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The responses of crop and tree species to future elevated [CO_2], temperature and drought stress

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The responses of crop and tree species to future elevated [CO_2], temperature and drought stress

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“Hayatta en hakiki mursit ilimdir, fendir.”

“Our true mentor in life is science.”

M. Kemal Atatürk

“İlim, ilim bilmektir.
İlim kendin bilmektir,
Sen kendini bilmezsen
Bu nice okumaktır.”

“Knowledge should mean a full grasp of knowledge:
Knowledge means to know yourself, heart and soul.
If you have failed to understand yourself,
Then all of your reading has missed its call.”

Yunus Emre

Declaration

I hereby declare that all the material presented for examination in this thesis is my own work and has not been written for me, in whole or in part, by any other person. I also undertake that any quotation or paraphrase from published or unpublished work of another person has been duly acknowledged in the thesis presented for examination at the University of Florence.

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Papers from the Thesis:

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Haworth, M., Hoshika, Y., **Killi, D**. (2016). Has the impact of rising CO_2 on plants been exaggerated? Meta-analysis of free air CO_2 enrichment studies. *Frontiers in Plant Science*, 7: 1153 doi: 10.3389/fpls.2016.01153

Haworth, M., **Killi, D**., Materassi, A., Raschi, A. (2015). Coordination of stomatal physiological behavior and morphology with carbon dioxide determines stomatal control. *American Journal of Botany*, *102*(5), 677-688.

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Killi D., Bussotti F., Acar O., 2014. "Physiological and antioxidant defences of urban trees in the context of climate change", Reactive oxygen and nitrogen species and environment: a new vision for 2020. October 14-17, Baeza, SPAIN.

Killi, D., Raschi, A., Bussotti, F., Haworth, M., 2015. "Photosynthetic Function Linked to Antioxidant Capacity on Drought Tolerant and Vulnerable Varieties of Sunflower and Maize Exposed to Water Deficit and Elevated Temperature. ", 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field, 24-26 June, Verona, ITALY, pp.75-75.

Killi, D., Merdamert, E., Bozyel, M. E., Bussotti, M.E., Raschi, A., Haworth, M., 2015. "Effects of Drought and Elevated Temperature on Chlorophyll-Carotenoid Contents in Drought Tolerant and Sensitive Sunflower and Maize Varieties. ", 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field, 24-26 June, Verona, ITALY, pp.89-89.

Index

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1. INTRODUCTION

Climatic changes associated with the rising atmospheric concentration of carbon dioxide ([CO_2]) are predicted to increase the frequency of extreme climatic events. Drought events are considered likely to increase in frequency, duration and severity in many parts of the world. Many of these drought events will be accompanied by heat-waves – transient increases in temperature above mean levels. Alongside increased atmospheric [CO_2] and mean global temperature, these extreme events will have direct effects on plant physiology, and therefore severe implications for both food security (Schmidhuber and Tubiello, 2007) and the maintenance of biodiversity and ecosystem services in natural and urban forests (Bonan, 2008; Bussotti et al., 2014). This thesis aims to investigate the photosynthetic and protective physiological responses to increased temperature, elevated [CO_2] and drought in Mediterranean crop and tree species. The Mediterranean region is predicted to be particularly at risk of the adverse effects of increased drought and heat-waves (Schär et al., 2004; Vautard et al., 2007). The identification of crop varieties and tree species with traits that confer resistance to these stresses will enable the maintenance of agricultural production and forest stability (Grierson et al., 2011).

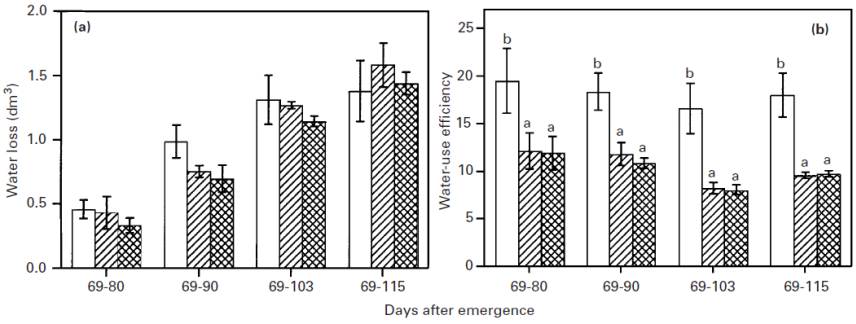

Figure 1 - Total water loss (a) and plant water-use efficiency (b) of drought stressed cherry (*Prunus avium*) seedlings grown in ambient [CO_2] of 350 ppm (shaded with single sloping lines), elevated [CO_2] of 700 ppm (open), or outside control (hatched shading). Data are the mean of 3 plants per treatment. Error bars indicate standard error and the letters (a, b) indicate significant statistical differences at the 5% significance level (ie. $P < 0.05$). From Centritto et al. (1999).

Higher atmospheric [CO_2] is not a stress factor; rather the increased availability of CO_2 is beneficial to plant growth, resulting in CO_2-fertilisation (Haworth et al., 2016c) and improved water use efficiency (WUE) (Figure 1) (Centritto et al., 1999; Ainsworth and Rogers, 2007). This enhanced WUE is the result of reduced transpirative water-loss caused by decreased stomatal conductance (G_s) and higher rates of photosynthesis (P_N) associated with greater availability of CO_2. This can have a positive effect in terms of improved growth and yield (Long et al., 2006) (Figure 2) alongside increased drought tolerance (Centritto et al., 1999; Wall, 2001; Wall et al., 2011). However, while rising [CO_2] may stimulate P_N and WUE under favourable growth conditions, it may also result in reduced stomatal sensitivity (Haworth et al., 2016b). *Fagus sylvatica*, *Castanea sativa* and *Quercus robur* all exhibit an inverse relationship between G_s and leaf to air vapour pressure deficit (VPD) under light conditions when grown at ambient [CO_2]. However, when grown in atmospheres enriched in [CO_2] to 710 ppm, *Q. robur* and *C. sativa* showed reduced stomatal sensitivity to VPD, while beech no longer altered G_s to VPD (Heath, 1998). Growth at elevated [CO_2] did not induce a reduction of G_s in *F. sylvatica*, but also reduced the speed (approximately -25% after four days) and tightness (approximately -22% over a soil water potential range of -200 to -250 hPa) of stomatal closure in response to soil drying. This impaired stomatal control was associated with reduced stomatal sensitivity to the drought stress hormone abscisic acid (ABA) in *F. sylvatica* grown at elevated [CO_2]. Furthermore, an increase in leaf area, alongside the loss of stomatal control incurred at high [CO_2], made the *F. sylvatica* trees more susceptible to drought stress when grown in atmospheres enriched in [CO_2] (Heath and Kerstiens, 1997). A loss of stomatal sensitivity to [CO_2] and ability to close was associated with photosynthetic down-regulation in the crop species *Avena sativa*, *Helianthus annuus*,

Gossypium hirsutum, *Hordeum vulgare* and *Triticum aestivum* when grown in elevated [CO_2] (Haworth et al., 2016b). This may suggest that the impact of elevated [CO_2] with drought and heat stress upon plants may be more complex than previously thought and dependent upon the possible combinations of these stresses and their timings and / or severity.

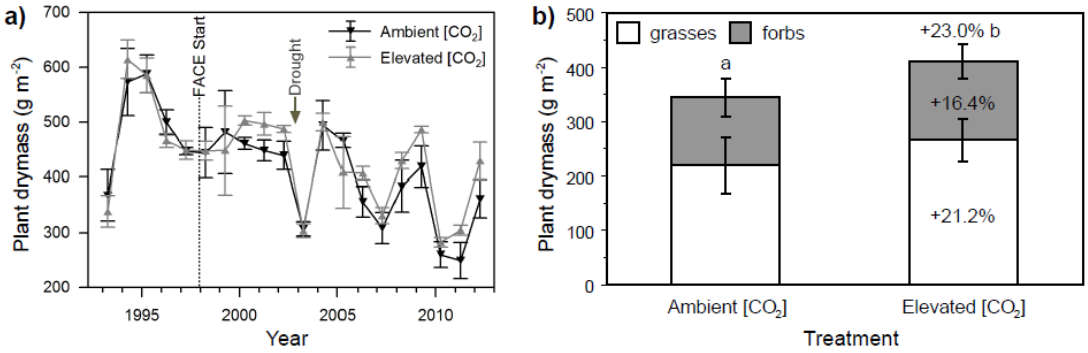

Figure 2 – a) above ground spring-time dry biomass production of the Giessen Free Air CO_2 Enrichment site before and after the instigation of FACE in 1998. b) Dry biomass of vegetation from the spring 2012 harvest. Error bars indicate one standard deviation either side of the mean. Percentage values indicate change after growth at moderate [CO_2] enrichment (480 ppm) relative to ambient [CO_2] (400 ppm). Letters indicate significant difference in total biomass between [CO_2] treatments from a one-way ANOVA and LSD *post-hoc* test. From Haworth et al. (2016c).

Increased temperatures generally have a contrary effect to elevated [CO_2]; reducing P_N relative to photorespiration by decreasing the affinity for CO_2 of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Berry and Björkman, 1980; Jordan and Ogren, 1984), reducing the activity of RubisCO (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000) and damaging the thylakoid membranes resulting in impaired photosynthetic electron transport (Havaux, 1993; Yordanov et al., 1999; Dekov et al., 2001). Higher temperatures also result in increased G_S and water-loss over the short-term; however, over longer time periods, increased temperatures may result in lower G_S as plants adapt to new growth conditions (Centritto et al., 2011). The effect of higher mean annual temperatures (rises of 2.5 to 4.5˚C are predicted for the Mediterranean: Boberg and Christensen

(2012)) will have long-term effects on P_N and growth. This effect of temperature should be considered distinct to 'heat-stress' incurred during heat-waves that are likely to have a more pronounced effect on plant stress.

Drought stress induces a reduction in G_s as plants close stomata to reduce transpirative water-loss (Flexas et al., 2002; Lauteri et al., 2014)(Figure 3). This reduction in G_s is frequently associated with an increase in the flow of abscisic acid (ABA) from the roots to the leaves (Davies and Zhang, 1991), and increased conversion of biologically inactive 'fixed' glucose-conjugated ABA (GE-ABA) in the vacuole to biologically active free ABA within the cytosol of the leaves (Dietz et al., 2000; Seiler et al., 2011). This increased foliar concentration of [ABA] causes stomatal closure (Zhang and Davies, 1990). This causes a reduction in the concentration of CO_2 within the sub-stomatal air space (C_i) resulting in lower availability of CO_2 for P_N (Centritto et al., 2003). As soil dries and drought stress progresses, less energy is utilised for photochemistry (Meyer and Genty, 1999). This energy is dissipated via the protective xanthrophyll cycle in a process known as non-photochemical quenching (Demmig-Adams and Adams, 2000). If drought stress persists for an extended period of time, the mechanisms to dissipate this excess energy become impaired leading to damage to the photosynthetic physiology and disruption to the chloroplast thylakoid membranes where electron transport takes place (Meyer and Genty, 1999). Under water deficit conditions this excess energy can result in the production of cytotoxic reactive oxygen species (ROS) (Pinheiro and Chaves, 2011). Plants possess a range of enzymatic and non-enzymatic anti-oxidant systems to detoxify these ROS (Türkan et al., 2005; Aganchich et al., 2009; Agati et al., 2009). These anti-oxidant cycles can also become overwhelmed during severe drought stress

leading to a loss of biochemical function and impaired P_N (Pinheiro and Chaves, 2011).

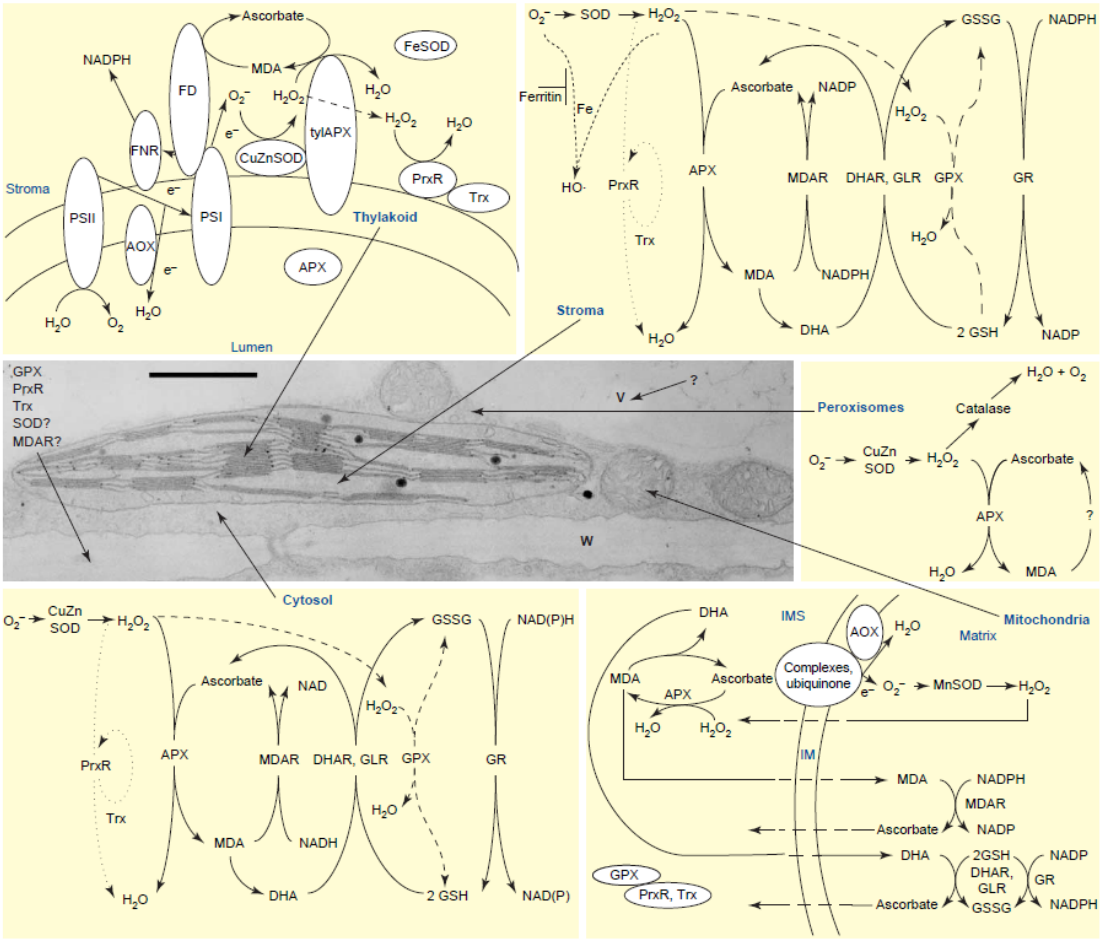

Figure 3 – Localization of reactive oxygen species (ROS) scavenging pathways in plant cell. The enzymatic pathways responsible for ROS detoxification are shown (from Mittler, 2006)

Drought (Flexas and Medrano, 2002; Kalaji et al., 2016) and heat (Jiang and Huang, 2001; Sekmen et al., 2014) stress induce oxidative damage to the thylakoid membranes where PSII occurs (Gounaris et al., 1983). This oxidative damage results in lipid peroxidation and a loss of structure and function in the thylakoid membranes (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000; Crafts-Brandner and Salvucci, 2002). To protect and stabilise the thylakoid membrane where PSII electron transport occurs, plants possess protective antioxidant mechanisms (Reddy et al.,

2004; Pinheiro and Chaves, 2011). A summary of plant antioxidant systems is provided in Figure 3. Glutathione reduces harmful ROS within the plant cell to produce glutathione disulphide which is then converted by glutathione reductase (GR) into glutathione and hydrogen peroxide (H_2O_2) (Foyer and Halliwell, 1976; Das and Roychoudhury, 2014). Superoxide dismutase (SOD) also reduces cytotoxic O_2^- to form H_2O_2. Hydrogen peroxide is then converted to water and oxygen by catalase (CAT) in peroxisomes, peroxidase (POX) in the chloroplast envelope and ascorbate peroxidase (APX) in the cytosol and chloroplast (Smirnoff, 1993; Das and Roychoudhury, 2014). Plants with increased tolerance of drought (Türkan et al., 2005) and heat (Sekmen et al., 2014) stress generally possess higher levels of antioxidant activity than their sensitive counterparts.

The effects of drought and increased temperature on plants have been studied extensively in isolation (eg. Berry and Björkman, 1980; Bunce, 2000; Centritto et al., 2009; Pinheiro and Chaves, 2011). However, despite commonly occurring together in agricultural and natural settings, their combined effects have been studied experimentally less frequently. Heat stress should in theory exacerbate the impact of drought stress through increased transpirative water-loss (as a result of higher leaf to air vapour pressure deficit – VPD) and increasing the energy balance of leaves resulting in greater damage to thylakoid membranes via enhanced generation of ROS (Kipp and Boyle, 2013). However, the biochemical pathways utilised by plants to heat and drought stress are largely distinct (Rizhsky et al., 2002; Rizhsky et al., 2004) and physiological and morphological adaptation to growth at high temperature (Centritto et al., 2011) may enable greater tolerance of the adverse effects of growth under water deficit conditions.

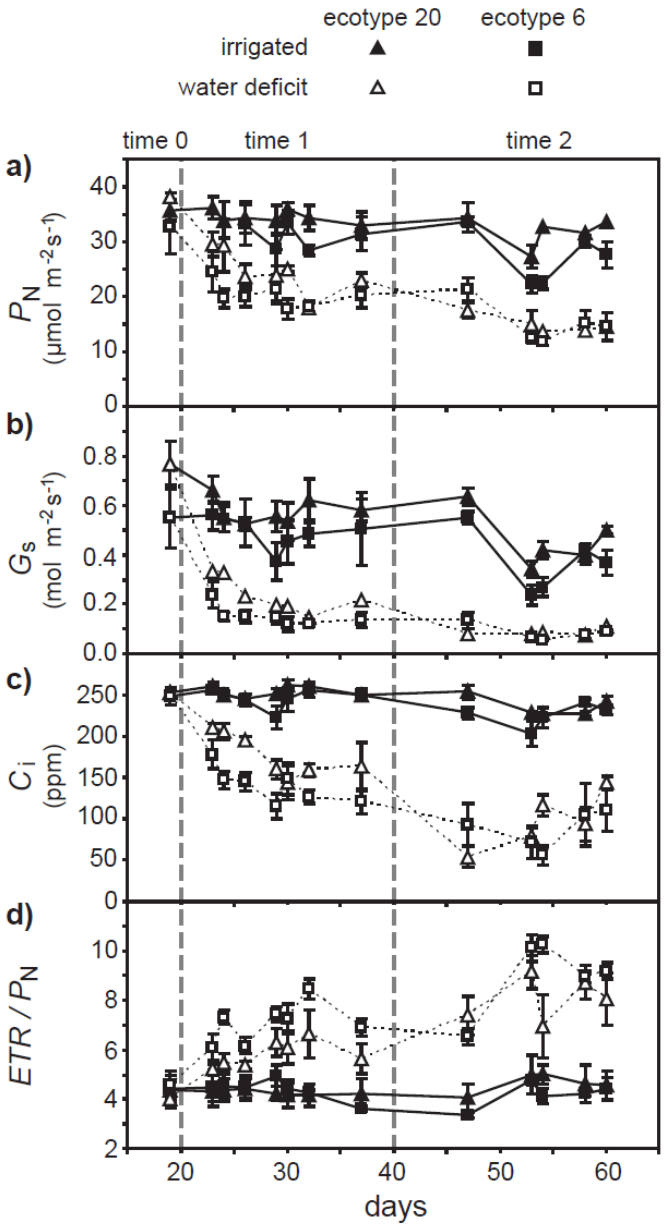

Figure 4 – Photosynthesis (P_N) (a), stomatal conductance (G_s) (b), internal sub-stomatal [CO_2] (C_i) (c) and the ratio of the electron transfer rate to photosynthesis (ETR/P_N) (d) of two *Arundo donax* ecotypes 6 (square symbols) and 20 (triangle symbols) under irrigated (solid symbols, solid line) and drought conditions (open symbols, dashed line). Error bars indicate one standard error either side of the mean. From Haworth et al. (2016a).

To investigate the differential effects of rising [CO_2], increased temperatures and drought stress on plants we undertook two experiments. The first involved analysis of the photosynthetic, protective and morphological responses to drought and a 10˚C increase in temperature (a simulated heat-wave) of C3 (*Helianthus annuus*) and C4 (*Zea mays*) crop varieties with contrasting drought tolerance. The second experiment involved exposing two *Quercus* species (*Quercus cerris* and *Quercus ilex*) that are

widespread in Mediterranean native and urban forests to growth under conditions predicted to occur in 2050 (ie. +2.5 ˚C and [CO_2] levels of +150-200 ppm relative t current ambient concentrations of 400 ppm). These related studies provide insights into the likely impact of climate change on plant ecophysiology and stress response in the Mediterranean region and beyond over the next 50 to 100 years. The project is split into three chapters, each representing a paper for publication in a peer reviewed scientific journal:

- Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance
- Lipid peroxidation and chlorophyll fluorescence of photosystem II performance during drought and heat stress is associated with the antioxidant capacities of C3 sunflower and C4 maize varieties
- Photosynthetic and morphological responses of oak species to temperature and [CO_2] increased to levels predicted for 2050

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2. AIMS

The presented PhD research was conducted for the following purposes:

1) To understand the physiological and morphological responses of drought tolerant and sensitive C3 sunflower and C4 maize varieties to a combination of heat and drought stress; since the effects of this combination of stress factors on crop species are largely absent in the literature.

2) To assess the influence of heat and drought stress on chlorophyll a fluorescence (ChlF) indicators of the maize and sunflower varieties with differing drought tolerance.

3) To describe the antioxidant response activity of the maize and sunflower varieties to drought and heat stress to elucidate stress tolerance mechanism to both stress factors and the combination.

4) To investigate physiological and morphological responses of different Oak species (*Quercus ilex* and *Quercus cerris*) to elevated [CO_2] and temperature predicted for 2050, to understand which species is more adapted to future climatic conditions.

"If we knew what it was we were doing,
it would not be called research, would it?"
Albert Einstein

Paper I

Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance

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Author contributions:

□ Designed and conducted the experiment: DK, MH

□ Provided equipment and consumables: FB, AR

□ Wrote the paper, analysed the data: DK

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Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance

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Heat and drought stress frequently occur together, however, their impact on plant growth and photosynthesis (P_N) is unclear. The frequency, duration and severity of heat and drought stress events are predicted to increase in the future, having severe implications for agricultural productivity and food security. To assess the impact on plant gas exchange, physiology and morphology we grew drought tolerant and sensitive varieties of C3 sunflower (*Helianthus annuus*) and C4 maize (*Zea mays*) under conditions of elevated temperature for 4 weeks prior to the imposition of water deficit. The negative impact of temperature on P_N was most apparent in sunflower. The drought tolerant sunflower retained ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity under heat stress to a greater extent than its drought sensitive counterpart. Maize exhibited no varietal difference in response to increased temperature. In contrast to previous studies, where a sudden rise in temperature induced an increase in stomatal conductance (G_s), we observed no change or a reduction in G_s with elevated temperature, which alongside lower leaf area mitigated the impact of drought at the higher temperature. The drought tolerant sunflower and maize varieties exhibited greater investment in root-systems, allowing greater uptake of the available soil water. Elevated temperatures associated with heat-waves will have profound negative impacts on crop growth in both sunflower and maize, but the deleterious effect on P_N was less apparent in the drought tolerant sunflower and both maize varieties. As C4 plants generally exhibit water use efficiency (WUE) and resistance to heat stress, selection on the basis of tolerance to heat and drought stress would be more beneficial to the yields of C3 crops cultivated in drought prone semi-arid regions.

Abbreviations – ABA, abscisic acid; FTSW, fraction of transpirable soil water; NPQ, non-photochemical quenching; PAR, photosynthetically active radiation; PSI, photosystem I; PSII, primary photochemistry/photosystem II (PSII); RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SM, drought sensitive maize variety; SS, drought sensitive sunflower variety; SWC, soil water content; TM, drought tolerant maize variety; TPU, triose phosphate utilisation; TS, drought tolerant sunflower variety; WUE_b, dry biomass change relative to water use; WUE_i, instantaneous water use efficiency.

Introduction

Climate change is predicted to induce an increase in the severity and duration of drought events in many regions. These droughts will often be accompanied by heat-waves – transient increases in temperature above normal levels lasting from days to weeks (Schär et al. 2004). This combination of water deficit and heat stress will have profound implications for food security through effects on crop growth and water-use (Battisti and Naylor 2009, Deryng et al. 2014, Stratonovitch and Semenov 2015). More than 70% of global arable land is rain-fed (Biradar et al. 2009). Temporal and/or spatial variations in precipitation associated with climatic change (Dore 2005) alongside the converging pressures of population growth and increased urbanisation/industrialisation will reduce the availability of fresh-water for irrigation (WWAP 2012). Analysis of the photosynthetic and growth responses of different varieties/species to drought and increased temperature will enable the identification and characterisation of traits that confer high productivity and stress resistance towards maintaining crop production under likely future growth conditions (e.g. Yeh et al. 2012, Lauteri et al. 2014).

The impacts of water deficit or temperature alone on plants have been extensively studied (e.g. Berry and Björkman 1980, Bunce 2000, Pinheiro and Chaves 2011). However, drought and heat stress often occur simultaneously in agricultural systems, and their combined effects on plant photosynthesis and growth are less well understood. As soil dries, the flow of abscisic acid (ABA) from the roots to the shoots increases (Davies and Zhang 1991) and within the leaves glucose-conjugated ABA stored within the vacuole is converted to free-ABA (Seiler et al. 2011). This increase in ABA induces stomatal closure to reduce transpirative water-loss (Pinheiro and Chaves 2011, Haworth et al. 2016b). The decline in stomatal (G_s) (Tardieu and Davies 1992) and mesophyll conductance (Sorrentino et al. 2016) associated with higher ABA concentration lowers the availability of CO_2 at the site of carboxylation resulting in a reduction in rates of net photosynthesis (P_N) and growth (Centritto et al. 2003). As the availability of water decreases, the proportion of light energy utilised by plants for photochemistry declines (Meyer and Genty 1999). This results in an increase in the dissipation of excess energy as heat via non-photochemical quenching (NPQ) (Demmig-Adams and Adams 2000). Over time, as drought stress develops and becomes more severe this can result in the degradation of the photosynthetic physiology through the generation of reactive oxygen species and disruption to the integrity of thylakoid membranes (Conroy et al. 1988, Fredeen et al. 1991, Türkan et al. 2005).

The effect of temperature on plants is dependent upon the pre-existing temperature acclimation and the type of photosynthetic physiology (Berry and Björkman 1980). As temperature rises, the rate of photorespiration relative to photosynthesis increases due to reductions in the solubility of CO_2 and affinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) for CO_2 relative to O_2 (Jordan and Ogren 1984). Furthermore, as temperature rises above 30°C the activation of RubisCO in the light declines, reducing P_N and necessitating an increase in the dissipation of excess light energy as heat (Feller et al. 1998, Law and Crafts-Brandner 1999). Nonetheless, any reduction in RubisCO activity at high temperatures would also have a corresponding effect on rates of photorespiration (Havaux et al. 1987), possibly reducing available electron sinks during heat stress (Havaux 1993, Sinsawat et al. 2004). The accumulation of oxaloacetic acid within the bundle sheath in C4 plants effectively concentrates CO_2 at the site of carboxylation and suppresses photorespiration. This allows C4 plants to maintain P_N at higher temperatures than their C3 counterparts, where the impact of impaired carboxylation efficiency is more apparent (Crafts-Brandner and Salvucci 2000, Crafts-Brandner and Salvucci 2002). Photosystem II (PSII) is generally considered to be more susceptible to the effects of heat stress than PSI (Havaux 1993). High temperature stress results in disruption of the thylakoid membranes. This effect is apparent as reductions of both the maximum (F_v/F_m) and actual (ΦPSII) quantum yields of PSII and concomitant increases in NPQ (Feller et al. 1998, Yordanov et al. 1999, Crafts-Brandner and Salvucci 2002). However, plants acclimated to higher temperatures exhibit greater PSII function during temperature stress (Havaux 1993), possibly due to an increase in protective proteins within the chloroplast (Heckathorn et al. 1998).

As temperature rises, stomata generally open and G_s increases (Heath and Meidner 1957, Drake et al. 1970, Raschke 1970, Schulze et al. 1975). However, under high leaf-to-air vapour pressure deficit (Schulze et al. 1974) or water deficit (Schulze et al. 1973) stomata do not open to the same degree as temperature increases. Nonetheless, Chinese lyme grass [*Leymus chinensis* (Trin.) Tzvel] grown at four levels of water deficit exhibited consistently greater G_s at higher temperatures irrespective of water availability (Xu and Zhou 2006). The combined influence of drought and high temperature stress on plants may be additive in their effects. The negative effect of drought on P_N, G_s and yield were more pronounced at higher temperatures in wheat (*Triticum aestivum*) (Shah and Paulsen 2003). Heat stress

also exacerbated the degradation of the thylakoid membrane and loss of PSII function in Chinese lyme grass (Xu and Zhou 2006). Hydroponically grown sunflower and maize exposed to physiological water-deficit and high temperature stress for 2 days showed reductions in P_N. High temperature and water deficit stress induced disruption to the integrity of cellular membranes. This damage was exacerbated when the stresses were combined, and more apparent in the C3 sunflower that exhibited lower foliar relative water content (RWC) than the C4 maize (Dekov et al. 2001); possibly due to the ability of C4 plants to maintain electron transport for photochemistry at higher temperatures (Crafts-Brandner and Salvucci 2002) and greater water use efficiency (WUE) than C3 plants (Sinclair et al. 1984). However, black poplar (*Populus nigra*) grown at 25 and 35°C for 2 months prior to the imposition of water deficit exhibited reduced G_s values before and during drought stress (Centritto et al. 2011). The contrast in these results may suggest that plant acclimation to a specific temperature may shape the extent of any stomatal response to drought stress.

Transcriptome analysis of *Arabidopsis thaliana* (L.) indicates that expression of comparatively few transcripts is altered under both heat stress and water deficit (Rizhsky et al. 2004). This may suggest that the response pathways elicited under drought and heat stress are largely distinct, despite the apparent frequency with which these stresses occur simultaneously. The identification of heat and drought tolerant crop varieties is of critical importance to ensuring food production (Lobell et al. 2013, Semenov et al. 2014). To investigate the potential impact of combinations of heat and drought stress on crop physiology, we exposed C3 sunflower and C4 maize to water deficit at two temperature regimes (25/20°C and 35/30°C). Drought tolerant and vulnerable varieties of each crop were chosen on the basis of observations of yield from field trials. Sunflower and maize are grown widely in regions predicted to experience increased drought and heat-wave events in the future (Schär et al. 2004, Lobell et al. 2011, Lobell et al. 2013). Varieties of sunflower (Virgona et al. 1990, Panković et al. 1999) and maize (Bolaños et al. 1993) have been shown to exhibit contrasting responses to drought. The quantitative trait loci regulating P_N, stomatal control and plant water status are located in adjacent regions of the genomes of sunflower (Hervé et al. 2001) and maize (Tuberosa et al. 2002), possibly indicating the occurrence of selective pressures favouring optimisation of WUE.

We hypothesise that: (1) increased temperature will exacerbate the impact of drought stress in both sunflower and maize, but that the effect will be less in the C4 species and (2) observations of increased tolerance to water deficit in the drought tolerant varieties may be associated with increased stomatal sensitivity to soil drying. This study aims to investigate the effect of heat stress and water deficit and their combination on drought tolerant and sensitive varieties of sunflower and maize to: (1) identify physiological traits associated with resistance to the deleterious effects of high temperature and water stress, (2) characterise the stomatal and photosynthetic responses of C3 (sunflower) and C4 (maize) crops with contrasting degrees of drought tolerance to soil drying and the effect of temperature on the kinetics of the drought response and (3) explore the implications of drought and heat-waves on the physiology and growth of these crops, and the potential for selection of varieties that are resistant to these stresses through phenotyping based upon physiological attributes.

Materials and methods

Plant growth conditions

The sunflower (*Helianthus annuus*) and maize (*Zea mays*) varieties were classified as drought tolerant or sensitive on the basis of observations of the effect of water deficit on yield in field trials. However, no detailed physiological analysis on the plants has been undertaken prior to this study. The drought tolerant (TS) (var. Bosfora) and sensitive (SS) (var. 08 TR 003) varieties of sunflower were provided by the Trakya Agricultural Research Institute, Edirne, Turkey. The drought tolerant (TM) (var. ADA-9516) and sensitive (SM) (var. ADA-523) varieties of maize were supplied by the Sakarya Maize Research Institute, Sakarya, Turkey. The seeds were germinated in trays of sand supplied with a commercially available nutrient solution (COMPO Concime Universale, NPK 7-5-7, B, Cu, Fe, Mn, Mo, Zn: COMPO Italia, Cesano Maderno, Italy) within a plant growth room with a day/night temperature of 28/24°C. Metal-halide lights were used to maintain a photosynthetically active radiation (PAR) of 800 μmol m^{-2} s^{-1} for 14 hours (h) each day. After 2 weeks, the plants were potted into square 9 l pots filled with a 90% sand to 10% v/v commercial compost mixture and placed into two large walk-in growth rooms with full control of light, temperature, [CO_2] (both chambers maintained ambient atmospheric [CO_2] of 400 ppm) and humidity (both chambers maintained a relative humidity of 50% to minimise the impact of variation in leaf to air vapour pressure deficit on stomatal behaviour) (for full technical details of the plant growth chambers see Materassi et al. 2005). Measurement of P_N/C_i response curves were performed 20 days after the plants were placed in the growth chambers. The plants were watered to pot capacity every 2 days and provided weekly with a commercial

liquid plant fertiliser (COMPO Concime Universale) to facilitate nutrient availability at free access rates. The growth chambers maintained conditions of 16 h of daylight (14 h at full PAR levels of 1000 μmol m^{-2} s^{-1} with two 1-h periods of simulated dawn/dusk where light intensity was incrementally increased/decreased). One chamber operated a day/night time temperature regime of 25/20°C (hereafter referred to as 25°C treatment) and the second chamber operated a day/night temperature of 35/30°C (hereafter referred to as 35°C treatment). Changes in temperature followed those of PAR, with a 1-h ramping period at dawn/dusk. To avoid any potential chamber effects the growth rooms were alternated every week – no significant differences were observed in the measurements conducted under the same conditions in different growth chambers. The plants were grown for 4 weeks in the respective growth chambers prior to the imposition of drought stress to allow acclimation to the growth temperatures. After 4 weeks of acclimation to the growth conditions within the chambers, the drought treatment was instigated in half of the 6-week-old plants for a further 3 weeks until the end of the experiment.

