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Different sensitivity of isoprene emission, respiration and photosynthesis to high growth temperature coupled with drought stress in black poplar (*Populus nigra*) saplings

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The effects of the interaction between high growth temperatures and water stress on gas-exchange properties of *Populus nigra* saplings were investigated. Water stress was expressed as a function of soil water content (SWC) or fraction of transpirable soil water (FTSW). Isoprene emission and photosynthesis (A) did not acclimate in response to elevated temperature, whereas dark (R_n) and light (R_d) respiration underwent thermal acclimation. R_d was ~30% lower than R_n irrespective of growth temperature and water stress level. Water stress induced a sharp decline, but not a complete inhibition, of both R_n and R_d . There was no significant effect of high growth temperature on the responses of A , stomatal conductance (g_s), isoprene emission, R_n or R_d to FTSW. High growth temperature resulted in a significant increase in the SWC endpoint. Photosynthesis was limited mainly by CO_2 acquisition in water-stressed plants. Impaired carbon metabolism became apparent only at the FTSW endpoint. Photosynthesis was restored in about a week following rewatering, indicating transient biochemical limitations. The kinetics of isoprene emission in response to FTSW confirmed that water stress uncouples the emission of isoprene from A , isoprene emission being unaffected by decreasing g_s . The different kinetics of A , respiration and isoprene emission in response to the interaction between high temperature and water stress led to rising R_d/A ratio and amount of carbon lost as isoprene. Since respiration and isoprene sensitivity are much lower than A sensitivity to water stress, temperature interactions with water stress may dominate poplar acclimatory capability and maintenance of carbon homeostasis under climate change scenarios. Furthermore, predicted temperature increases in arid environments may reduce the amount of soil water that can be extracted before plant gas exchange decreases, exacerbating the effects of water stress even if soil water availability is not directly affected.

Keywords: carbon balance, dark and light respiration, drought kinetics, isoprene, photosynthesis capacity, thermal acclimation, water stress.

Introduction

The Mediterranean basin has been identified as one of the most prominent hot spots of climate change in the world, where general circulation models predict a significant warming and decrease in precipitation (Giorgi and Lionello 2008). These global change constituents are likely to have both direct and indirect implications on photosynthesis, photorespiration and

respiration, i.e., the three main processes regulating the carbon balance of an ecosystem (Mahecha et al. 2010), as well as on the carbon emitted by many plant species in the form of isoprene and other biogenic volatile organic compounds (Guenther et al. 1993). These primary and secondary metabolisms are now accepted as important components of the biosphere's response to climate change (Way and Oren 2010, Centritto

et al. 2011) and, consequently, there is great interest in determining their sensitivity to the interaction between rising temperature and drought. This knowledge is particularly relevant for isoprene formation, which, besides having an intriguing ecological role in emitting plants (Sharkey et al. 2008, Vickers et al. 2009), is very reactive and influences both atmospheric chemistry composition and physics (Chameides et al. 1988, Kiendler-Scharr et al. 2009). Consequently, any change in the potential emission of isoprene will affect air chemistry and quality at regional and global level.

Isoprene formation occurs in the chloroplast and is closely connected to photosynthesis in non-stressed plants, because ~72–91% of the carbon in the isoprene molecule originates from fresh photosynthates, which are then the primary substrate for isoprene biosynthesis (Sharkey and Yeh 2001, Brilli et al. 2007). However, the early literature shows that isoprene formation and photosynthesis are uncoupled under drought because isoprene is resistant to this stress (Tingey et al. 1981, Sharkey and Loreto 1993, Brilli et al. 2007). However, the impact of drought is generally aggravated when interacting with rising temperature. Isoprene emission responds positively to a rapid increase in temperature because the Q_{10} of isoprene synthase (ISPS) ranges between 2 and 4 within the temperature range 20–45 °C (Monson et al. 1992, Sharkey and Yeh 2001). Although Fortunati et al. (2008) showed that elevated temperatures did not offset the decline of isoprene emission in water-stressed plants, the acclimatory response kinetics of isoprene emission to the interaction between rising temperature and water stress is unclear.

