments was quite different from 2015. Both KL and SA treatments lead to a lower accumulation of TP and o-DP, while the Fl concentration was not affected (Table 3).

In general terms, the antioxidant capacity of fruits from C trees was negatively affected in 2015, while it increases largely in 2016 (Table 3), following a similar trend of phenolic compounds. On the first harvest of 2015, the antioxidant capacity based on DPPH followed the order C < SA and KL (Table 3). By using the ABTS assay, the order was similar, C < SA < KL (Table 3). On the second harvest of 2015, the statistical differences recorded for DPPH assay was annulated and by using the ABTS assay the KL still stood out (C and SA < KL, Table 3). In 2016, the antioxidant capacity response to the applied treatments was also influenced by the method in test, but with a different trend from the previous year (Table 3). While with the DPPH assay the order was KL < C and SA, by using ABTS assay no differences were recorded (Table 3). The analysis of FRAP assay was also different with the order KL and SA < C (Table 3).

### 3.3. Phenolic compounds and antioxidant capacity of olive oil

The concentration of phenolic compounds in olive oil presented different patterns among years (Table 4). In 2015, the concentration of all quantified phenolics followed the order C < KL < SA (Table 4). On the other hand, the trend of phenolic compounds concentrations in 2016 was different, following the order SA and KL < C for TP and Fl and KL < SA < C for o-DP (Table 4). Moreover, the increase in phenolics noticed in fruits, from 2015 to 2016 (Table 3), was more evident in the olive oil, as well the huge increase in the C group (Table 4).

Following a similar trend of phenolic compounds concentrations, the olive oil analysis revealed a positive influence of KL and SA on the antioxidant capacity based on the antiradical activity in 2015, with the order C < SA and KL for DPPH assay and C < KL < SA for ABTS assay (Table 4). Following the fruit tendency, in 2016 the C group exhibited higher antioxidant capacity, with the order SA and KL < C for DPPH and ABTS assays and KL < SA < C for FRAP assay (Table 4).

**Table 4.** Influence of control (C), salicylic acid (SA) and kaolin (KL) treatments, on olive oil chemical parameters: total phenols, *ortho*-Diphenols and flavonoids content and antioxidant capacity (AC) based on DPPH, ABTS and FRAP assays. Means (n=9) followed by the same letter within the same harvest season are not significantly different at $P < 0.05$**.**

| | Date | C | SA | KL | Sig. |
|---|---|---|---|---|---|
| **Total Phenols (mg GAE kg$^{-1}$)** | **2015** | 84.0$^{c}$ | 123.0$^{a}$ | 113.1$^{b}$ | *** |
| | **2016** | 555.6$^{a}$ | 428.5$^{b}$ | 412.1$^{b}$ | *** |
| ***ortho*-Diphenols (mg GAE kg$^{-1}$)** | **2015** | 63.5$^{c}$ | 72.0$^{a}$ | 66.6$^{b}$ | *** |
| | **2016** | 195.1$^{a}$ | 152.4$^{b}$ | 136.8$^{c}$ | *** |
| **Flavonoids (mg CE kg$^{-1}$)** | **2015** | 74.7$^{c}$ | 116.0$^{a}$ | 92.7$^{b}$ | *** |
| | **2016** | 557.3$^{a}$ | 401.9$^{b}$ | 398.5$^{b}$ | *** |
| **AC - DPPH (mmol Trolox kg$^{-1}$)** | **2015** | 0.212$^{b}$ | 0.301$^{a}$ | 0.295$^{a}$ | *** |
| | **2016** | 1.23$^{a}$ | 0.910$^{b}$ | 0.827$^{b}$ | *** |
| **AC - ABTS (mmol Trolox kg$^{-1}$)** | **2015** | 0.367$^{c}$ | 0.451$^{a}$ | 0.395$^{b}$ | *** |
| | **2016** | 2.50$^{a}$ | 2.24$^{b}$ | 2.14$^{b}$ | *** |
| **AC - FRAP (mmol Trolox kg$^{-1}$)** | **2016** | 2.17$^{a}$ | 1.47$^{b}$ | 1.35$^{c}$ | *** |

*** - significant at p<0.001.

### 3.4. Correlation analysis

As presented in Table 5, in general terms the antioxidant capacity was significantly positive correlated with the phenolic concentrations, showing that phenols have a high association with the antioxidant capacity of olives and olive oil. In fruits, TP showed a significant positive correlation with all the antioxidant capacity methods tested, being this relationship strong for DPPH and very strong for both ABTS and FRAP methods, while Fl showed a very strong correlation with DPPH and ABTS methods and o-DP with FRAP method. In opposite to the fruits, in olive oil the correlation between the phenolic compounds and the antioxidant capacity methods was persistently very strong (Table 5).