Drought treatment and plant gas exchange

The fraction of transpirable soil water (FTSW) method (Sinclair and Ludlow 1986) was used to gauge the drought kinetics to soil drying of the drought tolerant and sensitive varieties of maize and sunflower. On the evening prior to the imposition of drought all of the plants were watered to pot capacity. The pots were then allowed to drain overnight and then weighed the next morning and sealed within plastic bags to eliminate evaporation from the soil. G_s of the plants was then recorded and assumed to represent 100% of potential G_s. Ninety-six plants (four different treatment × four replication for each treatment × two varieties × three sampling point) of each species were used in total, with half being allowed to dry and the other half maintained at pot capacity in both chambers (25 and 35°C), with the amount of water lost as transpiration replaced each day for the well-watered/control plants. The pots were weighed each day and G_s recorded every second day (alternating between chambers). When G_s fell to 10% of the starting G_s value or pot weight remained constant for 3 days it was considered that all water within the soil available for transpiration had been exhausted (i.e. 0% FTSW), and FTSW was calculated as:

$$\mathrm{FTSW} = \frac{\left(\mathrm{PW_{daily}} - \mathrm{PW_{final}}\right)}{\left(\mathrm{PW_{initial}} - \mathrm{PW_{final}}\right)}$$

where PW is the pot weight in grams. To prevent the soil becoming anoxic, the plastic bags were opened each day for approximately 10 min to permit the exchange of gases between the soil and the atmosphere. The soil water content (SWC) of the pots was also measured in this 10-min period using a FieldScout time domain reflectometry 100 Soil Moisture Meter (Spectrum Technologies, Aurora, IL). Values of SWC were correlated to FTSW for presentation in Figs 1 and 2; this varied slightly between species and varieties and so calibrations differ between plants and temperature treatments.

A PP-Systems Ciras-2 attached to a PLC6(U) leaf cuvette and LED light unit (PP-Systems, Amesbury, MA) was used to measure the gas exchange parameters of the plants. Four replicate plants were analysed for each species and variety in each of the temperature and drought treatments. The second fully expanded leaf from the flag leaf in maize and the second uppermost fully expanded leaf in sunflower were consistently used for gas exchange measurements. The leaf cuvette was set to the respective temperature of the plant growth chambers, external [CO_2] (C_a) was maintained at 400 ppm, vapour pressure deficit ranged from 1.5 to 1.8 kPa and light intensity was 2000 μmol m^{-2} s^{-1}. Instantaneous water use efficiency (WUE_i) was calculated as the ratio of P_N to evapotranspiration. Gas exchange measurements were conducted from 08:00 to 12:00 h. After 4 weeks of growth at the respective temperatures in the plant growth chambers, the response curve of P_N to increasing internal sub-stomatal [CO_2] (C_i) under a saturating light intensity of 2000 μmol m^{-2} s^{-1} (C_a sequence of 350, 250, 150, 50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 ppm) at a standard cuvette temperature of 25°C was recorded on four replicate plants for each variety and temperature treatment. It was not possible to record P_N/C_i response of drought stressed plants at the end of the experiment as rapid stomatal closure (Haworth et al. 2015) prevented biochemical limitations to CO_2 uptake being distinguished from diffusive constraints (Centritto et al. 2003). The maximum carboxylation rate of RubisCO (Vc_{max}), the maximum rate of electron transport for regeneration of ribulose-1,5-bisphosphate (J_{max}) and triose phosphate utilisation (TPU) were calculated from the P_N/C_i response curves following Sharkey et al. (2007). At the end of the drought stress at FTSW levels below 10%, the Kok method (Kok 1948) was used to estimate respiration in the light (R_d) by decreasing PAR at low levels of intensity (400, 300, 200, 150, 100, 75, 50, 30, 20 and 10 μmol m^{-2} s^{-1}) in both well-watered and drought stress plants. Respiration in the dark (R_n) was measured by switching off the LED light unit after the Kok protocol, shading the plant and recording the rate of CO_2 efflux from the leaf after values had remained stable for 5–10 min. Both R_d and R_n were determined at the

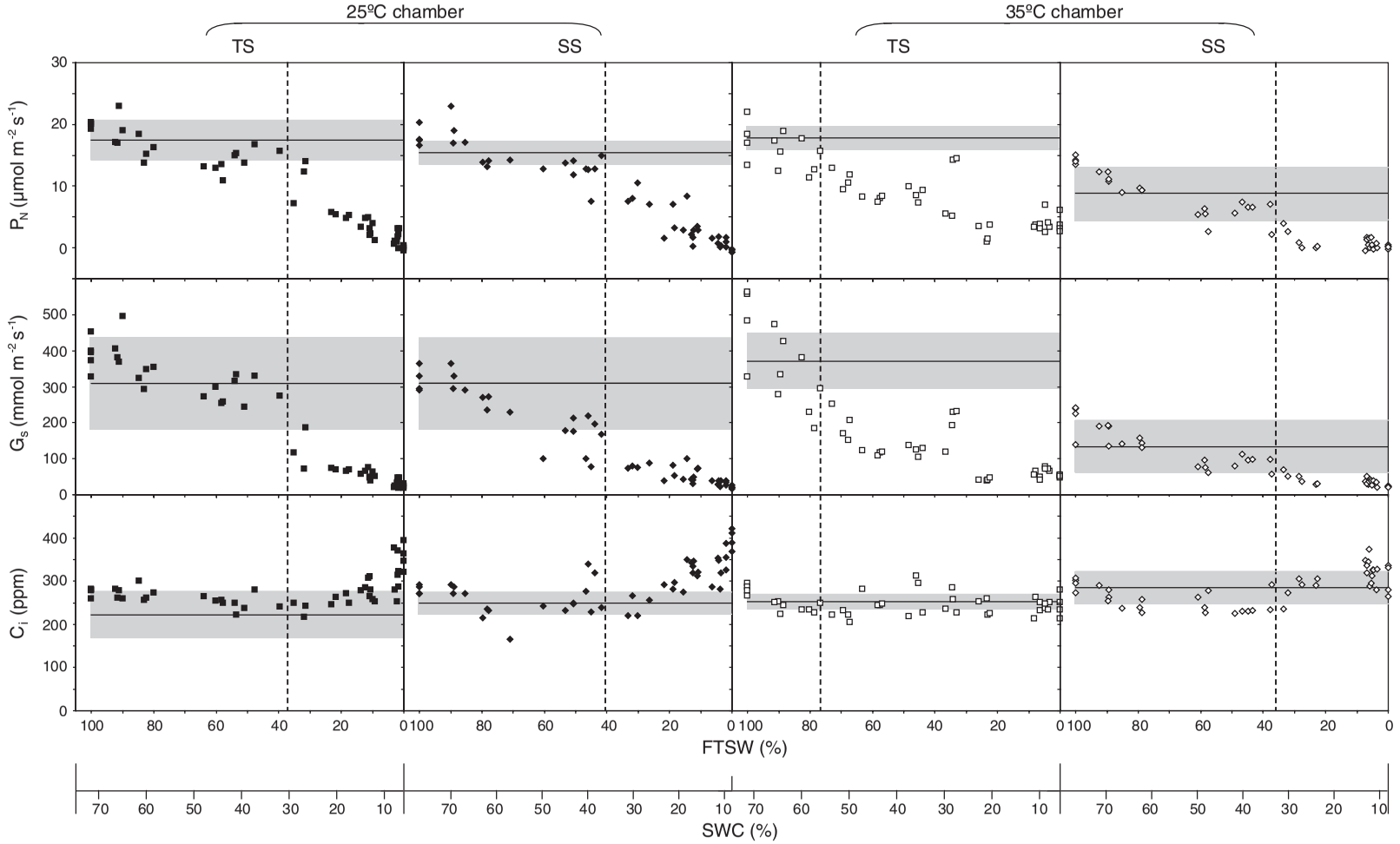

Fig. 1. The response of P_N, G_s and C_i in drought tolerant (TS) (square symbols) and sensitive (SS) (diamond symbols) varieties of sun flower to soil drying at 25°C (solid symbols) and 35°C (open symbols). Each data point represents a measurement from an individual plant exposed to water deficit. Data are plotted against the FTSW and a calibration between FTSW and SWC. The solid horizontal line indicates the mean value of the well-watered control plants (n = 4) during the experimental period and the grey shading indicates ±standard deviation either side of the mean.

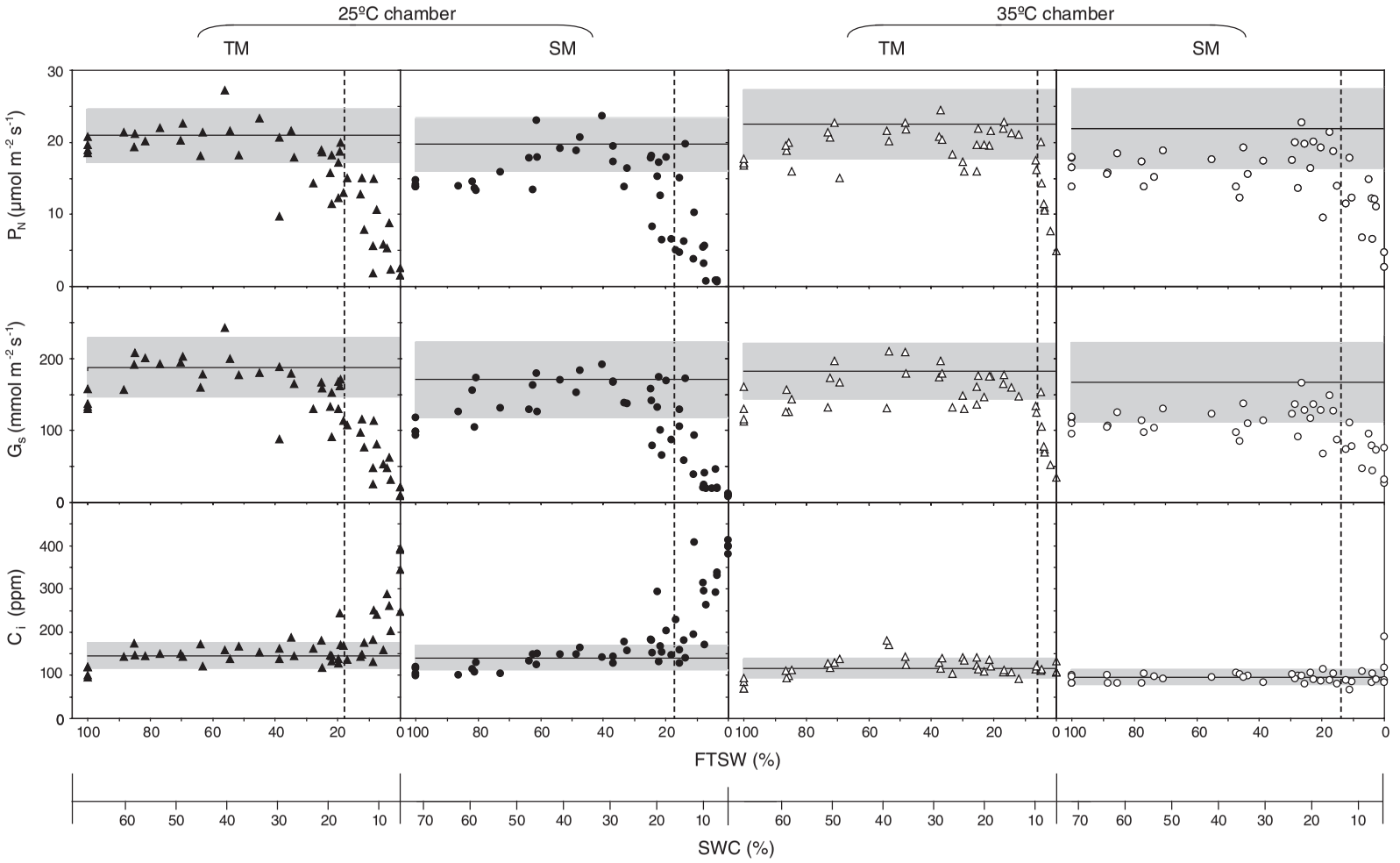

Fig. 2. The response of P_N, G_s and C_i in drought tolerant (TM) (triangle symbols) and sensitive (SM) (circle symbols) varieties of maize to soil drying at 25°C (solid symbols) and 35°C (open symbols). Data presented as in Fig. 1.

growth temperature of the plants in the controlled environment chambers. The effect of variation in C_i on R_d was corrected using the method of Kirschbaum and Farquhar (1987). Also at the end of the drought stress, the F_v/F_m, ΦPSII (ΔF/F′m) and NPQ were recorded using a modulate fluorimeter FMS-2 (Hansatech, Norfolk, King's Lynn, UK) (saturating pulse of 10 000 μmol m^{-2} s^{-1}) and dark adaptation clips after 30 min of dark adaptation and exposure to actinic light of 1000 μmol m^{-2} s^{-1} for a minimum of 10 min after the first saturating pulse (Genty et al. 1989, Maxwell and Johnson 2000).

Morphological analyses

Plant height was measured every 2 days during the course of the experiment. Before the onset of soil drying (time 0) four plants were destructively sampled from each species/variety in both temperature and drought treatments. Fresh leaf area was recorded using a LiCor Li3100C (Li-Cor, Inc., Nebraska, USA). Above and below ground dry biomass was determined after drying stems, leaves and roots in an oven at 60°C for 4 days. This was repeated when G_s values had declined by 50% (time 1) and at the end of the drought stress (time 2). The RWC of leaves from the sunflower and maize varieties was also sampled at these stages of the experiment. The second fully expanded leaf from the flag leaf in maize and the second fully expanded leaf from the top of the sunflower plant were used for determination of RWC using the approach of Diaz-Pérez et al. (1995).

Statistical analyses

Statistical analyses were performed using SPSS 20 (IBM, New York, NY). A one-way ANOVA with an LSD post hoc test was used to assess differences in variance between samples. Linear regression was used to investigate the relationship between P_N and G_s, and ANCOVA was used to test differences between the regression coefficients.

Results

Soil drying under two temperature regimes induced contrasting responses between the drought sensitive and tolerant varieties of C3 sunflower and C4 maize. At 25°C drought sensitive sunflower (SS) and tolerant sunflower (TS) varieties exhibited identical P_N, G_s and C_i responses to soil drying (Fig. 1). However, this similarity between the two sunflower varieties (TS and SS) in their response to drought did not occur at the higher temperature. At 35°C the TS exhibited greater leaf-level rates of P_N and G_s than the SS variety. Under well-watered conditions at 35°C the SS variety exhibited 42.8 and 56.7% respective reductions in P_N and G_s in comparison to growth at 25°C. In the TS, mean G_s over the duration of the soil drying period was 19.9% higher in the well-watered plants, while P_N was unaffected when grown at the higher temperature. However, at 35°C as soil dried the TS variety exhibited reductions in G_s at lower levels of soil water availability. In contrast, although lower at 35°C, G_s in the SS variety fell below the average level of the well-watered plants at similar FTSW values to plants at 25°C (Fig. 1). Growth at 25 and 35°C did not affect the P_N (one-way LSD ANOVA $F_{3,172} = 2.311$; $P = 0.0780$) or G_s (one-way LSD ANOVA $F_{3,172} = 1.481$; $P = 0.221$) of drought tolerant (TM) or sensitive maize (SM) varieties under well-watered conditions. It is noteworthy that the decline in G_s values of the TM occurred at lower FTSW levels at 35°C than 25°C (Fig. 2). Moreover, C_i values of both maize varieties (TM and SM) did not increase at lower levels of FTSW when grown at 35°C, whereas at 25°C an increase in C_i, indicative of damage to the photosynthetic physiology (Flexas et al. 2002) occurred at 10 and 20% FTSW in the TM and SM varieties, respectively.

At the end of the soil drying period, leaf-level rates of P_N were slightly lower at 35°C than 25°C in sunflower and maize plants under well-watered conditions, with this reduction more apparent in the drought sensitive varieties (Fig. 3). G_s of the TS was not affected by an increase in temperature, whereas the SS variety exhibited a 42.0% reduction in G_s under well-watered conditions. Rates of P_N and G_s were identical in the drought stressed sunflower varieties at 25 and 35°C. However, the maize plants exhibited lower reductions in P_N and G_s under water deficit at 35°C than occurred at 25°C (Fig. 3). WUE_i measured using gas exchange was generally lower at the higher temperature in both species, indicating lower carbon gain relative to water-loss on a leaf-area basis. Drought tolerant sunflower (TS) under well-watered conditions exhibited the highest level of WUE_i; however, when WUE was gauged as dry biomass change relative to water use (WUE_b) over the duration of the study, the C4 maize exhibited five- to sixfold greater WUE_b under well-watered and drought stressed conditions at both temperatures. Furthermore, WUE_b in maize during drought stress was not influenced by growth at 35°C (Fig. 3). This higher WUE and the greater retention of WUE at the higher growth temperature in maize may be evident in the relationship between P_N and G_s (Fig. 4). This relationship was steeper in the C4 plant (i.e. rates of $P_N > 20$ μmol m^{-2} s^{-1} were achieved at G_s levels of approximately 200 mmol m^{-2} s^{-1}) and did not become less steep when grown at 35°C. In contrast, equivalent rates of P_N in sunflower occurred at G_s levels above 350 mmol m^{-2} s^{-1} and were not achieved in the drought

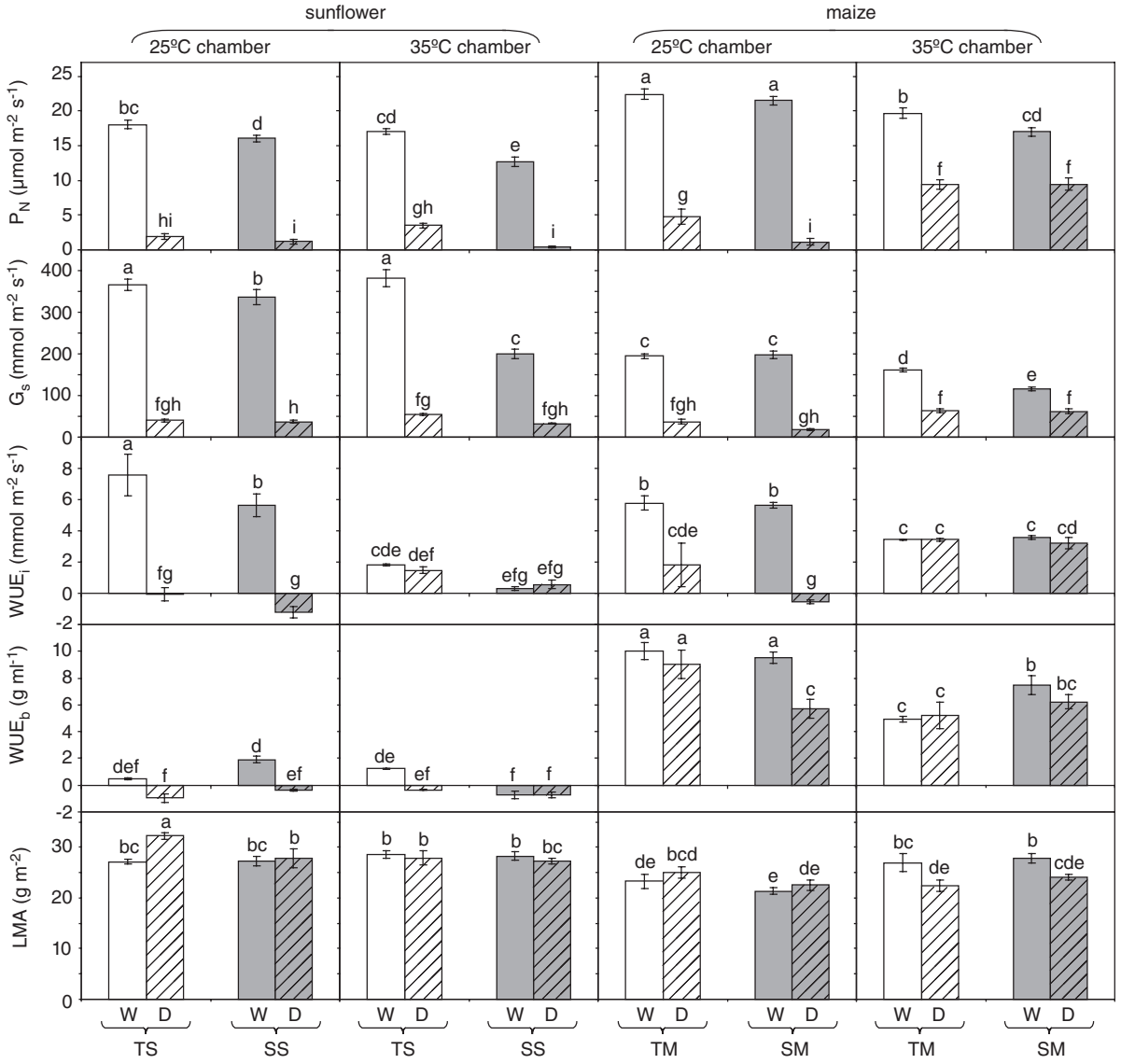

Fig. 3. P_N, G_s, WUE_i measured using gas exchange, WUE_b, and leaf mass per area (LMA) of drought tolerant (white) and sensitive (grey) varieties of sunflower (TS and SS) and maize (TM and SM). Gas exchange measurements represent the average of values collected at FTSW levels below 10% in drought stressed plants (hatched) and the corresponding well-watered control plants (open) measured during the same period. Error bars indicate one standard error either side of the mean (n = 4). Letters indicate homogenous groups between all of the varieties of both species determined using a one-way ANOVA and LSD post hoc test.

sensitive variety at 35°C. Nonetheless, the relationship between P_N and G_s was unaffected by growth at 35°C in both varieties of sunflower (TS and SS). The Vc_{max} was 25.5% greater in the SS than the TS variety at 25°C. However, at 35°C the SS exhibited a 61.1% reduction in Vc_{max} whereas its drought tolerant counterpart (TS) experienced a non-significant 19.9% decrease (Table 1). The J_{max} of the two sunflower varieties (TS and SS) showed a similar response to growth at 25 and 35°C; with no significant change observed in the J_{max}:Vc_{max} ratio. The SS variety showed a significant reduction of TPU at the higher temperature, consistent with a reduction of the efficiency of the transport of photosynthate out of the chloroplast envelope (Ellsworth et al. 2015), while TPU in the TS variety was unaffected. The Vc_{max} and J_{max} were unaffected by growth at 35°C in either of the maize varieties. The SM exhibited a significant 7.1% reduction in TPU at the higher growth temperature.

R_d was lower in well-watered sunflower grown at 35°C, while R_n was unaffected by temperature. Water

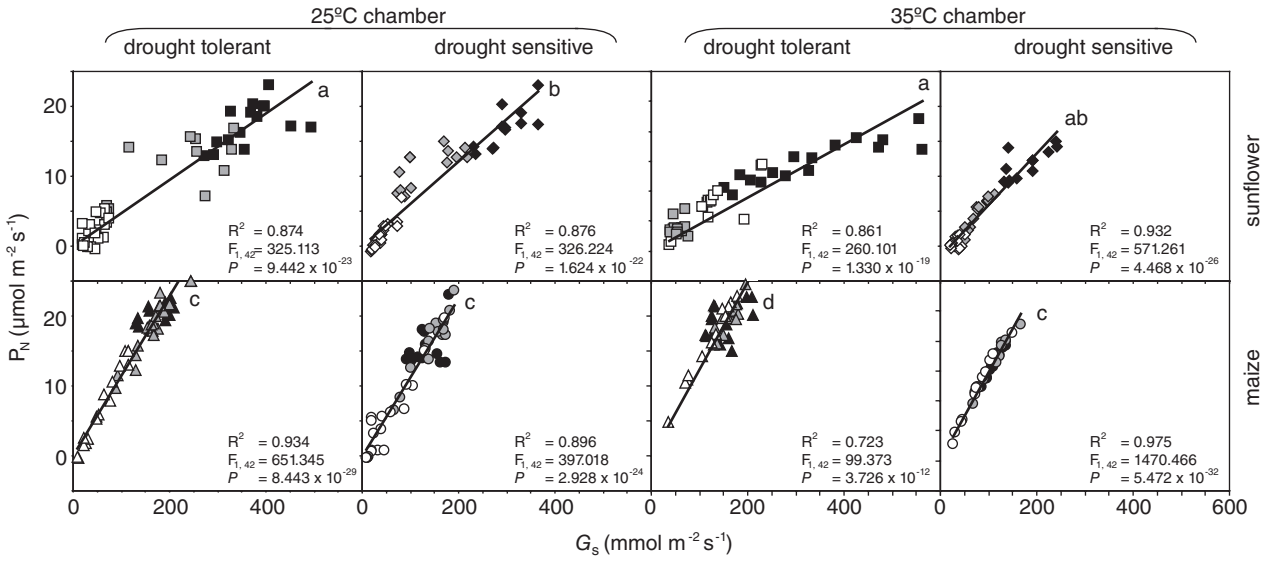

Fig. 4. The relationship between P_N and G_s in drought tolerant and sensitive varieties of sunflower and maize exposed to soil drying at 25 and 35°C. Symbol shapes as in Fig. 1. Solid symbols indicate >60% FTSW; grey symbols indicate <60–>20% FTSW, and; open symbols indicate <20% FTSW. R^2, F and *P* values and the best fit (solid line) given in figure panels were determined by linear regression. Regression coefficients with identical letters are statistically identical using ANCOVA (species/variety $F_{7,361} = 100.126$, $P = 8.255 \times 10{-}80$; temperature $F_{1,361} = 4.108$, $P = 0.0434$).

deficit induced respective 67.9 and 61.1% reductions in R_d values of TS and SS varieties. Soil drying did not affect R_n in the TS, but led to a significant 34.9% reduction in the SS at 25°C. At 35°C, drought did not affect R_d and R_n values in comparison to well-watered plants in the TS variety, but did induce significant reductions in respiration in the SS variety. The effect of temperature and water deficit on respiration in C4 maize was less clear. Drought stress induced a reduction in R_d in TM at 25°C, but not at 35°C. In contrast, SM showed reductions in R_d and R_n under water deficit at the higher temperature (Fig. 5).

The F_v/F_m in TS was unaffected by drought or temperature stress, while SS exhibited reduced F_v/F_m under water deficit at the higher temperature (Fig. 6). Despite the generally higher rates of P_N at 35°C (Fig. 2), both maize varieties (TM and SM) exhibited significant reductions in F_v/F_m values during drought at 35°C. The ΦPSII was more susceptible to drought in the sunflower varieties (TS and SS) at both temperatures. However, reductions in ΦPSII only occurred under water deficit at the higher temperature in the maize varieties (TM and SM). These reductions in ΦPSII during drought in both sunflower and maize were accompanied by corresponding increases in the dissipation of excess energy as heat via NPQ, with greater levels of NPQ occurring in the C4 species (Fig. 6).

Plant height was lower in the varieties of sunflower and maize when grown at 35°C (Fig. 7). Growth at the higher temperature reduced the impact of water deficit on plant height. At 25°C reduced growth in sunflower plants exposed to soil drying became apparent after 19 days, but maize plants showed impaired growth earlier after 8 days. This may be due to faster growth and biomass accumulation in the C4 species (Fig. 8C, G) exhausting the water available within the soil for plant uptake more rapidly. Under well-watered conditions, leaf area in maize (Fig. 8F) was respectively 60 and 50% greater at 25 and 35°C than in sunflower plants (Fig. 8B). High temperature reduced above ground biomass (Fig. 8C, G) and the rate of above ground biomass gain in both maize and sunflower plants under well-watered and water deficit conditions. The TS exhibited greater below-ground biomass than SS at both temperatures. A similar pattern was observed between the TM and SM plants at 25°C. However, at 35°C the SM exhibited greater root-growth under the water deficit treatment than TM.

Discussion

This study has shown contrasting responses to heat stress and water deficit in drought tolerant and sensitive varieties of C3 sunflower and C4 maize. Drought and high temperature frequently occur together (Ciais et al. 2005, Vautard et al. 2007), but plant responses to these stresses are controlled by largely distinct genetic pathways (Rizhsky et al. 2004). Photosynthesis and growth were directly affected by water deficit and temperature

Table 1. Gas exchange parameters of drought tolerant and sensitive varieties of sunflower and maize grown at day/night temperatures of 25/20°C (labelled 25°C) and 35/30°C (labelled 35°C). Photosynthetic response curves to internal [CO_2] were used to calculate the Vc_{max}, J_{max} and TPU as a possible indicator of limited capacity to transport photosynthate out of the chloroplast envelope following Sharkey et al. (2007). Due to diffusive limitations caused by stomatal resistance during drought it was not possible to accurately determine the state of biochemical capacity to assimilate CO_2 in the plants under water deficit conditions using gas exchange (see Centritto et al. 2003). Values are the mean of four measurements per variety/treatment and ± indicates standard error. Values followed by different letters indicate significant difference using a one-way ANOVA and LSD post hoc test.

		Sunflower				Maize			
		Vc_{max}	J_{max}	J_{max}:Vc_{max}	TPU	Vc_{max}	J_{max}	J_{max}:Vc_{max}	TPU
Sensitive	25°C	104.208 ± 10.833^{a}	160.332 ± 13.814^{a}	1.549 ± 0.045^{a}	9.751 ± 1.506^{a}	122.888 ± 20.181^{a}	142.326 ± 4.653^{a}	1.234 ± 0.155^{a}	11.409 ± 1.290^{a}
	35°C	40.480 ± 8.037^{c}	98.531 ± 11.111^{c}	2.616 ± 0.324^{a}	7.961 ± 0.789^{b}	90.078 ± 11.613^{a}	174.593 ± 34.765^{a}	1.909 ± 0.250^{a}	10.599 ± 1.199^{b}
Tolerant	25°C	77.574 ± 4.676^{ab}	136.130 ± 7.316^{ab}	1.764 ± 0.086^{a}	10.202 ± 0.506^{a}	132.688 ± 4.648^{a}	170.795 ± 11.772^{a}	1.296 ± 0.114^{a}	11.721 ± 0.180^{ab}
	35°C	62.127 ± 11.541^{bc}	111.966 ± 9.364^{bc}	1.914 ± 0.206^{a}	8.855 ± 0.650^{a}	125.151 ± 14.790^{a}	208.975 ± 43.657^{a}	1.655 ± 0.251^{a}	10.154 ± 0.883^{ab}

to a greater extent in the C3 crop. Nonetheless, a significant degree of genotypic variation in the response to heat and drought stress was observed within the two species.

The kinetics of the drought response

The concentration of HCO_3^- at the site of carboxylation within the bundle sheath allows C4 plants to possess generally higher rates of carbon gain due to the recapture of CO_2 released via photorespiration, maintain greater WUE and function at higher temperatures than C3 plants (Crafts-Brandner and Salvucci 2000, Crafts-Brandner and Salvucci 2002). The kinetics of the drought response of the plants, characterised by the shift in P_N and G_s to soil drying, was markedly different between C3 sunflower and C4 maize. The C3 sunflower showed reductions in P_N and G_s at higher FTSW levels than the C4 maize varieties (Figs 1 and 2); indicating either higher WUE (Farquhar et al. 1989) or more effective water uptake and transport (Haworth et al. 2016a). Leaf level rates of P_N were broadly similar between the sunflower and maize varieties, consistent with previous observations of P_N in the two crops (Hervé et al. 2001); however, G_s was greater in the sunflower, indicative of higher water-loss per unit leaf area (Fig. 3). Higher temperature did not alter G_s or the response to drought in either of the maize varieties; however, the point at which G_s declined in the TM occurred at a slightly lower FTSW, possibly due to lower growth and P_N reducing the transpirative demands of the whole plant (Liu et al. 2016).

