

Cannabis sativa genotypes with larger leaf areas have higher potential to adjust stomatal size and density in response to water deficit: The effect on stomatal conductance and physiological stomatal behaviour

Matthew Haworth^{a,*}, Andrea Carli^a, Vincenzo Montesano^a, Dilek Killi^a, André Fabbri^a, Raffaella Balestrini^{a,b}, Giovanni Marino^a, Mauro Centritto^a

^a National Research Council of Italy, Institute of Sustainable Plant Protection (CNR - IPSP), Italy

^b Present address: National Research Council, Institute of Biosciences and Bioresources (CNR - IBBR), Italy

ARTICLE INFO

Keywords:

Phenotyping
Hemp
Drought
Allometry
Stomatal spacing
Stomatal kinetic
Stomatal sensitivity

ABSTRACT

Effective stomatal control is fundamental to successful plant responses to water deficit stress. Control of stomatal conductance (G_s) can be exerted through modification of stomatal morphology (size and density) in newly developing leaves, or physiological adjustment of stomatal pore aperture. We investigated the potential coordination of stomatal morphological and physiological responses to water deficit in three varieties of hemp (*Cannabis sativa* L.) grown under field conditions. The three hemp varieties had contrasting leaf areas under well-watered irrigation (control): Earlina 8FC < Fedora 17 < Fibror 79. Stomatal size, density and the speed of physiological adjustment of G_s were not significantly different under full irrigation. Under water deficit conditions the leaf areas of the two varieties with the largest leaves decreased to match those of Earlina 8FC. This reduction in leaf area, alongside an increase in stomatal initiation, resulted in higher densities of smaller stomata. This effect was most evident in Fibror 79 that showed the largest reduction in leaf area, increase in stomatal density and decrease in stomatal size. This corresponded to the most rapid physiological adjustment of G_s to fluctuations in photosynthetic photon flux density of the hemp varieties occurring in Fibror 79 when subject to water deficit. The coincidence of the fastest velocity of G_s adjustment with the highest densities of small stomata may support interpretations of a functional advantage of high densities of small stomata in the physiological regulation of G_s to fluctuating conditions. The larger leaf area of Fibror 79 appeared to be associated with higher capacity to respond to water deficit through modification of stomatal morphology and physiological behaviour. This result indicates that phenotyping of crop species and genotypes to identify traits conducive to water deficit tolerance through effective stomatal control should consider the foliar plasticity of genotypes to water deficit, and the potential implications for stomatal morphological and physiological control of transpirative water loss and photosynthetic CO_2 -uptake.

1. Introduction

Stomata balance photosynthetic carbon dioxide (CO_2) uptake against transpirative water-loss (Cowan 1978). This central role in plant water-relations and carbon gain makes stomatal regulation of critical importance to the identification and development of more productive climate resilient crops (Bertolino et al., 2019; Haworth et al. 2021). Stomatal conductance (G_s) is largely a function of morphological (the number and size of stomatal complexes) and physiological (guard cell regulation of stomatal aperture) stomatal traits. The interaction between

stomatal morphology and physiology often reflects a number of trade-offs to optimise the carbon assimilation to transpiration ratio (water use efficiency - WUE) (Haworth et al. 2018c; Xiong and Flexas 2020). However, stomatal morphology (eg. Hu et al. 2019; Xu and Zhou 2008) and physiology (eg. Durand et al. 2019, Haworth et al. 2018a) are often considered in isolation when examining plant responses to their environment. To investigate the effect of water availability on stomatal control we assessed morphological and physiological stomatal responses to water deficit in three varieties of industrial hemp (*Cannabis sativa* L.) (hereafter referred to as 'hemp') with contrasting leaf

* Corresponding author.

E-mail address: matthew.haworth@ipspp.cnr.it (M. Haworth).

<https://doi.org/10.1016/j.stress.2024.100649>

Received 3 September 2024; Received in revised form 28 September 2024; Accepted 21 October 2024

Available online 28 October 2024

2667-064X/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC license (<http://creativecommons.org/licenses/by-nc/4.0/>).

macro-morphologies.

The close interaction between stomatal morphology and physiology is evident in stomatal distributions over the leaf surface and the speed of G_s adjustment (Haworth et al. 2023). Analysis of the rate of the initial 50 % of the reduction in G_s during a transition from saturating photosynthetic photon flux density (PPFD) to darkness (to stimulate stomatal closure) ($G_{s50\%}$), shows a close positive correlation with the proportion of the epidermis available for gas exchange ($A_{\%}$). The absolute and relative speeds of physiological adjustment correspond to the transition from hypostomatous to amphistomatous distributions, contributing to the higher potential rates of CO_2 uptake in monocot angiosperms (Haworth et al. 2018c). Stomatal size (SS) is also considered to influence physiological stomatal behaviour, as smaller stomatal complexes can achieve maximum potential changes in guard cell turgor with lower fluxes of ions than counterparts with larger guard cells (Raven 2014). Species with high densities of small stomata may therefore be able to adjust G_s more rapidly than those with low densities of large stomata (Drake et al. 2013). Higher densities of smaller stomata may represent a selective advantage for many angiosperms due to the capacity to sustain higher rates of G_s (Assouline and Or 2013; Franks and Beerling 2009). However, screening of the morphological and physiological traits of a large number of species suggests that biochemical guard cell properties and stomatal signalling strongly influence stomatal physiological responsiveness alongside SS to stomatal density (SD) scaling (Elliott-Kingston et al. 2016; Haworth et al. 2023). Stomata regulate the flow of gasses between plants and the atmosphere. It is therefore likely that physiological and morphological stomatal responses to changes in environmental conditions are co-ordinated. Indeed, plant species with active physiological stomatal behaviour (the active pumping of ions across the guard cell plasma membrane to regulate guard cell turgor) exhibit more constrained SD responses to the atmospheric concentration of carbon dioxide ($[\text{CO}_2]$) than species with passive physiological stomatal behaviour (where guard cell water potential follows leaf water potential) (Haworth et al. 2015). Despite the likelihood that drought events will become more frequent and severe in the future (Hoerling et al. 2012; Treydte et al. 2024), to the best of our knowledge, there are no studies that jointly examine both physiological and morphological stomatal responses to water deficit.

The characteristic response of plants to a reduction in soil water availability is a decrease in G_s as stomata close (Cowan 1978; Martin-StPaul et al., 2017; Tardieu and Davies 1992). This stomatal closure increases resistance to the uptake of CO_2 for photosynthesis (Flexas et al. 2000; Lauteri et al. 2014) and reduces the use of energy for photochemistry, potentially leading to photooxidative stress (Killi et al. 2020; Pinheiro and Chaves 2011). Under severe water deficit stress stomata will close to their maximum extent. However, under less severe water deficit stress G_s is not reduced to its maximum extent (Centritto et al. 2011; Killi et al. 2017; Sinclair and Ludlow 1986), indicating physiological regulation of guard cell turgor to partially reduce stomatal pore area and optimise WUE (Cai et al. 2017; Eisele et al. 2016). Alongside lower values of G_s compared to values when water availability is not limited, moderate water deficit may induce shifts in physiological stomatal behaviour. An increased foliar content of abscisic acid associated with water deficit in giant reed (*Arundo donax* L.) caused an increase in G_s sensitivity to fluctuations in PPFD and $[\text{CO}_2]$ (Haworth et al. 2018a). Water deficit induced a similar increase in G_s sensitivity to fluctuations in PPFD in tobacco plants (*Nicotiana tabacum* L.) (Gerardin et al. 2018). In contrast, G_s sensitivity of genotypes of two poplar species (*Populus euramericana* and *Populus nigra* L.) to PPFD and leaf to air vapour pressure deficit became less responsive under water deficit (Durand et al. 2019). Moreover, two genotypes of field grown tomato (*Solanum lycopersicum* L.) showed no difference in G_s sensitivity to fluctuations in PPFD when grown at two different levels of irrigation (Sillo et al. 2022). Application of exogenous abscisic acid to a sword fern (*Nephrolepis exalta* L.), cedar (*Calocedrus decurrens* (Torr.) Florin), and soybean (*Glycine max* L.) induced an increase in the speed of G_s decrease

associated with stomatal closure as PPFD was reduced. However, the effect of abscisic acid on stomatal opening as PPFD increased was less clear, as sword fern and soybean became more responsive, but cedar was unaffected (Grantz et al. 2019). This may suggest that inter-specific differences in the biochemical and hydraulic signals that regulate stomata (Brunetti et al. 2019; Rodriguez-Dominguez et al., 2016; Tombesi et al. 2015) may account for variation in stomatal physiological behaviour observed under water deficit. Moreover, differences in the type and severity of water deficit treatment also likely contribute to the differences in stomatal physiological sensitivity to reduced soil water availability reported in the literature.

The stomatal density (SD) and index (SI) responses of plants to atmospheric $[\text{CO}_2]$ have been widely examined (Hu et al. 2019; Körner 1988; Müller-Rushing et al. 2009). The effect of water availability on stomatal development is less well defined. This may be due to differences in the severity and duration of water deficit during leaf development alongside differential responses among species. Leaves of poplar (*Populus balsamifera*) (Hamanishi et al. 2012), soybean (*Glycine max*) (Tripathi et al. 2016), arabidopsis (*Arabidopsis thaliana*), oil palm (*Elaeis guineensis*), and rice (*Oryza sativa*) (Song et al. 2023) all showed reduced SD and SI values following sudden severe water deficit consisting of a cessation in the provision of water for seven to 21 days. Cotton (*Gossypium hirsutum*) plants receiving deficit irrigation of one-fifth of the volume of water provided to control plants for 15 days also developed leaves with lower SD and SI values (Dubey et al. 2023). In contrast, six genotypes of almond (*Prunus dulcis*) grown at three different soil water potential levels (control $\Psi = -0.33$ MPa; moderate $\Psi = -1.2$ MPa; severe $\Psi = -1.8$ MPa) for 35 days had no response in either stomatal size or density (Yadollahi et al. 2011). Arabidopsis maintained at a soil water content of 54.5 % of control plants for 10 days, then at 31.8 % for a further 10–11 days, exhibited higher SD values consistent with lower cells expansion, but no change in SI indicative of a maintenance of stomatal initiation rates in developing leaves (Clauw et al. 2015). Moreover, the SD of basil (*Ocimum basilicum*) increased on both the abaxial and adaxial leaf surfaces, SI was unaffected by 35-days growth at 50 % (control) and 20 % (water deficit) volumetric water content (Driesen et al. 2023). Likewise, C3 wheat (*Triticum aestivum*: 80 %, 60 %, and 40 % field capacity) (Yang and Wang 2001) and C4 maize (*Zea mays*: 70 %, 60 %, and 45 % soil water content) (Zhao et al. 2014) showed increases in SD as soil water availability declined. The SD and SI values of eudicot broad bean (*Vicia faba*) both increased as soil water content declined (75–85 %, 60–70 %, and 50–45 % field water content) (Gan et al. 2010). Sheepgrass (*Leymus chinensis* (Trin.) Tzvelev) grown for 20 days at five levels of field capacity showed an increase in SD at the initial levels of water deficit followed by a return to levels similar to those of well-watered plants at the lowest water potentials. Stomatal size showed a linear negative relationship with soil water availability. The SI values of sheepgrass across the same field capacity water availability treatments showed an initial increase at the higher water potentials before remaining constant at lower soil water potential levels, indicating that shifts in stomatal initiation were not responsible for the patterns in SD at the more severe levels of water deficit (Xu and Zhou 2008). This wide variation in stomatal density, size, and index responses to water deficit may reflect shifts in the activation of genes that regulate stomatal development (for example TOO MANY MOUTHS, STOMAGEN: Dubey et al. 2023, Tripathi et al. 2016) and leaf expansion (Zhou et al. 2015) elicited by different types of water treatment. The interaction between stomatal initiation with leaf expansion (eg. Clauw et al. 2015) and differences in stomatal physiological control (eg. Haworth et al. 2015) may contribute to the wide range of stomatal morphological responses to water deficit. The genetic regulation of stomatal initiation observed in leaves developed under water deficit may be related to shifts in transpiration rate and abscisic acid flux (Lake and Woodward 2008; Lü et al. 2013), or signalling from mature to developing leaves of higher internal sub-stomatal $[\text{CO}_2]$ associated with stomatal closure (Lake et al. 2001).