### 3.5. Olive oil parameters

The oil quality indices FA, PV, $K_{232}$ and $\Delta K$ were not affected by the applied treatments, whereas the $K_{270}$ coefficient was reduced by both SA and KL application (Table 6).

**Table 5.** Pearson correlation coefficients established between the antioxidant capacity and phenolics content.

| | TP (F) | Fl (F) | o-DP (F) | TP (O) | Fl (O) | o-DP (O) |
|---|---|---|---|---|---|---|
| **DPPH (F)** | 0.788*** | 0.858*** | 0.272 | | | |
| **ABTS (F)** | 0.923*** | 0.863*** | 0.109 | | | |
| **FRAP (F)** | 0.923*** | -0.246 | 0.947*** | | | |
| **DPPH (O)** | | | | 0.994*** | 0.994*** | 0.992*** |
| **ABTS (O)** | | | | 0.988*** | 0.982*** | 0.968*** |
| **FRAP (O)** | | | | 0.969*** | 0.973*** | 0.970*** |

TP (F) and TP (O) = total phenols content in fruits and oil, respectively; Fl (F) and Fl (O) = flavonoids content in fruits and oil, respectively; o-DP (F) and o-DP (O) = ortho-diphenols content in fruits and oil, respectively; DPPH (F) and DPPH (O) = antioxidant capacity based on DPPH assay in fruits and oil, respectively; ABTS (F) and ABTS (O) = antioxidant capacity based on ABTS assay in fruits and oil, respectively; FRAP (F) and FRAP (O) = antioxidant capacity based on FRAP assay in fruits and oil, respectively. *** - significant at $p<0.001$.

**Table 6.** Influence of control (C), salicylic acid(SA) and kaolin (KL) treatments on olive oil quality indices (FA - Free Acidity; PV – Peroxide Value; $K_{232}$ – specific extinction at wavelength 232; $K_{270}$ – specific extinction at wavelength 270; ΔK – Variation of the specific extinction). Means (n=3) followed by the same letter are not significantly different at $P < 0.05$.

| Oil quality indices in 2016 | C | SA | KL | Sig. |
|---|---|---|---|---|
| **FA (% oleic acid)** | 0.388 | 0.390 | 0.392 | n.s. |
| **PV (mgEq $O_2$ $kg^{-1}$ oil)** | 2.73 | 2.70 | 2.69 | n.s. |
| **$K_{232}$** | 2.03 | 1.96 | 1.96 | n.s. |
| **$K_{270}$** | 0.165[a] | 0.151[b] | 0.142[b] | ** |
| **ΔK** | 0.0025 | 0.0032 | 0.0027 | n.s. |

** - significant at $p<0.01$; n.s. – not significant at $p>0.05$.

## 4. Discussion

### 4.1. Yield and ripening degree of fruits

The increase in yield promoted by KL and SA agrees with previous studies in different plant species (Glenn *et al*., 2003; Saou and Makee, 2003; Jamali *et al*., 2011; Khalil *et al*., 2012; Abd El-Razek *et al*., 2013; Chamchaiyaporn *et al*., 2013; El-Shazly *et al*., 2013; Kazemi, 2013; Correia *et al*., 2014). Moreover, it is also important to notice that albeit the significant differences in yield in the 1st year, namely in KL-treated plants, the differences in olive yield in the 2nd year were notorious, since this species suffers from the peculiar phenomenon of alternate bearing, particularly under rainfed conditions, as in the present study. In addition, the severe summer stress verified in 2016 also contributed to limit potential crop yield in that year.

Meanwhile, the delay of maturation stage by the application of SA and KL was closely associated with the higher fruit yield. Similar relationship between crop load and fruit ripening was previously verified (Barone *et al*., 1994).

### 4.2. Influence of the harvest time on the phenolic compounds content and antioxidant capacity of olive fruits

An overall decrease in phenolic compounds concentrations and in antioxidant capacity of olive fruits during ripening is a common described phenomenon (Damak *et al*., 2008; Barros *et al*., 2013; Brahmi *et al*., 2013; Machado *et al*., 2013; Sousa *et al*., 2014a, 2015; Sousa *et al*., 2014b; Talhaoui *et al*., 2015), due to chemical and enzymatic reactions occurring during the ripening process (Ye *et al*., 2014). In the present study, this decrease was highly notorious in o-DP contents (Table 3). By other side, the increase in TP (8.8%) and Fl (23.7%) concentrations noticed in C plants over the fruit ripening (Table 3) may be justified by the appearance of other classes of phenolic compounds. Oleuropein, an ortho-diphenolic compound, is the most abundant phenolic during the green stage, but decreases sharply during the maturation, while by other side the black maturation phase is characterized by the appearance of other compounds, such as flavonoids, specially anthocyanins (Damak *et al*., 2008; Jemai *et al*., 2009; Machado *et al*., 2013; Sousa *et al*., 2014a; Talhaoui *et al*., 2015). This assumption is consistent with the discoveries of Barros *et al*. (2013) since they found an overall increase of flavonoids concentration from green to black stages in different olive cultivars, including "Cobrançosa".