Varieties of maize exhibit different sensitivity to low (Pietrini et al. 1999) and high temperatures (Lobell et al. 2011). Temperatures of 45–50°C are generally lethal to maize, but leaf initiation and shoot and root growth cease at lower levels of 35–45°C (Sánchez et al. 2014). The drought sensitive maize (SM) exhibited slightly lower and earlier declines in G_s and P_N under drought conditions at 35°C than the TM variety (Fig. 2), yet the genotypic difference was less apparent than in C3 sunflower. An increase in temperature up to 45°C over a 1-h period induced a rise in G_s in maize (Crafts-Brandner and Salvucci 2002). However, after 7 weeks of growth at elevated temperature, G_s was lower in both maize varieties (TM and SM) with the reduction most pronounced in the SM (Fig. 3). This suggests that longer-term adaptation to high temperature may influence stomatal behaviour, and as a consequence the impact of any water deficit. Nonetheless, growth at 35°C did not adversely affect the relationship between P_N and G_s in either of the maize varieties (Fig. 4). The effect of elevated temperature was more pronounced on the drought response of sunflower. Increased temperature is considered likely to exacerbate the impact of drought via higher water-loss (Shah and

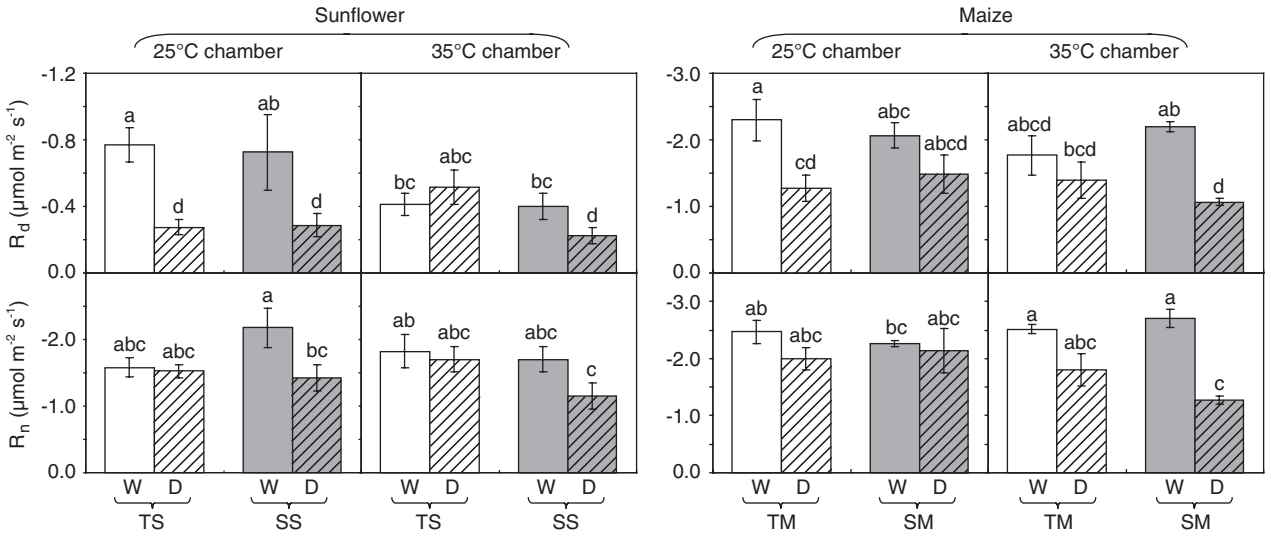

Fig. 5. R_d and R_n of drought tolerant (white) and sensitive (grey) varieties of sunflower (TS and SS) and maize (TM and SM) exposed to soil drying at 25 and 35°C. Plants experiencing water deficit (hatched bars) and well-watered control plants (open bars) were measured when the drought plants reached FTSW levels <10%. Error bars indicate one standard error either side of the mean (n = 4). Letters indicate homogenous groups determined using a one-way ANOVA and LSD post hoc test for each species.

Paulsen 2003, Xu and Zhou 2006). In contrast to previous studies (Bunce 2000, Dekov et al. 2001, Rizhsky et al. 2002, Shah and Paulsen 2003, Rizhsky et al. 2004, Xu and Zhou 2006) we did not observe an increase in G_s in the sunflower varieties after 7 weeks at the higher temperature (Fig. 3). Bunce (2000) found that G_s of sunflower increased by approximately 240% when temperature rose from 25 to 35°C. In our study, the TS exhibited greater variation in values but no significant alteration in G_s, while the SS variety showed a reduction in the level of G_s (Fig. 1). At 35°C the TS reduced G_s at a lower FTSW than at 25°C. However, while the G_s values of the SS variety were lower at the higher temperature, the pattern of decline to soil drying was identical to that at 25°C. This may suggest that not all plants will respond to an increase in temperature with a rise in G_s (Bunce 2000) and that the stomatal response to higher temperatures is not just a function of acclimation to those temperatures (e.g. Centritto et al. 2011) but may involve a degree of genotypic variation (Fig. 1). The more rapid decline in G_s at the higher temperature in the TS may represent a more conservative water-use strategy to maintain foliar water content (Maseda and Fernandez 2006) or the comparatively larger leaf area of the TS variety exhausting the water more rapidly (Liu et al. 2016). We suggest that future studies should vary the timings of drought and heat stress to unpick the likely impacts of increased G_s associated with a short-term rapid rise in temperatures (e.g. Bunce 2000) vs a reduction/consistency in G_s during a longer period of elevated temperatures (this study) on crop productivity. This is of particular importance to models of crop growth used to predict likely yield responses to climate change.

The drought tolerant sunflower (TS) invested a greater proportion of photosynthesis as below-ground biomass; with the root-systems of well-watered drought tolerant plants being approximately 75% greater than the drought sensitive variety at both 25 and 35°C. The more extensive root-network may enable the drought tolerant variety to more fully exploit the available water than its drought sensitive counterpart (Chloupek et al. 2010). Variation in transpiration rates and WUE in varieties of sunflower have been linked to biomass production (Virgona et al. 1990). During drought stress, varieties that maintain foliar water levels through osmotic adjustment exhibited the lowest declines in G_s and loss of PSII function (Conroy et al. 1988) alongside increases in the foliar concentration of RubisCO (Panković et al. 1999). However, no difference in RWC was observed between the two varieties under water deficit at either temperature (Fig. 8); suggesting that enhanced performance of the TS was associated with genotypic variation in photosynthetic physiology (e.g. Panković et al. 1999, Li et al. 2006).

Physiological adaptation to increased temperature and its interaction with drought

Reductions in the activity of RubisCO at higher temperature in sunflower are evident in the P_N/C_i curve conducted at the same temperature (Table 1). The TS

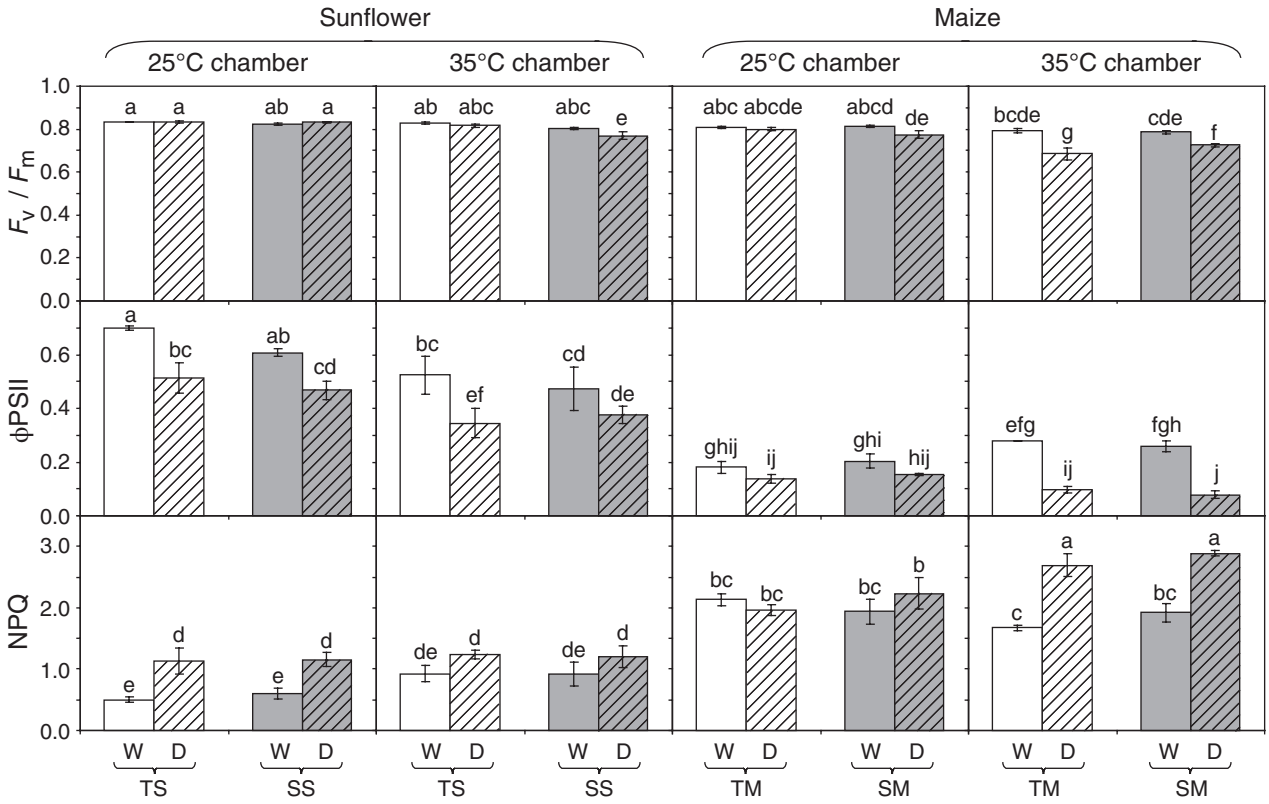

Fig. 6. F_v/F_m, ΦPSII and heat dissipation as NPQ determined by chlorophyll fluorescence in drought tolerant (white) and sensitive (grey) varieties of sunflower (TS and SS) and maize (TM and SM) exposed to soil drying at 25 and 35°C. Plants experiencing water deficit (hatched bars) and well-watered control plants (open bars) were measured when the drought plants reached FTSW levels <10%. Error bars indicate one standard error either side of the mean (n = 4). Letters indicate homogenous groups for all varieties of both species determined using a one-way ANOVA and LSD post hoc test.

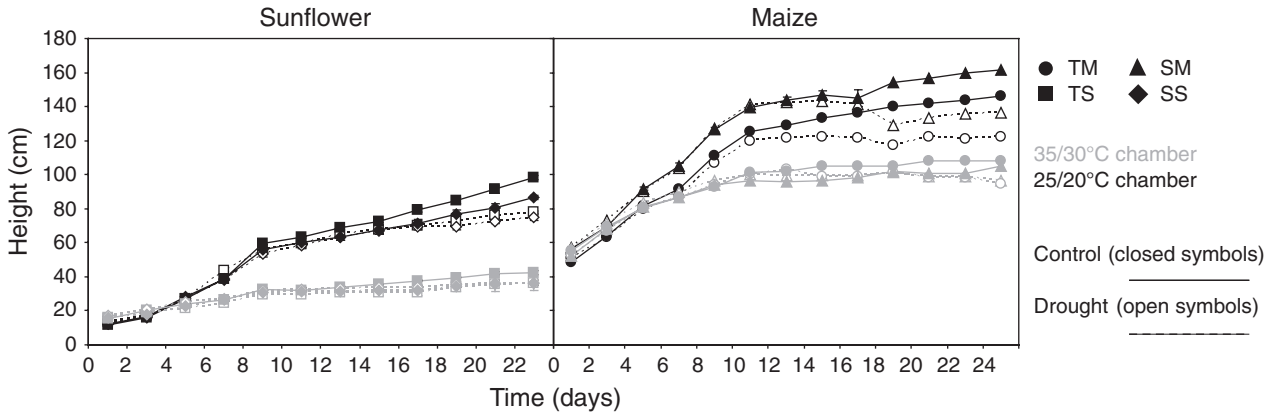

Fig. 7. Plant height of drought tolerant and sensitive varieties of sunflower (TS and SS) and maize (TM and SM) exposed to soil drying and their corresponding well-watered control counterparts at 25 and 35°C. Error bars indicate one standard error either side of the mean (n = 4).

exhibited a lower proportional 19.9% reduction in Vc_{max} at the higher temperature, in comparison to the 61.2% decline observed in the SS. At temperatures above 40°C, reductions in RubisCO activity were associated with reduced RubisCO activase activity in cotton (*Gossypium hirsutum*) and wheat (Feller et al. 1998). During water deficit, a drought tolerant variety of sunflower showed similar reductions in RubisCO activity to a more drought sensitive variety; however, this loss in RubisCO activity was compensated in the drought tolerant plants by an

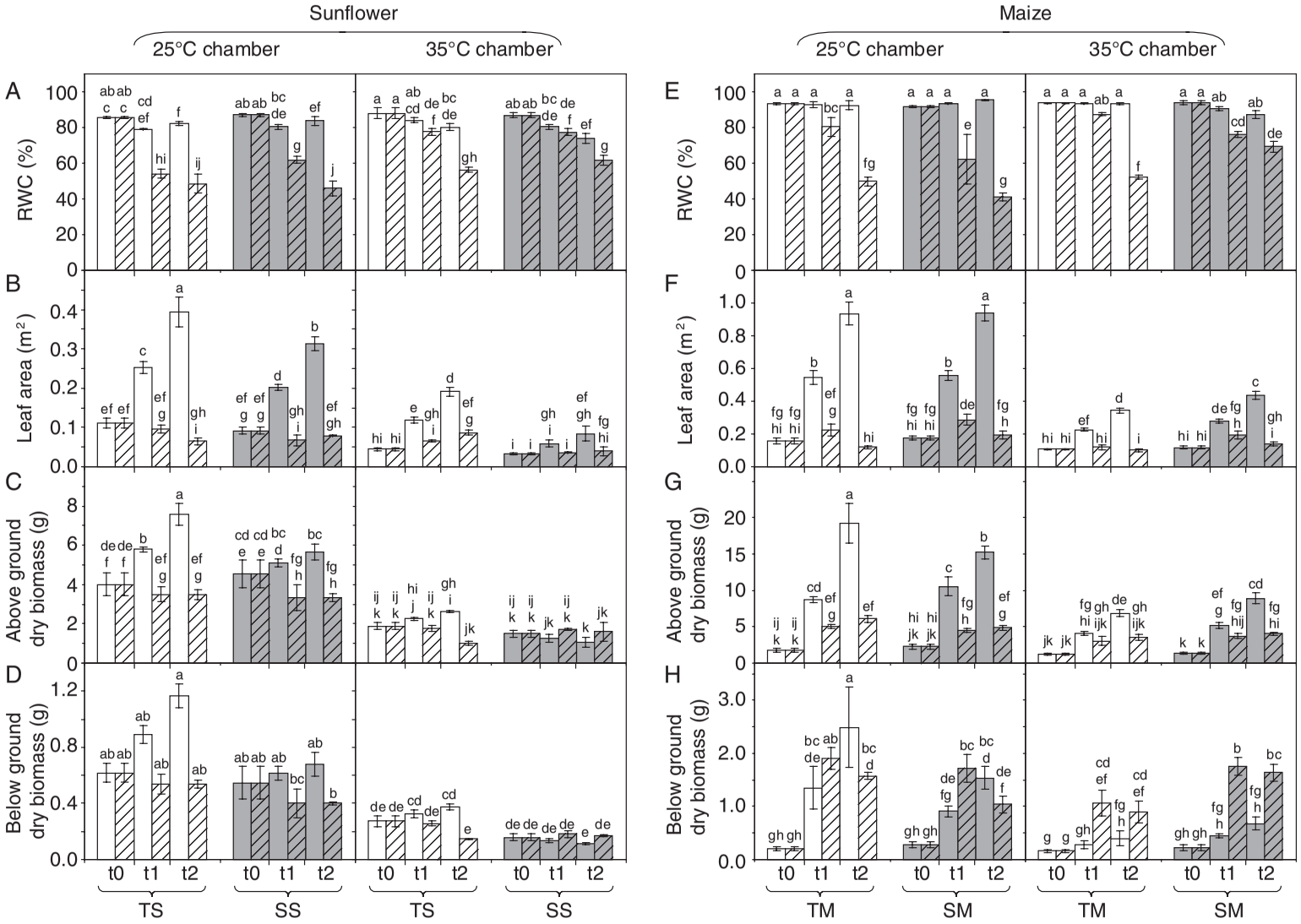

Fig. 8. Morphological parameters of drought tolerant (white) and sensitive (grey) varieties of sunflower (TS and SS) and maize (TM and SM) exposed to soil drying at 25 and 35°C: (A, E) RWC, (B, F) leaf area, (C, G) above ground dry biomass and (D, H) below ground dry biomass. Plants experiencing water deficit (hatched bars) and well-watered control plants (open bars) were measured at the start of the experiment (t0), when G_s conductance levels of the drought stressed plants reached 50% of their starting value (t1) and at the end of the drought stress period when G_s levels were <10% of their starting levels or pot weight had remained constant for 3 days (t2). Error bars indicate one standard error either side of the mean (n = 4). Letters indicate homogenous groups for each species determined using a one-way ANOVA and LSD post hoc test.

increase in total RubisCO content per unit leaf area (Panković et al. 1999). An increase in RubisCO content under drought stress would also counter the negative impact of elevated temperature on RubisCO activity (Feller et al. 1998, Crafts-Brandner and Salvucci 2000) and affinity for CO_2 (Jordan and Ogren 1984). Indeed, during heat stress the expression of assembly chaperones responsible for the construction of the RubisCO enzyme (Hauser et al. 2015) are increased (Rizhsky et al. 2002, Rizhsky et al. 2004). This may suggest that the retention of P_N rates (Figs 1 and 3) during drought and heat stress observed in the TS variety in this study may be due to enhanced RubisCO activity. Nonetheless, the capacity for field grown C3 crops to ameliorate the negative influence of higher temperatures through an increase in foliar RubisCO concentrations may be dependent upon the availability of nitrogen (Heckathorn et al. 1996, Martin et al. 2002) as the formation of RubisCO requires nitrogen (Spreitzer and Salvucci 2002) and the enzyme represents the major pool of nitrogen in most plants (Warren et al. 2000).

The high concentration of CO_2 at the site of carboxylation within the bundle sheathes of C4 species mitigates the impact of lower RubisCO activation and affinity for CO_2 at higher temperatures (Crafts-Brandner and Salvucci 2002), accounting for the lack of change in Vc_{max} values of the maize varieties at 25 and 35°C (Table 1). However, at the end of the experimental period, well-watered maize plants at 35°C exhibited 9.15–22.7% lower rates of leaf-level P_N than plants grown at 25°C (Fig. 3). Short-term exposure of maize plants grown at 28–35°C for 1 h induced a 20% reduction in RubisCO activity, but this did not correspond to a decline in P_N (Crafts-Brandner and Salvucci 2002). Longer-term growth at 35°C over a 7-week period may have led to reduced P_N via progressive loss of RubisCO activity and damage to the photosynthetic physiology of the maize varieties (Fig. 3). Maize plants exposed to a short-term heat stress over 20 min exhibited reduced ΦPSII and increased NPQ due to an inhibition of electron transport downstream of PSII (Sinsawat et al. 2004). Increases in temperature lasting 1 h induced reductions in P_N of maize at 40°C and above, which corresponded to reduced F_v/F_m indicating a loss of PSII function (Crafts-Brandner and Salvucci 2002). Despite short-term heat-stress inducing an increase in the dissipation of excess energy as heat in maize (Crafts-Brandner and Salvucci 2002, Sinsawat et al. 2004), no significant increase in NPQ was observed at 35°C in either maize variety used in this study (Fig. 6). This is consistent with longer-term adaptation to high temperatures through the production of heat shock proteins within the chloroplast to protect thylakoid membranes (Heckathorn et al. 1998) and observation of the maintenance of photochemistry at the higher temperature (Fig. 2). Under drought stress both varieties diverted electron transport away from photochemistry, and this was more pronounced at 35°C. However, there were not genotypic differences in the response of ΦPSII or NPQ to water deficit that may account for observations of differing drought tolerance between the two varieties. The maize varieties used in this study (TM and SM) showed no effect of growth at elevated temperature on the actual of maximum efficiency of PSII (Fig. 6). This is consistent with observations of no effect on PSII photochemistry in short-term temperature experiments but impaired photosynthetic physiology beyond PSII in maize (Crafts-Brandner and Salvucci 2002, Sinsawat et al. 2004); possibly suggesting that reduced P_N in this study may be associated with degradation of the PSI photosynthetic physiology incurred during longer-term growth at 35°C over a 7-week period.

The effect of growth at 35°C on PSII, and its interaction with drought, was more pronounced in the C3 sunflower. The higher temperature did not alter F_v/F_m but did induce reductions of approximately 24% in ΦPSII and 90% increases in NPQ. This is indicative of damage to the thylakoid membranes at high temperatures impairing photochemistry (Havaux 1993, Feller et al. 1998, Law and Crafts-Brandner 1999, Crafts-Brandner and Salvucci 2000) and became more apparent under water deficit (Fig. 6). However, no genotypic difference in the response of PSII to temperature or water deficit was evident between the TS and SS varieties; suggesting that other chlorophyll fluorescence parameters (such as the initial fluorescence, F_o) may be more useful in rapid phenotypic screening of tolerance to drought and heat stress (e.g. Li et al. 2006).

Morphological response to high temperature and water deficit

The sunflower and maize plants used in this study exhibited broadly similar leaf-level P_N at 25°C (Figs 1 and 2). However, both maize varieties (TM and SM) produced greater leaf area and biomass than sunflower (TS and SS) over the course of the study (Fig. 8). Plant height is a key determinant of competition for light (Ford and Diggle 1981, Craine and Dybzinski 2013) and closely related to biomass production (Haworth et al. 2016a) and yield (Doyce and Lessman 1966, Weng et al. 2014). Maize gained height more rapidly than sunflower, and the negative impact of drought and/or heat stress was lower (Fig. 7), possibly due to greater WUE in the C4 crop (Fig. 3) (Farquhar et al. 1989). The mean reduction in above ground biomass at 35°C was lower in maize (55.3%) than sunflower (69.6%). However, the lower

above ground biomass may have mitigated the impact of water deficit by reducing the amount of tissue from which transpirative water-loss could occur (Fig. 8). The lower than expected rate of water-loss at the higher temperature found in this study may reflect differential responses of plants adapted to increased temperatures, and those in other studies where the response to a sudden increase in temperature was recorded (e.g. Bunce 2000, Dekov et al. 2001, Crafts-Brandner and Salvucci 2002, Rizhsky et al. 2002). A sudden increase in temperature exacerbates the impact of drought via an increase in G_s (Shah and Paulsen 2003, Xu and Zhou 2006). However, after 7 weeks exposure to 35°C neither the C3 or C4 crop exhibited increased G_s (Fig. 3), analogous to observations in black poplar (Centritto et al. 2011). Alongside reduced growth, this adaptation of stomatal behaviour resulted in lower rates of water-loss at 35°C. The reduced growth rate at the higher temperature was not however associated with a reduction in respiration (Fig. 5) (Poorter et al. 1990); suggesting that growth at 35°C required the maintenance of metabolic processes to preserve membrane stability (Heckathorn et al. 1998) and maintain anti-oxidant activity (Türkan et al. 2005).

The results of this study suggest that heat and drought stress are likely to have a disproportionately negative impact upon C3 rather than C4 crops. In this context, the selection and development of varieties on the basis of tolerance to drought and heat stress is critical to ensure the productivity of C3 crops such as sunflower (Fig. 1) or wheat (Shah and Paulsen 2003, Semenov et al. 2014). This study suggests that the retention of P_N rates during heat and/or water deficit (Fig. 3 and Table 1) is crucial to plant growth (Fig. 8). The maintenance of P_N was not associated with greater foliar water content (Conroy et al. 1988), but the TS variety exhibited greater investment in root-systems that allowed the more complete exploitation of the available water (e.g. Chloupek et al. 2010). Despite the greater impact in sunflower, the growth of maize was also negatively affected by high temperatures (Fig. 8), and this is likely to have implications for food security in regions reliant upon maize production (Lobell et al. 2011, Lobell et al. 2013). This study showed no physiological (Figs 2–6) or morphological (Figs 7 and 8) parameter that may be associated with enhanced yield in the drought tolerant maize variety. As such, the respective yield characteristics of the drought and sensitive maize varieties may be the result of differences in the partitioning of photosynthate between reproduction and vegetative structures (e.g. Lauteri et al. 2014), metabolism (Ilahi and Dörffling 1982) or water transport (e.g. Haworth et al. 2016a).

In contrast to previous studies, elevated temperature did not exacerbate the impact of drought. Furthermore, the lower leaf area and above ground biomass (Fig. 8) induced by growth at 35°C ameliorated the impact of water deficit as overall transpiration was lower (Figs 1 and 2). While sudden rises in temperature are likely to induce a rise in G_s and compound the effect of water deficit, this study has shown that the interaction of heat and drought stress is complex, depending upon genotype and the temperature acclimation of the plant. Varietal effects were observed in the response of both species to elevated temperature and water deficit. Our findings suggest that an increased frequency of drought and heat stress events is likely to disproportionately affect P_N of C3 crops. As C4 maize exhibited tolerance to drought and heat stress in both varieties, to ensure food security priority should be given to the identification and development of varieties of C3 crops such as sunflower with tolerance to heat and drought stress.

Author contributions

D. K. wrote the paper, analysed the data and conducted the experiment. M. H. conducted the experiment. A. R. and F. B. provided equipment and consumables.

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"Science knows no country,
because knowledge belongs to humanity,
and is the torch which illuminates the world."
Louis Pasteur

Paper II

Lipid peroxidation and chlorophyll fluorescence of photosystem II performance during drought and heat stress is associated with the antioxidant capacities of C3 sunflower and C4 maize varieties.

Dilek Killi, Filippo Bussotti**,** Antonio Raschi and Matthew Haworth (*in preparation*)

Author contributions:

□ Designed and conducted the experiment: DK, MH

□ Provided equipment and consumables: FB, AR

□ Wrote the paper, analysed the data: DK

Abstract

Agricultural production in many regions is predicted to be adversely affected by an increased prevalence of simultaneous drought and heat-wave events. An understanding of how drought and heat stress affects the physiology of crops can assist in the identification and development of more tolerant phenotypes towards ensuring food security. However, there are comparatively few studies that analyse the impact of combined drought and heat stress in crops, and the mechanisms that underpin tolerance. Drought and heat stress damage plant cellular membranes; in particular, the chloroplast thylakoid membranes where photosystem II occurs (PSII). We investigated the functionality of PSII using chlorophyll fluorescence (*ChlF*) and protective antioxidant mechanisms in drought tolerant and sensitive varieties of C3 sunflower (*Helianthus annuus*) and C4 maize (*Zea mays*) that have been shown to exhibit contrasting varietal leaf gas exchange and whole-plant morphological responses to drought and heat stress. Drought and heat stress, in combination and isolation, had a more pronounced effect on *ChlF* parameters of the C3 sunflower than C4 maize. The maximum fluorescence (F_m) was the most effective *ChlF* measure in characterising varietal variation in the response of both C3 and C4 species to drought and heat stress. The greater retention of PSII function under drought in the drought tolerant sunflower at higher temperatures may have been associated with an increase in the activities of antioxidants (glutathione reductase, superoxide dismutase, catalase, peroxidase and ascorbate peroxidase), whereas antioxidant activity declined in the drought sensitive variety, possibly indicative of protective mechanisms being overwhelmed. In particular, peroxidase and ascorbate peroxidase that function within the chloroplast envelope were 2.5 to 5.5 times greater in the drought tolerant sunflower, possibly accounting for the maintenance of PSII performance. The drought tolerant and sensitive varieties of maize showed less pronounced differentiation in antioxidant activity during drought and heat stress. Nevertheless, the drought tolerant maize consistently up-regulated antioxidant activity during

drought at both temperatures, whereas the sensitive variety did not. This study indicates that antioxidants play a vital role in maintaining the functionality of photosynthetic membranes under drought and heat stress, and that improved tolerance to these stresses is associated with enhanced antioxidant function. The protective role of antioxidants is particularly important in the degree of tolerance exhibited by C3 crops that are more vulnerable to drought and heat stress than C4 species. Furthermore, *ChlF* is a highly effective tool in rapid non-destructive phenotyping of crop responses to drought and heat stress.

Key-words: phenotyping; glutathione reductase; superoxide dismutase; catalase; peroxidase; ascorbate peroxidase; *Helianthus annuus*; *Zea mays*; food security

Abbreviations;

FTSW, Fraction of transpirable soil water; SWC, Soil water content; P_N, Net Photosynthesis; G_s, Stomatal conductance; PSI, *Photosystem I;* PSII, Primary photochemistry/ Photosystem II (PSII); F_v/F_m (φ_{P0}), The maximum quantum yield of primary photochemistry (PSII), ((F_m-F_o)/F_m); PAR, Photosynthetically active radiation; *Chl*, chlorophyll; *ChlF*, chlorophyll fluorescence; F_0, minimal fluorescence; F_M, maximal fluorescence ; F_V, maximal variable fluorescence; ψ_{E0}, the efficiency of trapped energy to move an electron further than Q_A^-; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidases; POX, peroxidases; GR, glutathione reductase; TS, Drought tolerant sunflower variety (var. Bosfora); SS, Drought sensitive sunflower variety (var. 08 TR 003); TM, Drought tolerant maize variety (var. ADA-9516); SM, Drought sensitive maize variety (var. ADA-523).

4.1. INTRODUCTION

As average global temperatures rise, the frequency of drought events and heat-waves will increase (Schär et al., 2004; Vautard et al., 2007; Anderegg et al., 2013). This combination of drought and heat stress will have significant negative implications for agricultural production of both C3 (eg. Semenov et al., 2014; Stratonovitch and Semenov, 2015) and C4 (eg. Lobell et al., 2013) crops. The effect of drought (eg. Flexas et al., 2002) and higher temperatures (eg. Crafts-Brandner and Salvucci, 2000) on crop plants have been studied widely in isolation, but their potential combined impact is less clearly defined. To ensure food security, it is necessary to identify traits that confer resistance to drought and heat stress towards developing more tolerant and resistant varieties (Nguyen et al., 1997; Rizhsky et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007). Despite the frequent occurrence of drought events with heat-waves (Schär et al., 2004; Anderegg et al., 2013), the pathways regulating plant responses to drought and heat stress are largely distinct (Rizhsky et al., 2004). Analysis of the physiological mechanisms involved in crop responses to drought and heat stress may elucidate the photosynthetic and protective behaviours that underpin tolerance to these stresses. Chlorophyll fluorescence (*ChlF*) allows rapid non-destructive collection of data relating to the performance of photosystem II (PSII) (Kalaji et al., 2016) and is highly sensitive to the deleterious effects of drought (Genty et al., 1989) and heat (Crafts-Brandner and Salvucci, 2002) stress. To protect and stabilise the thylakoid membrane where PSII electron transport occurs, plants possess protective antioxidant mechanisms (Reddy et al., 2004; Pinheiro and Chaves, 2011). This study builds upon a previous investigation of the gas exchange and morphological responses of drought resistant and sensitive varieties of C3 sunflower (*Helianthus annuus*) and C4 maize (*Zea mays*) to heat and drought stress (Killi et al., 2016) by providing an in-depth examination of the *ChlF* and antioxidant characteristics associated with differential responses to drought and/or heat stress.

As the availability of water for uptake by plants declines, stomatal pores close to reduce transpirative water-loss and prevent desiccation (Flexas et al., 2002; Lauteri et al., 2014). A decrease in stomatal conductance (G_s) causes lower availability of CO_2 for photosynthesis (P_N). This results in a decrease in the use of energy for photochemistry and necessitates an increase in the dissipation of energy as heat to prevent damage to the photosynthetic apparatus (Flexas and Medrano, 2002; Kalaji et al., 2016). Reduced photochemical energy usage can also result in increased generation of reactive oxygen species (ROS) (Smirnoff, 1993; Pinheiro and Chaves, 2011). During the initial stages of drought stress the activity of antioxidant systems increases to protect against excessive levels of ROS (Reddy et al., 2004). During prolonged or severe drought stress the capacity of plants to dissipate excess energy or neutralise harmful ROS may become overwhelmed leading to damage to the structure and function of cellular membranes, in particular the thylakoid membranes within the chloroplast where PSII electron transport takes place (Flexas and Medrano, 2002; Pinheiro and Chaves, 2011). The thylakoid membranes are also vulnerable to heat stress (Gounaris et al., 1983; Havaux, 1993; Feller et al., 1998; Yordanov et al., 1999; Crafts-Brandner and Salvucci, 2002). Heat stress also induces a decrease in P_N by reducing the affinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) for CO_2 (Berry and Björkman, 1980) and the activity of RubisCO activase (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000), possibly reducing the capacity for photosystem I (PSI) to act as an electron receiver for PSII (Sinsawat et al., 2004), and thus impairing the use of energy for photochemistry through PSII (Kalaji et al., 2016). As such, the thylakoid membranes are particularly sensitive to the effects of both drought and heat stress, and any disruption to the structure and function of the thylakoid membranes is evident in reductions in the efficiency of electron transport during PSII (Feller et al., 1998; Crafts-Brandner and Salvucci, 2002; Haworth et al., 2016). Chlorophyll fluorescence measures of

PSII performance should therefore provide a quantitative measure of the impact of drought and heat stress on the functionality of thylakoid membranes. Moreover, comparison of different *ChlF* parameters may indicate the most sensitive or diagnostic measure(s) for use in rapid phenotyping for drought and heat tolerance using active *ChlF* methods (eg. Oukarroum et al., 2007).

During drought stress, the lipids that constitute cellular membranes become damaged via peroxidation (Jiang and Huang, 2001). This results in an increase in levels of malondialdehyde (MDA) within the plant. Glutathione reductase (GR) uses NADPH as a reductant to reduce GSSG (oxidized glutathione) to GSH (reduced glutathione) to allow binding GSH with hydrogen peroxide (H_2O_2) (Foyer and Halliwell, 1976; Das and Roychoudhury, 2014). Superoxide dismutase (SOD) also reduces cytotoxic O_2^- to form H_2O_2. Hydrogen peroxide is then converted to water and oxygen by catalase (CAT) in peroxisomes, peroxidase (POX) in the chloroplast envelope and ascorbate peroxidase (APX) in the cytosol and chloroplast (Smirnoff, 1993; Das and Roychoudhury, 2014). Plant species with increased tolerance to drought stress exhibit greater antioxidant activity than species that are more susceptible to drought (Türkan et al., 2005). Heat stress also increases the production of ROS and peroxidation of lipid membranes (Liu and Huang, 2000; Xu et al., 2006; Kumar et al., 2012). Varieties of wheat (*Triticum aestivum*)(Sairam et al., 2000) and cotton (*Gossypium hirsutum*) (Sekmen et al., 2014) with enhanced heat tolerance also exhibited significantly higher levels of antioxidant activity than their less tolerant counterparts. A combination of drought and heat stress increased the peroxidation of membrane lipids more rapidly than both stresses in isolation in Kentucky bluegrass (*Poa pratensis*) and tall fescue (*Festuca arundinacea*). This membrane degradation coincided with reductions in antioxidant activity, suggesting that the protective mechanisms functioning to stabilise plant membranes were overwhelmed when

drought and heat stress were combined (Jiang and Huang, 2001). The capacity of antioxidant mechanisms to protect plant physiology and membranes likely play an integral role in determining crop responses to drought and heat. Analysis of the activity of major antioxidants alongside *ChlF* should provide insights into the functioning of photosynthetic thylakoid membranes and the protective mechanisms that support the maintenance of P_N during episodes of high oxidative stress.