Effective stomatal control is fundamental to plant responses to water

deficit (Bertolino et al. 2019; Haworth et al. 2021). This is of particular importance where high rates of G_s under optimal conditions are closely correlated to leaf level rates of photosynthesis and the yield of crop plants (Lauteri et al. 2014). Analysis of either the physiological or morphological responses of stomata to water deficit in isolation risks missing vital information concerning the adaptative capacity of specific genotypes to reduced soil water availability. A holistic view of stomatal control is vital to our understanding of tolerance to water deficit and the effective phenotyping of crops for attributes conducive to high productivity and climate resistance in the context of climate change, rising populations, and the loss of agricultural land that imperil food and energy security (Costa et al. 2019; Grierson et al. 2011). We assessed the physiological and morphological stomatal characteristics of three genotypes of hemp grown under two irrigation regimes (100 and 50 % irrigation). Hemp requires less water than other fibre crops such as cotton (*Gossypium hirsutum* L.) (Wise et al. 2023), but its yield can be affected by water deficit during critical phenological stages (Gill et al. 2022). This study aimed to: i) assess photosynthetic and G_s responses of the three genotypes to water deficit; ii) characterise the physiological stomatal behaviour of the hemp genotypes in response to reduced soil water availability; iii) quantify possible SD to SS scaling relationships with respect to leaf macro-morphology and the impact of water deficit; iv) investigate potential co-ordination of stomatal physiological and morphological characters in the exertion of stomatal control under water deficit conditions, and v) explore 'ideotype' physiological and morphological characters towards the development of more productive and water deficit resilient genotypes of hemp suited for cultivation under hotter drier future climatic conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Three monoecious varieties of industrial hemp (*Cannabis sativa* L.) were grown under full (100 %) and water deficit (50 %) levels of irrigation in field conditions at the ENI-CNR Water Research Centre, Metaponto, Basilicata, Italy. The hemp varieties had contrasting leaf areas: Earlina 8FC < Fedora 17 < Fibror 79. Seeds of each variety were sown at a density of 35 kg seeds ha⁻¹ in rows spaced 20 cm apart within 10 m² plots in three replications (18 plots in total: nine full water and nine water deficit) on April 27, 2022. From seed sowing until 6 June 2022 (for a total of 40 days) all plots received the same volume of irrigation water until the plants were established. Water was supplied to the plants using an inline drip irrigation system with 15 drippers per m² (1.3 L/h;

16 mm inside diameter; 20 cm spacing). The amount of water supplied each day for 100 % irrigation was equivalent to daily levels of evapotranspiration (E_{Tc}):

$$E_{Tc} = E_o * K_p * K_c$$

where, E_o is the evaporation of water from a Class-A pan; K_p is the pan coefficient, and K_c is the crop growth stage. Rainfall was subtracted from the daily calculation of the volume of water supplied as irrigation (Allen et al. 1998). A weather station at the field site was used to record meteorological conditions during the cultivation of the hemp plants – the data is supplied in Supplementary Information Fig. 1.

2.2. Leaf gas exchange and stomatal kinetics

Leaf gas exchange measurements were performed after six weeks of water deficit treatment. Two PP-Systems Ciras-3 plant photosynthesis systems connected to a PLC3 Plant Leaf Cuvette with CFM-3 chlorophyll fluorescence modules were used for all leaf gas exchange measurements (PP-Systems, Amesbury MA, USA). Instantaneous point measurements of leaf gas exchange parameters were conducted on a minimum of four plants per plot on the uppermost fully expanded leaf. The leaves of hemp are palmate compound leaves (Anderson 1980). Measurements of leaf gas exchange were made using the 7 × 25 mm cuvette plate on the midpoint of the largest central leaflet – the same area of the leaf was also sampled for stomatal anatomy measurements. Cuvette conditions were maintained at a temperature of 30 °C, ambient atmospheric [CO₂] of 420 μmol mol⁻¹, photosynthetic photon flux density (PPFD) of 2000 μmol m⁻² s⁻¹ (40 % red, 20 % green, 10 % blue, and 30 % white light), and a relative humidity of 50–60 %. Leaves were allowed to stabilise to the controlled cuvette conditions for approximately 20 min before leaf gas exchange parameters were recorded.

To assess physiological stomatal behaviour, two stomatal kinetic measurements were performed (Haworth et al. 2018b) on a minimum of four replicate plants from each variety and treatment. The first stomatal kinetic involved measurement of stomatal conductance (G_s) response to a transition from saturating PPFD (2000 μmol m⁻² s⁻¹) to darkness (0 μmol m⁻² s⁻¹) to stimulate stomatal closure. The leaf was placed within the cuvette under identical conditions to those used for the instantaneous measurement of leaf gas exchange and allowed to acclimate for approximately 20 min. When leaf gas exchange parameters had remained stable for at least 5 min, the stomatal kinetic measurement was started. Leaf gas exchange parameters were automatically recorded every 10 s using the autolog function. After 1000 s, the illumination in the leaf cuvette was switched off, and the G_s response as stomata closed

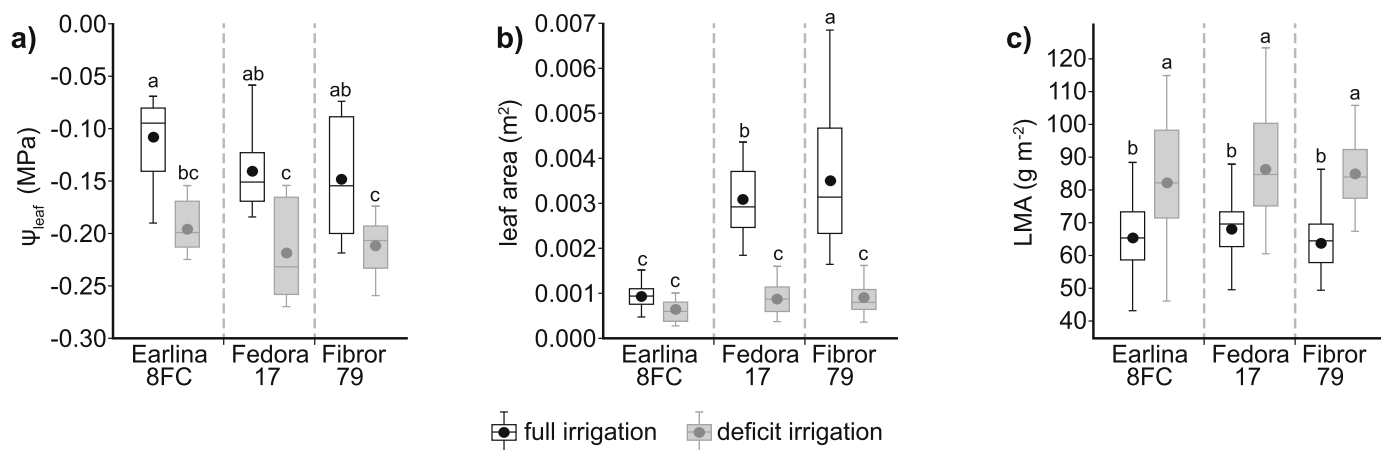


Fig. 1. Box plots illustrating the effect of 100 % well-watered control (white fill) and 50 % water deficit (grey fill) irrigation on three varieties of hemp: a) the midday leaf water potential (Ψ_{leaf}); b) leaf area, and c) leaf mass per area (LMA). The box signifies the distribution of the 25–75 % quartiles, the median is represented by a vertical line within the box, the mean is represented by a circle symbol, and horizontal bars indicate minimum/maximum values. Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post-hoc* test at the 0.05 significance level.

was recorded for a further 6000 s. The parameter $G_{s50\%}$ was calculated as the rate of the G_s change over the first 50 % of the total G_s response following Haworth et al. (2018b). A schematic illustration of the points in the stomatal kinetic used to calculate $G_{s50\%}$ is given in Supplementary Information Fig. 2. The percentage reduction in G_s from values before the cessation of illumination (when stomata were considered to be open) to the value when G_s had stabilised after darkness (stomata considered to be closed) was taken to reflect the ‘tightness’ of stomatal closure (ΔG_s). The second stomatal kinetic measurement involved transitions from high ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) to low ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) to high ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) levels of PPFD, simulating a sun fleck on a cloudy day (Sillo et al. 2022). Leaves were placed into the leaf cuvette under identical conditions to those used for instantaneous measurement of leaf gas exchange for a minimum of 20 min until all gas exchange values had remained constant for 5 min. Leaf gas exchange parameters were recorded automatically every 10 s for 1000 s at high PPFD, 1800 s at low PPFD, and then a further 1800 s at high PPFD. The $G_{s50\%}$ responses of G_s during the transition from high to low PPFD (stimulation of stomatal closure), and transition from low to high PPFD (stimulation of stomatal opening) were determined as outlined in Supplementary Information Fig. 2b.