Short-term increase in temperature within the functional range affects C3 photosynthesis (A) directly either by modifying the ratio between substrates (CO_2 and O_2) at Rubisco sites or by changing the catalytic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) with respect to its substrates, leading to an increase in photorespiration (Laisk 1977, Jordan and Ogren 1984). In general, in C3 plants from equitable habitats, 25 °C represents the typical optimum temperature for A (Berry and Björkman 1980). Thus, the effects of short-term rising temperatures on A depend on whether they increase beyond the A optimal temperature. However, acclimation to high growth temperatures that do not harm the photosynthetic apparatus, can cause an upward shift in the thermal optimum and, consequently, higher A at the new growth temperature (Berry and Björkman 1980). The degree of the acclimatory responses of the photosynthetic process to elevated temperatures is species specific, and may be linked to concomitant changes in CO_2 transport limitations (Fares et al. 2011) and, consequently, may be further affected by environmental stress.

Generally, the first event characterizing plant response to water shortage is progressive stomatal closure that directly determines higher restriction on CO_2 transport from the

atmosphere to the chloroplasts (Cornic 2000, Evans et al. 2009). As a consequence, during the first stages of drought stress, photosynthesis is primarily down-regulated by the lower concentration of CO_2 reaching the active carboxylation sites of Rubisco (Lawlor and Cornic 2002, Centritto et al. 2003, Aganchich et al. 2009, Evans et al. 2009). The importance of diffusive and metabolic limitations to the photosynthetic process during further steps of drought stress development is still under debate (Cornic 2000, Centritto et al. 2003, Evans et al. 2009).

Although the dominant source of CO_2 released by plants into the atmosphere is represented by respiration (Mahecha et al. 2010), studies examining the combination of drought and heat stress effects on this fundamental biochemical process are scarcer than those analyzing photosynthesis (Rennenberg et al. 2006). Progressive drought usually reduces the rate of leaf respiration to a lesser extent than photosynthesis, determining an increase in respiration to photosynthesis ratio (Lawlor and Cornic 2002). In contrast, mitochondrial respiration has been shown to be very sensitive to short-term temperature variation, increasing exponentially with temperature rising to 35–40 °C (Atkin et al. 2005, Fares et al. 2011). Nevertheless, recent studies confirmed that respiration undergoes thermal acclimation when long-term changes in growth temperature occur (Atkin et al. 2005, Ow et al. 2008a, 2008b, Way and Oren 2010).

Here we investigated, in isoprene-emitting *Populus nigra* plants of similar size, the impact of two different growing temperatures in combination with progressively limiting soil water content (SWC). High temperature may result in a shift in the threshold SWC at which carbon metabolism decreases, as compared with the threshold obtained under lower growth temperature, without altering the inherent plant response to equivalent levels of fraction of transpirable soil water (FTSW). This experiment was meant to (i) test the physiological acclimatory responses to elevated temperature; (ii) assess the kinetics of drought-induced variations in dark and light respiration, photosynthesis and isoprene emission when expressed as a function of FTSW or SWC; (iii) identify specific thresholds of SWC at which respiration, photosynthesis and isoprene emission were impaired; and (iv) estimate the relevance of isoprene emission rate to the total amount of carbon assimilated through photosynthesis when drought stress is combined with the occurrence of high temperature.

Materials and methods

Growth conditions and experimental design

Populus nigra L. saplings were cut during wintertime from the same clone CF40 in a provenance trial located in Monterotondo Scalo, Italy (42°04'N; 12°36'E), potted in 3-dm³ pots containing commercial soil and placed in the dark. When all the

collected plant material had rooted, it was transplanted to 6-dm³ pots filled with a mixture of 50% commercial soil and 50% sand and then moved to two Fitotron® growth chambers (Sanyo Gallenkamp, Loughborough, UK) under the same growing conditions of daylight (900 μmol m⁻² s⁻¹ for 12 h day⁻¹) and humidity (50–60%), but under different controlled temperature conditions (25 ± 0.2 and 35 ± 0.2 °C). When the light was switched off, the temperature decreased by 3–4 °C in both phytotrons.