### 4.3. Accumulation of phenolic compounds on fruits and olive oil in response to the applied treatments

The different trend in phenolic compounds accumulation recorded in 2015 and 2016 (Tables 3 and 4) may be justified by the distinctive environmental conditions that include frost events before harvest in 2015 and severe summer stress in 2016. Stress conditions influence the metabolism of phenolic compounds in fruits, mainly in two ways, which are not mutually exclusive and that may even interact. First, due to the reduction in net photosynthesis that may result in a decrease in carbohydrate supply to the fruits, the major source of precursors for the biosynthesis of these phytochemicals; second, the stress conditions may exacerbate the oxidative stress, promoting the biosynthesis of this group of antioxidant compounds (Wang and Frei, 201; Ripoll *et al*., 2014). To notice, phenolic compounds accumulate more, both in fruits and olive oil, in 2016 than in 2015 harvest (Tables 3 and 4). As can be seen in Figure 1, during

the fruit development, the accumulated precipitation was much higher in 2015 than in 2016, mainly in October (123.6 mm), close to the harvest time. Moreover, 2015 had a less hot summer. These data justify the higher accumulation of phenolic compounds in 2016, in agreement with other studies, which reported that stressful conditions increase the accumulation of phenolic compounds in olive fruits (Gómez-Rico *et al*., 2006; Machado *et al*., 2013). On the other side, in 2015 the plants were exposed to frost events before the harvest, coinciding one of those events with the second harvest of 2015. With temperatures below 0 °C occurs the freezing of extracellular water, and the thermodynamic equilibrium is achieved either by cellular dehydration and continued extracellular ice formation, or by intracellular ice formation. These effects seriously damage cell membranes, leading to cell death and the oxidative degradation of cell contents, as phenolic compounds, due to the contact between enzymes and their respective substrates (Morelló *et al*., 2003). Indeed, it is known that frost events can damage olive fruits and consequently the quality of the extracted olive oil, including the decrease in pigments and phenolic compounds concentrations (Morelló *et al*., 2003; Morelló *et al*., 2006; Houliston *et al*., 2007). As the early frosts are very common in this region, it is recommended to anticipate the harvest time, usually adopted by the local farmers. Interestingly, Morelló *et al*. (2006) reported that in crop seasons following the frost events, the concentration of phenolic compounds increases significantly, associating this response to the fact that the previously frost damaged olive trees were more sensitive to water deficit during summer.

Regarding the differences among treatments, it is important to refer that KL and SA ameliorate the olive photosynthetic rate during the fruit development (unpublished results). In the first year, this response not only contributed to higher crop yield, but also to a slight higher accumulation of phenolic compounds in the fruits (Table 3). Moreover, the plants sprayed with SA and KL were physiologically less damaged with the frost events (unpublished results). However, as in 2016 the plants face severest stress conditions during the summer period (Figure 1), the control plants, that were in worst physiological conditions (unpublished results), increase the investment in these phytochemicals in order to increase the free radical scavenging activity. Thus, these data demonstrate the effectiveness of the applied substances in reducing the degree of frost damage and in mitigating the extreme adverse conditions that were felt in 2016, namely in summer.

The present study showed that a very low percentage of total phenolic compounds were transferred from olive pulp to oil, being higher in 2016 in a closely association with the prevailing climatic conditions reported previously. Moreover, phenolic compounds changed

qualitatively and quantitatively during the transfer, as in the study of Ye *et al*. (2014), resulting in quite different phenolic composition of olive oil compared to that of olive flesh. To explain the different trend in the transference of phenolic compounds to oil between years and among the applied treatments (Tables 3 and 4) different hypothesis were raised: variations in pulp moisture (69.8% to 71.1% in 2015 and 60.5% to 61.4% in 2016), since higher moisture affects negatively the transfer of phenolics to the oil; changes in enzymes activities during the pressing and malaxation steps; and/ or changes in the transference of specific phenolics presented in olive stones, lignans, after whole olive fruits crushing and malaxation (Oliveras López *et al*., 2008; Talhaoui *et al*., 2016). Yet, the initial characteristics of the olive fruits, such as phenolic composition, is probably the most important variable involved in the quality of the final olive oil (Fregapane and Salvador, 2013). As stated previously, the information about the influence of both SA and KL in phenolic composition of fruits is scarce and sometimes not consensual. Indeed, a global analysis of the literature previously cited and our results reveal that other factors, such as genotypes or environmental conditions, may determine the influence of these mitigating agents in fruits quality.