The present study is a counterpart to the earlier study of Killi et al. (2016). The drought tolerant and sensitive varieties of sunflower and maize exhibited contrasting leaf gas exchange and morphological responses to drought when grown at 25 and 35˚C. Increased temperature had little effect on P_N in the maize varieties due to accumulation of oxaloacetic acid within the bundle sheath effectively concentrating CO_2 at the RubisCO carboxylation site thus eliminating the enhanced photorespiration at 35˚C that negatively affected sunflower (see Crafts-Brandner and Salvucci, 2002). The drought tolerant sunflower sustained greater P_N at 35˚C by retaining RubisCO activity (see Panković et al., 1999). Stomatal adaptation to the higher temperature allowed the sunflower and maize varieties to prevent excessive water-loss at 35˚C (Fig 1). Alongside reduced growth and leaf area at 35˚C this stomatal control ameliorated the impact of drought at the higher temperature (Killi et al., 2016). This study intends to explore the impact of drought and heat stress, in combination and isolation, on the functionality of PSII and the protective mechanisms that stabilise the photosynthetic membranes where electron transport occurs. We hypothesise that the more drought tolerant varieties of sunflower and maize will possess enhanced antioxidant activities (eg. Jiang and Huang, 2001; Türkan et al., 2005; Sekmen et al., 2014) and will retain PSII performance to a greater extent under drought and heat stress. This study aims to: i) investigate the impact of drought and heat stress on *ChlF* parameters of C3 sunflower and maize as an indicator of PSII performance; ii) identify the

most effective *ChlF* measures for the characterisation of drought and heat stress, and identification of tolerant varieties for phenotyping using active *ChlF*; iii) examine differences in antioxidant capacity and responses to drought and heat stress in the varieties of sunflower and maize, and; iv) explore whether the capacity of protective antioxidant systems is linked to the maintenance of PSII under drought and heat stress, and whether these attributes are desirable in the selection and development of crop varieties to maintain productivity in regions predicted to be affected by an increased frequency of drought events and heat-waves.

4.2. MATERIALS AND METHODS

4.2.1. Plant growth conditions and drought application

The sunflower *(Helianthus annuus L.)* and maize *(Zea mays L.)* varieties were classified as drought tolerant or sensitive according to observations of the effect of water deficit on yield in field trials. No analyses of *ChlF* or antioxidants of the plants have been undertaken prior to the present study. The drought tolerant (TS) (var. Bosfora) and sensitive (SS) (var. 08 TR 003) varieties of sunflower seeds were provided by the Trakya Agricultural Research Institute, Edirne, Turkey. The drought tolerant (TM) (var. ADA-9516) and sensitive (SM) (var. ADA-523) varieties of maize seeds were supplied by the Sakarya Maize Research Institute, Sakarya, Turkey. The seeds were germinated in trays of sand supplied with a commercially available nutrient solution (COMPO Concime Universale, NPK 7-5-7, B, Cu, Fe, Mn, Mo, Zn: COMPO Italia, *Cesano Maderno*, Italy) within a plant growth room with a day/night temperature of 28/24˚C. Metal-halide lights were used to maintain a photosynthetically active radiation (PAR) of 800 μmol m^{-2} s^{-1} for 14-hours each day. After two weeks, the plants were potted into nine litre square pots filled with a 90% fine-sand to 10% commercial compost mixture and placed into two large walk-in growth rooms with full control of light, temperature, [CO_2] (both chambers maintained ambient atmospheric [CO_2] of 400 ppm) and humidity (both chambers

maintained a relative humidity of 50%)(technical specifications of the growth chambers are given in Materassi et al., 2005). The plants were watered to pot capacity every two days and provided weekly with a commercial liquid plant fertiliser (COMPO Concime Universale) to facilitate nutrient availability at free access rates. The growth chambers maintained conditions of 16 hours of daylight (14 hours at full PAR levels of 1000 μmol m^{-2} s^{-1} with two one-hour periods of simulated dawn / dusk where light intensity was incrementally increased / decreased). One chamber operated a day/night time temperature regime of 25/20˚C (hereafter referred to as 25˚C treatment) and the second chamber operated a day/night temperature of 35/30˚C (hereafter referred to as 35˚C treatment). Changes in temperature followed those of PAR, with a one-hour ramping period at dawn/dusk. To avoid any potential chamber effects the growth rooms were alternated every week – no significant differences were observed in the measurements conducted under the same conditions in different growth chambers. The plants were grown for four weeks in the respective growth chambers prior to the imposition of drought stress to allow acclimation to the growth temperatures.

The fraction of transpirable soil water (FTSW) method (Sinclair and Ludlow, 1986) was applied to gauge the drought kinetics to soil drying of the drought tolerant and sensitive varieties of maize and sunflower. On the evening prior to the imposition of drought all of the plants (one hundred and ninety-two) were watered to pot capacity. The pots were then allowed to drain overnight and weighed the next morning and sealed within plastic bags to eliminate evaporation from the soil. Stomatal conductance of the plants was then recorded and assumed to represent 100% of potential $G_{s.}$ Stomatal conductance was measured using a using a PP-Systems Ciras-2 plant photosynthesis system attached to a PLC6(U) leaf cuvette and LED light unit (PP-Systems, Amesbury, Massachusetts, USA) – full details of leaf gas exchange measurements are provided in Killi et al. (2016). Ninety-six plants (four different treatments

x four replications for each treatment x two varieties x three sampling points) of each species were used in total, with half being allowed to dry and the other half maintained at pot capacity in both chambers (25 ˚C and 35 ˚C), with the amount of water lost as transpiration replaced each day for the well-watered/control plants. The pots were weighed each day and G_s recorded every second day (alternating between chambers). When G_s fell to 10% of the starting G_s value or pot weight remained constant for three days it was considered that all water within the soil available for transpiration had been exhausted (ie. 0% FTSW), and FTSW was calculated as:

$$\mathrm{FTSW} = \frac{\left(\mathrm{PW_{daily}} - \mathrm{PW_{final}}\right)}{\left(\mathrm{PW_{initial}} - \mathrm{PW_{final}}\right)}$$

Where PW is the pot weight in grams. To prevent the soil becoming anoxic, the plastic bags were opened each day for approximately 10 minutes to permit the exchange of gases between the soil and the atmosphere. The soil water content (SWC) of the pots was also measured in this 10-minute period using a FieldScout time domain reflectometry 100 Soil Moisture Meter (Spectrum Technologies, Aurora, Illinios, USA). Values of SWC were correlated to FTSW for presentation in Figures 1 and 2; this varied slightly between species and varieties and so calibrations differ between plants and temperature treatments.

4.2.2. Chlorophyll a fluorescence transient analysis and parameters

Chlorophyll a fluorescence transient analysis was carried out using a portable Handy-PEA (plant efficiency analyser) fluorimeter (Hansatech. Norfolk, UK). Measurements of *ChlF* were performed every two days, alternating between treatments throughout the imposition of drought stress. Leaves experienced 30-minutes dark adaptation and were then exposed to a saturating light pulse (intensity >3000 μmol photons m^{-2} s^{-1}, excitation light of 650 nm)(Strasser et al., 2004). The parameters considered in this study are defined in Appendix A1.

The software program Biolyzer 4 HP v.3 (Bioenergetics Laboratory, University of Geneva, Switzerland) was used to analyse H-PEA signals for OJIP test parameters. Plotted on a logarithmic time scale, the *ChlF* transient shows a polyphasic pattern. The different time steps of this polyphasic transient are labelled as O (20–50 µs), J (2 ms), I (30 ms) and P (peak). The latter indicates the highest fluorescence intensity (F_M), when all the reaction centres of photosystem II (PSII) are reduced by photosynthetic saturating light. For reviews of the theoretical background of the method used to analyse the structure and functionality of photosystem II (the JIP-test) and the *ChlF* parameters obtained from the *ChlF* induction curve (fast kinetics) of dark-adapted leaves see Strasser et al. (2004) and Kalaji et al. (2016). The *ChlF* parameters used in this study were F_o, F_m, F_v/F_m, Ψ_{Eo}, RC/ABS, $\Delta V_{IP,}$ K-band and the performance indices (PI_{abs} and PI_{tot}). Definitions of the parameters and the formulae used in their calculation are provided in Appendix A1.

4.2.3. Leaf sampling and assays of antioxidant enzyme activities

Fully developed leaves were taken for analyses of pigments, protein and antioxidant enzymes: the second leaf from the flag leaf was chosen for maize (if the second leaf was not sufficient size for all analyses the third leaf was also analysed) and the second to fourth uppermost fully expanded leaves from sunflower. Leaves were sampled, immediately frozen in liquid nitrogen before being stored at -80˚C prior to analysis. Leaves were sampled at three stages of the drought response: after four weeks of adaptation to the 25 and 35˚C treatments when G_s values are considered to be 100% (t0); when Gs values of the plants experiencing the drought treatment had declined by 50% (t1), and; the end point of the drought stress when G_s of the plants experiencing drought had decreased to 10% of the starting G_s value or pot weight remained constant for three days (t2).

Three replicate leaf samples of approximately 0.1g of fresh weight were taken from the middle part of each leaf, with four replicates per variety and treatment. The leaf samples were extracted at 4˚C in Na-phosphate buffer (pH 7.8) containing 1mM ethylenediaminetetraacetic acid (EDTA) with 2% polyvinyl pyrrolidone (PVP) (w/v). Samples were centrifuged at 14,000 × g for 30 min, and supernatants were used for the determination of protein content and enzyme activity. All spectrophotometric analyses were conducted on a Ultrospec 2100 Pro (UV-visible) spectrophotometer.

The total soluble protein content of the enzyme extracts were determined according to (Bradford, 1976) at 595 nm, using bovine serum albumin (BSA) as a standard. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed from its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm (Beauchamp and Fridovich, 1971). The reaction mixtures were incubated for 10 min under 300 µmol m^{-2} s^{-1} of light. One unit of SOD was defined as the amount of enzyme that inhibits 50% of the NBT photo reduction. Catalase **(**CAT**)** (EC 1.11.1.6) activity was estimated using the method of Bergmeyer and Gawehn (1970)**,** which measures the initial rate of decomposition of H_2O_2 at 240 nm for 3 min (extinction coefficient of 39.4 mM^{-1} cm^{-1}) with 1 µmol H_2O_2 min^{-1} considered to represent 1 unit of CAT. The activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was analysed using the method of Nakano and Asada (1981), where a decrease in absorbance at 290 nm and an extinction co-efficient of 2.8 mM^{-1} cm^{-1} for ascorbic acid is used to determine the oxidation of ascorbate. Determination of peroxidase (POX**)** (EC 1.11.1.7) activity was undertaken using the method of Kanner and Kinsella (1983). A unit of POX activity was defined as decomposition of µmol H_2O_2 min^{-1} at 470 nm using an extinction coefficient of 26.6 mM^{-1} $cm^{-1}$for guaiacol. Glutathione reductase (GR**)** (EC 1.6.4.2) activity

was measured according to Foyer and Halliwell (1976); where the oxidation of NADPH was measured at 340 nm, and GR activity calculated using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). One unit of GR was defined as 1 µmol glutathione disulfide/oxidized glutathione (GSSG) reduced min^{-1}.

4.2.4. Determination of lipid peroxidation

Oxidative damage to cellular membranes was gauged in terms of lipid peroxidation in the leaf samples. The accumulation of *malondialdehyde* (MDA) resulting from lipid peroxidation was determined by interference with thiobarbituric acid reactive substances (TBARS) measured at 532 and 600 nm according to the method of Rao and Sresty (2000). The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

4.2.5. Determination of pigment contents

The foliar content of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid pigments were determined following the method of Arnon (1949) using 80% acetone. Calculations were performed using the formulae of Lichtenthaler and Buschmann (2001).

4.2.6. Statistical analyses

Statistical analyses were performed using SPSS 20 (IBM, New York, USA). The Shapiro-Wilk test was applied to the *ChlF* data to assess normality, and the Levene test was used to investigate homogeneity of variance. The *ChlF* data was not normally distributed and the variance was not homogenous; therefore, the non-parametric Kruskal Wallis–H Test was applied to the *ChlF* data to assess rank-based differences of means between control and treatment groups. Three-Way ANOVA was used to determine interaction effects between three factors (variety, temperature and water status) on measured antioxidant enzymes (Table 3). A

one-way ANOVA with an LSD *post-hoc* test was used to assess differences in variance between treatments for all other data (see, Appendix A2). A 0.05 significance level ($P \leq 0.05$) was applied.

4.3. RESULTS

4.3.1. Chlorophyll fluorescence parameters

A summary of *ChlF* parameters in the final two days of the drought treatment and their relationship to the values of well-watered plants grown at 25˚C assessed using a non-parametric test are provided in Table 1. The effects of drought and heat stress were more apparent in the *ChlF* characteristics of the C3 sunflower varieties. Total photosynthetic index (PI_{tot}), IP-phase and K-band did not effectively differentiate the effects of drought stress on sunflower at 25˚C and drought on maize at both temperature treatments. The maximum quantum yield of PSII (F_v/F_m) was largely insensitive (although not universally) to the impact of drought stress in the 25˚C treatment. However, at the higher temperature significant reductions in the maximum quantum yield were apparent in both species. The fluorescence maximum (F_m) was the most sensitive parameter in both sunflower and maize varieties in terms of drought treatment and varietal effects at both temperatures.

Growth at 35˚C induced an increase in F_o and decrease in F_m values of both sunflower varieties under well-watered conditions (Fig 2). At 25˚C no difference was observed in the F_v/F_m values of the drought tolerant (TS) and sensitive (SS) sunflower; however, at 35˚C F_v/F_m values declined to a greater extent in the SS. Values of ΨE_o and $PI_{(abs)}$ allowed differentiation between tolerant and sensitive varieties at 25˚C, and these differences were more pronounced at 35˚C. The *ChlF* parameters ΨE_o, RC/ABS, PI_{abs}, IP-phase and k-band derived from the OJIP curve did not characterize the drought kinetics of the sunflower varieties at both temperatures.

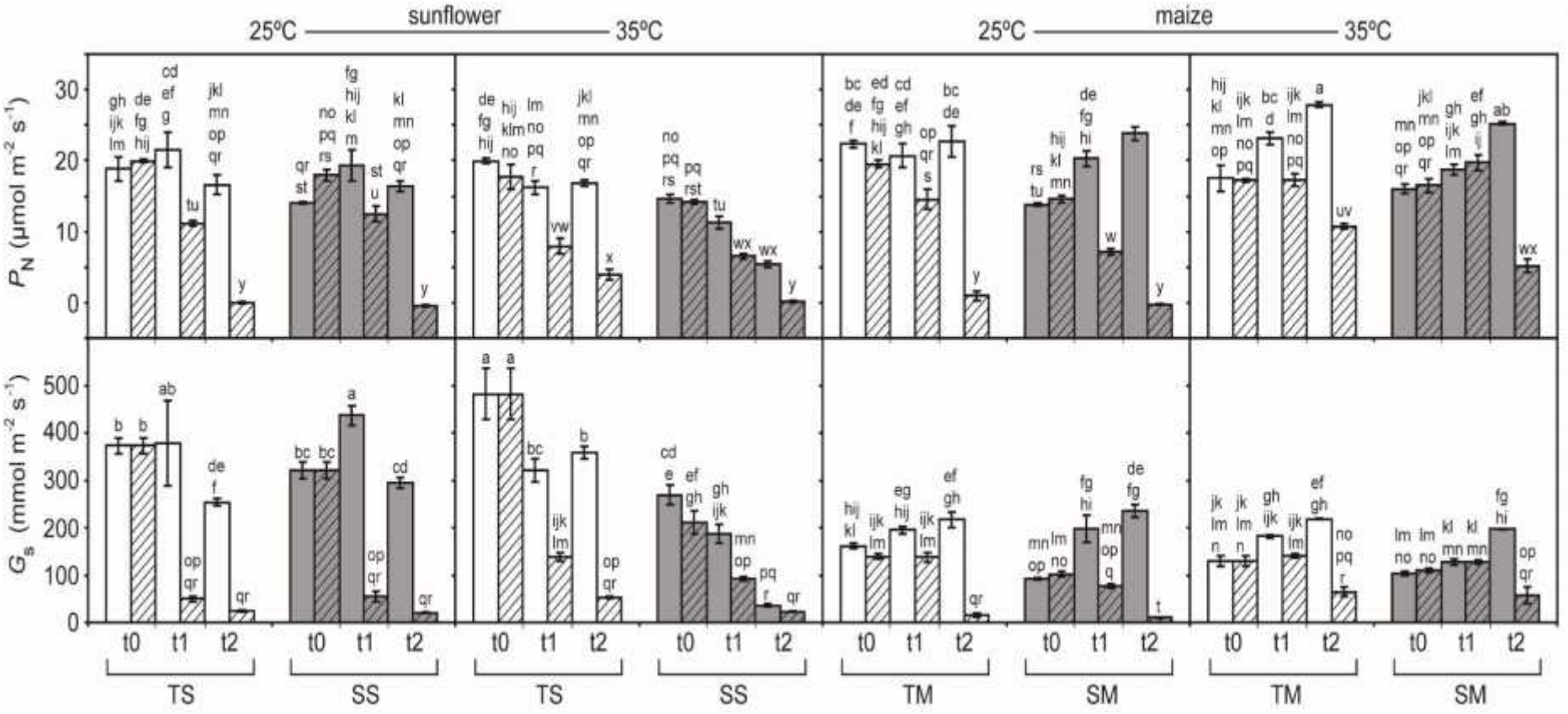

Figure 1: Photosynthesis (P_N), stomatal conductance (G_s), measured using gas exchange of drought tolerant and sensitive varieties of sunflower (TS and SS) and maize (TM and SM). Gas exchange measurements represent the average of values collected at three sampling point (t0, t1, t3). Drought stressed plants (hatched) and the corresponding well-watered control plants (open) measured during the same period. Error bars indicate one standard error either side of the mean. Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post-hoc* test.

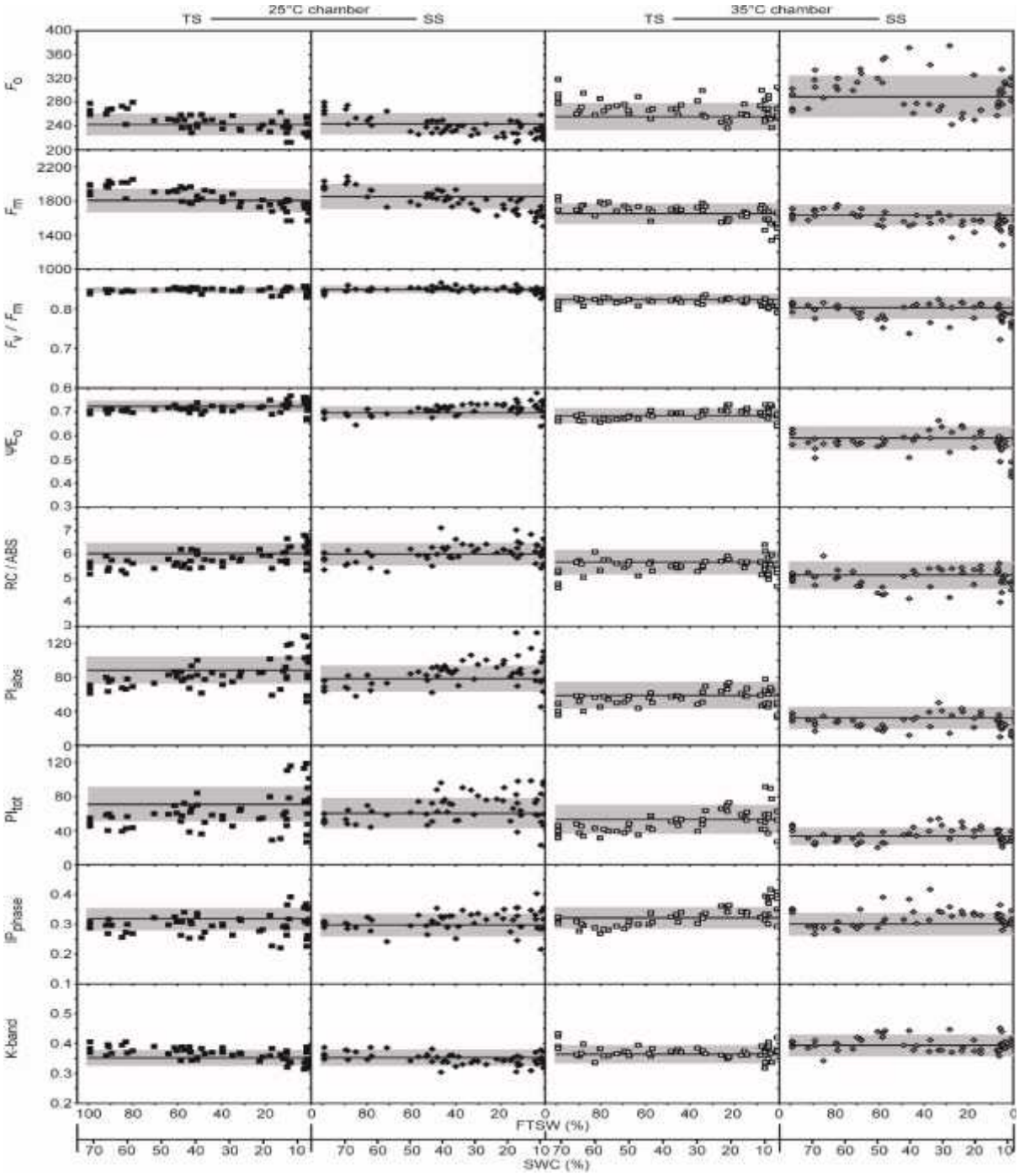

Figure 2: The response of *Chl a fluoresence* parameters in drought tolerant (TS) (square symbols) and sensitive (SS) (diamond symbols) varieties of sunflower to soil drying at 25˚C (solid symbols) and 35˚C (open symbols). Data are plotted against the FTSW and a calibration between FTSW and SWC. The solid horizontal line indicates the mean value of the well-watered control plants during the experimental period and the grey shading indicates ± standard deviation either side of the mean.

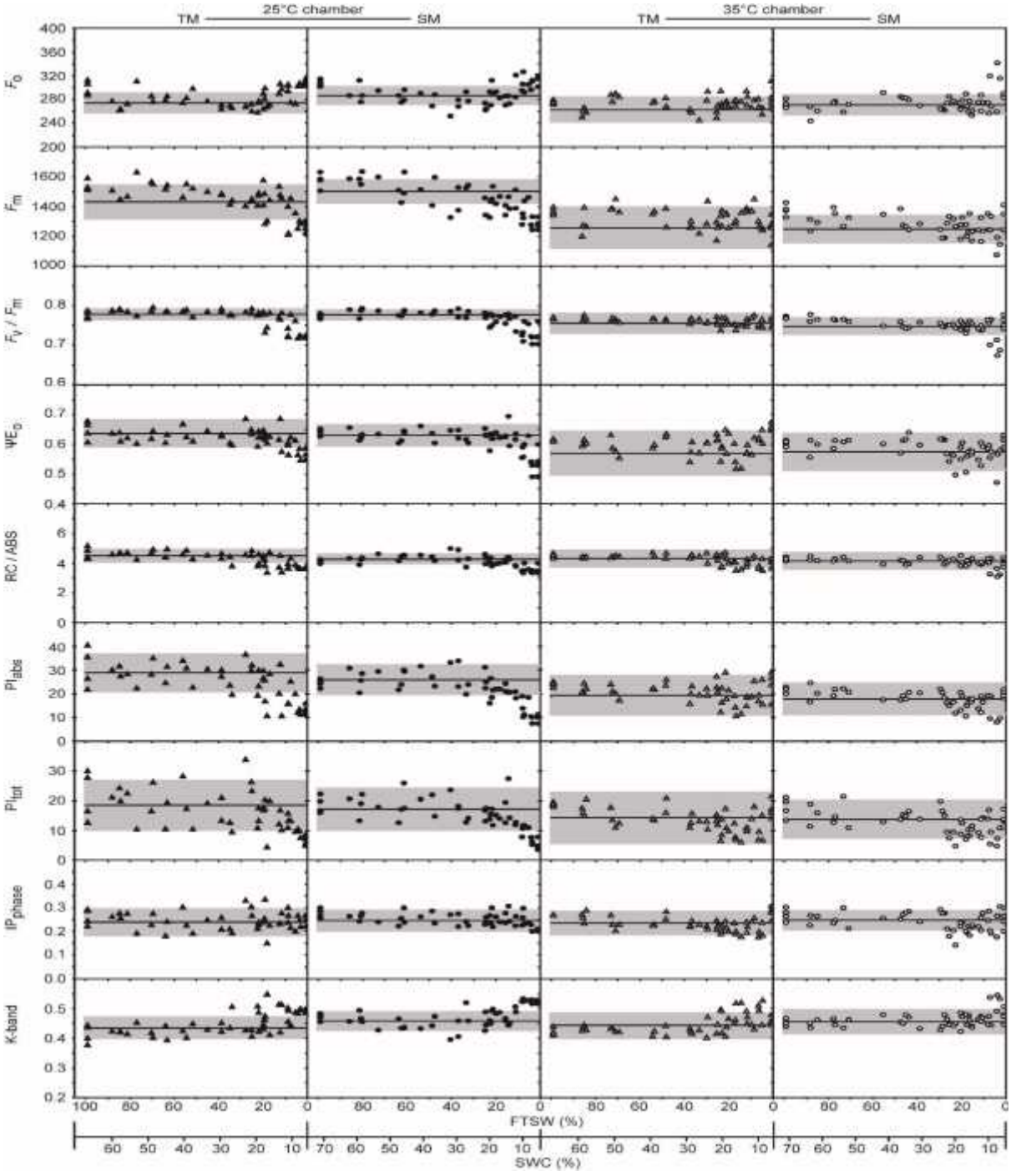

Figure 3: The response of *Chl a fluoresence* parameters in drought tolerant (TM) (triangle symbols) and sensitive (SM) (circle symbols) varieties of maize to soil drying at 25˚C (solid symbols) and 35˚C (open symbols). Data are plotted against the FTSW and a calibration between FTSW and SWC. The solid horizontal line indicates the mean value of the well-watered control plants during the experimental period and the grey shading indicates ± standard deviation either side of the mean.

Table 1: Results of Chlorophyll Fluorescence (ChlF) Parameters of each treatment, mean values represent averages of the last two days of the experiment (n=8). Symbols indicate significant difference of means of the treatments against to two control groups; (25°C: WW-TM(✖) and WW-SM(o)), (25°C: WW-TS(★) and WW-SS(●)) of tolerant and sensitive maize and sunflower varieties respectively, to assess rank-based differences of means between control and treatment groups by Kruskal-Wallis nonparametric test. Significance level 0.05 ($P \leq 0.05$) was applied.

Treatments			Fo			Fm			Fv/Fm			ψE0			RC/ABS			PI(abs)			PI(tot)			ΔV(I-P)			K Band		
Temperature	Variety	Water	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.
	TM	**WW**	263.4	± 13.1		1366.1	± 59.7	o	0.775	± 0.01		0.610	± 0.03		4.404	± 0.38		24.437	± 5.71		16.001	± 7.16		0.234	± 0.06		0.443	± 0.03	
25 °C	TM	D	287.6	± 24.7		1323.0	± 75.6	o	0.746	± 0.02	o	0.600	± 0.02		4.025	± 0.42		18.951	± 7.30		13.086	± 4.04		0.248	± 0.03	o	0.467	± 0.03	
	SM	**WW**	280.1	± 10.1		1455.4	± 63.8	✖	0.773	± 0.01		0.602	± 0.01		4.221	± 0.20		21.844	± 2.37		11.887	± 1.37		0.210	± 0.00		0.458	± 0.01	
	SM	D	302.8	± 13.7	✖ o	1322.9	± 59.0	o	0.728	± 0.02	✖ o	0.561	± 0.04	✖ o	3.569	± 0.30	✖ o	12.909	± 4.88	✖ o	9.171	± 2.49	o	0.234	± 0.02		0.512	± 0.02	✖ o
	TM	WW	258.3	± 13.5	o	1219.3	± 59.7	✖ o	0.755	± 0.00	✖ o	0.577	± 0.02		4.267	± 0.06		19.581	± 2.28		14.274	± 3.56		0.229	± 0.02		0.445	± 0.00	
35 °C	TM	D	276.0	± 15.0		1261.2	± 54.9	✖ o	0.749	± 0.02	✖ o	0.640	± 0.01	o	3.943	± 0.31	✖	21.678	± 5.22		13.402	± 3.54		0.240	± 0.02		0.477	± 0.02	
	SM	WW	276.4	± 7.3		1224.3	± 42.3	✖ o	0.735	± 0.00	✖ o	0.537	± 0.02		3.961	± 0.13		14.958	± 2.02	✖	12.216	± 4.40		0.233	± 0.04		0.469	± 0.01	
	SM	D	292.9	± 25.7	✖	1250.3	± 70.8	✖ o	0.726	± 0.01	✖ o	0.577	± 0.03		3.646	± 0.24	✖ o	14.338	± 1.21	✖ o	10.465	± 2.58		0.244	± 0.05		0.501	± 0.02	✖ o
	TS	**WW**	226.5	± 9.0		1650.4	± 40.3	●	0.847	± 0.00		0.749	± 0.01	●	6.477	± 0.25		108.032	± 9.44	●	98.229	± 12.29		0.355	± 0.02		0.328	± 0.01	
25 °C	TS	D	236.4	± 6.9		1716.0	± 58.2	★	0.845	± 0.00		0.724	± 0.02		6.185	± 0.32		93.674	± 13.73		74.735	± 18.56		0.308	± 0.03		0.343	± 0.01	
	SS	**WW**	232.5	± 13.5		1707.3	± 61.7	★	0.846	± 0.00		0.718	± 0.01	★	6.335	± 0.25		89.967	± 14.86	★	82.747	± 9.75		0.345	± 0.01		0.334	± 0.01	
	SS	D	231.6	± 10.9		1649.6	± 61.3	●	0.842	± 0.00		0.723	± 0.03		6.117	± 0.29		89.016	± 22.01		73.243	± 30.65		0.316	± 0.05		0.345	± 0.01	
	TS	WW	236.6	± 18.4		1534.6	± 100.3	★ ●	0.828	± 0.00	★ ●	0.717	± 0.01	★	6.151	± 0.46		75.968	± 12.30	★	73.436	± 16.29	★	0.350	± 0.02		0.338	± 0.02	
35 °C	TS	D	261.4	± 17.9	★ ●	1537.1	± 102.5	★ ●	0.811	± 0.00	★ ●	0.708	± 0.01	★	5.669	± 0.47	★ ●	59.649	± 11.68	★ ●	65.109	± 19.35	★	0.366	± 0.02		0.360	± 0.02	★
	SS	WW	292.2	± 38.9	★ ●	1529.2	± 61.2	★ ●	0.775	± 0.02	★ ●	0.593	± 0.03	★ ●	4.548	± 0.58	★ ●	26.128	± 10.52	★ ●	33.084	± 8.93	★ ●	0.340	± 0.02		0.432	± 0.04	★ ●
	SS	D	296.6	± 35.0	★ ●	1511.5	± 80.9	★ ●	0.773	± 0.01	★ ●	0.555	± 0.02	★ ●	4.791	± 0.41	★ ●	20.994	± 3.58	★ ●	28.027	± 4.22	★ ●	0.317	± 0.02	★ ●	0.405	± 0.02	★ ●

Rather, as water availability declined towards lower FTSW values, these *ChlF* parameters became more variable. The F_m was effective in demonstrating reduced photochemical energy usage at lower FTSW values in both TS and SS (Fig 2 and Table 1).

The minimal fluorescence values of well-watered maize were not as strongly affected by temperature as sunflower (Table 1). Nevertheless, F_m of maize was sensitive to growth at 25 and 35˚C. The *ChlF* parameters ΨE_o, PI_{abs} and PI_{tot} all showed non-significant declines (Table 1) at the higher temperature in the well-watered maize verities. The kinetics of drought stress were apparent in F_o, F_m, F_v/F_m and PI_{abs} values of maize at 25˚C but not 35˚C.

Lower PI_{abs} in sunflower (Fig 2) and maize (Fig 3) when grown at 35˚C coincided with reduced levels of photosynthetic pigments (Fig 4). Foliar levels of chlorophyll a, total chlorophyll and total carotenoids were lower in plants grown at the higher temperature. The TS generally possessed greater levels of photosynthetic pigments than its sensitive counterpart. In contrast, at t0 the SM exhibited higher levels of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids, but these declined over the course of the experiment in plants grown at 25˚C, while pigment concentrations generally increased in the TM (Fig 4).