2.3. Stomatal anatomy

The leaves used for stomatal kinetic measurements were labelled and cut immediately after completion of the gas exchange analysis of stomatal physiological behaviour. The leaves of the hemp varieties were hypostomatous, with stomata located on the abaxial surface. A negative impression of the abaxial leaf surface of the central area of the largest middle leaflet used for the leaf gas exchange measurements was created using dental impression gel (President Light Body Material, Coltene, Altstätten, Switzerland). Transparent nail varnish (Max Factor, Proctor and Gamble, Cincinnati OH, USA) was then used to create positive impressions of the leaf surface (Stein et al. 2024; Weyers and Lawson 1985). These nail varnish positives were then placed on a glass slide and imaged using a Leica DM2500 microscope attached to a Leica DFX300FX camera (Leica Microsystems, Wetzlar, Germany). To measure stomatal density (SD) and stomatal index (SI), a digital $0.4 \times 0.4 \text{ mm}$ grid was superimposed over each image, avoiding areas where leaf veins were present (Poole and Kürschner 1999). The number of stomata and epidermal cells were counted for each leaf. Stomatal index was calculated as the number of stomata divided by the total number of stomata and epidermal cells (Poole and Kürschner 1999). Measurements were

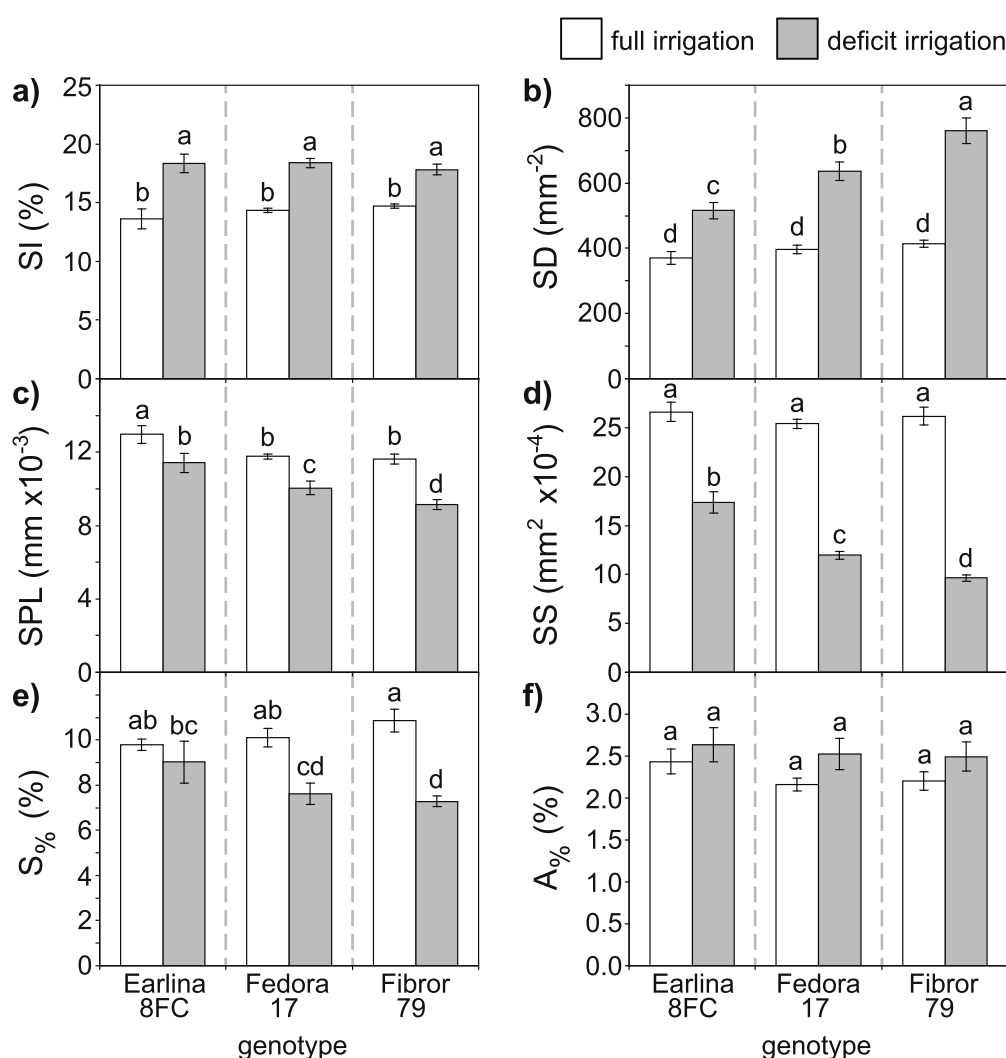


Fig. 2. The effect of 100 % well-watered control (white fill) and 50 % water deficit (grey fill) irrigation on leaf abaxial epidermal micro-morphology in three varieties of hemp: a) stomatal index (SI); b) stomatal density (SD); c) stomatal pore length (SPL); d) stomatal size (SS); e) the proportion of the epidermis devoted to stomatal complexes ($S_{\%}$), and f) the proportion of the epidermis allotted to stomatal pore area and available for gas exchange at theoretical maximal stomatal opening ($A_{\%}$). Error bars indicate one standard error either side of the mean. Letters indicate homogenous groups for each species determined using a one-way ANOVA and LSD *post-hoc* test.

taken from 20 stomatal complexes for each leaf: guard cell length (GCL), guard cell width (GCW), stomatal pore length (SPL) (Supplementary Information Fig. 3). Stomatal pore width at full stomatal opening was considered to be 0.5 SPL, with stomatal pore area at maximum stomatal opening calculated as the area of an ellipse following Beerling and Chaloner (1993). Stomatal size was calculated as GCL * 2GCW (Franks and Beerling 2009). The proportion of the epidermis available for gas exchange at full stomatal opening ($A_{\%}$) was calculated as the percentage of the epidermis occupied by stomatal pore area at theoretical full stomatal opening. The proportion of the epidermis allotted to stomatal complexes ($S_{\%}$) was determined by calculating stomatal complex area as GCL * 2GCW following Franks and Beerling (2009).

2.4. Leaf macro-morphology and water potential

The effect of the irrigation treatments on leaf macro-morphology and economics was assessed by collecting 10 fully expanded leaves from the uppermost part of the canopy in the centre of each plot. These leaves were then digitally photographed and their area calculated using the ImageJ program (National Institutes of Health, Bethesda MD, USA). The leaves were sealed in paper envelopes and then dried in an oven for 5 days at 60 °C until their weight remained stable. The weight of the leaves was then recorded and the leaf mass per area (LMA) calculated (Poorter et al. 2009). Leaf water potential (Ψ_{leaf}) was measured at

midday on two fully expanded leaves per plot using a Scholander pressure chamber (PMS Instruments, Albany OR, USA) (Scholander et al. 1965). No visual signs of leaf wilting were observed during the measurements. The soil of the experimental field has a wilting point of 13.9 % (Parton et al. 1998), soil water content in the deficit irrigation plots did not reach this level throughout the study (data not shown).

3. Results

3.1. Leaf water potential and macro-morphology

Deficit irrigation of 50 % of the water volume received by well-watered controls reduced the midday leaf water potential (Ψ_{leaf}) of the three hemp varieties by 42.5 to 76.0 % (Fig. 1a). This reduction in water availability induced significant decreases in leaf area in the varieties Fibror 79 (73.6 %) and Fedora 17 (73.0 %) that had the largest leaf areas under well-watered control conditions. Earlina 8FC had the smallest leaf area under full irrigation. Water deficit did not significantly affect leaf area in the Earlina 8FC genotype (Fig. 1b) that had the smallest leaves. No significant difference in LMA was observed between the hemp varieties. Deficit irrigation did induce significant ~25 to 30 % increases in LMA from 65 to 70 to 80–85 g m⁻² (Fig. 1c).

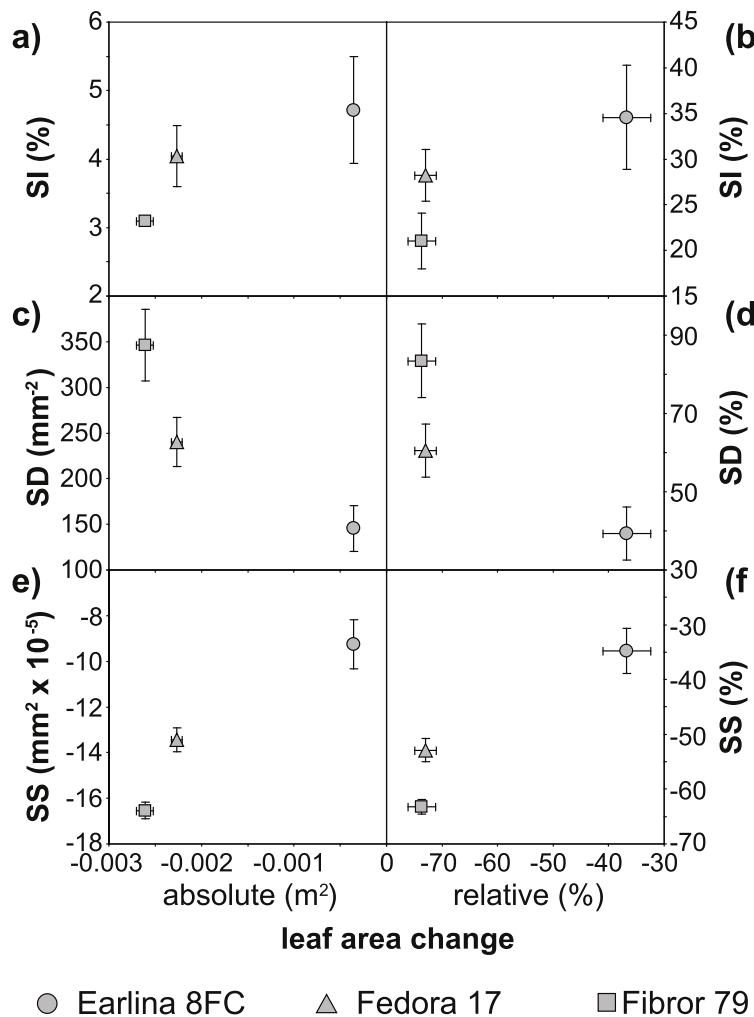


Fig. 3. The absolute and proportional effect of 50 % water deficit irrigation on stomatal morphological characters and leaf area of three varieties of hemp: Earlina 8FC (circle symbol); Fedora 17 (triangle symbol), and Fibror 79 (square symbol). The stomatal morphological characters assessed were: stomatal index (SI) (a, b); stomatal density (SD) (c, d) and stomatal size (SS) (e, f). Error bars indicate one standard error either side of the mean. Due to the low number of replicates, linear regression was not performed – any possible correlations should be considered preliminary.

3.2. Stomatal development

Rates of stomatal initiation were not significantly different between varieties under both full (13.6 to 14.7 %) and deficit irrigation (17.8 to 18.4 %), but SI values were 21.1 to 34.5 % higher under the water deficit treatment (Fig. 2a). Stomatal density, SPL and SS values showed no significant difference between the three hemp varieties under full irrigation. However, under water deficit irrigation, differences between the three varieties became apparent, with Earlina 8FC exhibiting the lowest relative change in SD (39.3 %), SPL (−11.9 %), and SS (34.8 %), while Fibror 79 showed the greatest proportional shift in SD (83.5 %), SPL (−21.4 %), and SS (63.2 %) (Fig. 2b–d). It is noteworthy that these changes in leaf micro-morphology under water deficit irrigation corresponded to varietal differences in the proportion of the epidermis devoted to stomatal complexes ($S_{\%}$) (Fig. 2e), but not the proportion of the epidermis available for gas exchange under theoretical maximum stomatal opening ($A_{\%}$) (Fig. 2f). Moreover, values of $A_{\%}$ were also unaffected by the irrigation treatment. It may be possible to infer that the changes in stomatal morphology associated with water deficit irrigation outlined in Fig. 2 correspond to changes in the leaf area of the hemp varieties. The largest absolute and proportional shifts in stomatal morphological parameters were observed in Fibror 79 (that had the largest leaf area under full irrigation) and smallest in Earlina 8FC (that had the smallest leaf area under well-watered control conditions - Fig. 3). As a note of caution, these potential relationships between leaf area and capacity to adjust stomatal morphology should be further evaluated, as given the number of varieties examined a robust linear regression to robustly test any putative relationships would not be feasible.