### 4.4. Antioxidant capacity of fruits and olive oil

The global antioxidant capacity of an extract reflects both the "antiradical" and the "antioxidant" activity, that not necessarily coincide (Tirzitis and Bartosz, 2010). The antiradical activity characterizes the ability of compounds to react with free radicals (e. g. ABTS and DPPH assays) and antioxidant activity represents the ability to inhibit the process of oxidation (e. g. FRAP assay) (Tirzitis and Bartosz, 2010; Moharram and Youssef, 2014). Therefore, to better understand the global antioxidant capacity of olives and olive oil, methanolic extracts were evaluated by three different assays to cover different mechanisms of the antioxidant defense system: DPPH and ABTS%+ radicals scavenging activity and FRAP. Indeed, according to the method tested the antioxidant capacity presented a different behavior (Tables 3 and 4), highlighting the importance of testing different methods. It is well established the influence of phenolic compounds on the antioxidant capacity of olive fruits and oil (Tripoli *et al*., 2005; Jemai *et al*., 2009; Brahmi *et al*., 2013; Gouvinhas *et al*., 2014; Sousa *et al*., 2014a). Nevertheless, other compounds with antioxidant capacity are known to be present in these products (Jemai *et al*., 2009; Sousa *et al*., 2014a), which may contribute to some different trends detected between phenols and the antioxidant capacity (Tables 3 and 4).

### 4.5. Correlation analysis

The correlation analysis suggest that the nature of phenolic compounds determine its ability to reduce the ABTS%+ and DPPH radicals and the ferric iron. Moreover, those data also suggest that in olive oil the phenolic compounds are the most responsible for the antioxidant capacity and that in fruits, the presence of other constituents in the extracts could also have an important contribution to the antioxidant capacity. For instance, Jemai *et al*. (2009) reported that the presence of sugar alcohols, as mannitol, enhances the ability of the extract to act as an antioxidant because it is known to be a quencher of ROS and scavenger of hydroxyl radicals. The correlation between the antioxidant capacity and the level of phenolic compounds has been largely described, but, in fact, it is very dependent on its structure, specially the number and the position of hydroxyl substituents in the aromatic ring (Rice- Evans *et al*., 1996; Bouaziz *et al*., 2005; Boskou *et al*., 2006; Silva *et al*., 2006; Gouvinhas *et al*., 2014; Sousa *et al*., 2014a).

### 4.6. Influence of the applied treatments on oil quality parameters

Since the harvesting, transportation, storage and extraction process were equal among the treatments, the similar values of olive oil FA (Table 6) reflect the same quality of the olives (Khaleghi *et al*., 2015). As far as we know, no study access previously the influence of SA application in olive oil quality indices. Regarding KL application, our results corroborate the study of Saour and Makee (2003) whereas Khaleghi *et al*. (2015) reported a reduction of FA with the application of kaolin.

The higher PV means the greater degradation of the oil due to oxidation (Khaleghi *et al*., 2015). The absence of KL effects in this index contradicts the results of Saour and Makee (2003) and Khaleghi *et al*. (2015), that showed a lower PV in olive oil from KL-treated trees.

The lower $K_{270}$ absorption coefficient in oils from KL and SA treatments (Table 6) indicates a fewer formation of secondary products of oxidation in the oils from sprayed trees (Limón *et al*., 2015). A reduction in both $K_{232}$ and $K_{270}$ absorption coefficients with KL application was already described in the literature (Saour and Makee, 2003; Khaleghi *et al*., 2015). According to the European Community EEC/2568/91 (Regulation, 1991) the evaluated indices of all the analyzed olive oils fall within the ranges established for "extra virgin olive oil" category. FA (0.388–0.392%) are below the limit of 0.8%, PV (2.693–2.733 mEq $O_2$ $kg^{-1}$) are below the limit of 20 mEq $O_2$ $kg^{-1}$ and the UV spectrophotometric coefficients are also below the stablished limits, 2.5 for K232 (1.957‑2.026), 0.22 for K270 (0.142‑0.165) and 0.01 for ΔK (0.0025-0.0032).

## 5. Conclusions

This study provided evidences that yield, phenolics compounds and antioxidant capacity of olive fruits and olive oil are modulated by the applied mitigating agents. Both SA and KL lead to an increase in olive yield. However, it is hard to conclude their influence on the phenolic composition and antioxidant capacity, since the climatic conditions determine different responses by the plants. The lower concentration of phenolic compounds under higher maturation index was accelerated by KL and SA. In general, both stress mitigating agents increased the concentration of phenolic compounds and antioxidant capacity in both fruits and oil on the first year, but decreased their levels on the second year, in a closely association with the prevailing climatic conditions. The quality indices of olive oils set by the European Community EEC/ 2568/91(Regulation, 1991) were not negatively affected by KL and SA. On the contrary, oils from KL and SA treatments presented lower formation of secondary products oxidation. Both KL and SA compounds were effective in reducing the degree of frost damage and in mitigating the adverse summer conditions, typical of Mediterranean olive growing areas.