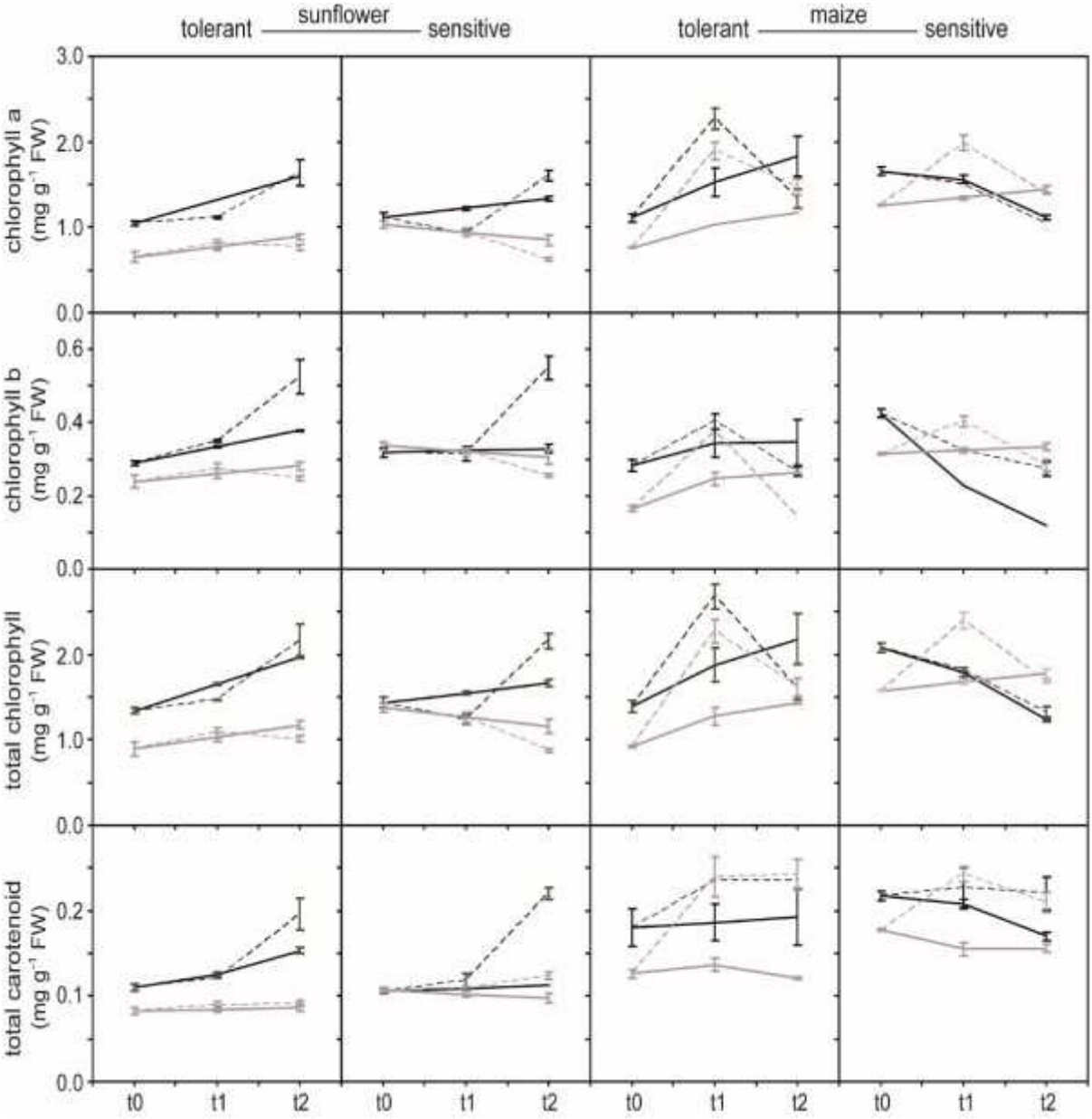

Figure 4: Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content in drought tolerant and sensitive varieties of sunflower and maize exposed to soil drying at 25 and 35˚C at three sampling point (t0, t1, t3). Straight line indicates well-watered/control plants, hatched line indicates drought plants. Black line indicates 25˚C treatment, grey line indicates 35˚C treatment. Differences in homogenous groups for each species (maize and sunflower separately) determined using a one-way ANOVA and LSD *post-hoc* test are provided in supplementary data (see Appendix A2).

4.3.2. Antioxidant Enzyme Activities

At t0, levels of all antioxidants were lower in tolerant and sensitive sunflower varieties grown at 35˚C (Figure 5). This coincided with greater MDA levels in the sunflower varieties from the lower temperature treatment (Fig 6). Levels of antioxidants declined in well-watered sunflower varieties over the course of the study at 25˚C. Drought stress induced more rapid reductions in antioxidant levels in TS at 25˚C, but increases in APX, POX, GR and SOD activity in the SS (Fig 5). Both TS and SS exhibited similar levels of lipid peroxidation during drought stress at 25˚C (Fig 6). At 35˚C, the TS exhibited more pronounced increases in CAT, APX, POX and SOD activities under both well-watered and drought conditions. Drought stress induced increased membrane damage in both sunflower varieties; however, the increase at 35˚C was lower in TS than SS (Fig 6).

The maize varieties exhibited lower levels of antioxidant activity than the sunflower varieties at the start of the experimental period (Fig 5). At 25˚C the well-watered TM exhibited no change or declines in antioxidant levels over the course of the experiment, whereas the SM showed increases in CAT, POX and GR levels (Fig 5). Over the duration of the study, both tolerant and sensitive maize exhibited increased lipid peroxidation under well-watered conditions. Tolerant maize largely showed no difference in the significance or direction (ie. elevated, decreased or maintained) of antioxidant response between plants grown at 25 and 35˚C (Fig 5). In contrast, SM showed reduced levels of CAT and POX activity and increased activities of APX, GR and SOD at the higher temperature. Drought induced increased CAT, APX, POX and GR activities in TM at 25 and 35˚C, but SM showed reduced CAT, POX and SOD at 25ºC during the drought stress. At 35˚C, the SM when subject to drought showed increased CAT, APX, POX, GR and SOD activities at t2. While the impact of temperature on the response of antioxidants is less pronounced between the tolerant and sensitive maize

varieties at 35˚C than between the contrasting sunflower varieties (Fig 5), it is noteworthy that drought induced an increase in activities of CAT, APX, POX and GR at both temperatures, whereas such a consistent up-regulation of antioxidant activities during drought stress was not present in SM indicative of varietal differences in maize. The impact of temperature, drought and variety on the level of antioxidant activity was more apparent in sunflower than maize – a three-way ANOVA was used to assess these interactions and the results are summarised in Table 2.

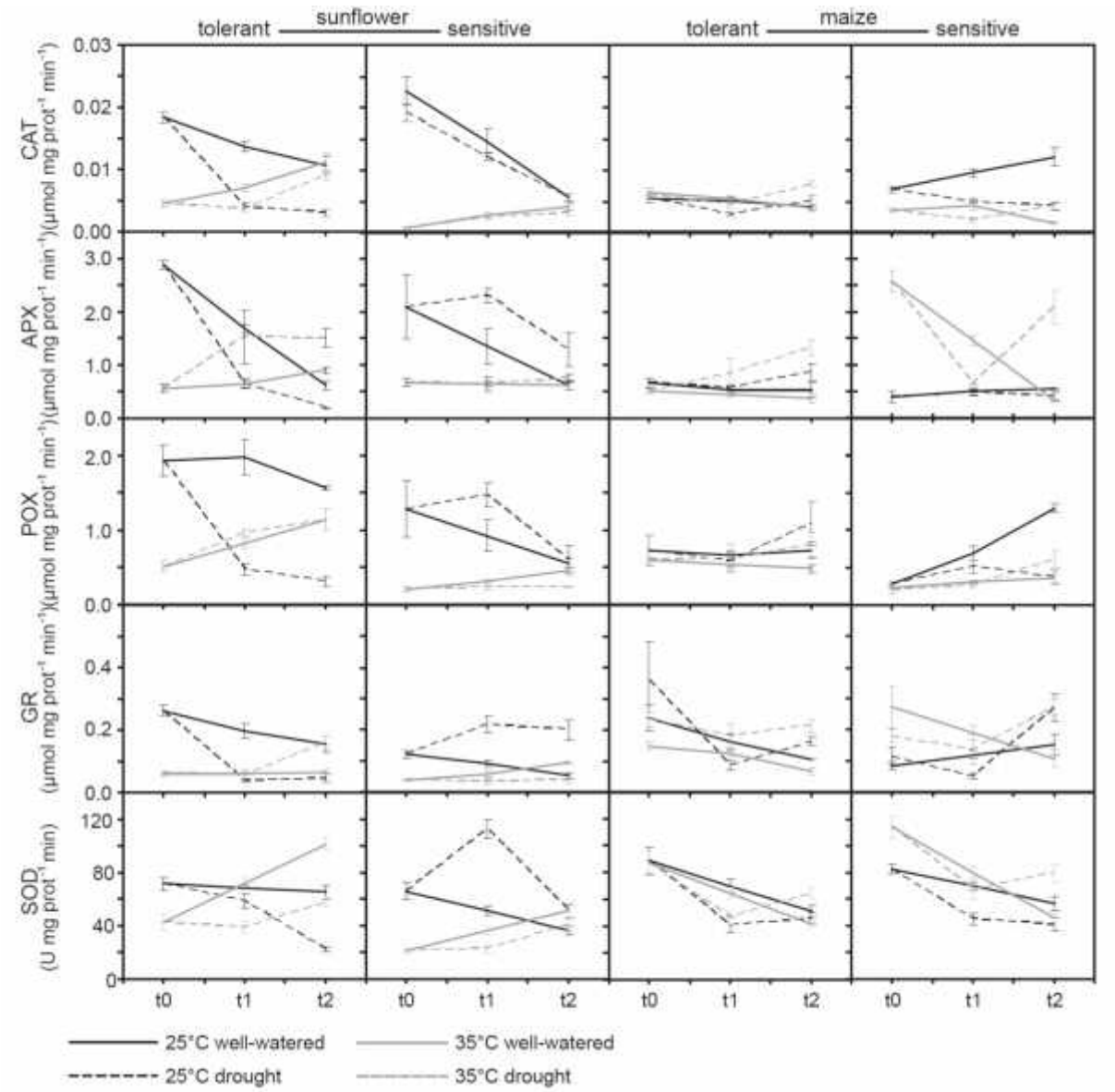

Figure 5: Antioxidant enzyme (CAT, APX, POX, GR) activities in drought tolerant and sensitive varieties of sunflower and maize exposed to soil drying at 25 and 35˚C at three sampling point (t0, t1, t2). Line indications same as Fig. 4. Differences in homogenous groups for each species (maize and sunflower separately) determined using a one-way ANOVA and LSD *post-hoc* test are provided in supplementary data (see Appendix A2).

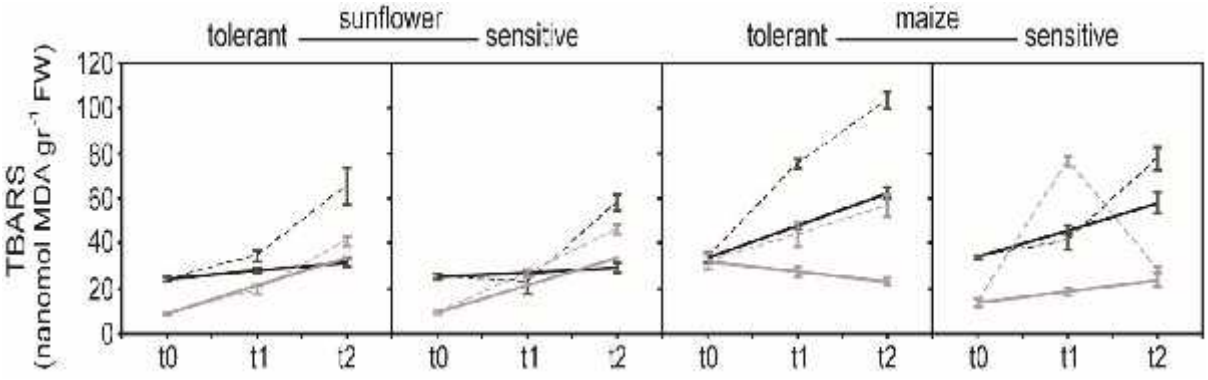

Figure 6: Lipid peroxidation level (TBARS content) in drought tolerant and sensitive varieties of sunflower and maize exposed to soil drying at 25 and 35˚C at three sampling point (t0, t1, t3). Line indications same as Fig. 4 Differences in homogenous groups for each species (maize and sunflower separately) determined using a one-way ANOVA and LSD *post-hoc* test are provided in supplementary data (see Appendix A2).

Table 2. Effects of factors on measured parameters at the end of the experiment (t2) by three-way ANOVA test. Symbols; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ represent significance levels of stress factors and their interactions on parameters; antioxidant enzymes, chlorophyll and carotenoid content of maize and sunflower for each species independently.

		CAT	APX	POX	GR	SOD	TBARS	Chl.a	Chl.b	Tot. Chl	Tot. Carot.
Maize	Variety (V)	n.s.	n.s.	n.s.	**	n.s.	***	**	n.s.	*	n.s.
	Temperature (T)	***	***	***	n.s.	**	***	n.s.	n.s.	n.s.	n.s.
	Water (W)	n.s.	***	n.s.	**	**	***	n.s.	n.s.	n.s.	***
	V * T	***	*	n.s.	n.s.	n.s.	n.s.	***	***	***	n.s.
	V * W	***	n.s.	**	n.s.	n.s.	***	n.s.	***	n.s.	n.s.
	T * W	***	***	*	n.s.	***	*	**	***	n.s.	n.s.
	V * T * W	***	**	***	n.s.	n.s.	n.s.	**	*	**	n.s.
Sunflower	Variety (V)	***	n.s.	***	***	***	***	***	***	**	***
	Temperature (T)	n.s.	***	**	n.s.	***	***	***	***	***	***
	Water (W)	n.s.	***	**	***	*	***	n.s.	*	n.s.	***
	V * T	***	***	**	n.s.	***	***	***	***	***	**
	V * W	***	**	***	*	***	***	n.s.	***	n.s.	*
	T * W	***	***	***	n.s.	**	***	n.s.	***	n.s.	n.s.
	V * T * W	n.s.	***	***	*	***	n.s.	***	**	***	**

4.4. DISCUSSION

Drought and heat stress frequently occur together in agricultural systems causing impaired plant growth and loss of productivity. This study has shown contrasting *ChlF* and antioxidant responses to drought and/or heat stress in drought tolerant and sensitive varieties of C3 sunflower and C4 maize. The impact of drought and heat stress was more pronounced in sunflower and less evident in maize; consistent with the leaf gas exchange (Fig 1) and morphological responses reported by Killi et al 2016. Nonetheless, antioxidants also played a significant role in determining the PSII response of C4 maize to drought stress.

4.4.1. Chlorophyll fluorescence analysis of the effects of drought and heat stress

The maximum quantum yield of PSII is relatively stable under drought stress (Baker and Rosenqvist, 2004; Bukhov and Carpentier, 2004), and F_v/F_m declines only in the most severe stages of drought stress (Kalaji et al., 2016). Studies carried out by analyzing the OJIP transient (JIP test: Oukarroum et al., 2007; Pollastrini et al., 2014) indicate that the depression of the IP phase was the most relevant effect of drought stress assessed on crop and tree plant species. Heat stress may be more detrimental than drought stress to PSII, particularly with temperatures above 40ºC (Oukarroum et al., 2007; Martinazzo et al., 2012). Heat stress induces irreversible damage to PSII, with a strong rise of F_o (Baker and Rosenqvist, 2004; Kalaji et al., 2016). In the OJIP transient, heat stress produces an increase in relative variable fluorescence at 300 μs (K-band) that indicates a break-down of the oxygen evolving complex (Srivastava et al., 1997). In the present study, the antagonistic effect of the two stressors was detected on maize. In this crop species, the PSII appeared to be vulnerable to severe drought (SWC< 20%) at the temperature of 25°C, with the increase of F_o, decline of F_m and subsequent reduction of F_v/F_m. According to Krause and Weis (1991) the increase of F_o is indicative of irreversible damage to PSII. These effects were more pronounced in the sensitive varieties, and included a significant

rise of K-band. Detrimental effects of drought stress were alleviated on maize subjected to higher temperature 35°C, with few differences between the two varieties. These results are consistent with the behaviour of P_N reported by Killi et al. (2016) (Fig 1).

Sunflower at 25ºC did not show any change in chlorophyll fluorescence parameters also in the most severe drought conditions; although the decrease of P_N at SWC<40% (Killi et al., 2016), so indicating the stability of PSII under drought stress and the prominent role of stomatal limitation in the regulation of P_N (Pinheiro and Chaves, 2011; Killi et al., 2016). Significant changes of *ChlF* parameters in the most severe drought conditions (SWC<10%) were observed in sunflower plants at 35°C, both in sensitive and tolerant varieties. However, a different pattern in *ChlF* responses was observed in maize. The contrasting behaviours observed between maize and sunflower suggest that the interactive effects of high temperatures and drought stress are species specific depending upon the photosynthetic pathway.

To maintain and increase food production in hot arid regions it is necessary to identify crop varieties that tolerate drought and heat stress. Chlorophyll-fluorescence allows the rapid non-invasive screening of large numbers of crop varieties to characterise their phenotypic responses under stress conditions. Despite the potential of high throughput *ChlF* screening for categorising phenotypic responses to drought and heat stress, few studies have investigated which *ChlF* parameters are effective in gauging the negative impacts of drought and/or heat stress on PSII as a basis for identifying resistance. The *ChlF* measurements performed in this study are considered to be 'active' and require dark-adaptation and then exposure to a saturating pulse of PAR to provide information about the capacity for photochemistry in the PSII reaction centres (Strasser et al., 2004). Our measurements suggest that the most effective parameter in gauging the kinetics of the drought response in both sunflower (Fig 2) and maize

(Fig 3) is the F_m. The reduction in F_m values at lower FTSW levels indicates a reduction in the potential use of energy for photochemistry. The maximum quantum yield of PSII (F_v/F_m), ΨEo and PI_{abs} were effective in gauging the impact of heat stress on photosynthetic performance in both species. The RC/ABS parameter declined at 35˚C in the C3 sunflower and to a greater extent in the SS (Fig 2), possibly due to lower levels of photosynthetic pigments (Fig 4) and antioxidant activity (Fig 5) in the sensitive variety resulting in less functional reaction centres to absorb light energy for photochemistry. Differences in photosynthetic pigment concentrations were not so pronounced between the TM and SM varieties at 35˚C, possibly accounting for the lack of difference in RC/ABS values at 25 and 35˚C in the maize plants.

4.4.2. Antioxidant enzyme responses

Plant responses to a combination of stresses are different to those of the stresses individually (Jiang and Huang, 2001; Rizhsky et al., 2004; Mittler, 2006; Rampino et al., 2012). Stomatal regulation is one of the most important adaptation mechanisms to environmental changes such as water deficit (Hetherington and Woodward, 2003; Lauteri et al., 2014; Haworth et al., 2016). However, the limitation of CO_2 availability for photosynthesis due to stomatal closure results in an increase in un-utilized light energy (Smirnoff, 1993; Demmig-Adams and Adams, 2000; Flexas and Medrano, 2002). This creates an imbalance between the generation and the utilization of electrons by PSII and PSI (Peltzer et al., 2002; Reddy et al., 2004). These excessive electrons cause the formation of ROS in plant sub-cellular compartments under stress conditions (Elstner, 1982; Halliwell and Gutteridge, 1984). The formation and antioxidant scavenging of ROS also differs under single stress and combined stresses conditions (Jiang and Huang, 2001; Mittler, 2002; Sekmen et al., 2014). These mechanisms are unclear in the

literature, especially when comparing C3 and C4 plant species and may account for the differences in antioxidant responses of sunflower and maize recorded in this study.

C4 metabolism has been suggested to serve as a mechanism for the avoidance of ROS production (Mittler, 2002, 2006). C4 species such as maize exhibit greater WUE than C3 species and can maintain P_N during drought and heat stress to a greater extent (Crafts-Brandner and Salvucci, 2002; Killi et al., 2016). This reduces the generation of ROS, possibly accounting for the lower levels of antioxidant activity and less pronounced varietal differences observed in maize in this study. At 25ºC the drought tolerant and sensitive varieties of sunflower exhibited largely similar responses to soil drying in terms of antioxidant activity (Fig 5) and lipid membrane peroxidation (Fig 6). However, at 35ºC antioxidant activity of SS declined over the duration of the experiment, possibly indicating that the protective mechanisms were being overwhelmed in the drought sensitive variety. In contrast, antioxidant activities generally rose over the course of the study in the TS. This may possibly account for higher levels of TBARs in the drought stressed SS at 35ºC. The activity of SOD was greater in the TS than SS at 35ºC (Fig5), indicative of a greater capacity to neutralise O_2^- (Smirnoff, 1993). Catalase activity was also greater in the TS, consistent with greater capacity to convert H_2O_2 produced by GR and SOD into water and oxygen (Foyer and Halliwell, 1976). In particular, POX and APX that act within the chloroplast envelope (Smirnoff, 1993; Das and Roychoudhury, 2014), where the thylakoid membranes are located (Gounaris et al., 1983), were respectively 2.5 and 5.5 times higher in the TS than SS (Fig 5). This may account for the greater retention of PSII electron transport of TS exposed to drought at the higher temperature. A drought tolerant C3 cotton variety also exhibited increased POX activity in comparison to a drought sensitive variety, but no differences were recorded in APX activity between the contrasting cotton varieties (Sekmen et al., 2014); possibly suggesting that antioxidant

characteristics, and how these manifest as drought tolerance, vary between C3 species. The results of this study may suggest that antioxidant capabilities play a more pronounced role in determining the PSII performance of C3 rather than C4 species; as the C4 photosynthetic system minimises the production of ROS (Mittler, 2002, 2006; Gambarova and Gins, 2008). Nevertheless, antioxidants do play an important role in the prevention of membrane lipid peroxidation in maize as evidenced by the activities of CAT, APX, POX and GR being increased under drought stress in TM at 25 and 35ºC; whereas this consistent up-regulation of antioxidant activities during drought stress was not present in SM (Fig 5). As such, the selection of drought and heat tolerant varieties on the basis of antioxidant capabilities is likely to be most beneficial to C3 species such as sunflower, but it is also a component in determining varietal tolerance to heat and drought stress in C4 species such as maize.

4.5. CONCLUSIONS

Drought events and heat waves frequently occur together causing widespread damage to crops. Both drought and heat stress disrupt the structure and function of plant membranes, such as the thylakoid membranes within the chloroplast where PSII takes place. To stabilise and protect these membranes from lipid peroxidation, plants possess antioxidant systems to neutralise ROS that are produced by an imbalance of energy usage experienced by plants during drought and/or heat stress. Analysis of PSII electron transport using *ChlF* indicated that drought and heat stress had a greater impact in C3 sunflower than C4 maize (Fig 2 and 3); a result consistent with gas exchange analysis of CO_2 assimilation in the same varieties (Fig 1)(Killi et al., 2016). The TS retained PSII performance to a greater extent during drought stress at 35˚C due to enhanced antioxidant activities (Fig 5); in particular, POX and APX that function within the chloroplast envelope where PSII occurs. The levels of antioxidant activity in the SS declined during drought stress at the higher temperature, possibly accounting for impaired PSII.

Moreover, the TS exhibited increases in photosynthetic pigments over the duration of the study (Fig 4), consistent with a greater use of energy in photochemistry, reducing the production of harmful ROS that may have overwhelmed the protective antioxidant systems in the SS. Varietal differences in antioxidant activities were less pronounced in maize; yet, TM did consistently increase antioxidant activities during drought stress at both temperatures, whereas the SM did not respond in the same manner. This may indicate that while antioxidants play an important role in both crops, selection on the basis of enhanced antioxidant function would be more beneficial in the development of drought and heat tolerant sunflower phenotypes. Chlorophyll fluorescence was highly effective in screening the sunflower and maize varieties during drought and heat stress. Indeed, the F_m was the most sensitive parameter in discerning varietal effects of drought and heat stress in both species.

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"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more,
so that we may fear less."
Marie Curie

Paper III

Photosynthetic and morphological responses of oak species to temperature and [CO_2] increased to levels predicted for 2050.

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Author contributions:

□ Analysed the data, conducted the experiment: DK

□ Wrote the paper: DK, FB

□ Conducted M-PEA measurements: DK, EG, MP

□ Conducted H-PEA measurements: DK, MP, FB

□ Conducted leaf gas-exchange measurements: AF, DK, JM

□ Conducted electron microscope analysis: CT, AP

□ Designed the experiment: AF, FF

Abstract

Urban forests are environmentally, climatically, socially and economically important. An understanding of the response of urban trees to future climate change is crucial to the maintenance of urban forests and the ecosystem services they support. We conducted a controlled environment experiment to investigate the impact of elevated CO_2 and temperature to levels predicted for 2050 in urban areas of central Italy on two common Mediterranean oak species: the evergreen *Quercus ilex* and the deciduous *Quercus cerris.* *Quercus cerris* initially increased net-photosynthesis (P_N) under elevated 2050 conditions (EC) compared to present ambient conditions (AC), before P_N declined, possibly indicative of down-regulation of photosynthetic physiology. *Quercus ilex* P_N was not influenced by EC throughout the three-month duration of the study. Levels of P_N and G_s were generally lower in *Q. ilex* than *Q. cerris*. *Quercus ilex* also reduced G_s during growth at EC. This reduced transpirative water-loss caused a significant increase in the water use efficiency (WUE) of *Q. ilex*. This reduction in G_s may have been associated with reduced stomatal density in *Q. ilex* grown under EC, while the number of stomata on leaves developed under the experimental conditions were unaffected by the EC treatment in *Q. cerris*. Over the course of the experiment, above (stem dry weight: SDW) and below-ground biomass (root dry weight: RDW) and foliar starch increased in *Q. cerris* (in both EC and AC equally) but not *Q. ilex*. Chlorophyll a fluorescence *(ChlF);* Prompt Fluorescence (PF), Delayed Fluorescence (DF) and Modulated Reflectance (MR) also indicated that a greater resilience of photochemistry to growth under EC was more apparent in *Q. ilex* than *Q. cerris*. In particular, the reduction of the quantum yield efficiency (F_V/F_M) in *Q. ilex* may also be considered functional to maintain constant P_N levels in elevated temperature and [CO_2]. The results of this study suggest that *Q. ilex* exhibits greater plasticity and adaptation

to EC, and may therefore perform more favourably under future 2050 climatic conditions.

Keywords: elevated CO_2, heat stress, *Quercus ilex*, *Quercus cerris*, urban forest, TreeCity.

Highlights

Short-term photosynthetic responses of oak species to increased temperature and [CO_2] were assessed.

Down-regulation mechanisms of photosynthesis act differently in deciduous and evergreen species.

Quercus ilex is better adapted than *Quercus cerris* to future climates of Mediterranean cities.

Abbreviations;

P_N, Net Photosynthesis; G_s, Stomatal conductance; C_a, Ambient/external CO_2; PSI, *Photosystem I (P700);* PSII, Photosystem II (PSII, P680); ΦPSII, Actual quantum yields of primary photochemistry ; WUE_i, Instantaneous water use efficiency (ratio of P_N to transpiration); *Chl,* chlorophyll; *ChlF,* chlorophyll fluorescence; PF,prompt fluorescence*;* F_0, minimal fluorescence ; F_J, F_I, fluorescence intensity at 2 ms or 30 ms, respectively; F_M, maximal fluorescence ; F_V, maximal variable fluorescence; *OEC,* oxygen-evolving complex; *RC,* reaction centers; *RE,* reduction of end-electron acceptors; δ_{R0}, the efficiency of electron transfer between intermediate carriers to the RE of PSI; F_V/F_M (φ_{P0}), maximal quantum yield of PSII primary photochemistry ψ_{E0}, the efficiency of trapped energy to move an electron further than Q_A^-; PC, plastocyanin; MR, modulated reflectance (820nm) of P700; V_{ox}, the rate of P700 and

PC oxidation, calculated as the maximum slope decrease of MR_t/MR_0;V_{red},the rate of P700 and PC re-reduction, calculated as the maximum slope increase of MR_t/MR_0; MR_{min}, minimum value of the normalized MR curve (MR_t/MR_0), a transitory steady state with equal oxidation and re-reduction rates of P700 and PC; MR_t/MR_0, ratio between modulated 820 nm reflection intensity at time *t*; (MR_t), and value of the 820 nm reflection of the sample at the onset of the actinic illumination (between 0.3 and 1 ms, MR_0); DF, delayed fluorescence; I_1, first maximum of the DF induction curve; I_2, second maximum of the DF induction curve; I_4,the final maximum of the DF induction curve.

5.1. INTRODUCTION

Mediterranean trees grow in habitats characterized by high levels of photosynthetically active radiation (PAR), high temperatures and low water availability that frequently results in photo-inhibition. In urban areas these factors are amplified, increasing the energy balance of leaves, but impairing the utilization of this energy for photosynthesis (P_N). Rising levels of atmospheric [CO_2] are likely to increase water use efficiency (WUE), possibly alleviating some of the negative impacts of growth in high energy Mediterranean environments (Battipaglia *et al.*, 2013; Bussotti *et al.*, 2013; Keenan *et al.*, 2013). However, temperature is also predicted to rise alongside [CO_2], negatively affecting plant carbon-water balance through increased leaf to air vapor pressure deficit (VPD) and a reduction in the ratio of photosynthesis to photorespiration. How rising [CO_2] and temperatures will interact to affect the growth of Mediterranean tress is currently unclear.

The effects of climate change will become more apparent and prominent over the upcoming decades. Mean global surface temperature is expected to rise up to 2.6 °C by 2050 (IPCC, 2014); moreover, IPCC reports show that decadal average temperature over land area for 2002–2011 is already 1.3° ± 0.11°C greater than average of second half of the 19th century (1850-1899) (Brohan *et al.*, 2006; Kovats *et al.*, 2014). The concentration of [CO_2], the most abundant greenhouse gas, has increased from pre-industrial levels of 270 ppm to 400 ppm at present. Current estimates predict a further increase to 463-623 ppm [CO_2] by 2050 (IPCC, 2014). In cities, [CO_2] and temperatures are usually higher than in the surrounding rural areas (Oke, 1973; Rizwan *et al.*, 2008; Bowler *et al.*, 2010; Onishi *et al.*, 2010; Oliveira *et al.*, 2011; Loughner *et al.*, 2012). In particular, night-time temperatures can be up to 4-5 °C greater in urban areas than in surrounding rural areas (i.e. the "Urban Heat Island" effect) (Nichol, 2005; Kardinal Jusuf *et al.*, 2007). Forests contribute around 50% of terrestrial net primary productivity (NPP) and store approximately 45% of terrestrial carbon; thus playing a crucial role in atmospheric carbon cycling (Silva and Anand, 2013). Urban trees contribute significantly to global carbon storage and climate change mitigation (Brack, 2002; Nowak, 2002; Davies *et al.*, 2011; Horn *et al.*, 2014; Lwasa *et al.*, 2014). However, the impacts of climate change will be most pronounced in cities and their environmental conditions can affect the ecological and physiological efficiency of urban trees, reducing their capacity to deliver ecosystem services (Lindner *et al.*, 2010; Allen *et al.*, 2011; Zeppel *et al.*, 2012; Brown *et al.*, 2015; Calfapietra *et al.*, 2015; Savi *et al.*, 2015).

Elevated concentrations of atmospheric [CO_2] stimulate photosynthesis, plant growth and productivity (Drake et al., 1997; Eamus and Jarvis, 1989; Haworth et al., 2016a;

Stinziano and Way, 2014; Warren et al., 2015). The carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is generally limited at current ambient [CO_2] levels, thus rising [CO_2] will likely increase photosynthetic rates in C3 plants by increasing the rate of carboxylation and inhibiting oxygenation (Long *et al.*, 2004; Bernacchi *et al.*, 2005; Sage and Kubien, 2007; Yajun *et al.*, 2015; Flexas *et al.*, 2016). However, rising temperature also decreases the affinity of RuBisCO for CO_2 and the solubility of [CO_2] relative to O_2. Therefore, temperature increases reduce the rate of carboxylation to oxygenation and enhance photorespiration (Long, 1991; Sage and Kubien, 2007; Carrera *et al.*, 2016; Killi *et al.*, 2016), possibly counteracting the stimulation of P_N by rising [CO_2](Farquhar *et al.*, 1980). Higher [CO_2] generally reduces stomatal conductance (G_s), decreasing plant water-loss and increasing water use efficiency (WUE) (Haworth *et al.*, 2013, 2015; Keenan *et al.*, 2013; Silva and Anand, 2013; Tang *et al.*, 2014; Frank *et al.*, 2015). These physiological responses to rising [CO_2] do not necessarily induce increased growth in tree species (Stinziano and Way, 2014), rather an increase in the ratio of carbon gain to water loss that potentially enhances drought tolerance and adaptation to a water-limited environment (Wullschleger *et al.*, 2002; Ainsworth and Long, 2005). However, rising temperatures increase leaf to air vapor pressure deficit (VPD), inducing higher potential rates of water loss in the absence of stomatal regulation (Heath, 1998). The effect of rising temperature and atmospheric [CO_2] on trees is largely unclear. The meta-analyses performed by Baig et al. (2015), however indicated a large variability in the responses of trees subjected to increased [CO_2] and temperature, ranging from positive interactions between these two factors to no interactions. Salzer et al. (2009), observed that in areas where photosynthesis was limited by temperature, a combination of [CO_2] and higher temperatures increased forest productivity. However many studies

report that warmer temperatures and an accompanying increase in the number of heat waves associated with rising atmospheric [CO_2] lead to decreased forest growth and increased tree mortality (Silva *et al.*, 2010; Perry *et al.*, 2012; Anderegg *et al.*, 2013; Stinziano and Way, 2014). Coumou et al., 2013 reported that the duration of heat waves is increasing and may have contributed to a 0.5 °C increase in mean global temperature. Moreover, under a medium global warming scenario, by the 2040s, the number of global monthly heat records and high-temperature events will likely become more pronounced and frequent. The impact on trees of a combination of elevated [CO_2] with other environmental factors such as rising temperature (Slot and Winter, 2016) and drought (Killi *et al.*, 2016) is highly variable. Therefore, it is difficult to definitively state whether the effects of elevated [CO_2] and temperature will be additive, antagonistic or independent. We hypothesize that different plant species are able to implement individual strategies to cope with these factors, including the modification of the photosynthetic physiology. These responses may depend on the evolutionary history of a given species due to the prevailing ecological conditions that have affected plant physiology.