3.3. Leaf gas exchange and stomatal kinetics

Rates of photosynthesis were positively related to G_s in all three hemp varieties, with water deficit irrigation inducing reductions in P_N (60.4 to 77.4 %) and G_s (79.0 to 89.2 %) (Fig. 4a to 4c). Water deficit irrigation reduced the internal sub-stomatal $[CO_2]$ (C_i) of the hemp varieties by 11.6 to 21.9 % (Fig. 4d). The maximum rate of stomatal conductance decrease ($G_{s50\%}$) during a transition from saturating PPFD to darkness was not significantly different between the three hemp varieties under full irrigation. However, under water deficit irrigation, $G_{s50\%}$ in Fibror 79 was respectively 17.4 and 33.2 % greater than values observed in Earlina 8FC and Fedora 17 (Figs. 5a and 6). It is noteworthy that under water deficit the highest value of $G_{s50\%}$ was observed in Fibror 79, the species with the shortest SPL (Fig. 2c) and smallest SS (Fig. 2d). Alongside the faster rate of change of G_s values, the ΔG_s (indicative of ‘tightness’ of stomatal closure’) was greater in Fibror 79 (Fig. 5b). The rates of stomatal opening and closing during a simulated sunfleck did not differ significantly between the three varieties under full irrigation. Under deficit irrigation, Fibror 79 also exhibited faster

adjustment of G_s rates than Earlina 8FC and Fedora 17 to fluctuations of PPFD during a simulated sunfleck (Fig. 5b, and 5d). Rates of P_N associated with the transition from 2000 to 200 to 2000 $\mu mol\ m^{-2}\ s^{-1}$ during the simulated sunfleck are shown in Supplementary Information Fig. 3.

3.4. Potential coordination of stomatal morphology and physiology

The speed of G_s responses to a transition from light to dark (Fig. 6) or a simulated sunfleck (Fig. 7) did not correlate to SD, SPL, or the SS:SD ratio under well-watered conditions. The ΔG_s of the well-watered plants during the stomatal closure inducing transition from saturating PPFD to darkness was negatively associated with SD, and positively correlated to both SPL and the SS:SD ratio (Fig. 8). Under water deficit irrigation, these correlations between ΔG_s and stomatal morphological parameters were not present. The $G_{s50\%}$ of stomatal closing and opening during the simulated sunfleck did correlate to SD under water deficit irrigation (Fig. 9). No significant correlations were observed between $G_{s50\%}$ and stomatal morphology characters during the saturating PPFD to darkness stomatal kinetic.

4. Discussion

Stomatal control is central to plant adaptive capacity to reduced water availability and the effectiveness of deficit irrigation technologies (Dbara et al. 2016; Rodrigues et al. 2008). The three hemp varieties used in this study modified both stomatal physiology and morphology in response to a sustained period of water deficit (Figs. 8 and 9). It is noteworthy that under well-watered full irrigation, the three hemp varieties exhibited no significant differences in their stomatal morphological (Fig. 2) and physiological (Fig. 5) attributes, despite their diverse leaf macro-morphologies (Fig. 1b and 1c). Varietal differences in both stomatal morphology and physiology only became apparent under water deficit irrigation (Figs. 2 and 5). The variety with the largest leaf area (Fibror 79) showed the greatest modification in SD, SPL, and SS in response to deficit irrigation (Fig. 2). This coincided with the most rapid rate of G_s adjustment during stomatal kinetic analysis of the three hemp varieties under water deficit irrigation (Figs. 5–7). Fibror 79 also showed the highest values of ΔG_s under both irrigation treatments. This may suggest that Fibror 79 possessed the greatest plasticity, or adaptive capacity to water deficit stress, in the exertion of stomatal control under water deficit conditions.

Stomatal size, density, and initiation responses to water deficit vary dependent upon genotype alongside the type, severity, and duration of water deficit stress (eg. Gan et al. 2010, Song et al. 2023, Xu and Zhou 2008). The three hemp varieties did not show significant differences in increased SI under reduced water availability (Fig. 2a). This increase in stomatal initiation may be associated with signalling from mature to developing leaves of a reduction in $[CO_2]$ in the sub-stomatal air-space (C_i) (Lake et al. 2001). It is worth noting that the measurements of C_i

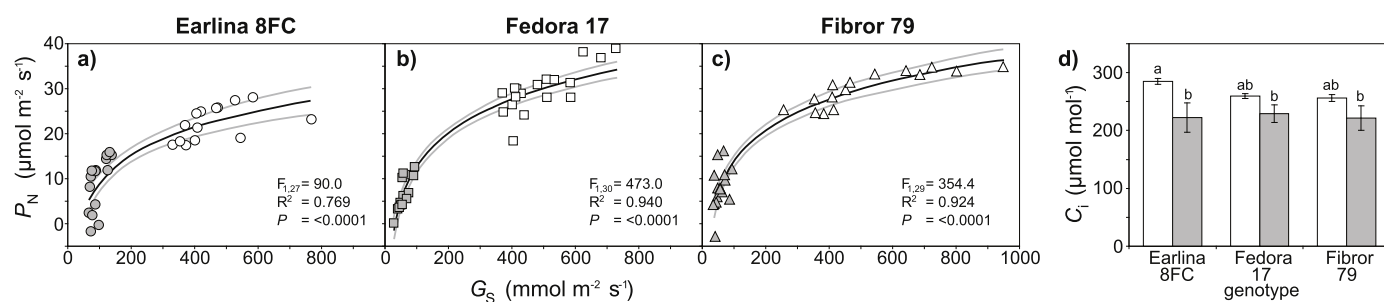


Fig. 4. The effect of 100 % well-watered control (white fill) and 50 % water deficit (grey fill) irrigation on leaf gas exchange parameters of three hemp varieties. The relationship between photosynthesis (P_N) and stomatal conductance (G_s) in: a) Earlina 8FC (circle symbol); b) Fedora 17 (square symbol), and c) Fibror 79 (triangle symbol). d) values of sub-stomatal $[CO_2]$ (C_i) - error bars indicate one standard error either side of the mean. Letters indicate homogenous groups for each species determined using a one-way ANOVA and LSD *post-hoc* test.

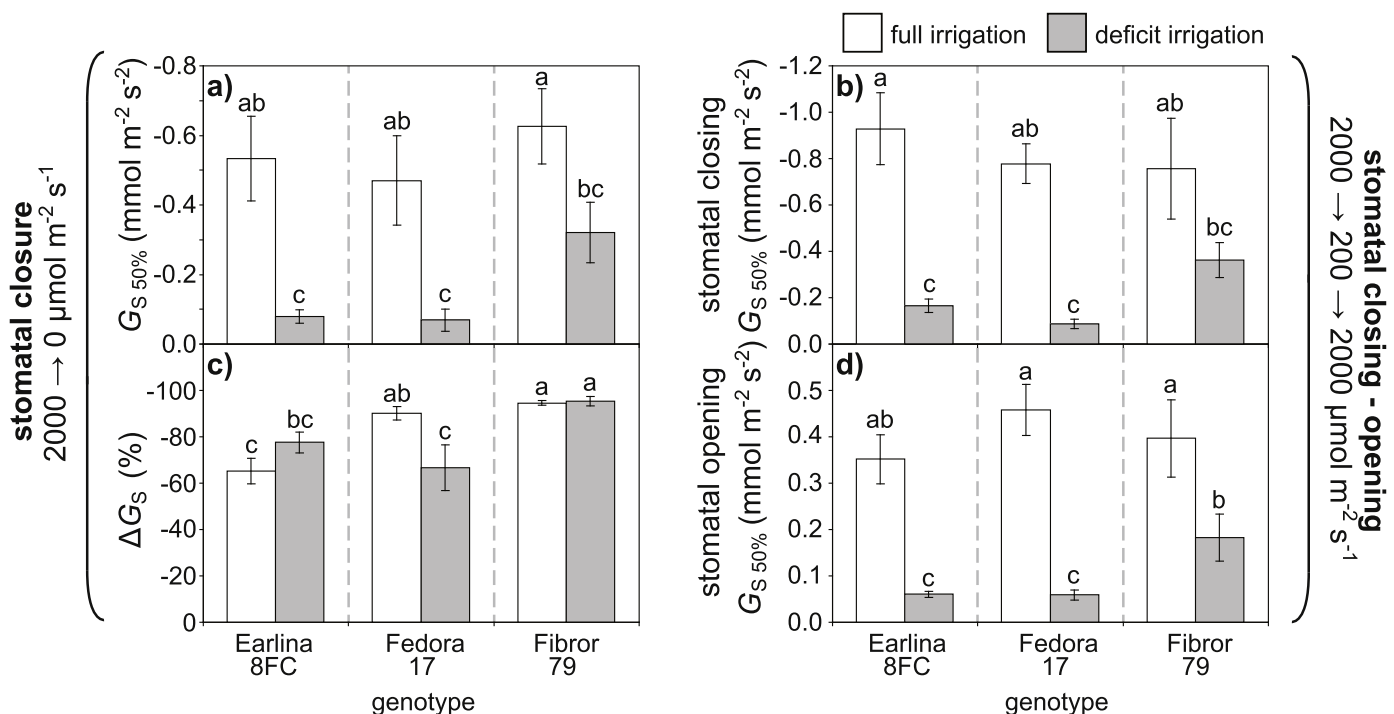


Fig. 5. The effect of 100 % well-watered control (white fill) and 50 % water deficit (grey fill) irrigation on the velocity of stomatal conductance adjustment ($G_{s50\%}$) during the stomatal kinetic measurements of the three hemp varieties: a) $G_{s50\%}$ during a transition from saturating PPFD of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ to darkness (Fig. 6) to stimulate stomatal closure; b) $G_{s50\%}$ during a PPFD transition from 2000 to $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 7) to stimulate stomatal closure; c) ΔG_s as a proxy for tightness of stomatal closure during a transition from saturating PPFD of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ to darkness, and d) $G_{s50\%}$ during a PPFD transition from 200 to $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 7) to stimulate stomatal opening. Error bars indicate one standard error either side of the mean. Letters indicate homogenous groups for each species determined using a one-way ANOVA and LSD *post-hoc* test.