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# CHAPTER 7

General Discussion

## General Discussion

The changing climate is a major challenge for agriculture, especially in particularly susceptible areas, as the Mediterranean Basin (IPCC, 2013), where olive tree is one of the emblematic crops, and where is produced about 97% of world olive oil (IOC, 2017), the top product among vegetable oils. Olive oil is widely known as the main source of fat in the so-called Mediterranean diet, being related with several beneficial effects in human health, due to its balanced fatty acid composition and antioxidant proprieties (Ghanbari *et al*., 2012). As a consequence, the consumption and the demand for olive oil is increasing all over the world (IOC, 2017). Thus, it is important to act in order to make this crop more sustainable, productive and resilient under the severe conditions of summer season in the Region.

Understanding how olive trees respond to environmental stresses is the first step to improve its profitability, allowing the selection of more resistant cultivars, to identify tolerant characteristics useful in breeding programs and in genetic engineering and to develop accurate mitigation strategies according to the necessities. Several studies addressed the olive tree response to the common summer stress conditions, highlighting drought (Bacelar *et al*., 2009; Petridis *et al*., 2012; Torres-Ruiz *et al*., 2013; Perez-Martin *et al*., 2014) that is commonly associated with heat and high irradiances (Sebastiani *et al.*, 2002; Cansev, 2012; Koubouris *et al.*, 2015). These studies revealed that olive is a highly tolerant species, due to large investment of resources in defence mechanisms, but compromising the productivity. Still, several mechanisms by which the species respond to drought conditions remain least discussed, such as the physiological mechanisms that occur during the night period. As nighttime temperatures will increase at higher extent than daytime temperatures (IPCC, 2013), and as evidences suggest a substantial nighttime stomatal open in different species (Dawson *et al*., 2007; Ogle *et al*., 2012; Escalona *et al.*, 2013; Resco de Dios *et al*., 2015), the first concern of this thesis was to understand the olive tree nighttime water balance. The results obtained demonstrate that nighttime water balance should not be neglected. Moreover, more attention should be paid under drought conditions, as water-stressed olive tree presented higher nighttime stomatal open in the first hours of the night and superior proportion of nighttime stomatal transpiration in relation to whole-day losses. Still, until certain levels of water deficit, potential benefits to the plant can be associated to this response, as the reduction in respiration rate, possibly by the effect of evaporative cooling (Coupel-Ledru *et al*., 2016), that reduce the depletion of carbon reserves, and the improvement of minerals absorption, by the maintenance of the transpiration stream (Snyder *et al*., 2003). This study, as others in different plant species, claim the necessity

to incorporate the nighttime water balance in simulation models and to increase the water use efficiency in olive orchards through the adoption of agronomic practices that might contribute to reduce the nighttime transpiration under severe drought conditions.

As the increase in the severity of summer seasons in arid and semi-arid areas of the globe is already a reality, urge the necessity to concentrate efforts on the study and identification of efficient short-term adaptation strategies for the exposed orchards. The application of exogenous products on the leaf surface can be considered in this sense. Thus, in this study were evaluated the use of foliar sprays of kaolin (KL) and salicylic acid (SA), as summer stress mitigating agents. The choice was based in their mode of action. KL forms a white protective particle film, which increases the reflection of excess radiation, reducing the heat load (Glenn *et al*., 2005; Nanos, 2015), while SA is a signaling phytohormone with diverse regulatory roles in plant metabolism, as in antioxidant defense system activation, osmolytes synthesis and optimization of mineral nutrients status (Khan *et al*., 2015). Although KL has been studied in some fruit trees, the plant induced changes are still not fully understood. Regarding SA, the knowledge about its mode of action is wider, but it is highly dependent on applied concentration (Kang *et al*., 2012; Agami *et al*., 2013; Jesus *et al*., 2015), and the investigation remains essentially in herbaceous species (Kang *et al*., 2012; Agami *et al*., 2013; Fayez and Bazaid, 2014). Moreover, the efficiency of mitigation strategies against water stress, usually the most important component of summer stress, should not only must include the increase in drought resistance, being also important to include the recovery capacity. Undeniably, the recovery capacity has been proved to be determinant for a successful drought adaptability (Chen *et al*., 2016). Thus, it was primarily evaluated the response of young-treated olive trees to repeated cycles of drought-rewatering, representing the typical cycles observed in the Mediterranean region. In the case of SA, three different concentrations were tested (10, 100 and 1000 µM), since concentration highly determine its effectiveness (Kang *et al*., 2012; Jesus *et al*., 2015) and no manufacture recommended dosage is available, as for KL (5% w/v).