The effect of simultaneous increases in [CO_2] and temperature on Mediterranean forest and urban trees is relatively unexplored. Therefore we conducted a short term experiment to gauge the likely impact of climate change on WUE, morphology and physiology of two common, but contrasting, Mediterranean oak species (evergreen schlerophyll *Quercus ilex* L. and deciduous *Quercus cerris* L.), under growth conditions representing the present (AC) and predicted future 2050 (EC) conditions of central Italy. Evergreen species are supposed to be slower in their acclimation to change in environmental parameters than deciduous species (Niinemets *et al.*, 2011). These

two selected species are common in rural areas of central Italy, and are abundant in urban streets and forests (Ugolini *et al.*, 2012). This study aims to: i) investigate the combined impact of higher [CO_2] and temperatures on the photosynthetic physiology of *Q. ilex* and *Q. cerris*; ii) quantify the combination of elevated [CO_2] and temperature on stomatal regulation of water-loss; iii) gauge the impact of predicted 2050 growth conditions on leaf energy partitioning using chlorophyll fluorescence techniques, and; iv) characterize the likely impact of rising [CO_2] and growth temperatures on evergreen and deciduous oak species and identify the attributes that are likely to be favourable to growth in the urban environments of 2050.

5.2. MATERIALS AND METHODS

5.2.1. Plant growth conditions

Four-year-old deciduous *Q. cerris* and evergreen *Q. ilex* L. were grown in 50 liter pots in a substrate composed of a mixture of peat and pumice (2:1) and fertilized with 3kg/m^3 of controlled release fertilizer (Osmocote exact, 15: 9: 12, Everris, Geldermalsen, the Netherlands) for six months prior to the experiment. The plants were grown in full sunlight and watered regularly to pot capacity. In June (10^{th}) 2015 (t_0), three plants of each species were placed into large walk-in climatic growth-chambers. The growth chambers maintained conditions of 16 hours of daylight (14 hours at full PAR levels of 1200 µmol m^{-2} s^{-1} with two one-hour periods of simulated dawn/dusk where the light intensity was incrementally increased/decreased) and 75% relative humidity. Temperature and [CO_2] differed between the chambers: the ambient chamber operated a [CO_2] level of 400 ppm (C_a:400 ppm) and a day/night temperature regime of 28/24°C (T_a: 28/24°C), while the elevated chamber maintained a [CO_2] level of 550 ppm (C_e:550 ppm) and temperature level of 30.5/28°C (T_e: 30.5/28°C) (ie. ambient

+2.5 °C during the day and +4 °C at night). The ambient chamber/condition (AC: C_a+T_a) replicates current average summer conditions, while the elevated chamber/condition (EC: C_e+T_e) was intended to simulate average predicted summer weather conditions for 2050 (Meehl *et al.*, 2007). Plants were exposed to an acclimation period in the growth chambers starting from t0 until t1 (40 days). Physiological measurements were carried out three times on fully expanded leaves that had developed in the chambers (i.e., under experimental conditions) in the spring – summer 2015: time 1 (t1; 21st July); time 2 (t2; 4th August) and time 3 (t3; 20th August).

5.2.2. *Simultaneous leaf gas-exchange and chlorophyll fluorescence measurements*

Gas-exchange measurements were taken at t1 and t3. A PP-Systems Ciras-2 attached to a PLC6(U) leaf cuvette and LED light unit with a CFM chlorophyll fluorescence module (PP-Systems, Amesbury, Massachusetts, USA) was used to simultaneously measure gas-exchange and chlorophyll fluorescence parameters of the plants. Three replicate plants and three representative leaves for each plant were analyzed for each species. Fully expanded leaves grown under full PAR during the acclimation period were consistently used for gas exchange measurements. The measurements were carried out from 10:00 to 13:00. The cuvette conditions used for the simultaneous measurements of leaf gas-exchange and chlorophyll fluorescence were identical to growth conditions inside the respective growth chambers (ie. PAR of 1200 µmol m^{-2} s^{-1}, leaf temperature of 28 or 30°C and reference [CO_2] concentrations of 400 ppm and 550 ppm for AC and EC plants, respectively). The actual quantum efficiency of photosystem II (ΦPSII: ΔF/F'm; (Genty *et al.*, 1989) was determined following application of a saturating pulse of light of 10000 µmol m^{-2} s^{-1} of 0.8 s duration.

5.2.3. Chlorophyll a fluorescence transient analysis and parameters

Prompt Fluorescence (PF) measurements were carried out with a Plant Efficiency Analyzer (PEA) portable fluorimeter Handy-PEA (Hansatech. Norfolk, UK) on the following dates t1:21st July, t2: 4th August, t3: 20th August, 2015, during the experiment. Prompt Fluorescence measurements were taken from three leaves per plant. Leaves were exposed to 30-minutes dark adaptation then exposed to a saturating light pulse (intensity >3000 µmol photons m^{-2} s^{-1}, excitation light of 650 nm) (Strasser *et al.*, 2000, 2004, 2010). The parameters considered in this study are provided in supplementary data B1 (Appendix B1). H-PEA signals for OJIP test parameters were analyzed by Biolyzer 4 HP v.3 (the chlorophyll fluorescence analyzing program by Bioenergetics Laboratory, University of Geneva, Switzerland).

Plotted on a logarithmic time scale, the ChlF transient shows a polyphasic pattern. The different time steps of this polyphasic transient are labelled as O (20–50 µs), J (2 ms), I (30 ms) and P (peak). The latter indicates the highest fluorescence intensity (F_M), when all the reaction centres of photosystem II (PSII) are reduced by saturating light. For reviews of the theoretical background of the method used to analyze the structure and functionality of photosystem II (the JIP-test) and the ChlF parameters obtained from the ChlF induction curve (fast kinetics) of dark-adapted leaves, (see Kalaji et al., 2014a; Strasser et al., 2004). ChlF parameters used in this study were F_V/F_M, ψ_{Eo}, ΔV_{IP} and the performance indices (PI_{ABS} and PI_{TOT}) (formulas are provided in Appendix B1). F_V/F_M is the maximum quantum yield for primary photochemistry of a dark-adapted sample; ψ_{Eo} is the probability of an electron to reduce the primary Quinone acceptor (Q_A) and to move into the electron transport chain beyond PSII; ΔV_{IP} represents the amplitude of the relative contribution of the I-to-P rise to the OJIP transient, is an

indicator of the abundance of photosystem I (PSI) with respect to PSII, and is related to the electron transport chain beyond PSI (Ceppi *et al.*, 2012). Finally, the performance indices (PIs) measure the potential energy conservation of photons in the intersystem between PSII and PSI (PI_{ABS}) and the potential energy conservation from photons absorbed by PSII to the reduction flux of PSI end acceptors (PI_{TOT}).

5.2.4. Analysis of double-hit protocol

The 'double-hit' protocol (Appenroth *et al.*, 2001) was applied to leaves at t3 by applying a 10 second of dark-adaptation after PF measurement followed by a second saturating light pulse. During the first flash saturating (first hit) all PSII reaction centers (RCs) are closed (reduced), after 10 seconds of darkness the second flash F_0 value is higher than the first F_0. The difference of the relative variable fluorescence between the second and the first flash corresponds to the fraction of RC that are not reopened during the 10 seconds of darkness. This fraction is called "very slow re-opening RCs" or "no Q_B-binding centre" Based on these principles the fraction of so-called "Q_B-binding centers" are calculated as follows:

$$1-\Delta V_o = (1- F_0/ F_M)_{\text{second hit}} - (1- F_0/ F_M)_{\text{first hit}}$$

Another expression of the rate of oxidation of plastoquinone velocity is given by the relative amplitude of the J-point in two saturating flashes (ΔV_J) (Toth *et al.*, 2007). This analysis is carried out as the difference between V_J of the normalized curve of the second flash and the first flash.

5.2.5. Simultaneous measurements of the kinetics of modulated 820 nm reflection (MR) and delayed fluorescence (DF)

A Multichannel Plant Efficiency Analyzer, M-PEA (Hansatech Instruments, Norfolk, UK) was used (at t1 and t3) to measure the modulated reflection (MR) change and delayed fluorescence (DF). Modulated reflection was measured near 820 nm (MR) and is related to the redox state of PSI (reaction center P700, and plastocyanin, PC). The minimal MR_t/MR_0 value (MR_{min}), the velocity of oxidation (V_{ox}) and the following re-reduction (V_{red}) (Schansker *et al.*, 2003). Full technical details of the M-PEA instrument and the type of parameters measured by the instrument are provided in Oukarroum et al. (2013) and Strasser et al. (2010).

Delayed fluorescence (DF) indicates the light emission by plants (Goltsev *et al.*, 2005) in the infrared region, after they were exposed to a saturating light, and is emitted mainly from the PSII. The trend of the DF is represented by a curve indicating the decay of fluorescence at different time periods. Delayed fluorescence was also obtained by interrupting the actinic illumination during the measurements according to Goltsev et al. (2009a). The parameters derived from the analysis of the curve, which indicate the various stages of recombination of the states of oxidation-reduction, are related to the different peaks (indicated with the letter I) and bent (indicated with the letter D). The results are expressed with the ratios I_1/I_2 and I_4/D_2. The ratio I_1/I_2 is inversely connected to electron flow in PSII, and was found to be sensitive to mild drought stress (Goltsev et al. 2012); the second ratio I_4/D_2 is related to the transmembrane proton gradient (Goltsev et al. 2005). The signals measured with the M-PEA were analyzed using the M-PEA-data analyzer version 5.4 software (Hansatech, Poole, UK)(Kalaji *et al.*, 2014*a*). Details are provided in Appendix B1 in supplementary data.

5.2.6. Biomass measurements

At the start of the acclimation process (t0), four plants per species were selected for the determination of above ground biomass/stem dry weight (SDW) and below ground biomass/root dry weight (RDW). Plants were cut above the insertion point of the first root and the above ground biomass was separated. Roots were washed with water under pressure to eliminate the retained substrate for 30 minutes. Once above-ground and below-ground biomass was separated, samples were kept in an oven at 70 °C until they reached a constant weight considered to be representative of the dry weight. The biomass weights measured at this stage represent the initial biomass (t0). At the end of the experiment (t3), final above (SDW) and below (RDW) ground dry biomass were measured from the plants that were placed into climatic chambers to gauge monitor the growth.

5.2.7. Stomatal density and ultrastructural analysis

Optical microscope (MO) and transmission electron microscope (TEM) ultrastructure observations were carried at t3, on the leaves of *Q. ilex* and *Q. cerris* that grew under the controlled environmental conditions. Foliar samples were collected at the end of the experimental period. The number of stomata (stomatal density) of the leaves (3 branches from each pot x 3 leaves from each branch x 2 section from each leaf x 5 view from different part of a section = 90 views for each pot, 270 views per treatment) were counted using Fluorescence Microscopy (Leica, Leitz DMRB, LabCommerce, Inc., San Jose, CA).

The cross-sectional observations of leaf ultra-structure by light microscopy were performed with a Zeiss Axioplan (Oberkochen, Germany) microscope on sections

embedded in historesin (2 μm thick) cut with an ultramicrotome Ultracut S (Reichert-Yung, Wien, Austria). The tests performed (O'Brien and McCully, 1981) included: toluidine blue, pH 4.4 for the structure and a first explorative survey. For electron transmission microscope (TEM), leaves were prefixed in a phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde + 4% paraformaldehyde. After 20 h at 5 °C, samples were rinsed twice (2x10 min) in the same buffer, then post-fixed (2 h) in 2% osmium tetroxide prepared in the same buffer. Subsequently, samples were dehydrated in a series of solvents containing increasing proportions of ethanol (10 min at each stage of the fixation series). Finally, after two 5 min rinses in propylene oxide (100%), the samples were embedded in resin, according to Spurr (1969). A Reichert Ultracut S (Leica, Heerhrugg, CH) microtome was used to cut ultra-thin sections (0.09 μm) with a diamond knife. These sections were stained with uranyl acetate (500 mg in 10 ml of distilled water) and lead citrate (saturated solution), and then observed with a EM-300 Philips (Amsterdam, NL) microscope.

5.2.8. Statistical analyses

Statistical analyses were performed using SPSS 20 (IBM, New York, USA). The Shapiro-Wilk test was applied to the ChlF data (PF, MR, DF) to assess the normality, and the Levene test was used to the investigate homogeneity of variance. ChlF data was not normally distributed and the variance was not homogenous, and therefore the non-parametric Wilcoxon Signed-Rank Test was applied to the ChlF data to assess the mean rank differences. A one-way ANOVA with an LSD *post-hoc* multiple comparison test were used to assess differences in variance between treatments for biomass, leaf gas-exchange and stomatal density data. A 0.05 significance level was applied. The

stomatal density data was analyzed using R statistical software v3.1.3 (R Development Core Team, 2015) and represented as box-plots to observe skewness and outliers.

5.3. RESULTS

5.3.1. Leaf gas exchange

The deciduous species, *Q. cerris*, grown in the elevated conditions (EC) showed significantly higher net photosynthesis at t1, than any other time (P = 0.000); P_N increased 73.9% compared to ambient condition (AC) (Fig. 1a), coinciding with a significant increase in stomatal conductance (G_s) (P = 0.003). *Quercus cerris* showed evidence of possible down-regulation of photosynthetic physiology under EC between t1 and t3 by reducing P_N by 19.0% (P = 0.000) (Fig. 2a). However, *Q. cerris* at EC-t3 increased P_N 40.7% relative to AC-t1 (P = 0.001) and by 25% relative to AC-t3 (P = 0.028). Photosynthesis of *Q. ilex* was not influenced significantly by growth in the EC and was identical at both t1 and also t3 (Fig. 1a). Generally, under AC *Q. ilex* showed similar levels of P_N to *Q. cerris*. However, *Q. ilex* at EC-t1 exhibited significantly lower P_N (P = 0.000) in comparison to *Q. cerris* under EC at t1. Due to the reduction of P_N levels in *Q. cerris* under EC by t3, the difference in P_N values between *Q. ilex* and *Q. cerris* at t3 was no longer statistically significant (P = 0.106) (Fig. 1a).

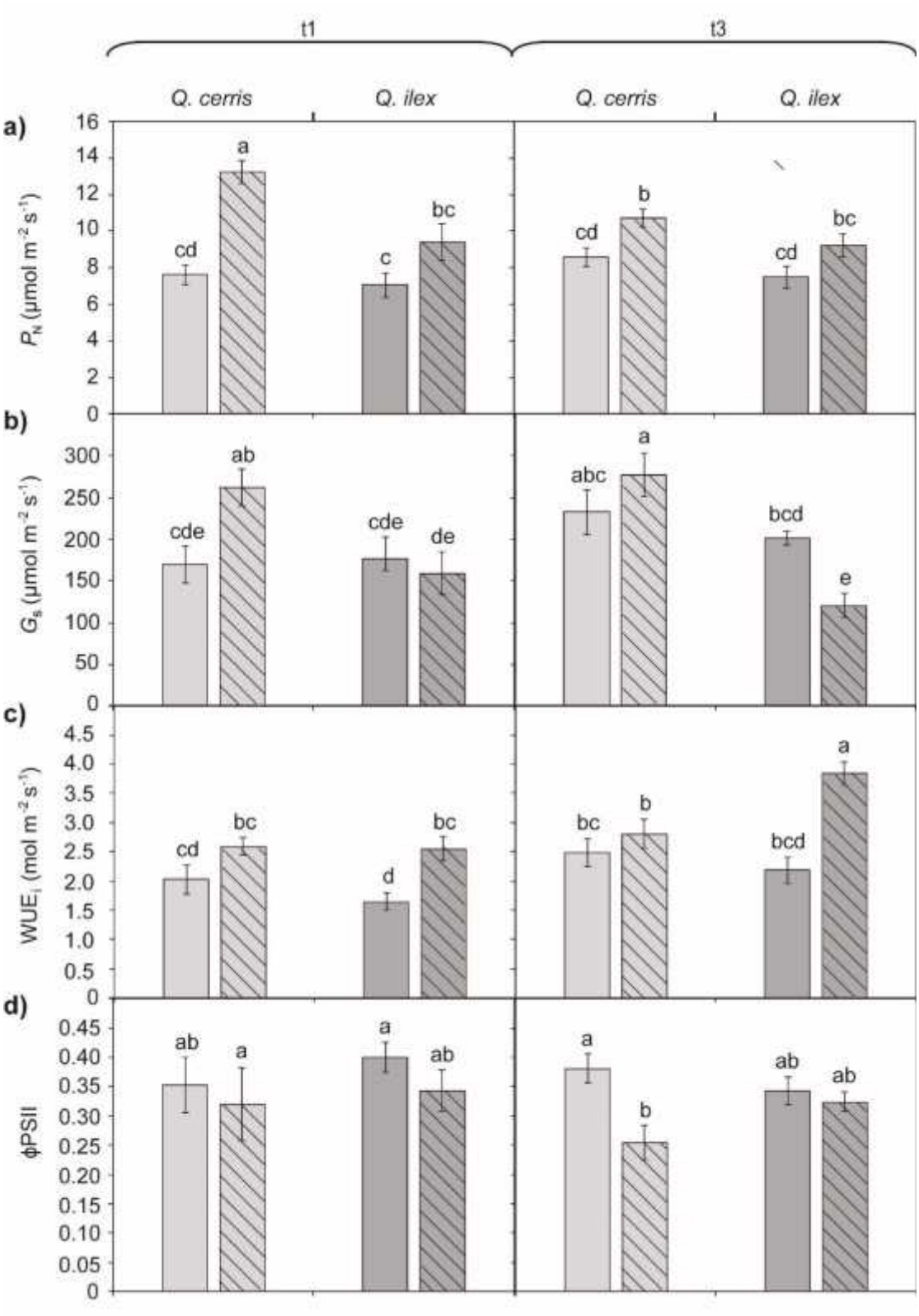

Figure 1: a) Photosynthesis (P_N); b) stomatal conductance (G_s); c) instantaneous water use efficiency (WUE_i), and; (d) the actual quantum yield of PSII (ϕPSII) measured using simultaneous gas exchange and chlorophyll *a* fluorescence of *Quercus cerris*

(light grey) and *Quercus ilex* (dark grey) grown under ambient (open) and elevated (hatched) conditions. Error bars indicate standard error either side of the mean (n = 3). Letters indicate homogenous groups between all of the varieties of both species determined using a one-way ANOVA and LSD *post-hoc* test.

Quercus cerris increased G_s significantly (P = 0.001) by 54.6% at EC-t1 in comparison to AC-t1, coinciding with increased P_N at EC-t1 (Fig. 1b). Despite the fact that *Q. cerris* exhibited significantly greater G_s under both AC and EC at t3, the increase of G_s values was not significantly higher at EC-t3 than EC-t1. Even though *Q. cerris* exhibited 37.3% higher G_s at AC-t3 than AC-t1, this increase was not statistically significant (Fig. 1b). *Quercus ilex* exhibited identical levels of G_s at both AC and EC at t1, and these rates of Gs were comparable to those of *Q. cerris* at AC-t1. *Quercus ilex* showed a significant 40.2% reduction of G_s at EC-t3 in comparison to AC-t3 (P = 0.008); however, the 31.9% in AC (P = 0.06) and 24.3% in EC (P = 0.19) lower G_s values at t1 were not significantly different to those recorded at EC-t3. The reduction in G_s under EC at t3 induced an increase in the WUE_i of *Q. ilex* at EC-t3 (Fig. 1c). This WUE_i is significantly higher than all other treatments (P =< 0.0001). The increase of WUE_i observed in *Q. ilex* at EC-t3 was greater than values observed at AC-t3 and also AC-t1 by 76.1% and 134.1% respectively. In contrast, the WUE_i of *Q. cerris* was unaffected by growth in elevated conditions when compared to both AC-t1 and AC-t3 (Fig. 1c). The actual quantum yield of PSII (ΦPSII) measured during leaf gas exchange was unaffected in *Q. ilex* when grown under elevated conditions at both t1 and t3 and also *Q. cerris* at t1 (Fig. 1d). However, ΦPSII values of *Q. cerris* were reduced at EC-t3 by 34.2% in comparison to AC-t3 (P = 0.008). *Quercus cerris* also experienced reduced ΦPSII of 28.5% at EC-t1 in comparison to AC-t1 (P = 0.03).

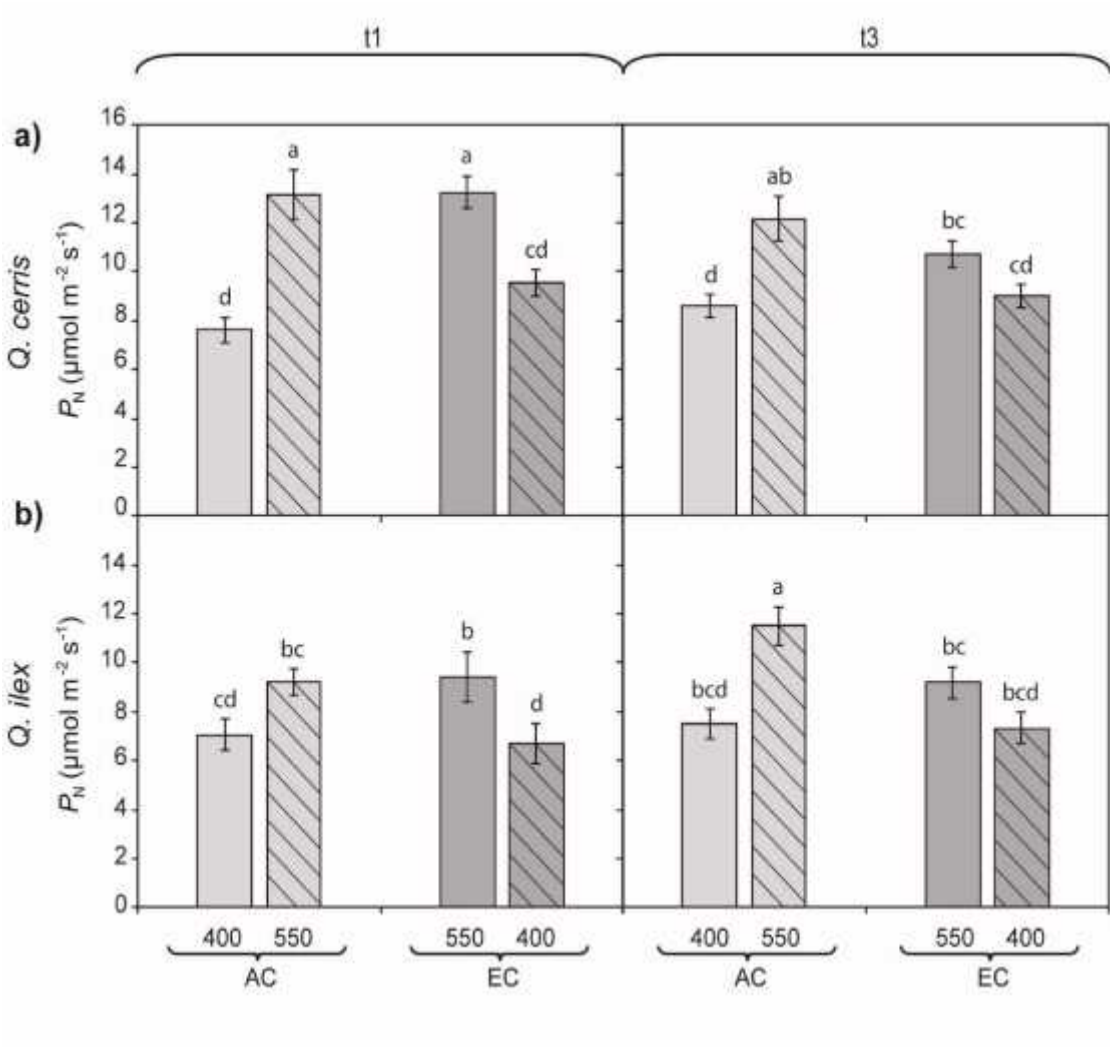

Figure 2: Instantaneous leaf gas exchange measurements of P_N of *Q. cerris* and *Q. ilex* grown under ambient and elevated conditions measured under the [CO_2] level at which the plant was grown (open) and the [CO_2] level of the opposite chamber (hatched). The degree of stimulation of P_N between 400 and 550 ppm [CO_2] should provide evidence for possible down-regulation of the photosynthetic physiology of plants grown in EC – the lower level of P_N at 550 in EC plants compared to those grown at AC may be indicative of down-regulation. Error bars indicate standard error either side of the mean (n = 3). Letters indicate homogenous groups between all of the varieties of both species determined using a one-way ANOVA and LSD *post-hoc* test.

At the end of the experiment, the stem dry weight (SDW) and root dry weight (RDW) of *Q. ilex* were not significantly influenced by growth under EC in comparison to ambient conditions (Fig. 3a and b). However, *Q. cerris* SDW and RDW increased by 55.0% ($P = 0.010$) and by 48.4% ($P = 0.027$) respectively under the EC. Under AC, *Q. cerris* exhibited a 75% increase of SDW ($P = 0.001$) while RDW showed a non-significant 33.6% ($P => 0.05$) increase in comparison to the initial (t0) biomass. Root and shoot ratios (RSR) showed identical ($P > 0.05$) results for all the treatments with the exception of the initial (t0) *Q. cerris* and ambient *Q. ilex* values that are significantly different to one another ($P = 0.012$) (Fig. 3c).

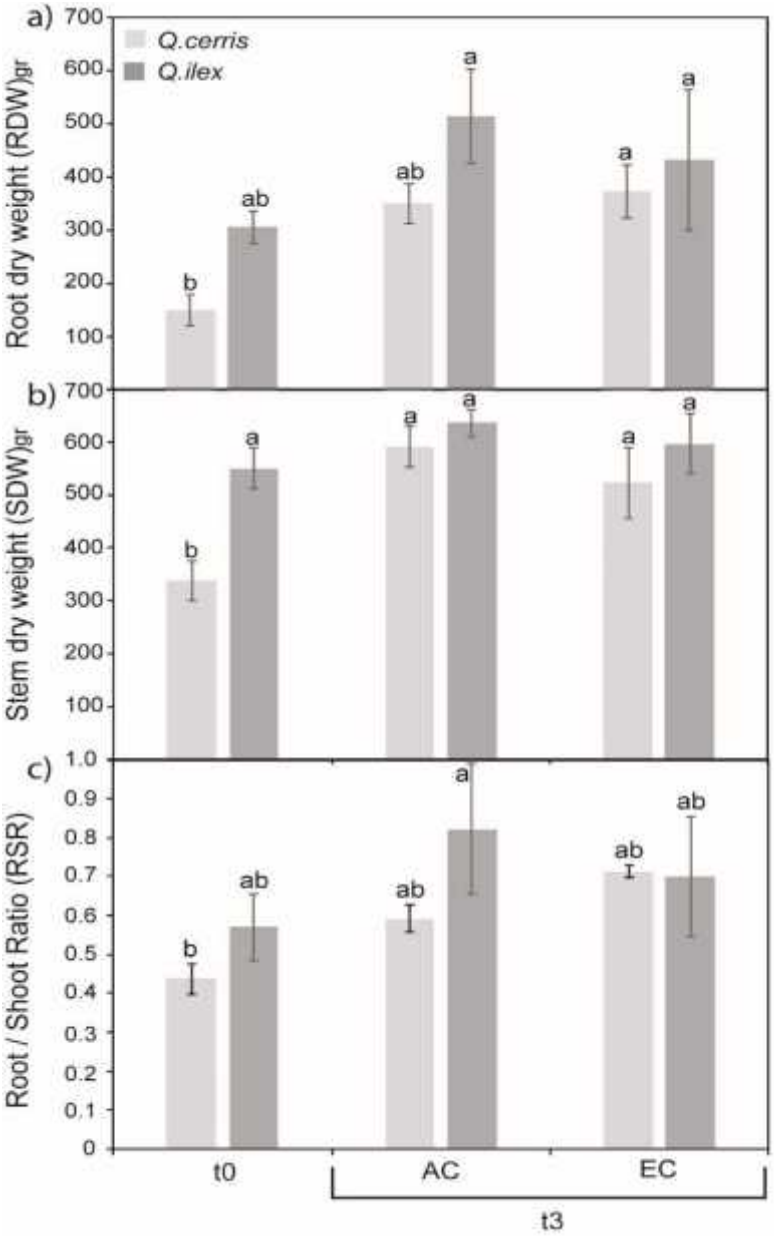

Figure 3: a) Below-ground (RDW); b) above-ground (SDW) dry biomass, and; c) root/shoot ratio (RSR) of *Quercus cerris* (light grey) and *Quercus ilex* (dark grey) at the beginning (t0) of the study and following three months of growth (t3) under AC and EC. Error bars indicate standard error either side of the mean (n = 3). Letters indicate homogenous groups between all of the varieties of both species determined using a one-way ANOVA and LSD *post-hoc* test.

5.3.2. Stomatal density and ultrastructural analysis

Fluorescence microscopy indicated that *Q. cerris* did not alter the number stomata in leaves developed under EC (Fig. 4), consistent with observations of higher G_s at EC. Under the AC, *Q. ilex* showed higher, but not significantly, stomatal density than *Q. cerris*. *Quercus ilex* significantly reduced the number of stomata in leaves formed under EC by 11.4% ($P = 0.000$) in comparison to leaves developed in AC. *Quercus ilex* grown under EC exhibited the lowest stomatal density lower of all other treatment ($P = 0.000$).

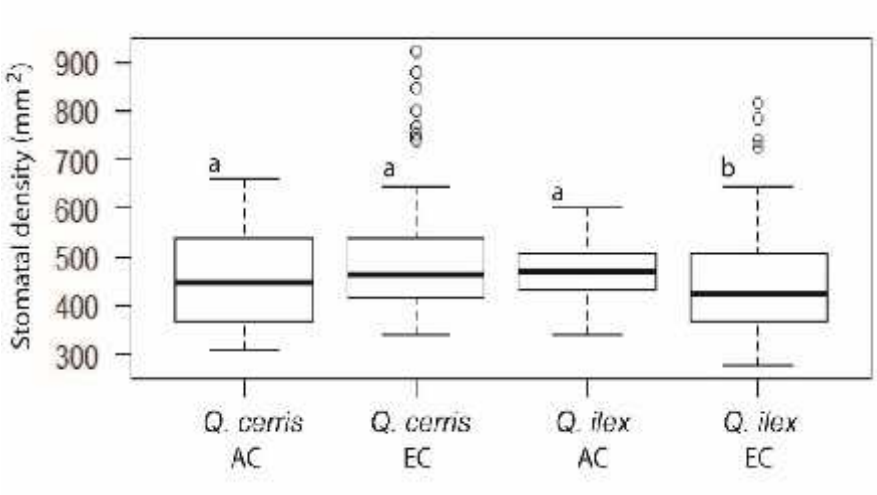

Figure 4: Box-plots representing the range of stomatal density (number of stomata/mm^2) values of leaves of *Quercus cerris* and *Quercus ilex* (270 views per treatment) that developed under experimental conditions. The central box represents 50% of the variation, with the upper whisker (third quartile) and lower whisker (first quartile) representing 25% of the scatter. The horizontal band represents the median value.

The explorative observations with light microscope revealed an accumulation of primary starch in the mesophyll cells of *Q. cerris* plants grown in EC conditions (Fig 5d) with respect to the AC ones (Fig. 5c), whereas no evident differences were observed

in *Q. ilex* grown in AC and EC chambers (Fig. 5a and 5b). The different behaviour of *Q. cerris* in AC and EC conditions was confirmed by mean of TEM observations (Fig 6). Starch grains in the chloroplasts (black arrows) and lipidic bodies (plastoglobuli, white arrows), possible indicators of early senescence, were observed in both conditions, but were more abundant in the EC chamber.

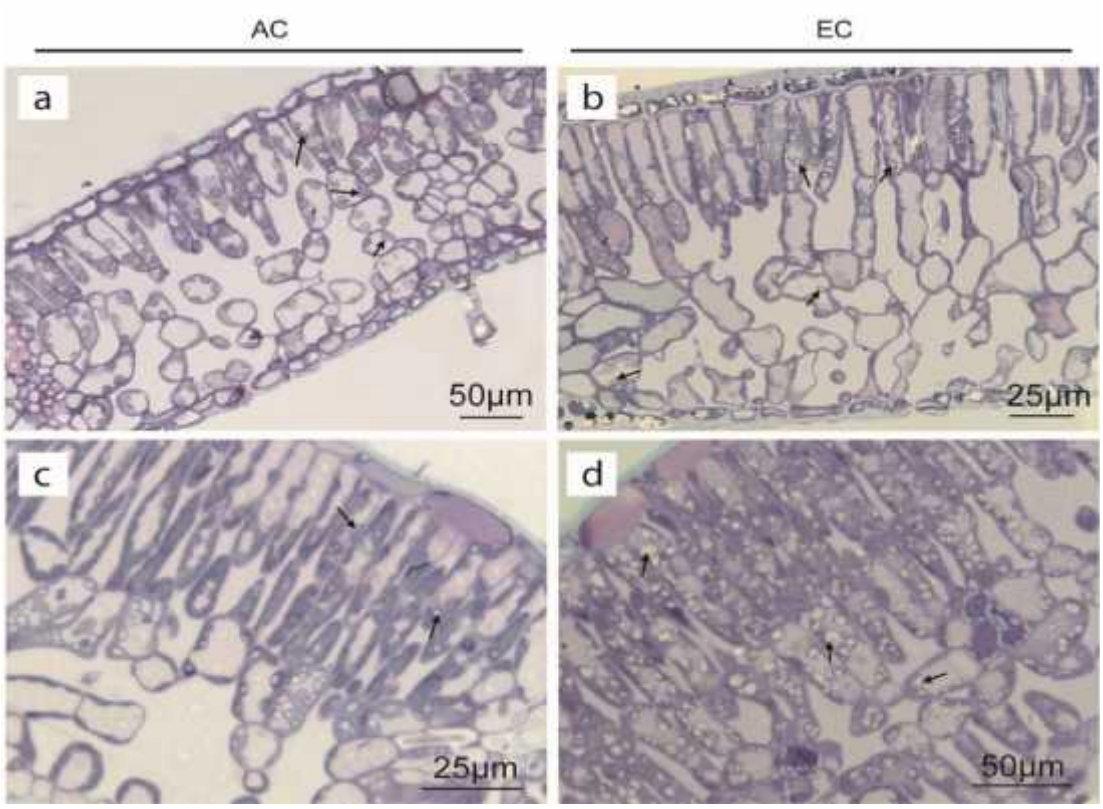

Figure 5: Light Microscope observations. Cross sections of *Quercus ilex* (a, b) and *Quercus cerris* (c, d) in control (AC chambers, panels a and c) and CO_2+temperature treatment (EC chambers, panels b and d). Leaves were stained with Toluidine blu for explorative survey. Arrows indicate starch grains accumulation.