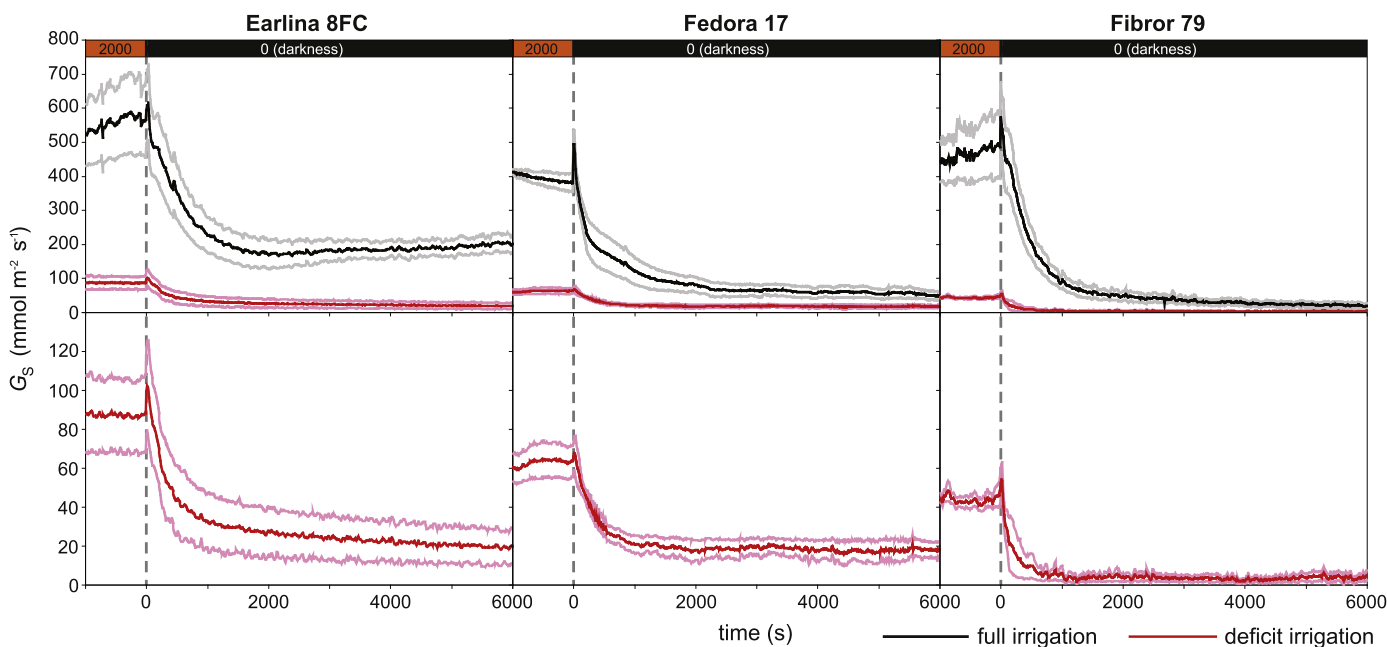


Fig. 6. Stomatal kinetic responses of the three varieties of hemp to a transition from saturating PPFD of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (orange bar) to darkness (black bar) to stimulate stomatal closure when grown under 100 % well-watered control (black line indicates the mean; grey lines either side of the mean indicate one standard error) and 50 % water deficit (red line indicates the mean; pink lines either side of the mean indicate one standard error) irrigation. Stomatal conductance values of plants receiving water deficit irrigation are also shown on a lower panel with a different scale y-axis to illustrate their response more clearly. The dashed vertical line at time 0 indicates the point at which PPFD inside the leaf cuvette was switched off. An illustration of the data points used to extract $G_{s50\%}$ data presented in Fig. 5 from these stomatal kinetics is shown in Supplementary Information Fig. 2.

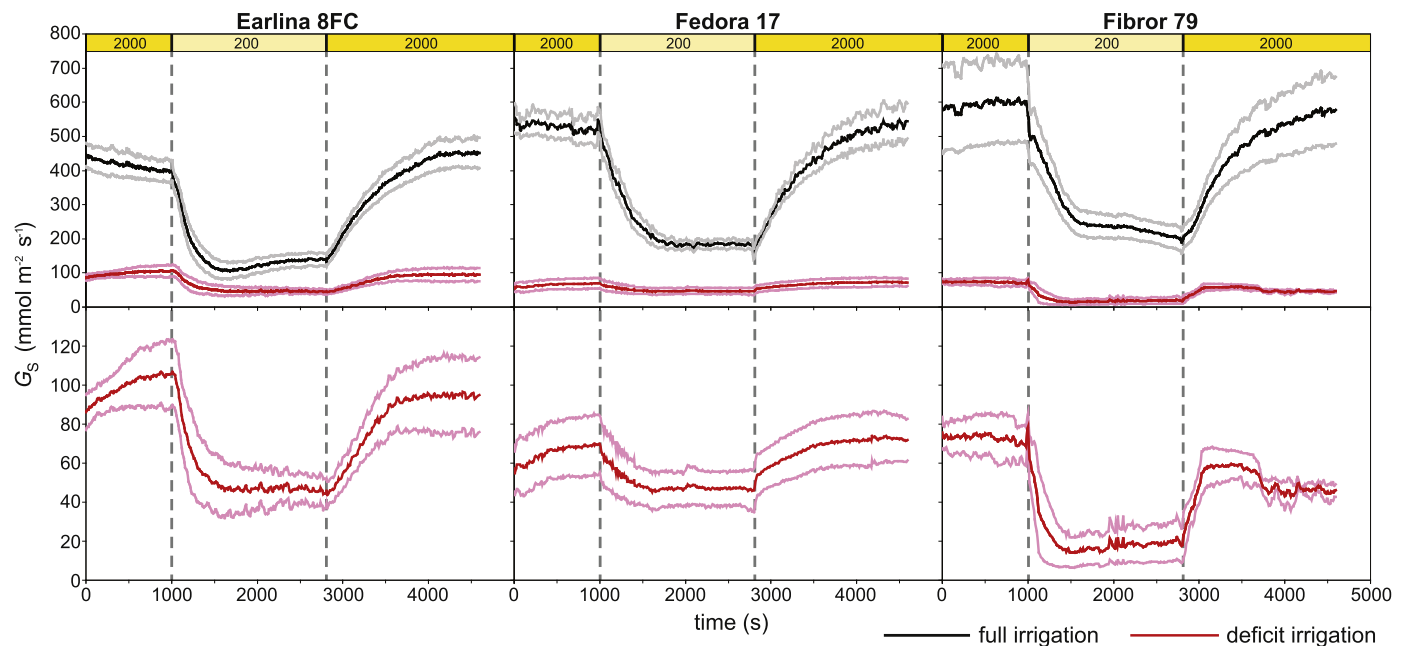


Fig. 7. Stomatal kinetic responses of the three varieties of hemp to PPFD transitions from 2000 to 200 to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to stimulate a stomatal closing and opening cycle during a simulated sunfleck. The point at which changes in PPFD occurred are marked by the colour changes in the bar at the top of the figure and vertical dashed lines. The hemp varieties were grown under 100 % well-watered control (black line indicates the mean; grey lines either side of the mean indicate one standard error) and 50 % water deficit (red line indicates the mean; pink lines either side of the mean indicate one standard error) irrigation.

presented in Fig. 4 were performed on leaves that had fully developed under the irrigation treatments, rather than mature leaves that had developed under full irrigation and then signalled to newly developing leaves any change in C_i as soil water availability declined. The increase in SD observed in the hemp leaves under water deficit conditions was also associated with a reduction in leaf expansion (Figs. 1 and 3). Arabidopsis varieties exposed to 'mild' water deficit did not adjust SI, but reduced cell expansion resulted in higher SD values (Clauw et al. 2015). In contrast, SI of sheepgrass (Xu and Zhou 2008) and broad bean (Gan et al. 2010) exhibited a step increase in SI as water deficit developed and then constant SI values at subsequent increasing levels of water deficit stress. The further increases in SD with water deficit were likely the result of reduced leaf expansion indicated by a positive relationship between LMA and SD (Xu and Zhou 2008). Water deficit negatively affects the rate of foliar cell expansion, resulting in lower leaf areas (Alves and Setter 2004, Granier and Tardieu 1999). The greatest increase in SD occurred in the Fibror 79 genotype that had the largest leaf area under well-watered full irrigation and greatest proportional decrease in leaf area under water deficit irrigation (Figs. 1 and 2). Under well-watered full irrigation conditions there was no difference in cell size between the three hemp varieties (Fig. 2a and 2d), indicating that allometric scaling between epidermal micro-morphology and leaf area did not occur (Grace et al. 2013). The genotype with the smallest leaf area, Earlina 8FC, had the lowest reduction in leaf expansion and smallest increase in SD of the hemp varieties. These patterns of higher SD also coincided with a reduction in SS under water deficit irrigation, with the greatest reduction in SS occurring in Fibror 79 (Fig. 2d). The negative relationship between SS and SD has been widely observed (Franks and Beerling 2009; Hetherington and Woodward 2003) and its functional significance debated (Haworth et al. 2023). The coordinated adjustment of SS and SD observed in the hemp varieties under water deficit may reflect spatial geometric constraints influencing stomatal spacing (eg. Croxdale 2000, Gan et al. 2010, Korn 1993), or serve an adaptive role in facilitating CO_2 -uptake (Assouline and Or 2013; Franks and Beerling 2009) and / or enabling more rapid adjustment of guard cell turgor to regulate stomatal pore area (Drake et al. 2013; Lawson and Blatt 2014; Raven 2014). The putative relationships between leaf area

and the capacity to adjust stomatal size and density observed in the hemp genotypes may reflect a greater genetic capacity to regulate stomatal development (eg. Dubey et al. 2023) in genotypes with larger leaves, or the greater proportional effect of water deficit on cell expansion on larger leaves. Water deficit does not affect the duration of leaf expansion, but does influence the rate and extent of cellular expansion during the period of leaf development (Alves and Setter 2004). In 20 poplar (*Populus nigra* L.) varieties collected from diverse habitats, the number of epidermal cells per leaf were correlated to leaf area (Allwright et al. 2016). If the number of cells scales to leaf area in hemp genotypes, this may account for the greater proportional reduction in leaf area observed under water deficit in Fibror 79 (Fig. 1b), but not the greater reduction in cell expansion evident in the smaller stomatal complexes of Fibror 79 (Fig. 2d), given that Ψ_{leaf} (and presumably any turgor related impact on cellular expansion Ali et al. 2023, Dupuy et al. 2010) was not significantly different under water deficit in the three varieties (Fig. 1a). Analysis of the genetic regulation of stomatal development and leaf expansion, and differential gene expression that may occur under water deficit conditions, may further elucidate the patterns in leaf area and stomatal morphology observed in this study.