The results showed the efficacy of both SA and KL to improve drought resistance, although a suitable SA concentration was more efficient than KL. Moreover, the data also revealed that the recovery capacity was stimulated in a different extent by the applied products, as SA induce a better response again. Each product modulates different plant responses, what justify the different results obtained. During drought periods, KL application contributed to increase stomatal conductance, but without substantial increase in transpiration. Meanwhile, a positive impact in net photosynthesis was recorded, in line with other studies (Jifon and Syvertsen, 2003;

Nanos, 2015; Dinis *et al*., 2018), due to lower stomatal and non-stomatal limitations, that include lower photochemical damages. However, with the course of the experiment KL effects in net photosynthetic rate were disappearing, possibly by the developed shade-related characteristics in sprayed leaves or by the prevalence of stressful conditions, highly accentuated by the containers. Indeed, a similar response was previously reported with the increase of stress severity (Shellie and Glenn, 2008; Nanos, 2015). Still, the alleviation of the stressful conditions around the leaves contribute to keep water status during the most stressful period. Moreover, KL influenced positively the primary metabolism and reduced the signals of oxidative stress and the investment in antioxidant defense strategies. The positive influence in water status was widely described (Nanos, 2015; AbdAllah, 2017; Dinis *et al.*, 2018), but the influence in primary and, specially, in secondary metabolism and oxidative biomarkers were poorly investigated, revealing a key role of KL in stress alleviation. Regarding recovery, although the more comfortable conditions conferred during the drought episodes reduce the necessity to invest in extra repair damages after rewatering, the particle film on the leaves limited gas exchange restauration. Thus, despite the induced positive changes in plant nutritional status, the long recovery events might have determined the absence of significant influence in whole-plant water use efficiency and biomass accumulation. Regarding SA, the results corroborated that the influence in olive tree responses is concentration-dependent. The influence of the lowest concentration, 10 µM, was barely evident, revealing the sprayed plants a close similar response as the droughted controls. The more significant induced responses were the huge increase in total soluble proteins and $H_2O_2$ during drought, also shared with the other applied concentrations. These results, in association with the restauration of soluble protein levels to well-watered controls after stress relief, revealed a SA role in protein metabolism, as already proposed in other studies (Jalal *et al*., 2012; Kang *et al*., 2012; Kabiri *et al*., 2014), and a possible influence of SA in inducing $H_2O_2$ production, as signaling hormone (Belkadhi *et al*. 2014). The two higher concentrations (100 and 1000 µM) influence to a great extent the olive tree responses to drought and rewatering, being quite similar. However, the higher concentration, 1000 µM, stands out from the other concentration by the tendency to negatively influence water status, possibly by the early induction of stomatal open. The higher efficiency of 100 µM in improving drought resistance was achieved by higher detoxification and scavenging of ROS, leaf osmolytes accumulation, leaf water status maintenance, reduced photosynthetic systems drought-associated damages, and by optimizing shoot/root ratio. Some of these responses are in line with other studies that showed positive effects of SA in redox

status (Fayez and Bazaid, 2014; Jesus *et al*., 2015; Nazar *et al*., 2015), leaf water status (Kang *et al*., 2012; Jesus *et al*., 2015; Nazar *et al*., 2015) and photosynthetic capacity (Wang *et al*., 2014; Jesus *et al*., 2015; Nazar *et al*., 2015) of drought stressed plants. The better plant fitness induced by 100 µM SA during drought periods allowed a fast recovery of the physiological functions upon rewatering and reduced the necessity to invest in extra repair damages, allowing the regrowth. Although the influence of SA in recovery processes was poorly accessed, there are some evidences that induce a faster recovery of photosynthetic capacity after heat and high light stress (Wang *et al*., 2010, 2014; Zhao *et al*., 2011). Moreover, under recovery this concentration reduces the permanence of ABA signal in leaves, while increases the signal of IAA. This SA concentration was also the most effective in plant ionome regulation, confirming the SA role in mineral nutrient status improvement addressed in previous studies (Gunes *et al*., 2007; Nazar *et al*., 2015). Altogether, these changes contributed to increase whole-plant water use efficiency and to attenuate the limitation of total biomass accumulation imposed by drought, mainly in root system, as already described (Umebese *et al*., 2009; Kang *et al*., 2012).

To validate the pot experiment results under realistic field conditions and to evaluate the influence on yield and harvests quality, rainfed olive trees were sprayed with the most effective SA concentration (100 µM) and KL (5%) for two consecutive growing seasons. The results of the field experiment demonstrated, in both years, the efficacy of KL and SA in mitigating the stressful conditions of rainfed orchards. In addition, the protection conferred during the summer season was also determinant for higher recovery capacity during autumn and winter seasons. Although the improvement of leaf water status and photosynthetic capacity, due to lower stomatal and non-stomatal limitations, was a general picture with both products, the depth analysis performed confirms that each product alleviates stress effects by distinct action modes. Kaolin-sprayed leaves showed, again, shade-related leaf structural changes, in association with a reduced investment in secondary metabolism. The capacity to keep similar photosynthetic rates as SA plants, in spite of lower absorption of irradiance and reduced investment in palisade parenchyma, means that KL particle film allows a good performance of photochemical and biochemical processes in chloroplasts. On the other hand, the lower respiratory construction and maintenance costs of KL-treated leaves allows the use of more resources in growth and yield. Indeed, in line with the intense leaf IAA signal in KL-sprayed plants, these trees also exhibited higher canopy growth, what is determinant to the productivity of the following seasons. Meanwhile, the protective action of SA was also associated with the induction of some stress tolerance responses and the enhancement of specific minerals concentrations in leaves.