Twelve plants were placed in each growth chamber. During the growing period prior to the experiment (i.e., 2 months after the appearance of the first fully expanded leaf), all the plants were regularly watered and fertilized with Hoagland solution once a week to supply mineral nutrients at free access rates (Centritto et al. 1999a, Centritto 2005). On the afternoon preceding the initiation of the experiment, plants were fully irrigated and excess water was allowed to drain overnight. After draining, the pots were weighed to 1-g precision on a digital balance (model QS32A; Sartorius Instrumentation Ltd, Göttingen, Germany) to determine the weight at pot water capacity (Initial_{pot weight}). Each pot was then enclosed in a plastic bag that was tied around the stem to prevent soil evaporation. Six plants were water-stressed by withholding water, while the other six saplings continued to be well watered to pot capacity (control). The development of drought stress was followed and parameterized by recording the daily pot weight (Brilli et al. 2007). The physiological lower limit of available soil water (FTSW_{endpoint}) was defined as the FTSW at which stomatal conductance approached zero (i.e., soil water decreased to a level where there was no longer water available to support transpiration) (Sinclair and Ludlow 1986). The water-stressed pots were then weighed to determine the final pot weight. Thereafter, every morning the plastic bags were unwrapped to weigh the water-stressed saplings (daily pot weight) and to water the control plants. Then, FTSW was calculated as the ratio between the amount of transpirable water remaining in the pot and the total amount of stored transpirable water determined for that pot:

$$\text{FTSW} = \frac{(\text{Daily}_{\text{pot weight}} - \text{Final}_{\text{pot weight}})}{(\text{Initial}_{\text{pot weight}} - \text{Final}_{\text{pot weight}})}$$

At the FTSW endpoint, all the drought-stressed plants were daily rewatered to pot water capacity over an 8-day recovery period (DAR). At the end of the experiment, plants were removed from each pot and the respective soil was oven-dried at 105 °C for 48 h to determine the dry mass. Then, SWC, expressed as water fraction (g g⁻¹) (Kramer and Boyer 1995), was determined as

$$\text{SWC} = \frac{(\text{Daily}_{\text{pot weight}} - \text{Soil}_{\text{dry mass}})}{(\text{Initial}_{\text{pot weight}} - \text{Soil}_{\text{dry mass}})}$$

Gas-exchange measurements

During the drought stress period (25–30 days) and after rewatering (8 days), all measurements were carried out at the growing temperatures using the LI-6400 portable gas-exchange system (Li-Cor, Lincoln, NE, USA) by enclosing a portion of a single fully expanded leaf in a gas-exchange cuvette under a constant saturating light intensity of 1000 μmol m⁻² s⁻¹.

Physiological parameters of photosynthesis (*A*), stomatal conductance (*g_s*) and internal CO₂ concentration (*C_i*) were daily measured between 10:00 and 17:00 h. During the measurements, leaf tissues were exposed to a 300 μmol s⁻¹ flux of synthetic contaminant-free air (O₃, NO and other pollutants free) by flowing the main LI-6400 inlet with a mixture of pure gases of N₂, O₂ and CO₂ in constant ratios (80%, 20% and 380 μmol mol⁻¹, respectively) through mass flow controllers (Brooks Instruments, Ede, The Netherlands). Before entering the LI-6400, relative humidity was adjusted to ~40–55% by first bubbling the synthetic CO₂-free air flow through water and then condensing the excess water in a trap immersed in a thermostatted water bath.

Following CO₂ and H₂O gas-exchange evaluation, isoprene emission was in-line detected by connecting the cuvette outflow to a proton transfer reaction mass spectrometer (PTR-MS; Ionicon, Innsbruck, Austria). Details on the theory and practice of the PTR-MS technique were reported by Lindinger et al. (1998). After each set of measurements, the PTR-MS was calibrated by measuring the same isoprene gaseous standard cylinder (70 nl l⁻¹) (Rivoira, Milano, Italy).