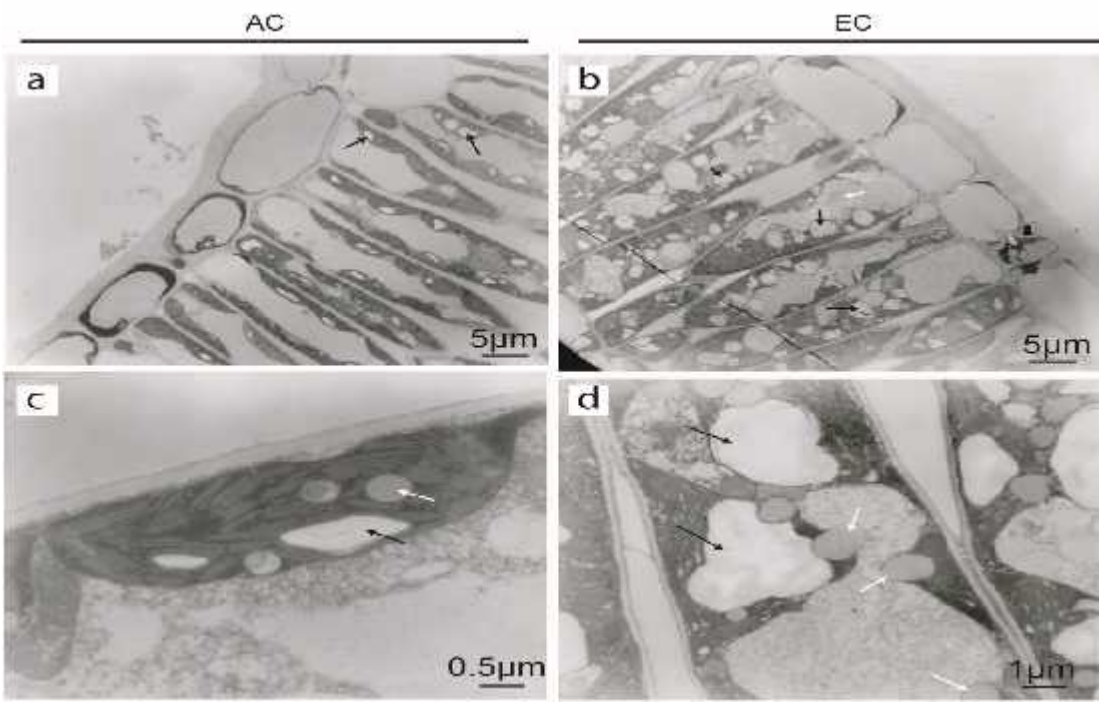

Figure 6: Transmission Electron Microscope (TEM) observations. Ultrastructural cross sections of *Quercus cerris* grown in control (AC chambers, panels a and c) and CO_2+temperature treatment (EC chambers, panels b and d). Black arrows indicate starch grain accumulation in chloroplasts; white arrows indicate plastoglobuli (lipidic bodies).

5.3.3. Chlorophyll a fluorescence analysis

Chlorophyll *a* fluorescence analyses of prompt fluorescence (PF), modulated reflectance of P700 (MR) and delayed fluorescence (DF) were conducted during the experiment and the descriptive statistics of the considered parameters are shown in Table 1, 2 and 3 respectively. Concerning the prompt fluorescence (Table 1), *Q. ilex* showed a significant difference of F_M, ψ_{Eo}, RC/ABS, PI_{ABS}, PI_{TOT} and K Band at t1, while *Q. cerris* shows significant difference in ΔV_{IP} and PI_{TOT} values under EC with respect to AC. *Quercus ilex* under EC exhibited significantly lower F_M (P = 0.023), ψ_{Eo} (P = 0.020), RC/ABS (P = 0.027), PI_{ABS} (P = 0.03), PI_{TOT} (P = 0.011) and significantly

higher K-Band level (P = 0.047) in comparison to AC. However, *Q. cerris* showed significant reductions of PI_{TOT} (*P* = 0.020) and ΔV_{IP} (*P* = 0.012) under EC. Both *Q. ilex* and *Q. cerris* did not show significant difference on any of the ChlF - PF parameters at t2. At the end of the experiment at t3, *Q. cerris* did not show any significant difference in any PF-ChlF parameters between EC and AC. However, *Q. ilex* exhibited significant increases of F_0 (*P* = 0.038) while F_V/F_M, ΔV_{IP} and PI_{TOT} significantly declined (*P*= 0.038, 0.038, 0.028 respectively) at t3. Under EC, *Q. ilex* showed significantly higher F_0 (*P* = 0.008) and F_M (*P* = 0.028) in comparison to *Q. cerris* at t3 (Table 1). The double-hit protocol indicated that *Q. ilex* (but not *Q. cerris*) showed a significant decrease in the active Q_B-binding centres under EC conditions at t3 (Table 4), while both *Q. ilex* and *Q. cerris* had identical ΔV_J under EC and AC.

The analysis of MR revealed no effects on both *Q. ilex* and *Q. cerris* under EC, with the exception of a significantly lower value of V_{red} (*P* = 0.008) in *Q. cerris* at t1; however, this significant decline became identical at t3 (Table 2). Under EC at t3, *Q. ilex* showed significantly higher V_{ox} than *Q. cerris* (*P* = 0.015). Among the DF parameters, the only significant increase was observed in *Q. ilex* on I_4/D_2 under EC at t3; however, no significant difference was found in *Q. cerris* at both t1 and t3 (Table 3).

Table 1: Results of Prompt Chlorophyll Fluorescence Parameters (PF) of each treatment (A_QI: Ambient *Q. ilex*, A_QC: Ambient *Q. cerris*, E_QI: Elevated *Q. ilex*, E_QC: Elevated *Q. cerris*) at time t1 (21st July), t2 (4th August) and t3 (20th August). Letters indicates mean ranks difference between the replicates for each treatment (n=3) of Wilcoxon Signed-Rank Test to assess matched observation of each treatment for each time independently. Different letters indicate significant difference (P≤ 0.05) between matched treatments.

Treatment		Fo			F_M			F_V/F_M			ψ_{E0}			RC/ABS		
Time	Name	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.
t1	A_QI	313	± 45.7	a	1487	± 270.8	a	0.763	± 0.033	a	0.673	± 0.083	a	4.971	± 0.65	a
t1	A_QC	256	± 21.7	b	1160	± 82.5	c	0.749	± 0.026	a	0.608	± 0.055	c	4.525	± 0.34	b
t1	E_QI	289	± 22.3	a	1249	± 257.6	b	0.722	± 0.098	a	0.586	± 0.089	bc	4.446	± 0.74	b
t1	E_QC	270	± 21.9	b	1193	± 151.4	bc	0.740	± 0.043	a	0.606	± 0.028	bc	4.589	± 0.48	b
t2	A_QI	259	± 11.1	a	1480	± 74.8	a	0.799	± 0.011	ab	0.606	± 0.065	a	5.611	± 0.67	a
t2	A_QC	241	± 22.6	bc	1404	± 108.2	b	0.801	± 0.010	bc	0.606	± 0.041	a	5.348	± 0.41	a
t2	E_QI	252	± 22.4	ac	1388	± 101.4	ab	0.793	± 0.007	a	0.591	± 0.051	a	5.662	± 0.43	a
t2	E_QC	237	± 15.3	b	1418	± 99.0	ab	0.806	± 0.004	c	0.587	± 0.025	a	5.614	± 0.25	a
t3	A_QI	416	± 28.2	a	2419	± 235.6	ac	0.807	± 0.013	a	0.619	± 0.077	a	6.788	± 0.66	a
t3	A_QC	404	± 43.6	a	2287	± 254.0	ab	0.801	± 0.007	ac	0.588	± 0.039	a	6.240	± 0.41	a
t3	E_QI	457	± 27.7	b	2397	± 138.8	a	0.789	± 0.010	b	0.622	± 0.091	a	6.265	± 0.45	a
t3	E_QC	401	± 41.2	a	2237	± 193.8	bc	0.800	± 0.009	bc	0.586	± 0.030	a	6.628	± 0.54	a

Table 1; continued.

Treatment		PI_{ABS}			ΔV_{IP}			PI_{TOT}			K-Band		
Time	Name	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.
t1	A_QI	37.9	± 14.43	a	0.255	± 0.049	ab	23.46	± 10.22	a	0.390	± 0.052	a
t1	A_QC	22.2	± 6.39	bc	0.276	± 0.045	b	18.98	± 7.15	a	0.416	± 0.026	b
t1	E_QI	20.5	± 8.27	b	0.204	± 0.075	ac	11.64	± 6.27	b	0.410	± 0.029	b
t1	E_QC	22.3	± 8.83	bc	0.216	± 0.054	c	11.96	± 4.98	b	0.406	± 0.032	b
t2	A_QI	37.5	± 15.95	a	0.177	± 0.034	a	16.07	± 8.62	a	0.353	± 0.103	ab
t2	A_QC	33.9	± 7.14	a	0.192	± 0.027	a	16.04	± 5.16	a	0.412	± 0.097	a
t2	E_QI	32.5	± 8.14	a	0.182	± 0.027	a	14.76	± 4.81	a	0.329	± 0.094	b
t2	E_QC	33.5	± 4.73	a	0.176	± 0.023	a	14.47	± 3.37	a	0.360	± 0.015	ab
t3	A_QI	51.3	± 20.81	a	0.195	± 0.055	a	25.16	± 13.59	a	0.300	± 0.027	a
t3	A_QC	36.7	± 8.55	a	0.180	± 0.028	ac	16.41	± 5.31	ab	0.322	± 0.018	a
t3	E_QI	41.8	± 14.82	a	0.176	± 0.048	bc	17.13	± 8.45	b	0.316	± 0.021	a
t3	E_QC	38.5	± 8.85	a	0.186	± 0.027	ac	18.26	± 5.77	ab	0.303	± 0.023	a

Table 2: Results of Modulated Reflectance (MR) Chlorophyll Fluorescence Parameters of each treatment (A_QI: Ambient *Q. ilex*, A_QC: Ambient *Q. cerris*, E_QI: Elevated *Q. ilex*, E_QC: Elevated *Q. cerris*) at t1 and t3. Letters indicates mean ranks difference (n=3) of Wilcoxon Signed-Rank Test to assess matched observation of each treatment for each time independently. Different letters indicate significant difference ($P \leq 0.05$) between matched treatments.

		MR								
Treatment		Vox			Vred			MRmin		
Time	Name	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.
t1	A_QI	1.30	± 0.24	a	0.07	± 0.03	a	56478.2	± 3270	ab
t1	A_QC	1.13	± 0.12	a	0.14	± 0.027	b	55731.6	± 1961	b
t1	E_QI	1.21	± 0.17	a	0.06	± 0.015	a	58888.4	± 2194	a
t1	E_QC	1.08	± 0.17	a	0.06	± 0.012	ac	57115.8	± 2565	ab
t3	A_QI	1.44	± 0.18	a	0.06	± 0.015	a	57870.1	± 3693	a
t3	A_QC	1.15	± 0.28	b	0.05	± 0.008	b	57032.3	± 5848	a
t3	E_QI	1.49	± 0.18	a	0.06	± 0.031	ab	59901.8	± 2367	a
t3	E_QC	1.18	± 0.14	b	0.05	± 0.022	ab	58134.7	± 2600	a

Table 3: Results of Delayed Chlorophyll Fluorescence (DF) Parameters of each treatment (A_QI: Ambient *Q. ilex*, A_QC: Ambient *Q. cerris*, E_QI: Elevated *Q. ilex*, E_QC: Elevated *Q. cerris*) at t1 and t3. Letters indicates mean ranks difference (n=3) of Wilcoxon Signed-Rank Test to assess matched observation of each treatment for each time independently. Different letters indicate significant difference ($P \leq 0.05$) between matched treatments.

		DF																
Treatment		I1			I2			I4			D2			I1/I2			I4/D2		
Time	Name	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.	Mean	StDev	Sig.
t1	A_QI	6135.3	± 694	a	5132.1	± 758	a	3557	± 633	a	3272.4	± 709	a	1.20	± 0.07	a	1.10	± 0.061	a
t1	A_QC	5598.4	± 614	a	4362.2	± 496	b	3113	± 328	a	2526.0	± 319	b	1.28	± 0.04	b	1.24	± 0.03	b
t1	E_QI	6216.1	± 815	a	4649.6	± 1427	ab	3256	± 809	a	3099.5	± 862	ab	1.40	± 0.24	ab	1.06	± 0.039	a
t1	E_QC	6201.1	± 837	a	4393.5	± 772	ab	3158	± 569	a	2649.3	± 570	ab	1.42	± 0.09	ab	1.20	± 0.05	b
t3	A_QI	15838.8	± 4477	ab	9519.3	± 3514	ab	5115	± 2406	a	4907.7	± 2318	a	1.71	± 0.16	a	1.04	± 0.012	a
t3	A_QC	13403.0	± 1307	a	7115.5	± 915	a	3738	± 777	a	3377.9	± 741	a	1.90	± 0.17	b	1.11	± 0.063	b
t3	E_QI	15879.7	± 2894	ab	8773.5	± 2086	ab	4830	± 1506	a	4437.2	± 1512	a	1.83	± 0.12	ab	1.10	± 0.056	b
t3	E_QC	16322.9	± 1700	a	8298.6	± 963	a	3765	± 393	a	3484.0	± 376	a	1.98	± 0.20	ab	1.08	± 0.028	b

Table 4: Results of Double Hit Chlorophyll Fluorescence Parameters (QB-bc; active QB-binding centres, ΔVJ; relative amplitude of the J-point in second hit and the first hit) of each treatment (A_QI: Ambient *Q. ilex*, A_QC: Ambient *Q. cerris*, E_QI: Elevated *Q. ilex*, E_QC: Elevated *Q. cerris*) at t3. Letters indicates mean ranks difference (n=3) of Wilcoxon Signed-Rank Test to assess matched observation of each treatment for each time independently. Different letters indicate significant difference ($P \leq 0.05$) between matched treatments.

Treatment		Q_{B}-bc			ΔV_J		
Time	Name	Mean	StDev	Sig.	Mean	StDev	Sig.
t3	A_QI	0.89	± 0.023	a	0.29	± 0.060	a
t3	A_QC	0.87	± 0.035	b	0.31	± 0.046	a
t3	E_QI	0.88	± 0.030	b	0.31	± 0.090	a
t3	E_QC	0.87	± 0.030	b	0.29	± 0.078	a

5.4. DISCUSSION

The results of this study suggest that the oak species *Q cerris* and *Q. ilex* will likely respond differently to growth conditions predicted for Mediterranean urban environments in the year 2050. Higher [CO_2] and temperatures induced contrasting physiological and morphological responses between evergreen *Q. ilex* and deciduous *Q. cerris*. This will have implications for the performance of urban trees and provide valuable insights into the choice of species for urban habitats/forests in anticipation of future climate changes.

5.4.1. Leaf gas exchange effects of 2050 conditions

Increased [CO_2] and higher temperatures have confounding effects on the carboxylation of RuBisCO (Berry and Björkman, 1980; Farquhar *et al.*, 1980). The 150 ppm increase in [CO_2] and +2.5 °C day and +4 °C temperature rise used in this study are

comparatively small compared to other controlled environment and free air [CO_2] enrichment studies (Nijs *et al.*, 1997; Ainsworth and Rogers, 2007; Killi *et al.*, 2016). Nevertheless, we observed contrasting leaf gas exchange responses of *Q. ilex* and *Q. cerris* induced by growth in 2050 conditions. *Quercus ilex* showed no photosynthetic response to EC (Fig. 1a), but did reduce G_s via a reduction in stomatal density (Fig. 1b); indicative of the maintenance of carboxylation associated with enhanced diffusion of CO_2, while transpirative water-loss was decreased via a reduction in stomatal initiation in leaves developed under controlled environmental conditions. The reduction in stomatal density observed in *Q. ilex* in this study is consistent with observations recorded in other elevated [CO_2] experiments (Woodward and Kelly, 1995). In contrast, *Q. cerris* did not modify *Gs* or stomatal morphology (Fig. 1b), but did exhibit an initial stimulation in P_N followed by a reduction in P_N values consistent with down-regulation of the photosynthetic apparatus (Van Oosten *et al.*, 1994; Drake *et al.*, 1997). This may be indicative of contrasting mechanisms driving the response of the *Quercus* species to elevated [CO_2] and temperature; the evergreen *Q. ilex* utilizes stomatal control of P_N, while the deciduous *Q. cerris* adjusted photosynthetic physiology.

Species with active physiological stomatal behaviour generally do not adjust the number of stomata on the leaf surface in response to [CO_2], while plants with passive stomatal behaviour alter stomatal initiation (Haworth *et al.*, 2015). Moreover, active physiological control of stomatal aperture is closely linked to rates of P_N in the mesophyll (Messinger *et al.*, 2006; Engineer *et al.*, 2016), and down-regulation of photosynthetic physiology is associated with a loss of stomatal sensitivity to [CO_2] (Haworth *et al.*, 2016*b*). This may suggest that differences in the physiological stomatal behaviours of the two species may have contributed to their responses to a rise in [CO_2].

The reduction in stomatal density observed in *Q. ilex* is consistent with passive stomatal behaviour (Fig. 4), while the lack of modification in G_s values of *Q. cerris* may indicate a loss of stomatal sensitivity alongside the down-regulation of the photosynthetic physiology. This study has produced evidence to suggest that the photosynthetic physiology of *Q. cerris* experienced down-regulation following growth in atmospheres of 550 ppm [CO_2] (Fig. 2a); an accumulation of photosynthate within the leaf (Madsen, 1968), observed in *Q. cerris* but not in *Q. ilex* (Figs. 5, 6), has been proposed to act as a 'bottle-neck' inducing down-regulation through the suppressed expression of photosynthetic genes (Van Oosten *et al.*, 1994). This would suggest that the lack of stomatal response in *Q. cerris* is not due to the relatively low increase in [CO_2], as the physiology is obviously sensitive, but possibly due to an associated loss of stomatal function. An increase in atmospheric [CO_2] is widely considered to preferentially favour species with thick schlerophyllous leaves as the increased diffusion gradient would influence CO_2 availability within the chloroplast envelope of thick leaves to a greater extent than thin leaves (Centritto *et al.*, 2011; Niinemets *et al.*, 2011; Shi *et al.*, 2015). This may account for the lack of evidence indicative of down-regulation in *Q. ilex* observed in this study. The greater increase in WUE induced by reduced transpirative water-loss in *Q. ilex* could constitute a competitive advantage in drought-prone regions such as the Mediterranean (Field *et al.*, 1983; Bombelli and Gratani, 2003; Maseyk *et al.*, 2008; Wall *et al.*, 2011).

Higher temperatures are generally considered to increase G_s via a rise in leaf to air VPD (Drake *et al.*, 1970; Raschke, 1970; Schulze *et al.*, 1975). The reduction in stomatal density observed in *Q. ilex* would limit any water-losses that might be associated with increased average growth temperature or larger transient increases during heat-waves

(Killi *et al.*, 2016). *Quercus cerris* would instead rely upon physiological regulation of guard cell turgor to determine stomatal aperture size and thus constrain water-loss during high temperatures. However, any loss of stomatal function associated with photosynthetic down-regulation and previous observations of impaired stomatal closure in deciduous tree species grown at elevated [CO_2](Heath and Kerstiens, 1997; Heath, 1998) may suggest that *Q. cerris* would be vulnerable to drought or heat-stress in a probable 2050 climate scenario with higher atmospheric [CO_2].

5.4.2. Chlorophyll fluorescence analysis of PSII performance under a 2050 Mediterranean climate scenario

The actual quantum yield of PSII (ΦPSII) measured alongside leaf gas exchange did not alter in *Q. ilex* under EC but did decline by 32.4% in *Q. cerris*, consistent with the gas exchange analysis of P_N. These reductions in ΦPSII indicate that electron transport in the light-adapted state of *Q. cerris* was significantly affected by the EC at t3, while electron transport of *Q. ilex* was not affected (Fig. 1d). Plants grown under long-term (15-year) moderate [CO_2] enrichment of ~50 ppm did not show any modification of quantum yield of CO_2 determined by fluorescence (Haworth *et al.*, 2016*c*). However, growth at higher temperatures induces pronounced reductions in the ΦPSII values of C3 species (Feller *et al.*, 1998; Crafts-Brandner and Salvucci, 2000; Killi *et al.*, 2016). This may suggest that the foliage of evergreen *Q. ilex* possessed a higher capacity for protective mechanisms to maintain PSII electron transport through the thylakoid membranes during heat stress such as the production of heat shock proteins (Heckathorn *et al.*, 1998).

Prompt Chlorophyll Fluorescence parameters are sensitive to many stress factors that are responsible for a decline in photosynthetic efficiency (Kalaji *et al.*, 2014*b*, 2016). The two *Quercus* species displayed contrasting chlorophyll fluorescence responses to elevated [CO_2] and temperature; the PSII performance of deciduous *Q. cerris* was relatively insensitive to growth under EC, while the evergreen *Q. ilex* was more sensitive. This is somewhat surprising given the reduction in ΦPSII observed in *Q. cerris* and lack of response in *Q. ilex* measured alongside gas exchange parameters. Nonetheless, *Q. ilex* is highly adapted to stress conditions and is the dominant tree species in many Mediterranean forests (Barbero *et al.*, 1992). At t1, ψ_{E_0} and $\Delta V_{I\text{-}P}$ were reduced in *Q. ilex* under EC but not in *Q. cerris* (Table 1). The decline in the effectiveness of electron transport at steps J and I is reflected in the reduction fn PI_{ABS} and PI_{TOT} in *Q. ilex*. However, this initial response is likely transitory, as at t2 these differences disappeared, possibly indicating acclimation. At the end of the experiment (t3) we recorded a decline in F_V/F_M of *Q, ilex* grown in the EC chamber, whereas no effects were detected in *Q. cerris* (Table 1). The suggestion that the maximum rate of PSII electron transport was reduced in *Q. ilex* but not in *Q. cerris* is not consistent with indications of photosynthetic down-regulation in *Q. cerris* determined using gas exchange. The lowered F_V/F_M observed in *Q. ilex* may be consistent with the reductions in G_s (and unchanged P_N) to limit the flux of electrons to RuBisCO where carboxylation activity is enhanced by an increased abundance of CO_2 (Parry *et al.*, 2008; Galmés *et al.*, 2013).

Other responses assessed in this experiment suggest that this reduction in PSII efficiency is an active response of *Q. ilex* grown in EC chambers. The application of the "double-hit protocol" (Appenroth *et al.*, 2001) showed a significant reduction of the

"Q_B-binding centers" under EC in *Q. ilex* but not *Q. cerris*. Plastoquinone re-oxidation velocity (ΔV_J) also showed identical values for both *Q. ilex* and *Q. cerris* under EC and AC (Table 4). This is consistent with a decrease in the fraction of Q_B-binding centers and an identical velocity of re-oxidation in treated (EC) *Q. ilex* plants, showing that RCs may have experienced damaged but no evidence of re-oxidation was observed (Table 4).The analysis of the delayed fluorescence (DF) evidenced an increase of the transmembrane proton gradient in leaves of *Q. ilex* under EC conditions (I_4/D_2), suggesting that an over-excitation was produced at the thylakoid level by the EC growth conditions (Table 3). Among the parameters of DF the only difference observed concerned I_4/D_2 that was higher in *Q. ilex* under EC treatment at t3, but not in *Q. cerris*. Delayed fluorescence is considered sensitive to different stress conditions (Goltsev *et al.*, 2009*b*, 2012), however, there are comparatively few studies to compare with our findings, nevertheless (Salvatori *et al.*, 2014) found a similar behaviour of I_4/D_2 in *Viburnum* plants under drought stress, possibly suggesting that electron transport was impaired in *Q. ilex* plants growing under elevated temperature and [CO_2].

The analysis of MR revealed no effects with the exception of a significantly lower value of V_{red} in *Q. cerris* at t1 but not at t3 (Table 2). The decrease in MR_t/MR_0 (fast phase, V_{ox}) corresponded to an increase in the concentration of oxidized states of the PSI reaction centre ($P700^+$) and plastocyanin (PC^+), while the MR_t/MR_0 increase (slow phase, V_{red}) indicates $P700^+$ and PC^+ re-reduction, where electrons arrive from PSII through the inter-system electron carriers (Strasser *et al.*, 2010; Oukarroum *et al.*, 2013). Decreasing values of V_{ox} in plants treated with various kind of stress from different experiments were reported by Salvatori *et al.* (2014), but Fusaro *et al.* (2015) found an opposite trend in *Q. ilex* seedlings treated with ozone. Finally Oukarroum *et*

al. (2013), working with *Pisum sativum* plants treated with high temperature, found a decrease of V_{ox} in the same temperature range as our experiment.

5.4.3. Implications for Mediterranean urban forests in 2050

Exposure to elevated CO_2 generally results in increased below and above-ground biomass (Körner and Miglietta, 1994; Curtis and Wang, 1998; Jastrow *et al.*, 2000; Pendall *et al.*, 2004). In this study, *Q. cerris* exhibited increased above- and below-ground biomass in the EC, but the biomass gain of *Q. ilex* was unaffected (Fig. 3a and 3b). The accumulation of carbohydrates as starch in *Q. cerris* may have represented a sink limitation inducing down-regulation of the photosynthetic physiology (Körner and Miglietta, 1994). *Quercus ilex* did not show any alteration of RDW and SDW between AC and EC, and also in respect to initial (t0) biomass (Fig. 3a, 3b), consistent with the observed lack evidence to suggest CO_2-fertilisation in the EC due to reduced G_s. The root and shoot ratio (RSR) for both species in all treatments was identical, suggesting that higher [CO_2] and temperature did not affect biomass partitioning (Fig. 3c). The stomatal responsiveness, modification of PSII performance and lack of evidence indicative of photosynthetic down-regulation in *Q. ilex* suggest that the evergreen species would most likely perform more favourably than *Q. cerris* under warmer higher [CO_2] conditions. This may be due to greater thermal tolerance (Loreto *et al.*, 1998) and a greater proportional impact of an improved diffusion gradient from the external atmosphere to the chloroplast envelope (Centritto *et al.*, 2011; Niinemets *et al.*, 2011) in the thick schlerolophyllous leaves of *Q. ilex*.

5.5. CONCLUSIONS

The two species considered in this experiment displayed different strategies to cope with increased [CO_2] and temperature. The findings presented in this paper are derived from a short-term experiment using well-watered plants. The behaviours described here cannot be generalized as the response to climate change is largely species specific and can vary with the age and the stage of development of a given species (Baig *et al.*, 2015). The results of this study suggest that *Q. ilex* has a greater resilience to increasing [CO_2] and temperature than *Q. cerris*. *Quercus ilex* displayed strategies towards the maintenance of identical rates of photosynthesis and growth but lower loss of water in the EC. In *Q. cerris* the enhanced growth rates, and lack of stomatal control of P_N may make the trees more susceptible to recurrent drought in hot semi-arid Mediterranean habitats (Allen *et al.*, 2015). This may suggest that *Q. ilex* is more suited to growth under 2050 conditions of elevated [CO_2] and temperature; moreover, consideration should be given to the planting of *Q. ilex* instead of *Q. cerris* in Mediterranean urban forests where drought and temperature effects are likely to be more pronounced.

Authors Contributions

DK and FB wrote the paper, DK analyzed the data and prepared the figures. DK, EG, MP conducted M-PEA measurements. DK, MP and FB conducted H-PEA measurements. AF, DK and JM took leaf gas-exchange measurements. CT and AP conducted electron microscope analysis. DK conducted fluorescence-microscope stomatal counts. AF and FF designed the experiment. All the authors contributed to the manuscript.

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6. GENERAL CONCLUSIONS

Climate change offers particular challenges to sessile organisms such as plants that have to rely upon phenotypic adaptation to respond to higher temperatures, increased [CO_2] and more frequent / severe drought. Climate change poses a severe challenge to food security via the maintenance of crop production and the sustainability of ecosystem services provided by urban trees and forests. This thesis has attempted to understand and address some of these challenges by studying the physiological, morphological and biochemical responses of different species / varieties of crops and Mediterranean urban trees to increased temperature, elevated [CO_2] and drought. Indeed, the selection of crop varieties and urban tree species with desirable phenotypic responses to these conditions is a key component in efforts to mitigate the negative impact of climate change on agricultural services and urban environment. The major outcomes of these works are summarised below:

Leaf gas exchange and morphological responses of drought tolerant and sensitive varieties of sunflower (*Helianthus annuus*) and maize (*Zea mays*) to heat and drought stress

- A significant degree of genotypic variation in the response to heat and drought stress was observed within C3 sunflower and C4 maize. However, photosynthesis (P_N) and growth were directly affected by water deficit and temperature to a greater extent in the C3 crop.
- Increased temperature had little effect on P_N in the maize varieties due to accumulation of oxaloacetic acid within the bundle sheath effectively concentrating CO_2 at the RubisCO carboxylation site thus eliminating the enhanced photorespiration at 35˚C that negatively affected sunflower (see Crafts-Brandner and Salvucci, 2002). The drought tolerant sunflower sustained greater P_N at 35˚C by retaining ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity (see Panković et al., 1999).

)The kinetics of the response to drought were not affected by temperature in the different varieties of the C4 species. However, while the drought tolerant and sensitive varieties of sunflower exhibited identical responses to drought at 25˚C, the kinetics of the drought response diverged at 35˚C with the sensitive variety exhibiting lower stomatal conductance (G_s) and declines in foliar gas exchange indicative of severe drought stress at higher fraction of transpirable soil water levels than its drought tolerant counterpart.

)An increase in G_s has been observed in large numbers of previous studies following short term increases in temperature (Heath and Meidner, 1957; Drake et al., 1970; Raschke, 1970; Schulze et al., 1975; Crafts-Brandner and Salvucci, 2002; Shah and Paulsen, 2003; Xu and Zhou, 2006). However, we observed that stomatal adaptation to the higher temperature over four weeks of exposure to the higher temperature allowed the sunflower and maize varieties to prevent excessive water-loss at 35˚C (Fig 1). Alongside reduced growth and leaf area at 35˚C this stomatal control ameliorated the impact of drought at the higher temperature.

)Models of crop growth used to predict likely yield responses to climate change require input of plant water-use parameters in respect to increases in temperature associated with heat-waves. The findings of our study suggest that future studies should vary the timings of drought and heat stress to unpick the likely impacts of increased G_s associated with a short-term rapid rise in temperatures (eg. Bunce, 2000) versus a reduction/consistency in G_s during a longer period of elevated temperatures (our study) on crop productivity.

)The drought tolerant sunflower variety invested a greater proportion of P_N as below-ground biomass; with the root-systems of well-watered drought tolerant plants being ~75% greater than the drought sensitive variety at both 25 and 35˚C. The more extensive root-network of the drought tolerant sunflower variety may enable it to more fully exploit the available water than its drought sensitive counterpart (Chloupek et al., 2010). No difference was observed in biomass partitioning in the maize varieties, possibly suggesting that the respective yield

characteristics of the drought and sensitive maize varieties may be the result of differences in the partitioning of photosynthate between reproduction and vegetative structures (eg. Lauteri et al., 2014), metabolism (Ilahi and Dörffling, 1982) or water transport (eg. Haworth et al., 2016a).

Plant height is a key determinant of competition for light (Ford and Diggle, 1981; Craine and Dybzinski, 2013) and closely related to biomass production (Haworth et al., 2016a) and yield (Doyce and Lessman, 1966; Weng et al., 2014). Maize gained height more rapidly than sunflower, and the negative impact of drought and/or heat stress was lower, possibly due to greater water use efficiency in the C4 crop (Farquhar et al., 1989).

The results of our experiment suggest that more frequent drought and heat stress events aare likely to disproportionately affect the P_N of C3 crops (eg. Semenov et al., 2014; Stratonovitch and Semenov, 2015). As C4 maize exhibited tolerance to drought and heat stress in both varieties, to ensure food security priority should be given to the identification and development of varieties of C3 crops such as sunflower with tolerance to heat and drought stress.

Photosystem II performance and antioxidant capabilities in drought tolerant and sensitive varieties of C3 sunflower and C4 maize grown at high temperature and experiencing water deficit

Chlorophyll fluorescence (*ChlF*) allows rapid non-destructive collection of data relating to the performance of photosystem II (PSII) (Kalaji et al., 2016) and is highly sensitive to the deleterious effects of drought (Genty et al., 1989) and heat (Crafts-Brandner and Salvucci, 2002) stress. To protect and stabilise the thylakoid membrane where PSII electron transport occurs, plants possess protective antioxidant mechanisms (Reddy et al., 2004; Pinheiro and Chaves, 2011). This study builds upon the previous investigation of the gas exchange and

morphological responses of drought resistant and sensitive varieties of C3 sunflower and C4 maize to heat and drought stress, published in *Physiologia Plantarum* as Killi et al. (2016), by providing an in-depth examination of the *ChlF* and antioxidant characteristics associated with differential responses to drought and/or heat stress.