For instance, the SA-sprayed plants showed an improvement in ABA signal, without reducing stomatal conductance, claiming the role of this phytohormone in signaling processes. Moreover, also presented an improvement of leaf total soluble proteins and ascorbate pools, reinforcing the role in antioxidant responses. Therefore, the higher increase in canopy volume in KL plants and the increase in yield in both sprayed plants resulted from the favourable changes induced by KL and SA in some of the biochemical and physiological processes discussed above. Although the influence of KL in plant growth and/or biomass accumulation are not consistent in the literature (Cantore *et al*., 2009; Roussos *et al*., 2010; Correia *et al*., 2014; Segura-Monroy *et al*., 2015), the positive effect on yield is recurrent, even with the reduced investment in growth in some cases (Saour and Makee 2003; Cantore *et al*., 2009; Correia *et al*., 2014; Segura-Monroy *et al*., 2015). In its turn, the positive influence of SA on plant growth under stressful conditions has been largely described (Shakirova, *et al*., 2003; Kang *et al*., 2012; Fayez and Bazaid 2014; Aliniaeifard *et al*., 2016; Nazar *et al*., 2015), although some of this increase was mainly related with the stimulation of root development (Shakirova, *et al*., 2003; Aliniaeifard *et al*., 2016). Meanwhile, the positive influence of SA application on yield has also been reported on the literature (Shakirova *et al*., 2003; Javaheri *et al*., 2012; Abd El-Razek *et al*., 2013). One possible explanation for the higher accumulated yield and canopy increase in KL than in SA plants, despite the similar photosynthetic capacities recorded in individual leaves, is the increased light distribution within the canopy, due to the reflective properties of kaolin. By increasing the incident radiation on inner-canopy leaves, previously shaded or partially-shaded, an increased carbon gain at the whole-plant scale is observed, even when the individual photosynthesis is negatively affected (Rosati *et al*., 2007; Glenn, 2009).

Interestingly, the KL and SA influence in olives and olive oil quality depends on the prevailing climatic conditions. In general, both KL and SA increased the concentration of phenolic compounds and antioxidant capacity in both fruits and oil on the first year, but decreased their levels on the second year. The discussed positive influences of KL and SA on plants physiological performance might contributed to an increased phenolic concentration. Moreover, also conferred protection against the severe frost events, that is known to affect phenolic compounds (Houliston *et al*., 2007; Morelló *et al*., 2003, 2006). By other side, the increased severity of summer stress during the second year might affected seriously control plants, that being in worst physiological conditions need to increase largely the investment in

secondary metabolism, a common response of olive tree to severe conditions (Gómez-Rico *et al*., 2006; Machado *et al*., 2013).

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# CHAPTER 8

## Concluding Remarks

The main outcomes of this thesis are summarized below.

1. The results of this study increased the understanding about the olive tree responses to drought in terms of nighttime water balance. Early nighttime stomatal open and the proportion of nighttime water losses in relation to whole-day losses increased under moderate drought, indicating that olive tree nighttime water balance is modulated by water status. Moreover, until certain level of water deficit, nighttime stomatal open could be coupled with some potential benefits to the plant, as the reduction of respiration rate and the improvement of nutrients uptake. Therefore, nighttime water balance cannot be neglected and must be considered in simulation models and in the selection of cultivars and stress mitigation agronomic practices.
2. The studies performed increased the knowledge about how physiological and biochemical responses are modulated by KL particle film on leaves surface. In response to the shade effect of the particle film, sprayed leaves developed shade-related characteristics. Generally, in potted plants, KL (5% w/v) was suitable to alleviate the drought effects by improving water and mineral status, cellular functions, reducing the drought-induced stomatal and non-stomatal limitations to photosynthesis and reducing the necessity to invest in extra repair damages after drought recovery. However, the shaded effect conferred by kaolin interfered with gas exchange restauration, minimizing the influence of water stress on plant biomass accumulation. Under rainfed field conditions, KL also improved plant water status and photosynthetic activity and reduce the necessity to invest in secondary metabolism during the summer months, while contributed to improve gas exchange responses during the autumn and winter months, what contributed to increase canopy growth and yield. Taking together, the results suggest that KL was effective in stress alleviation, but the system where the study was carried on determines the level of efficacy. Hereof, it was more effective in older trees under rainfed field conditions than in potted younger trees, certainly a response to the less cloudy days that occurred on rainfed field experiment and to the light redistribution within the denser canopy of older trees.
3. The studies performed increased the knowledge about how physiological and biochemical responses are modulated by SA. The influence of SA in potted plants was concentration-dependent. Among the applied concentrations, 10, 100 and 1000 µM, 100 µM was the most efficient in inducing positive responses. While the influence of 10 µM SA was less noticeable, 1000 µM induced some negative responses. 100 µM SA improved water