In order to generate *A/C_i* response curves, external CO₂ was manipulated from 50 to 2500 μmol mol⁻¹. To remove the effect of stomatal limitation on *A* (and to estimate photosynthetic capacity at high *g_s*), leaves were first pre-conditioned at low [CO₂] (50 μmol mol⁻¹) up to 1.5 h to force stomatal opening as described by Centritto et al. (2003). Then, [CO₂] was progressively increased up to 2500 μmol mol⁻¹. The maximum rate of Rubisco carboxylation (*V_{cm, max}*), the apparent maximum rate of electron transport at saturating irradiance (*J_{max}*) and the maximum photosynthesis (*A_{max}*) were obtained by fitting the mechanistic model of CO₂ assimilation proposed by Farquhar et al. (1980), as described by Centritto et al. (2004).

Measurements of *R_n* (dark respiration) were made at ambient CO₂ concentration in the dark after switching off the light for 15–20 min. CO₂ evolution in light conditions, an indicator of the respiration rate in the light (*R_d*), was assessed by exploiting the very low sensitivity of LI-6400 gas analyzers to ¹³CO₂ (Loreto et al. 2001). Under light conditions, in the humidified N₂ and O₂ mixture entering the LI-6400 the external ¹²CO₂ source was rapidly replaced by an equal concentration of pure ¹³CO₂ (Sigma Aldrich, Milwaukee, WI, USA), and the flux of ¹²CO₂ emitted from the leaf tissue was considered as representative of respiration (but see Loreto et al. (2001) for possible pitfalls of this approach).

To reduce the level of variability, the gas-exchange parameters shown in Figures 2 and 3 were normalized against the respective mean rates, registered on the same day, of the well-watered saplings subjected to the same temperature treatment. The R_n and R_d values were not normalized because their variability during the dehydration cycle was limited.

Statistics

All the measurements were carried out on four to six different plants. Two-way analyses of variance were used to test the effect of different temperatures (25 or 35 °C) on drought treatments. Means were compared and separated by Tukey's test using SigmaStat® software version 3.1 (Systat Software Inc., San Jose, CA, USA). Different letters reported in Tables 1 and 2 indicate statistically different means within the same group (different FTSW levels at the same temperature), and asterisks (*) indicate statistically different means between the two groups (same level of FTSW at two different temperatures of 25 and 35 °C).

Results

Thermal acclimation of gas-exchange parameters in the absence of drought

High growth temperature significantly decreased photosynthesis of well-watered (FTSW₁₀₀) saplings by ~33% when measured at the growth conditions. However, no significant differences were found in g_s and C_i of well-watered plants grown at 25 and 35 °C (Table 1). Temperature-dependent decline in A was mirrored by inhibition of leaf photosynthetic

capacity, as indicated by the A/C_i curves (Figure 1), in the high-temperature treatment. Analysis of the photosynthetic capacity of FTSW₁₀₀ saplings grown at 35 °C showed significantly lower values of both A_{max} (~–22%) and J_{max} (~–20%), and a non-significant reduction in V_{cmax} (~–1%) than in plants grown at 25 °C (Table 2). Differently from carbon assimilation, growth in high temperature did result in acclimatory responses of respiration rates in well-watered plants, since R_n and R_d were not significantly different between the temperature treatments at FTSW₁₀₀ (Table 1). It is noteworthy that in well-watered plants R_d was lower than R_n irrespective of temperature treatment, but especially at 35 °C. As expected, high growth temperature had a pronounced effect on isoprene emission, which was ~60% higher than at 25 °C (Table 1). Consequently, because of the contrasting effects on A and isoprene emission, a higher percentage (i.e., ~140%) of photosynthetic carbon was lost as isoprene at 35 °C than at 25 °C in well-watered saplings.