Under well-watered full irrigation, the three hemp varieties exhibited statistically similar rates of stomatal opening and closing (Fig. 5) alongside statistically similar SD and SS (Fig. 2). Varietal differences in the speed of G_s adjustment only became apparent in leaves developed under water deficit irrigation. Fibror 79, that had the highest SD and smallest SS arrangement under water deficit, also exhibited the most rapid adjustment of G_s during the stomatal kinetic measurements (Fig. 5). This would be consistent with observations of more rapid G_s adjustment in phylogenetically closely related species with higher densities of smaller stomata (Drake et al. 2013). Due to the highly variable nature of stomatal physiological (Haworth et al. 2018b) and morphological (eg. Hu et al. 2019, Miller-Rushing et al. 2009) measurements, it is worth noting that significant correlations were only observed between SD and $G_{s50\%}$ for the stomatal kinetic replicating a sunfleck under water deficit irrigation (Fig. 9). The analysis of hemp varieties in this work precludes the drawing of firm conclusions regarding the functional significance of stomatal size to density scaling

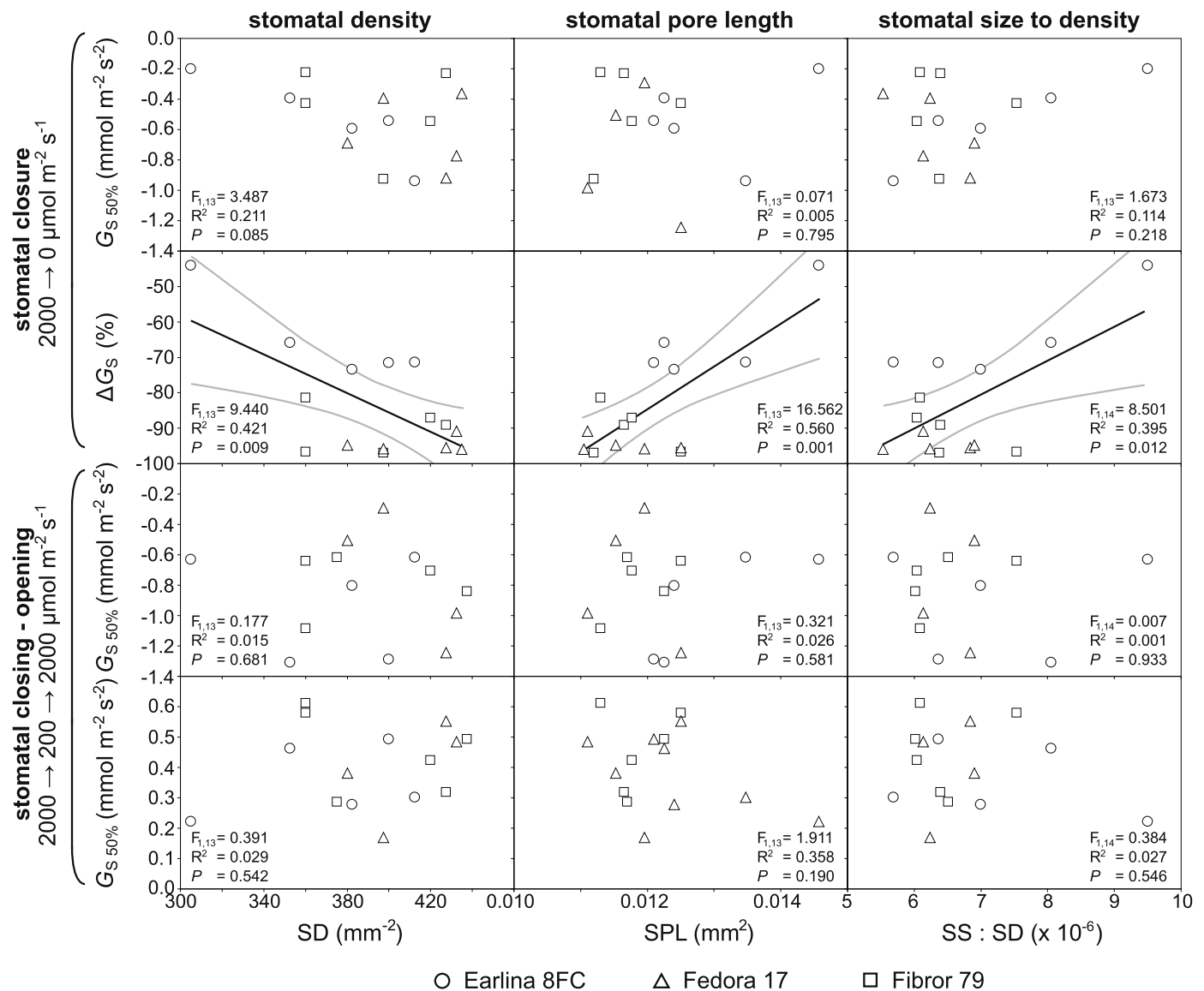


Fig. 8. examination of potential correlations between stomatal physiological responses (Fig. 6 and 7) and stomatal morphology (Fig. 2) of three hemp varieties grown under 100 % well-watered control irrigation. Symbols as in Fig. 3. The solid black line indicates the line of best-fit using linear regression. The two light grey lines either side represent the 95 % confidence intervals of the mean.

relationships, or the potential of higher densities of smaller stomata to serve an adaptive role in speedy adjustment of G_s to fluctuations in growth conditions across a wider range of plant species (Elliott-Kingston et al. 2016; Haworth et al. 2015). It remains unclear whether the increased rate of $G_{s50\%}$ observed in Fibror 79 under water deficit is associated with higher densities of smaller stomata (Drake et al. 2013; Raven 2014), or reflects genotypic differences in stomatal signalling (Franks 2013; Haworth et al. 2023) or biochemical properties of the guard cell plasma membrane (eg. Dietrich et al. 1998, Saito and Uozumi 2019).

The greater the decrease in leaf area of the three hemp varieties, the greater the increase in SD and reduction in SS (Fig. 3). This was reflected in significant reductions in the area of the epidermis allocated as stomatal complexes ($S_{\%}$) in Fedora 17 and Fibror 79 that had the largest leaf areas, but not the smaller leaved Earlina 8FC (Fig. 2). It is noteworthy that while water deficit generally reduced $S_{\%}$, the proportion of the epidermis available for gas exchange at theoretical maximal stomatal opening ($A_{\%}$) was unaffected by variety or irrigation level (Fig. 2f). This may suggest that $A_{\%}$, as a trait, is strongly conserved across the three varieties. The lack of any effect of water deficit on $A_{\%}$

may reflect the influence of selective pressures to ensure sufficient gas exchange potential if soil water availability becomes no longer limited (see Miyashita et al. 2005, and, Xu et al. 2010 for an analysis of photosynthetic recovery after water deficit stress) enabling recovery of photosynthetic function (Jahan et al. 2023). The conservation of $A_{\%}$ would not limit potential P_N from leaves formed under water deficit, although the available leaf area for photosynthetic light capture would be less (Alves and Setter 2004). More severe water deficit than that imposed in this study may have further affected stomatal initiation and leaf expansion potentially affecting $A_{\%}$, as evidenced by reductions in SD at severe levels of water deficit stress (eg. Dubey et al. 2023, Song et al. 2023, Tripathi et al. 2016, Xu and Zhou 2008).

5. Conclusion

Water deficit affected both the stomatal morphology and physiological behaviour of the three hemp varieties. An increase in stomatal initiation and reduced leaf expansion resulted in higher densities of smaller stomata in leaves developed under reduced soil water availability. This effect was most evident in the Fibror 79 genotype which had

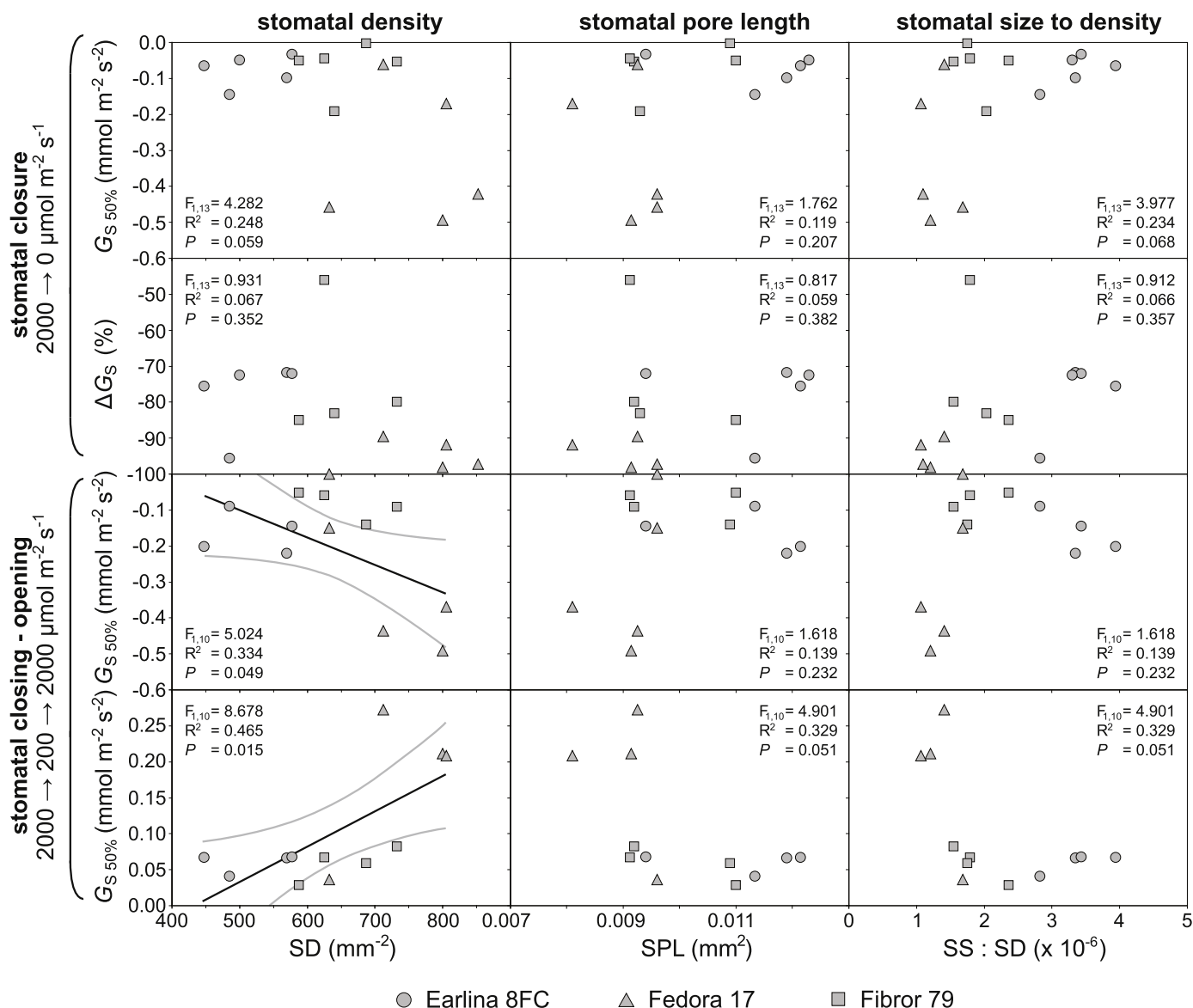


Fig. 9. Examination of potential correlations between stomatal physiological responses (Fig. 6 and 7) and stomatal morphology (Fig. 2) of three hemp varieties grown under 50 % water deficit irrigation. Symbols as in Fig. 3. The solid black line indicates the line of best-fit using linear regression. The two light grey lines either side represent the 95 % confidence intervals of the mean.