The effects of drought and heat stress were more apparent in the *ChlF* characteristics of the C3 sunflower than in the C4 maize. Moreover, varietal differences in parameters were more clearly evident between the drought tolerant and sensitive varieites of sunflower.

The maximum quantum yield of Photosystem II (F_v/F_m) declined only in the most severe dehydration conditions (Kalaji et al., 2016). However, heat stress may have a stronger effect on PSII with a strong rise of F_o (Baker and Rosenqvist, 2004; Kalaji et al., 2016), and the increase of the relative variable fluorescence at 300 μs (K-band)in the OJIP transient indicating the break-dawn of the oxygen evolving complex (Srivastava et al., 1997).

To maintain and increase food production in hot arid regions it is necessary to identify crop varieties that tolerate drought and heat stress. High through-put *ChlF* allows the rapid non-invasive screening of large numbers of crop varieties to characterise their phenotypic responses under stress conditions; however, it is not currently clear which *ChlF* parameters are effective in gauging the negative impacts of drought and/or heat stress on PSII as a basis for identifying resistance. Our measurements suggest that the most effective parameter in gauging the kinetics of the drought response in both sunflower and maize is the F_m due to a decline in potential energy usage for photochemistry.

The drought tolerant sunflower possessed higher concentrations of photosynthetic pigments than its drought sensitive counterpart; however, this effect was less clear in the maize varieties. Moreover, the drought tolerant variety increased the abundance of photosynthetic pigments during the experiment, whereas the sensitive sunflower exhibited declines. This may suggest

that retention of light harvesting pigments plays a role in the maintenance of *ChlF* parameters and may be used as an indicator of drought tolerance in C3 sunflower varieties.

The activities of the antioxidants catalase, superoxide dismutase, ascorbate peroxidases, peroxidases and glutathione reductase were generally higher in the drought tolerant sunflower variety than in the sensitive variety. Furthermore, over the course of the experiment in the 35˚C treatment, the level of antioxidant activity rose to a greater extent in the drought tolerant variety than in the drought sensitive counterpart. These effects were less marked in the C4 maize, reinforcing the *ChlF* (see above) and gas exchange (Killi et al., 2016) observations.

These findings indicate that varietal differences in tolerance to drought and heat stress were more evident in the C3 species. The retention of photosynthetic activity via the maintenance of RubisCO activity (Killi et al., 2016) and PSII electron transport (this study) are likely linked to enhanced protective antioxidant capabilities.

Impact of elevated [CO_2] and temperature to predicted 2050 levels on the Mediterranean oak species *Quercus ilex* and *Quercus cerris*

Urban forests are environmentally, climatically, socially and economically important but are more at risk of the deleterious effects of climate change than non-urban woodland. Urban trees experience elevated temperatures, higher atmospheric [CO_2] and lower water availability.

Evergreen *Quercus ilex* and deciduous *Quercus cerris* are commonly planted in Mediterranean urban parks. To assess the impact of climate change on these species we grew them under controlled environment conditions predicted to occur by the year 2050: 550 ppm [CO_2] (as oppose to present day levels of 400 ppm [CO_2]) and a day/night increase in temperature of +2.5/+4.0˚C.

- Rates of P_N in *Q. cerris* declined over the duration of the study indicative of down-regulation, whereas photosynthetic capacity was unaltered in *Q. ilex*. This 'down-regulation' was associated with an increase in the abundance of starch within the chloroplasts of *Q. cerris* and reduced PSII electron transport.

- *Quercus ilex* reduced stomatal conductance via a reduction in the numbers of stomatal pores over the surface of leaves developed in the chambers under the growth conditions. This resulted in improved water use efficiency in *Q. ilex*. In contrast, G_s was unaffected in *Q. cerris*, yet P_N was reduced via physiological down-regulation, suggesting that the carbon and water balance of *Q. cerris* would be adversely affected by conditions predicted to occur in 2050.

- *Quercus cerris* increased above ground biomass rapidly. While elevated [CO_2] is considered to improve water use efficiency under well-watered conditions, recent research suggests that species with active physiological stomatal behaviour (Haworth et al., 2016b) and those that increase leaf area as [CO_2] increases (Faralli et al., 2016) are likely to be more vulnerable to severe drought and heat-waves.

- The results of this study indicate that the phenotypic adaptations of evergreen *Q. ilex* are more likely to successfully adjust to growth conditions predicted for Mediterranean urban environments in 2050 than deciduous *Q. cerris*. Stomatal control of P_N in *Q. ilex* is likely to improve the water use efficiency of *Q. ilex* allowing it to withstand drought and heat stress more effectively than its deciduous counterpart.

General Conclusion of the Thesis:

The impact of higher temperature on plants depends upon the scale and duration of any increase in temperature. Heat waves are likely to have a more negative effect than smaller rises in mean annual temperature.

The beneficial effect of CO_2-fertilisation associated with elevated [CO_2] is likely to be reduced by the effect of higher temperatures increasing photorespiration.

The interaction of drought and heat stress depends upon the timing of temperature rises – adaptation to high temperature may minimise the impact of drought stress via adaptation of stomatal behaviour and reductions in leaf expansion.

The tolerance of plant species, and varieties of the same species, to drought and heat stress is likely associated with the functionality of protective antioxidant systems.

The impacts of drought, higher temperature and elevated [CO_2] are largely specific to species or varieties. Phenotyping of plant responses to these factors is an important component in ensuring agricultural food security and the maintenance of ecosystem services in urban forests.

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Killi et al. "Lipid peroxidation and chlorophyll fluorescence of photosystem II performance during drought and heat stress is associated with the antioxidant capacities of C3 sunflower and C4 maize varieties**"**

SUPPLEMENTARY INFORMATION (Appendix A1)

Chlorophyll *a* fluorescence parameters used in the fluorescence transient analysis

Technical fluorescence parameters	
$\mathbf{F_t}$	Fluorescence emission from a dark-adapted leaf at the time t
$\mathbf{F_0}$	Minimal fluorescence from a dark-adapted leaf
$\mathbf{F_M}$	Maximal fluorescence from a dark-adapted leaf
$\mathbf{F_J}$	Fluorescence intensity at the J-step (at 2 ms)
$\mathbf{F_I}$	Fluorescence intensity at the I-step (at 30 ms)
$\mathbf{F_V}$	Maximal variable fluorescence from a dark-adapted leaf. $F_V = F_M - F_0$
$\mathbf{V_J}$	Relative variable fluorescence at 3ms. $V_J = (F_{2ms} - F_0) / (F_M - F_0)$
$\mathbf{V_I}$	Relative variable fluorescence at 30ms. $V_I = (F_{30ms} - F_0) / (F_M - F_0)$
$\mathbf{M_0}$	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300\mu s} - F_0) / (F_M - F_0)$
Derived parameters	
$\boldsymbol{F_v/F_m}$ =	$[F_m - F_0]/F_m = \varphi_{Po} = TR_0/ABS$ = maximum quantum yield of PSII primary photochemistry, measured in samples in dark-adapted state. F_v/F_m expresses the probability that an absorbed photon will be trapped by the PSII reaction centre.
$\boldsymbol{\Psi_{Eo}}$ =	$ET_0/TR_0 = 1 - V_J = 1 - (F_{2\ ms} - F_0)/ (F_m - F_0)$. Ψ_{Eo} expresses the probability that the energy of a trapped excitation is used for electron transport beyond Q_A. V_J represents the relative variable fluorescence at 2 ms (step-J) ($V_J = (F_J - F_o)/(F_m - F_o)$).
$\boldsymbol{\Delta V_{I\text{-}P}}$ =	$1 - V_I = (F_m - F_{30\ ms})/ (F_m - F_0)$, I-P phase (Oukarroum et al., 2009). This parameter indicates relative contribution of the I-P phase to the fluorescence transient OJIP; it is regarded as a measure of the efficiency of electron flux through PSI to reduce the final acceptors of the electron transport chain, i.e. ferredoxin and NADP. V_I indicates the relative variable fluorescence at 30 ms (step-I) ($V_I = (F_I - F_o)/(F_m - F_o)$).
RC/ABS=	$(1-(F_o/F_m))/(M_o/V_J) = \varphi_{Po}\ (V_J/M_0)$. This parameter represents the total number of active reaction center per absorbtion. $Mo = 4(F_{300\ \mu s} - F_o)/(F_m - F_o)$. M_0 represents the initial slope of the double normalised fluorescence induction curve, and is a proxy of the net rate of PSII closure.
$\mathbf{PI_{ABS}}$ =	(RC/ABS) $[\varphi_{Po}\ /(1- \varphi_{Po})]\ [\Psi E_o/(1- \Psi E_o)]$. Performance index on absorption bases; absorption of antenna Chls of PSII. This measure incorporates photochemical and non-photochemical processes, such as absorption and trapping of excitation energy, electron transport beyond the primary plastoquinone (QA) and dissipation of excess excitation energy.
$\mathbf{PI_{TOT}}$=	Performance Index total (PI_{TOT}) is the potential for energy conservation from photons absorbed by PSII to the reduction flux (RE) of PSI end acceptors. The Pi_{tot} is a multi-parametric indicator of four measures of photosynthetic electron transport: (1) the concentration of reaction centres; (2) the quantum yield of PSII photochemistry; (3) the capacity for uptake of electrons in the electron chain between PSII and PSI; (4) the efficiency with which an electron can transfer from the reduced intersystem electron acceptors to the PSI end electron (Strasser et al., 2010, 2004; Tsimilli-Michael and Strasser, 2008). $PI_{TOT} = PI_{ABS}\ [\delta_{Ro}/(1- \delta_{Ro})]$ where $\delta_{Ro} = (1- V_J)/ (1- V_J) = (F_m - F_I)/ (F_m - F_J)$. δ_{Ro} is the efficiency of an electron can transport from a reduced PQ to PSI end electron acceptor.

K-band=	$V_{OJ}300$= ($F_{300\ \mu s}$ –F_o)/(F_J-F_o). K band indicate relative variable fluorescence at 300 μs (transient normalized between F_o and F_K). This parameter express the breakdown of the oxygen-evolving system (Srivastava et al., 1997).
ChlF steps=	The ChlF induction phase has different time steps called as: 20–50 μs (O-step), 2 ms (J-step), 30 ms (I-step), around 0.8 s (P-step; peak) and generally denoted F_O, F_J and F_I. The last step (P-step) indicates the highest fluorescence intensity (F_m), when saturating light is used.

SUPPLEMENTARY INFORMATION (Appendix A2)

Least Significant Difference (LSD) *Post-Hoc* Test Results:

Analysis performed; maize and sunflower seperatly for each antioxidant enzymes,

Maize (8 treatments) within the all sampling point.

Sunflower (8 treatments) within the all sampling point.

Treatments	CAT								
	t0			t1			t2		
1C-TM	0.0054	±0.0006	defgh	0.0052	±0.0005	defgh	0.0041	±0.0003	ghi
1D-TM	0.0054	±0.0006	defgh	0.0029	±0.0003	ikl	0.0051	±0.0006	defgh
1C-SM	0.0069	±0.0005	cd	0.0096	±0.0007	b	0.0121	±0.0014	a
1D-SM	0.0069	±0.0005	cd	0.0049	±0.0006	efgh	0.0043	±0.0007	fghi
2C-TM	0.0064	±0.0007	cde	0.0056	±0.0005	defg	0.0040	±0.0005	ghi
2D-TM	0.0061	±0.0006	cdef	0.0045	±0.0005	efghi	0.0078	±0.0004	c
2C-SM	0.0035	±0.0003	hik	0.0044	±0.0008	fghi	0.0016	±0.0002	l
2D-SM	0.0036	±0.0003	ghik	0.0021	±0.0003	kl	0.0043	±0.0004	fghi
1C-TS	0.0009	±0.0000	b	0.0008	±0.0000	cd	0.0015	±0.0000	ef
1D-TS	0.0184	±0.0009	b	0.0040	±0.0007	ij	0.0030	±0.0005	ijk
1C-SS	0.0226	±0.0022	a	0.0146	±0.0020	c	0.0056	±0.0006	hi
1D-SS	0.0192	±0.0014	b	0.0121	±0.0006	cde	0.0056	±0.0006	hi
2C-TS	0.0006	±0.0000	hij	0.0006	±0.0000	gh	0.0013	±0.0000	def
2D-TS	0.0006	±0.0000	hij	0.0004	±0.0000	ijk	0.0007	±0.0000	fg
2C-SS	0.0008	±0.0000	k	0.0029	±0.0005	ijk	0.0041	±0.0004	ij
2D-SS	0.0008	±0.0000	k	0.0024	±0.0003	jk	0.0032	±0.0003	ijk

	APX								
Treatments	t0			t1			t2		
1C-TM	0.664	±0.09	def	0.534	±0.07	def	0.525	±0.13	def
1D-TM	0.664	±0.09	def	0.572	±0.03	def	0.862	±0.15	d
1C-SM	0.400	±0.10	ef	0.499	±0.05	def	0.554	±0.02	def
1D-SM	0.400	±0.10	ef	0.478	±0.05	def	0.413	±0.08	ef
2C-TM	0.504	±0.04	def	0.442	±0.03	def	0.381	±0.09	f
2D-TM	0.504	±0.04	def	0.838	±0.28	de	1.324	±0.14	c
2C-SM	2.567	±0.20	a	1.451	±0.09	c	0.337	±0.02	f
2D-SM	2.567	±0.20	a	0.634	±0.01	def	2.078	±0.31	b
1C-TS	2.881	±0.09	a	1.677	±0.07	bcd	0.611	±0.07	fg
1D-TS	2.881	±0.09	a	0.650	±0.06	fg	0.189	±0.01	g
1C-SS	2.087	±0.59	bc	1.352	±0.33	cdef	0.617	±0.08	fg
1D-SS	2.087	±0.59	bc	2.304	±0.13	ab	1.282	±0.31	def
2C-TS	0.563	±0.07	fg	0.653	±0.09	fg	0.905	±0.05	efg
2D-TS	0.563	±0.07	fg	1.520	±0.50	bcde	1.510	±0.17	bcde
2C-SS	0.673	±0.08	fg	0.643	±0.03	fg	0.613	±0.08	fg
2D-SS	0.673	±0.08	fg	0.637	±0.13	fg	0.744	±0.07	fg

	POX								
Treatments	t0			t1			t2		
1C-TM	0.200	±0.00	b	0.061	±0.00	bc	0.108	±0.00	b
1D-TM	0.726	±0.20	b	0.579	±0.12	bcde	1.089	±0.29	a
1C-SM	0.279	±0.03	def	0.694	±0.09	b	1.295	±0.05	a
1D-SM	0.279	±0.03	def	0.514	±0.08	bcdef	0.382	±0.09	bcdef
2C-TM	0.029	±0.00	bcd	0.036	±0.00	bcdef	0.057	±0.00	bcdef
2D-TM	0.029	±0.00	bcd	0.191	±0.00	bcd	0.159	±0.00	bc
2C-SM	0.228	±0.01	ef	0.310	±0.01	cdef	0.355	±0.04	bcdef
2D-SM	0.198	±0.03	f	0.270	±0.03	def	0.615	±0.11	bcd
1C-TS	1.934	±0.20	ab	1.977	±0.24	a	1.566	±0.03	abc
1D-TS	1.934	±0.20	ab	0.475	±0.07	fgh	0.315	±0.06	gh
1C-SS	1.280	±0.38	cde	0.920	±0.21	ef	0.560	±0.07	fgh
1D-SS	1.280	±0.38	cde	1.471	±0.15	bcd	0.606	±0.18	fgh
2C-TS	0.514	±0.06	fgh	0.825	±0.06	efg	1.136	±0.14	cde
2D-TS	0.514	±0.06	fgh	0.967	±0.04	def	1.136	±0.14	cde
2C-SS	0.205	±0.02	h	0.309	±0.02	gh	0.452	±0.05	fgh
2D-SS	0.205	±0.02	h	0.235	±0.05	h	0.235	±0.01	h

	GR								
Treatments	t0			t1			t2		
1C-TM	0.237	±0.04	bc	0.162	±0.02	bcdefg	0.106	±0.00	defg
1D-TM	0.360	±0.12	a	0.087	±0.01	efg	0.164	±0.01	bcdefg
1C-SM	0.085	±0.01	efg	0.119	±0.01	cdefg	0.152	±0.03	bcdefg
1D-SM	0.116	±0.02	ab	0.051	±0.01	g	0.270	±0.04	cdefg
2C-TM	0.148	±0.01	bcdefg	0.126	±0.01	cdefg	0.068	±0.01	fg
2D-TM	0.235	±0.02	bcd	0.183	±0.03	bcdef	0.213	±0.01	bcde
2C-SM	0.272	±0.06	ab	0.189	±0.02	bcdef	0.106	±0.02	defg
2D-SM	0.180	±0.02	ab	0.137	±0.04	cdefg	0.273	±0.02	bcdefg
1C-TS	0.261	±0.01	a	0.196	±0.02	bc	0.155	±0.02	cd
1D-TS	0.261	±0.01	a	0.038	±0.00	g	0.047	±0.01	fg
1C-SS	0.121	±0.01	de	0.090	±0.01	ef	0.052	±0.00	fg
1D-SS	0.121	±0.01	de	0.215	±0.02	b	0.200	±0.03	bc
2C-TS	0.060	±0.00	fg	0.061	±0.00	fg	0.062	±0.00	fg
2D-TS	0.060	±0.00	fg	0.056	±0.01	fg	0.159	±0.02	cd
2C-SS	0.038	±0.00	g	0.056	±0.00	fg	0.093	±0.00	ef
2D-SS	0.038	±0.00	g	0.036	±0.01	g	0.036	±0.00	g

	SOD								
Treatments	t0			t1			t2		
1C-TM	88.6	±10.1	b	69.9	±4.6	bcd	51.2	±4.6	def
1D-TM	88.6	±10.1	b	40.7	±5.6	f	45.6	±4.5	f
1C-SM	82.6	±3.4	bc	69.7	±3.3	bcd	56.8	±5.0	def
1D-SM	82.6	±3.4	bc	45.4	±4.5	f	41.1	±4.9	f
2C-TM	87.3	±7.7	b	64.4	±3.8	ef	41.6	±1.6	cde
2D-TM	87.3	±7.7	b	46.4	±5.1	f	64.7	±4.3	cde
2C-SM	114.1	±8.4	a	80.1	±3.7	bc	46.2	±3.6	ef
2D-SM	114.1	±8.4	a	67.4	±7.2	cd	79.5	±6.5	bc
1C-TS	71.6	±4.8	b	68.4	±1.4	b	65.2	±5.2	bc
1D-TS	71.6	±4.8	b	58.5	±5.3	bc	21.8	±1.7	e
1C-SS	66.0	±6.3	bc	51.1	±3.4	cd	36.1	±3.7	de
1D-SS	66.0	±6.3	bc	112.8	±7.0	a	51.2	±4.7	cd
2C-TS	42.3	±6.0	d	71.9	±4.5	b	101.4	±5.1	a
2D-TS	42.3	±6.1	d	38.8	±4.6	d	58.0	±4.2	bc
2C-SS	21.6	±1.7	e	36.4	±3.5	de	51.1	±6.2	cd
2D-SS	21.6	±1.7	e	22.9	±3.1	e	40.7	±4.2	d

	TBARS								
Treatments	t0			t1			t2		
1C-TM	33.5	±2.0	ef	48.0	±1.8	d	62.4	±2.4	c
1D-TM	33.5	±2.0	ef	75.4	±2.1	b	103.5	±4.0	a
1C-SM	33.9	±1.0	ef	45.9	±2.1	d	58.0	±4.5	c
1D-SM	33.9	±1.0	ef	41.3	±4.0	de	77.6	±5.1	b
2C-TM	31.9	±3.2	f	27.5	±2.0	fg	23.0	±1.7	gh
2D-TM	31.9	±3.2	f	44.0	±5.2	d	56.5	±5.0	c
2C-SM	13.7	±1.4	i	18.8	±1.7	hi	23.8	±3.0	gh
2D-SM	13.7	±1.4	i	76.6	±2.0	b	28.2	±1.6	fg
1C-TS	24.5	±1.0	ghij	28.0	±1.2	defghi	31.5	±1.7	defg
1D-TS	25.5	±1.1	ghij	34.5	±2.6	d	65.6	±8.3	a
1C-SS	25.4	±1.0	ghij	27.2	±0.8	efghi	29.1	±2.0	defgh
1D-SS	23.8	±0.9	hij	26.2	±4.2	ghij	57.8	±3.5	b
2C-TS	9.0	±0.6	k	21.3	±1.4	ij	33.6	±3.0	def
2D-TS	9.0	±0.6	k	19.5	±1.7	i	41.0	±1.7	c
2C-SS	9.4	±0.8	k	21.6	±0.6	ij	33.8	±1.4	de
2D-SS	9.4	±0.8	k	26.8	±1.6	fghi	46.3	±2.2	c

Treatments	Chl.a								
	t0			t1			t2		
1C-TM	1.108	±0.05	hi	1.530	±0.16	def	1.833	±0.23	bc
1D-TM	1.108	±0.05	hi	2.273	±0.12	a	1.334	±0.11	efgh
1C-SM	1.654	±0.05	cd	1.560	±0.05	de	1.117	±0.02	hi
1D-SM	1.654	±0.05	cd	1.493	±0.02	def	1.043	±0.07	i
2C-TM	0.762	±0.00	j	1.029	±0.08	i	1.163	±0.00	ghi
2D-TM	0.762	±0.00	j	1.895	±0.10	bc	1.477	±0.09	def
2C-SM	1.257	±0.00	fghi	1.348	±0.02	efgh	1.440	±0.04	def
2D-SM	1.257	±0.00	fghi	1.986	±0.08	b	1.395	±0.01	defg
1C-TS	1.049	±0.02	de	1.316	±0.01	b	1.597	±0.01	a
1D-TS	1.049	±0.02	de	1.116	±0.01	cd	1.636	±0.10	a
1C-SS	1.118	±0.04	cd	1.221	±0.01	bc	1.335	±0.03	b
1D-SS	1.118	±0.04	cd	0.927	±0.03	efg	1.605	±0.06	a
2C-TS	0.653	±0.06	i	0.772	±0.04	ghi	0.890	±0.03	efgh
2D-TS	0.653	±0.06	i	0.809	±0.05	fgh	0.764	±0.03	hi
2C-SS	1.034	±0.04	de	0.942	±0.04	ef	0.849	±0.06	fgh
2D-SS	1.034	±0.04	de	0.941	±0.00	ef	0.622	±0.02	i

	Chl.b								
Treatments	t0			t1			t2		
1C-TM	0.283	±0.01	defg	0.343	±0.03	bcd	0.346	±0.06	bcd
1D-TM	0.283	±0.01	defg	0.404	±0.02	ab	0.266	±0.01	fg
1C-SM	0.425	±0.01	a	0.228	±0.01	g	0.119	±0.00	h
1D-SM	0.425	±0.01	a	0.323	±0.00	efg	0.273	±0.02	cdef
2C-TM	0.164	±0.00	h	0.246	±0.01	g	0.264	±0.00	fg
2D-TM	0.164	±0.00	h	0.377	±0.03	abc	0.140	±0.00	h
2C-SM	0.315	±0.00	cdef	0.325	±0.00	cdef	0.335	±0.00	cde
2D-SM	0.315	±0.00	cdef	0.403	±0.01	ab	0.283	±0.01	defg
1C-TS	0.290	±0.00	defg	0.334	±0.00	bcd	0.377	±0.00	b
1D-TS	0.290	±0.00	defg	0.350	±0.00	bc	0.525	±0.04	a
1C-SS	0.317	±0.01	cde	0.324	±0.00	cde	0.328	±0.01	bcd
1D-SS	0.317	±0.01	cde	0.315	±0.01	cde	0.550	±0.03	a
2C-TS	0.239	±0.01	g	0.260	±0.01	fg	0.282	±0.01	defg
2D-TS	0.239	±0.01	g	0.273	±0.01	efg	0.246	±0.00	g
2C-SS	0.336	±0.01	bcd	0.320	±0.01	cde	0.304	±0.01	cdef
2D-SS	0.336	±0.01	bcd	0.321	±0.00	cde	0.254	±0.00	fg

	Tot.Chl								
Treatments	t0			t1			t2		
1C-TM	1.391	±0.06	efgh	1.873	±0.20	cd	2.179	±0.29	b
1D-TM	1.391	±0.06	efgh	2.677	±0.14	a	1.599	±0.12	defg
1C-SM	2.078	±0.06	bc	1.788	±0.05	cd	1.236	±0.02	h
1D-SM	2.078	±0.06	bc	1.817	±0.02	cd	1.317	±0.07	fgh
2C-TM	0.927	±0.01	i	1.275	±0.10	gh	1.426	±0.00	efgh
2D-TM	0.927	±0.01	i	2.272	±0.10	b	1.617	±0.10	def
2C-SM	1.572	±0.00	defg	1.673	±0.02	de	1.774	±0.05	cd
2D-SM	1.572	±0.00	defg	2.389	±0.09	b	1.678	±0.01	de
1C-TS	1.340	±0.03	cdefg	1.650	±0.01	b	1.974	±0.01	a
1D-TS	1.340	±0.03	cdefg	1.466	±0.01	bcd	2.160	±0.19	a
1C-SS	1.434	±0.06	cde	1.545	±0.02	bc	1.662	±0.04	b
1D-SS	1.434	±0.06	cde	1.241	±0.05	efgh	2.155	±0.08	a
2C-TS	0.892	±0.08	jk	1.032	±0.05	ijk	1.172	±0.04	fghi
2D-TS	0.892	±0.08	jk	1.082	±0.06	hij	1.011	±0.03	ijk
2C-SS	1.370	±0.05	cdef	1.261	±0.06	defgh	1.153	±0.08	ghi
2D-SS	1.370	±0.05	cdef	1.261	±0.00	defgh	0.876	±0.02	k

	Tot.Carotenoid								
Treatments	t0			t1			t2		
1C-TM	0.180	±0.02	cdefg	0.186	±0.02	bcdefg	0.193	±0.03	abcdef
1D-TM	0.180	±0.02	cdefg	0.236	±0.03	abc	0.236	±0.02	ab
1C-SM	0.217	±0.00	abcde	0.208	±0.00	abcdef	0.170	±0.00	efghi
1D-SM	0.217	±0.00	abcde	0.227	±0.02	abcd	0.220	±0.02	abcde
2C-TM	0.126	±0.00	hi	0.137	±0.00	ghi	0.121	±0.00	i
2D-TM	0.126	±0.00	hi	0.239	±0.02	ab	0.242	±0.01	a
2C-SM	0.177	±0.00	defgh	0.155	±0.00	fghi	0.156	±0.00	fghi
2D-SM	0.177	±0.00	defgh	0.243	±0.00	a	0.210	±0.01	abcde
1C-TS	0.110	±0.00	defg	0.125	±0.00	d	0.153	±0.00	c
1D-TS	0.110	±0.00	defg	0.121	±0.00	d	0.196	±0.01	b
1C-SS	0.106	±0.00	defgh	0.109	±0.00	defg	0.113	±0.00	def
1D-SS	0.106	±0.00	defgh	0.118	±0.00	de	0.220	±0.00	a
2C-TS	0.082	±0.00	i	0.085	±0.00	ij	0.087	±0.00	ij
2D-TS	0.082	±0.00	i	0.090	±0.00	hij	0.091	±0.00	ghij
2C-SS	0.107	±0.00	defg	0.102	±0.00	efghi	0.097	±0.00	fghij
2D-SS	0.107	±0.00	defg	0.109	±0.00	defg	0.124	±0.00	d

Supplementary Data (Appendix B1)

Chlorophyll *a* fluorescence parameters used in the fluorescence transient analysis

Technical fluorescence parameters	
$\mathbf{F_t}$	Fluorescence emission from a dark-adapted leaf at the time t
$\mathbf{F_0}$	Minimal fluorescence from a dark-adapted leaf
$\mathbf{F_M}$	Maximal fluorescence from a dark-adapted leaf
$\mathbf{F_J}$	Fluorescence intensity at the J-step (at 2 ms)
$\mathbf{F_I}$	Fluorescence intensity at the I-step (at 30 ms)
$\mathbf{F_V}$	Maximal variable fluorescence from a dark-adapted leaf. $F_V = F_M - F_0$
$\mathbf{V_J}$	Relative variable fluorescence at 3ms. $V_J = (F_{2ms} - F_0) / (F_M - F_0)$
$\mathbf{V_I}$	Relative variable fluorescence at 30ms. $V_I = (F_{30ms} - F_0) / (F_M - F_0)$
$\mathbf{M_0}$	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300\mu s} - F_0) / (F_M - F_0)$
Derived parameters	
$\boldsymbol{F_v/F_m}$ =	$[F_m - F_0]/F_m = \varphi_{Po} = TR_0/ABS$ = maximum quantum yield of PSII primary photochemistry, measured in samples in dark-adapted state. F_v/F_m expresses the probability that an absorbed photon will be trapped by the PSII reaction centre.
$\boldsymbol{\Psi_{Eo}}$ =	$ET_0/TR_0 = 1 - V_J = 1 - (F_{2\ ms} - F_0)/ (F_m - F_0)$. Ψ_{Eo} expresses the probability that the energy of a trapped excitation is used for electron transport beyond Q_A. V_J represents the relative variable fluorescence at 2 ms (step-J) ($V_J = (F_J - F_o)/(F_m - F_o)$).
$\boldsymbol{\Delta V_{I\text{-}P}}$ =	$1 - V_I = (F_m - F_{30\ ms})/ (F_m - F_0)$, I-P phase (Oukarroum et al., 2009). This parameter indicates relative contribution of the I-P phase to the fluorescence transient OJIP; it is regarded as a measure of the efficiency of electron flux through PSI to reduce the final acceptors of the electron transport chain, i.e. ferredoxin and NADP. V_I indicates the relative variable fluorescence at 30 ms (step-I) ($V_I = (F_I - F_o)/(F_m - F_o)$).
RC/ABS=	$(1-(F_o/F_m))/(M_o/V_J) = \varphi_{Po}\ (V_J/M_0)$. This parameter represents the total number of active reaction center per absorbtion. $Mo = 4(F_{300\ \mu s} - F_o)/(F_m - F_o)$. M_0 represents the initial slope of the double normalised fluorescence induction curve, and is a proxy of the net rate of PSII closure.
$\mathbf{PI_{ABS}}$ =	(RC/ABS) $[\varphi_{Po}/(1-\varphi_{Po})]$ $[\Psi E_o/(1-\Psi E_o)]$. Performance index on absorption bases; absorption of antenna Chls of PSII. This measure incorporates photochemical and non-photochemical processes, such as absorption and trapping of excitation energy, electron transport beyond the primary plastoquinone (QA) and dissipation of excess excitation energy.
$\mathbf{PI_{TOT}}$=	Performance Index total (PI_{TOT}) is the potential for energy conservation from photons absorbed by PSII to the reduction flux (RE) of PSI end acceptors. The Pi_{tot} is a multi-parametric indicator of four measures of photosynthetic electron transport: (1) the concentration of reaction centres; (2) the quantum yield of PSII photochemistry; (3) the capacity for uptake of electrons in the electron chain between PSII and PSI; (4) the efficiency with which an electron can transfer from the reduced intersystem electron acceptors to the PSI end electron (Strasser et al., 2010, 2004; Tsimilli-Michael and Strasser, 2008). $PI_{TOT} = PI_{ABS}\ [\delta_{Ro}/(1-\delta_{Ro})]$ where $\delta_{Ro} = (1 - V_J)/ (1 - V_J) = (F_m - F_I)/ (F_m - F_J)$. δ_{Ro} is the efficiency of an electron can transport from a reduced PQ to PSI end electron acceptor.

K-band=	$V_{OJ}300= (F_{300\ \mu s} – F_o)/(F_J-F_o)$. K band indicate relative variable fluorescence at 300 μs (transient normalized between F_o and F_K). This parameter express the breakdown of the oxygen-evolving system (Srivastava et al., 1997).
ChlF steps=	The ChlF induction phase has different time steps called as: 20–50 μs (O-step), 2 ms (J-step), 30 ms (I-step), around 0.8 s (P-step; peak) and generally denoted F_O, F_J and F_I. The last step (P-step) indicates the highest fluorescence intensity (F_m), when saturating light is used.

- I_1, first maximum of the DF induction curve; appears during J-I increase of the PF and in the oxidation phase of MR measurements
- I_2, second maximum of the DF induction curve, or 'shoulder'; ; appears in I-P phase of PF and in the re-reduction phase of MR measurements.
- I_1/I_2 ; ratio is inversely related to the flow of electrons in the PSII and is sensitive to stress conditions (Goltsev et al., 2012);
- D_2 ; indicates the reduction of PSII complex activity.
- I_4; the final maximum of the DF induction curve; appears during the decrease of the PF intensity and oxidation of PSI.
- I_4/D_2 ; ratio is connected to the trans-membrane proton gradient (Goltsev et al., 2005)
- **MR**, modulated reflectance of 820 nm light;
- MR_t/MR_0, ratio between modulated 820 nm reflection intensity at time *t*
- MR_t, and value of the 820 nm reflection of the sample at the onset of the actinic illumination (between 0.3 and 1 ms, MR_0);
- MR_{min}, minimal MR_t/MR_0 value, a transitory steady state, with equal oxidation and re-reduction rates of P700 and PC;
- **PF**, prompt fluorescence;
- v_{ox}, the rate of P700 and PC oxidation, calculated as the maximum slope decrease of MR_t/MR_0;
- v_{red}, the rate of P700 and PC re-reduction, calculated as the maximum slope increase of MR_t/MR_0