Impact of drought stress on the biochemical limitations of photosynthesis

The biochemical limitations to photosynthesis during dehydration and after rewatering were investigated in vivo by measuring A as a function of C_i in plants grown at 25 °C (Figure 1a and c) and 35 °C (Figure 1b and d). The A/C_i curves were dramatically affected by water stress at both growth temperatures. This indicates that the progressive intensity of water stress leads to increasing metabolic impairment of photosynthesis (Figure 1a and b). At early stages of drought stress (i.e., FTSW₅₀), V_{cmax} , A_{max} and J_{max} decreased to similar levels at 25 °C and at 35 °C (Table 2), indicating that the photosynthetic

Table 1. Assimilation rate (A), stomatal conductance (g_s), intercellular CO₂ concentration (C_i), mitochondrial respiration in the light (R_d), mitochondrial respiration in the dark (R_n), isoprene emission (I) and percentage of photosynthetic carbon lost as isoprene of *P. nigra* plants growth at 25 °C (a) and at 35 °C (b).

	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	R_d ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	R_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	I ($\text{nmol m}^{-2} \text{s}^{-1}$)	C%
25 °C							
FTSW ₁₀₀	16.13 ± 0.77a*	0.185 ± 0.014a*	239 ± 12a	1.35 ± 0.09a	1.89 ± 0.15a	19.47 ± 0.85a*	0.60 ± 0.08d*
FTSW ₈₀	16.60 ± 0.85a*	0.166 ± 0.018a*	238 ± 9a	NA	1.96 ± 0.10a	16.78 ± 1.01ab*	0.51 ± 0.09d*
FTSW ₅₀	12.43 ± 1.52b*	0.111 ± 0.020b	194 ± 14b*	NA	1.54 ± 0.07b	15.72 ± 1.97ab*	0.63 ± 0.08d*
FTSW ₃₀	6.69 ± 1.07c	0.068 ± 0.010c*	189 ± 6b*	1.21 ± 0.06ab*	1.45 ± 0.11b	19.21 ± 1.18a*	1.44 ± 0.12c*
FTSW ₁₀	2.72 ± 0.86d	0.020 ± 0.005d	181 ± 25b*	1.19 ± 0.03b*	0.87 ± 0.13c	13.40 ± 1.75b	2.46 ± 0.30b*
FTSW ₅	0.48 ± 0.17e	0.006 ± 0.001e	237 ± 24a*	0.57 ± 0.07c	0.80 ± 0.09c	8.51 ± 1.56c	8.87 ± 0.86a*
35 °C							
FTSW ₁₀₀	10.78 ± 0.77a	0.142 ± 0.023a	243 ± 4b	1.38 ± 0.11a	2.17 ± 0.18a	31.07 ± 0.42a	1.44 ± 0.40d
FTSW ₈₀	9.47 ± 0.74ab	0.127 ± 0.020ab	228 ± 4c	NA	NA	30.18 ± 1.94a	1.59 ± 0.34d
FTSW ₅₀	7.90 ± 0.66b	0.103 ± 0.012bc	226 ± 5c	1.10 ± 0.20ab	1.58 ± 0.18bc	30.78 ± 0.94a	1.95 ± 0.80cd
FTSW ₃₀	4.20 ± 0.71c	0.025 ± 0.005d	110 ± 5d	0.95 ± 0.14b	1.23 ± 0.23c	23.85 ± 1.80b	2.84 ± 0.51c
FTSW ₁₀	1.45 ± 0.28d	0.017 ± 0.001e	254 ± 12b	0.85 ± 0.18bc	1.18 ± 0.24cd	14.63 ± 0.51c	5.05 ± 0.39b
FTSW ₅	0.27 ± 0.12e	0.007 ± 0.001f	364 ± 19a	0.52 ± 0.14c	0.85 ± 0.10d	9.40 ± 1.14d	17.41 ± 0.63a

The measurements, made during the water stress cycle, are shown at 100% (FTSW₁₀₀), 80% (FTSW₈₀), 50% (FTSW₅₀), 30% (FTSW₃₀), 10% (FTSW₁₀) and 5% (FTSW₅) FTSW, respectively. Gas-exchange measurements were made at 25 °C under a saturating photon flux of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Values are means of four plants ±1 SE. Means were compared and separated by Tukey's test; different letters (a–f) in the same column indicate significant differences at $P < 0.05$.

*Indicates significant differences between the two different growing temperature (25 and 35 °C) at $P < 0.05$; NA, not available.