the largest leaf area under well-watered full irrigation, and the greatest reduction in leaf area resulting in the highest SD and lowest SS values. This combination of high SD and low SS in leaves of Fibror 79 developed under water deficit irrigation coincided with the most rapid rates of G_s adjustment to fluctuations in PPFD during the stomatal kinetic measurements. This result may be indicative of a selective advantage of high densities of small stomata under conditions of limited water availability in the capacity to optimise photosynthetic gas exchange and WUE to fluctuations in growth conditions. The Fibror 79 variety possessed a greater capacity to adjust stomatal morphology and physiological behaviour to water availability. We are performing analysis of differential gene expression across the hemp genotypes with a wide range of leaf shapes and areas to further elucidate whether a higher number of epidermal cells associated with larger leaf areas, enabling greater plasticity in stomatal size and spacing, may contribute to the adaptive capacity to optimise stomatal control in environments constrained by water availability. The interaction between leaf expansion and stomatal size to density relationships should be considered in phenotyping efforts to enhance crop water use efficiency through improved stomatal

control.

CRediT authorship contribution statement

Matthew Haworth: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Andrea Carli:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Vincenzo Montesano:** Writing – review & editing, Investigation, Data curation. **Dilek Killi:** Writing – review & editing, Data curation. **André Fabbri:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Raffaella Balestrini:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Giovanni Marino:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Mauro Centritto:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully acknowledge funding from: ENI-CNR joint Research Center “Water - Hypatia of Alexandria” (Metaponto, Italy); MUR-PRIN2020 (EvoPlant: The biochemical and diffusive optimisation of photosynthesis: evolutionary implications for the development of climate resilient productive plants); European Union Next-GenerationEU (Piano Nazionale di Ripresa e Resilienza - PNRR) projects Agritech National Research Centre - Spoke 4 Multifunctional and resilient agriculture and forestry systems for the mitigation of climate change risks (Missione 4 Componente 2, Investimento 1.4 (D.D. 1032 17/06/2022, CN00000022) and Italian Integrated Environmental Research Infrastructures System (Missione 4, Componente 2, investimento 3.1- D.D. 130 21 June 2022, IR0000032).. This manuscript reflects only the authors’ views and opinions, neither the European Union nor the European Commission can be considered responsible. The comments of two anonymous referees significantly improved this manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.stress.2024.100649](https://doi.org/10.1016/j.stress.2024.100649).

Data availability

Data will be made available on request.

References

- Ali, O., Cheddadi, I., Landrein, B., Long, Y., 2023. Revisiting the relationship between turgor pressure and plant cell growth. *New Phytol.* 238, 62–69.
- Allen, R.G., Pereira, L.S., Raes, D., Smith, M., 1998. Crop evapotranspiration-Guidelines for computing crop water requirements-FAO Irrigation and drainage paper 56. FAO, Rome 300, D05109.
- Allwright, M.R., Payne, A., Emiliani, G., Milner, S., Viger, M., Rouse, F., Keurentjes, J.J., Bérard, A., Wildhagen, H., Faivre-Rampant, P., Polle, A., Morgante, M., Taylor, G., 2016. Biomass traits and candidate genes for bioenergy revealed through association genetics in coppiced European *Populus nigra* (L.). *Biotechnol. Biofuels* 9, 195.
- Alves, A.A.C., Setter, T.L., 2004. Response of cassava leaf area expansion to water deficit: cell proliferation, cell expansion and delayed development. *Ann. Bot.* 94, 605–613.
- Anderson, L.C., 1980. Leaf variation among cannabis species from a controlled garden. *Bot Mus Leaf Harv Univ* 28, 61–69.
- Assouline, S., Or, D., 2013. Plant water use efficiency over geological time – evolution of leaf stomata configurations affecting plant gas exchange. *PLoS One* 8, e67757.
- Beerling, D.J., Chaloner, W.G., 1993. Evolutionary responses of stomatal density to global CO₂ change. *Biol. J. Linn. Soc.* 48, 343–353.
- Bertolino, L.T., Caine, R.S., Gray, J.E., 2019. Impact of stomatal density and morphology on water-use efficiency in a changing world. *Front. Plant Sci.* 10, 225–225.
- Brunetti, C., Gori, A., Marino, G., Latini, P., Sobolev, A.P., Nardini, A., Haworth, M., Giovannelli, A., Capitani, D., Loreto, F., Taylor, G., Mugnozza, G.S., Harfouche, A., Centritto, M., 2019. Dynamic changes of ABA content in water-stressed *Populus nigra*: effects on carbon fixation and soluble carbohydrates. *Ann. Bot.* 124, 627–643.
- Cai, S., Chen, G., Wang, Y., Huang, Y., Marchant, D.B., Wang, Y., Yang, Q., Dai, F., Hills, A., Franks, P.J., Nevo, E., Soltis, D.E., Soltis, P.S., Sessa, E., Wolf, P.G., Xue, D., Zhang, G., Pogson, B.J., Blatt, M.R., Chen, Z.-H., 2017. Evolutionary conservation of ABA signaling for stomatal closure. *Plant Physiol.* 174, 732–747.
- Centritto, M., Brillì, F., Fodale, R., Loreto, F., 2011. Different sensitivity of isoprene emission, respiration and photosynthesis to high growth temperature coupled with drought stress in black poplar (*Populus nigra*) saplings. *Tree Physiol.* 31, 275–286.
- Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K., Storme, V., Clement, L., Gonzalez, N., Inzé, D., 2015. Leaf responses to mild drought stress in natural varieties of *Arabidopsis*. *Plant Physiol.* 167, 800–816.
- Costa, J.M., Marques da Silva, J., Pinheiro, C., Barón, M., Mylona, P., Centritto, M., Haworth, M., Loreto, F., Uzilday, B., Turkan, I., Oliveira, M.M., 2019. Opportunities and limitations of crop phenotyping in southern European countries. *Front. Plant Sci.* 10, 1125.
- Cowan, I.R., 1978. Stomatal behaviour and environment. In: Preston, R.D., Woolhouse, H.W. (Eds.), *Advances in Botanical Research*. Academic Press, pp. 117–228 eds.
- Croxdale, J.L., 2000. Stomatal patterning in angiosperms. *Am. J. Bot.* 87, 1069–1080.
- Dbara, S., Haworth, M., Emiliani, G., Mimoun, M.B., Gómez-Cadenas, A., Centritto, M., 2016. Partial root-zone drying of olive (*Olea europaea* var. ‘Chetoui’) induces reduced yield under field conditions. *PLoS One* 11, e0157089.
- Dietrich, P., Dreyer, I., Wiesner, P., Hedrich, R., 1998. Cation sensitivity and kinetics of guard-cell potassium channels differ among species. *Planta* 205, 277–287.
- Drake, P.L., Froend, R.H., Franks, P.J., 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *J. Exp. Bot.* 64, 495–505.
- Driesen, E., De Proft, M., Saeys, W., 2023. Drought stress triggers alterations of adaxial and abaxial stomatal development in basil leaves increasing water-use efficiency. *Hortic Res* 10, uhad075.
- Dubey, R., Pandey, B.K., Sawant, S.V., Shirke, P.A., 2023. Drought stress inhibits stomatal development to improve water use efficiency in cotton. *Acta Physiologica Plantarum* 45, 30.
- Dupuy, L., Mackenzie, J., Haseloff, J., 2010. Coordination of plant cell division and expansion in a simple morphogenetic system. *Proc. Natl. Acad. Sci.* 107, 2711–2716.
- Durand, M., Brendel, O., Buré, C., Le Thiec, D., 2019. Altered stomatal dynamics induced by changes in irradiance and vapour-pressure deficit under drought: impacts on the whole-plant transpiration efficiency of poplar genotypes. *New Phytol.* 222, 1789–1802.
- Eisele, J.F., Fäßler, F., Bürgel, P.F., Chaban, C., 2016. A rapid and simple method for microscopy-based stomata analyses. *PLoS One* 11, e0164576.
- Elliott-Kingston, C., Haworth, M., Yearsley, J.M., Batke, S.P., Lawson, T., McElwain, J.C., 2016. Does size matter? Atmospheric CO₂ may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO₂. *Front. Plant Sci.* 7, 1253.
- Flexas, J., Briantais, J.-M., Cerovic, Z., Medrano, H., Moya, I., 2000. Steady-state and maximum chlorophyll fluorescence responses to water stress in grapevine leaves: a new remote sensing system. *Remote Sens. Environ.* 73, 283–297.
- Franks, P.J., 2013. Passive and active stomatal control: either or both? *New Phytol.* 198, 325–327.
- Franks, P.J., Beerling, D.J., 2009. Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *Proc. Nat. Acad. Sci. U.S.A.* 106, 10343–10347.
- Gan, Y., Zhou, L., Shen, Z.-J., Shen, Z.-X., Zhang, Y.-Q., Wang, G.-X., 2010. Stomatal clustering, a new marker for environmental perception and adaptation in terrestrial plants. *Botanical Studies* 51.
- Gerardin, T., Douthe, C., Flexas, J., Brendel, O., 2018. Shade and drought growth conditions strongly impact dynamic responses of stomata to variations in irradiance in *Nicotiana tabacum*. *Environ. Exp. Bot.* 153, 188–197.
- Gill, A.R., Loveys, B.R., Cowley, J.M., Hall, T., Cavagnaro, T.R., Burton, R.A., 2022. Physiological and morphological responses of industrial hemp (*Cannabis sativa* L.) to water deficit. *Ind. Crops Prod.* 187, 115331.
- Grace, J.P., Scoffoni, C., Sack, L., 2013. Allometry of cells and tissues within leaves. *Am. J. Bot.* 100, 1936–1948.
- Granier, C., Tardieu, F., 1999. Water deficit and spatial pattern of leaf development. Variability in responses can be simulated using a simple model of leaf development. *Plant Physiol.* 119, 609–620.
- Grantz, D.A., Linscheid, B.S., Grulke, N.E., 2019. Differential responses of stomatal kinetics and steady-state conductance to abscisic acid in a fern: comparison with a gymnosperm and an angiosperm. *New Phytol.* 222, 1883–1892.
- Grierson, C.S., Barnes, S.R., Chase, M.W., Clarke, M., Grierson, D., Edwards, K.J., Jellis, G.J., Jones, J.D., Knapp, S., Oldroyd, G., Poppy, G., Temple, P., Williams, R., Bastow, R., 2011. One hundred important questions facing plant science research. *New Phytol.* 192, 6–12.
- Hamanishi, E.T., Thomas, B.R., Campbell, M.M., 2012. Drought induces alterations in the stomatal development program in *Populus*. *J. Exp. Bot.* 63, 4959–4971.
- Haworth, M., Cosentino, S.L., Marino, G., Brunetti, C., Riggi, E., Avola, G., Loreto, F., Centritto, M., 2018a. Increased free abscisic acid during drought enhances stomatal sensitivity and modifies stomatal behaviour in fast growing giant reed (*Arundo donax* L.). *Environ. Exp. Bot.* 147, 116–124.
- Haworth, M., Killi, D., Materassi, A., Raschi, A., 2015. Co-ordination of stomatal physiological behavior and morphology with carbon dioxide determines stomatal control. *Am. J. Bot.* 102, 677–688.
- Haworth, M., Marino, G., Centritto, M., 2018b. An introductory guide to gas exchange analysis of photosynthesis and its application to plant phenotyping and precision irrigation to enhance water use efficiency. *Journal of Water and Climate Change* 9, 786–808.
- Haworth, M., Marino, G., Loreto, F., Centritto, M., 2021. Integrating stomatal physiology and morphology: evolution of stomatal control and development of future crops. *Oecologia* 197, 867–883.
- Haworth, M., Marino, G., Materassi, A., Raschi, A., Scutt, C.P., Centritto, M., 2023. The functional significance of the stomatal size to density relationship: interaction with atmospheric [CO₂] and role in plant physiological behaviour. *Sci. Total Environ.* 863, 160908.
- Haworth, M., Scutt, C.P., Douthe, C., Marino, G., Gomes, M.T.G., Loreto, F., Flexas, J., Centritto, M., 2018c. Allocation of the epidermis to stomata relates to stomatal physiological control: stomatal factors involved in the diversification of the angiosperms and development of amphistomaty. *Environ. Exp. Bot.* 151, 55–63.
- Hetherington, A.M., Woodward, F.I., 2003. The role of stomata in sensing and driving environmental change. *Nature* 424, 901–908.
- Hoerling, M., Eischeid, J., Perlwitz, J., Quan, X., Zhang, T., Pegion, P., 2012. On the increased frequency of Mediterranean drought. *J. Clim.* 25, 2146–2161.