status, in line with osmolites accumulation, increased total soluble proteins concentration and ROS detoxification, plant mineral nutrition and photosynthetic activity during drought, contributing also to improve recovery capacity upon rehydration. These responses culminated in higher biomass accumulation at root level. Under rainfed field conditions, 100 µM SA induced a closest response in sprayed trees. In addition to improved plant performance during the stressful summer months, SA also improved the physiological functions during the autumn and winter months, culminating in higher yields. Taking together, the results suggest that SA, by inducing osmotic adjustment and improving metabolites dynamics, was effective in inducing stress tolerance and improving recovery capacity.

**4.** The influence of KL and SA in olive harvests quality was modulated by the stressful conditions severity. Under moderate summer stressful conditions, KL and SA improved antioxidants and antioxidant capacity of olives and olive oil, while under severe stress, KL attenuated the investment in secondary metabolites. Moreover, both products contributed to protect the phenolic compounds against severe frost events during fruit maturation. By other side, the foliar sprays did not significantly affect the oil quality indices regulated by the European Community EEC/2568/91.

**5.** This thesis increased the knowledge about how olive tree responds to drought, revealing new hints that should be considered in agronomic management. Moreover, revealed cost-effective strategies to attenuate the negative effects of summer stress in olive trees. These results are of utmost importance for the management of the rainfed olive orchards, contributing to increase the sustainability and competitiveness of this sector and anticipating solutions to the negative effects of climate change.

# CHAPTER 9

Future Prospects

The research presented in this thesis have raised some interesting questions awaiting further investigation. Hence, were identified several lines of research which should be pursued:

1. Although we showed new hints about the causes and cost-benefits of nighttime stomatal conductance, it is important to confirm and explore new factors that can determine stomatal conductance during the night, such as the "hydraulic activation of stomata". Moreover, urge the necessity to evaluate a continuous response over severe drought periods and subsequent recovery, to confirm the hypothesis that stress severity might determine stomatal conductance behavior. The implications and opportunities derived from nighttime stomatal open also deserve more attention. Besides to confirm the benefits related to respiration rate and to minerals uptake, other lines must be explored. For instance, the implications in global water cycle and in hydraulic redistribution, an important mechanism in olive tree; the possible involvement with direct water uptake via stomata; the role in nighttime sap flux maintenance for oxygen delivery for internal sapwood respiration and/or stem corticular photosynthesis. In fact, much more efforts are needed to understand the effects of this phenomena from plant to ecosystem levels and its impact under a changing world.
2. Regarding KL and SA effects in plants under drought and rewatering events, many different tests and experiments have been left due to lack of time. A deeper analysis of particular mechanisms influenced by those products could improve the understanding about how induce drought resistance, helping to determine the conditions where they might be more suitable. For instance, in addition to the distribution within different plant organs, it might be interesting to evaluate the hormonal levels dynamics during drought and recovery, and a possible crosstalk influence. The study of aquaporins response and leaf hydraulic conductance could help to understand the influence of both products in water relations and in the stomatal conductance dynamics under drought and upon rewatering. Moreover, their influence in mesophyll conductance and Rubisco activity will elucidate about the capacity to supply $CO_2$ to the carboxylation sites and to use it. By other side, the enzymatic antioxidant responses will provide a more comprehensive picture of redox status and stress severity.
3. The positive influence of KL and SA in yield raises the question about their influence in oil content. Moreover, as those products interfered with plant ionome, it might be interesting to evaluate the influence in olive fruit and oil mineral composition.

4. Since KL have aluminium in its composition, ($Al_2Si_2O_5(OH)_4$), to screen a possible contamination of the olive related products it is important to test its concentration in fruits and olive oil and to compare with the limits imposed in the regulation (EEC). As well, the levels of aluminium in soils must be evaluated in long-term trials.
5. A study under filed conditions deserves to be performed for more than two years. In fact, as could be observed in this thesis, the environmental conditions can change from one to another year, making difficult to standardize the arising knowledge. However, due to the lack of time it was unable to repeat the experiment for at least one more year. Still, the general positive influence of KL and SA during the different growing seasons make the results very promising, encouraging to repeat the experiment.
6. The promising results related with KL and SA effectiveness as summer stress mitigating agents also encourages to evaluate its effect in different fruit tree species and cultivars. Moreover, it might be interesting to test their effects in different locations, as in hotter and drier agroecosystems.

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