- Hu, J.-J., Xing, Y.-W., Su, T., Huang, Y.-J., Zhou, Z.-K., 2019. Stomatal frequency of *Quercus glauca* from three material sources shows the same inverse response to atmospheric $p\text{CO}_2$. *Ann. Bot.* 123, 1147–1158.
- Jahan, E., Sharwood, R.E., Tissue, D.T., 2023. Effects of leaf age during drought and recovery on photosynthesis, mesophyll conductance and leaf anatomy in wheat leaves. *Front. Plant Sci.* 14.
- Killi, D., Bussotti, F., Raschi, A., Haworth, M., 2017. 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. *Physiol. Plant.* 159, 130–147.
- Killi, D., Raschi, A., Bussotti, F., 2020. 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. *Int. J. Mol. Sci.* 21, 4846.
- Korn, R.W., 1993. Evidence in dicots for stomatal patterning by inhibition. *Int. J. Plant Sci.* 154, 367–377.
- Körner, C., 1988. Does global increase of CO_2 alter stomatal density? *Flora* 181, 253–257.
- Lake, J.A., Quick, W.P., Beerling, D.J., Woodward, F.I., 2001. Plant development: signals from mature to new leaves. *Nature* 411, 154–154.
- Lake, J.A., Woodward, F.I., 2008. Response of stomatal numbers to CO_2 and humidity: control by transpiration rate and abscisic acid. *New Phytol.* 179, 397–404.
- Lauteri, M., Haworth, M., Serraj, R., Monteveddi, M.C., Centritto, M., 2014. Photosynthetic diffusional constraints affect yield in drought stressed rice cultivars during flowering. *PLoS One* 9, e109054.
- Lawson, T., Blatt, M.R., 2014. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiol.* 164, 1556–1570.
- Lü, P., Kang, M., Jiang, X., Dai, F., Gao, J., Zhang, C., 2013. RhEXPA4, a rose expansin gene, modulates leaf growth and confers drought and salt tolerance to *Arabidopsis*. *Planta* 237, 1547–1559.
- Martin-StPaul, N., Delzon, S., Cochard, H., 2017. Plant resistance to drought depends on timely stomatal closure. *Ecol. Lett.* 20, 1437–1447.
- Miller-Rushing, A.J., Primack, R.B., Templer, P.H., Rathbone, S., Mukunda, S., 2009. Long-term relationships among atmospheric CO_2 , stomata, and intrinsic water use efficiency in individual trees. *Am. J. Bot.* 96, 1779–1786.
- Miyashita, K., Tanakamaru, S., Maitani, T., Kimura, K., 2005. Recovery responses of photosynthesis, transpiration, and stomatal conductance in kidney bean following drought stress. *Environ. Exp. Bot.* 53, 205–214.
- Parton, W.J., Hartman, M., Ojima, D., Schimel, D., 1998. DAYCENT and its land surface submodel: description and testing. *Global Planet. Change* 19, 35–48.
- Pinheiro, C., Chaves, M.M., 2011. Photosynthesis and drought: can we make metabolic connections from available data? *J. Exp. Bot.* 62, 869–882.
- Poole, I., Kürschner, W.M., 1999. Stomatal density and index: the practise. In: TP, Jones, Rowe, NP (Eds.), *Fossil Plants and Spores: Modern Techniques*. Geological Society, London, pp. 257–260 eds.
- Poorter, H., Ü, Niinemets, Poorter, I., Wright, I.J., Villar, R., 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytol.* 182, 565–588.
- Raven, J.A., 2014. Speedy small stomata? *J. Exp. Bot.* 65, 1415–1424.
- Rodrigues, M.L., Santos, T.P., Rodrigues, A.P., de Souza, C.R., Lopes, C.M., Maroco, J.P., Pereira, J.S., Chaves, M.M., 2008. Hydraulic and chemical signalling in the regulation of stomatal conductance and plant water use in field grapevines growing under deficit irrigation. *Funct. Plant Biol.* 35, 565–579.
- Rodriguez-Dominguez, C.M., Buckley, T.N., Egea, G., de Cires, A., Hernandez-Santana, V., Martorell, S., Diaz-Espejo, A., 2016. Most stomatal closure in woody species under moderate drought can be explained by stomatal responses to leaf turgor. *Plant Cell and Environment* 39, 2014–2026.
- Saito, S., Uozumi, N., 2019. Guard cell membrane anion transport systems and their regulatory components: an elaborate mechanism controlling stress-induced stomatal closure. *Plants* pp.
- Scholander, P.F., Bradstreet, E.D., Hemmingsen, E., Hammel, H., 1965. Sap pressure in vascular plants: negative hydrostatic pressure can be measured in plants. *Science* 148, 339–346.
- Sillo, F., Marino, G., Franchi, E., Haworth, M., Zampieri, E., Pietrini, I., Fusini, D., Mennone, C., Centritto, M., Balestrini, R., 2022. Impact of irrigation water deficit on two tomato genotypes grown under open field conditions: from the root-associated microbiota to the stress responses. *Italian Journal of Agronomy* 17.
- Sinclair, T., Ludlow, M., 1986. Influence of soil water supply on the plant water balance of four tropical grain legumes. *Funct. Plant Biol.* 13, 329–341.
- Song, Z., Wang, L., Lee, M., Yue, G.H., 2023. The evolution and expression of stomatal regulators in C3 and C4 crops: implications on the divergent drought tolerance. *Front. Plant Sci.* 14.
- Stein, R.A., Sheldon, N.D., Smith, S.Y., 2024. Comparing methodologies for stomatal analyses in the context of elevated modern CO_2 . *Life* 14, 78.
- Tardieu, F., Davies, W.J., 1992. Stomatal response to abscisic acid is a function of current plant water status. *Plant Physiol.* 98, 540–545.
- Tombesi, S., Nardini, A., Frioni, T., Soccolini, M., Zadra, C., Farinelli, D., Poni, S., Palliotti, A., 2015. Stomatal closure is induced by hydraulic signals and maintained by ABA in drought-stressed grapevine. *Sci. Rep.* 5, 12449.
- Treydte, K., Liu, L., Padrón, R.S., Martínez-Sancho, E., Babst, F., Frank, D.C., Gessler, A., Kahmen, A., Poulter, B., Seneviratne, S.I., 2024. Recent human-induced atmospheric drying across Europe unprecedented in the last 400 years. *Nat. Geosci.* 17, 58–65.
- Tripathi, P., Rabara, R.C., Reese, R.N., Miller, M.A., Rohila, J.S., Subramanian, S., Shen, Q.J., Morandi, D., Bücking, H., Shulaeva, V., 2016. A toolbox of genes, proteins, metabolites and promoters for improving drought tolerance in soybean includes the metabolite coumestrol and stomatal development genes. *Bmc Genomics [Electronic Resource]* 17, 1–22.
- Weyers, J.D.B., Lawson, L.G., 1985. Accurate estimation of stomatal aperture from silicone rubber impressions. *New Phytol.* 101, 109–115.
- Wise, K., Baziotopoulos, E., Zhang, C., Leaming, M., Shen, L-H, Selby-Pham, J., 2023. Comparative study of water requirements and water footprints of fibre crops hemp (*Cannabis sativa*) and cotton (*Gossypium hirsutum* L.). *Journal of Agrometeorology* 25, 392–396.
- Xiong, D., Flexas, J., 2020. From one side to two sides: the effects of stomatal distribution on photosynthesis. *New Phytol.* 228, 1754–1766.
- Xu, Z., Zhou, G., 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *J. Exp. Bot.* 59, 3317–3325.
- Xu, Z., Zhou, G., Shimizu, H., 2010. Plant responses to drought and rewetting. *Plant Signaling and Behaviour* 5, 649–654.
- Yadollahi, A., Arzani, K., Ebadi, A., Wirthensohn, M., Karimi, S., 2011. The response of different almond genotypes to moderate and severe water stress in order to screen for drought tolerance. *Sci. Hortic.* 129, 403–413.
- Yang, H.-M., Wang, G.-X., 2001. Leaf stomatal densities and distribution in *Triticum aestivum* under drought and CO_2 enrichment. *Chinese Journal of Plant Ecology* 25, 312.
- Zhao, W., Sun, Y., Kjelgren, R., Liu, X., 2014. Response of stomatal density and bound gas exchange in leaves of maize to soil water deficit. *Acta Physiologiae Plantarum* 37, 1704.
- Zhou, S., Han, Y-y, Chen, Y., Kong, X., Wang, W., 2015. The involvement of expansins in response to water stress during leaf development in wheat. *J. Plant Physiol.* 183, 64–74.