Table 2. Maximum response of photosynthesis (A_{\max}), maximum rate of electron transport at saturating irradiance (J_{\max}) and maximum rate of carboxylation (V_{cmax}) of *P. nigra* plants grown at 25 and 35 °C.

	A_{\max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	J_{\max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	V_{cmax} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
25 °C			
FTSW ₁₀₀	29.77 ± 0.12b*	149.39 ± 3.90a*	95.13 ± 3.79a
FTSW ₅₀	15.37 ± 0.50e*	74.58 ± 3.69c*	48.61 ± 1.03c
FTSW ₁₀	0.70 ± 0.03g*	10.15 ± 0.67e*	5.59 ± 0.26g*
DAR ₁	8.20 ± 0.70f*	33.51 ± 3.91d*	6.73 ± 0.19f*
DAR ₂	9.52 ± 0.82f*	38.05 ± 1.65d*	7.18 ± 0.26f*
DAR ₃	18.61 ± 1.44d	82.04 ± 4.64c	31.06 ± 1.59e
DAR ₆	22.48 ± 0.77c*	103.21 ± 2.94b*	41.50 ± 0.27d*
DAR ₈	33.14 ± 0.94a*	154.99 ± 2.25a*	77.66 ± 5.86b
35 °C			
FTSW ₁₀₀	23.31 ± 0.75b	119.39 ± 3.40b	92.65 ± 6.98a
FTSW ₅₀	18.33 ± 1.28c	91.76 ± 4.47c	52.00 ± 2.85c
FTSW ₁₀	3.88 ± 0.09e	21.85 ± 1.02e	11.51 ± 0.76e
DAR ₁	15.55 ± 0.42d	76.93 ± 1.45d	24.28 ± 2.21d
DAR ₂	16.99 ± 1.44cd	79.20 ± 4.73d	28.27 ± 2.56d
DAR ₃	NA	NA	NA
DAR ₆	26.65 ± 0.91a	130.16 ± 3.63a	80.12 ± 2.13b
DAR ₈	26.84 ± 0.62a	132.85 ± 2.70a	82.15 ± 3.37ab

During the drying cycle, values are shown at 100% (FTSW₁₀₀), 50% (FTSW₅₀) and 10% (FTSW₁₀) FTSW, respectively; after the drying cycle, during the recovery period values are shown at 1, 2, 3, 6 and 8 DAR. Values are means of four plants ± 1 SE. Means were compared and separated by Tukey's test; different letters (a–g) in the same column indicate significant differences at $P < 0.05$.

*Indicates significant differences between the two growing temperatures (25 and 35 °C) at $P < 0.05$; NA, not available.

capacity of poplar saplings was more affected by drought at the lower growth temperature. These higher apparent biochemical limitations to photosynthesis at 25 °C than at 35 °C were further amplified under rather severe drought stress (FTSW₁₀), because A_{\max} was about fourfold lower and V_{cmax} and J_{\max} were twofold lower at 25 °C than at 35 °C. Plant photosynthetic capacity, as described by A/C_i curve, was not only fully restored but even increased after a week into plant stress relief (Figure 1c and d). However, the recovery of photosynthetic capacity was much faster at 35 °C than at 25 °C. In plants grown at 35 °C, V_{cmax} recovered by ~30% and A_{\max} and J_{\max} by ~58% of their respective values at 8 DAR already 1 day after rewatering (Table 2), and maximum photosynthetic capacity was reached already at 6 DAR (Figure 1d). However, at 25 °C, V_{cmax} , A_{\max} and J_{\max} recovered only ~10, 26 and 23%, respectively, after 1 DAR, and ~63, 72 and 70%, respectively, after 6 DAR of the capacity shown at 8 DAR (Table 2).

Gas-exchange parameters of leaves at decreasing SWC

The reduction in SWC determined, overall, a concurrent reduction in A (Figure 2a and b) and g_s (Figure 2c and d) at both growth temperatures, despite A showing a relatively constant trend up to an SWC threshold of ~75% at 25 °C. However, the decline of both parameters was faster at 35 °C than at 25 °C. Consequently, A and g_s were reduced to their minimal values at SWC endpoints of ~45.16 ± 2.84% at 35 °C and

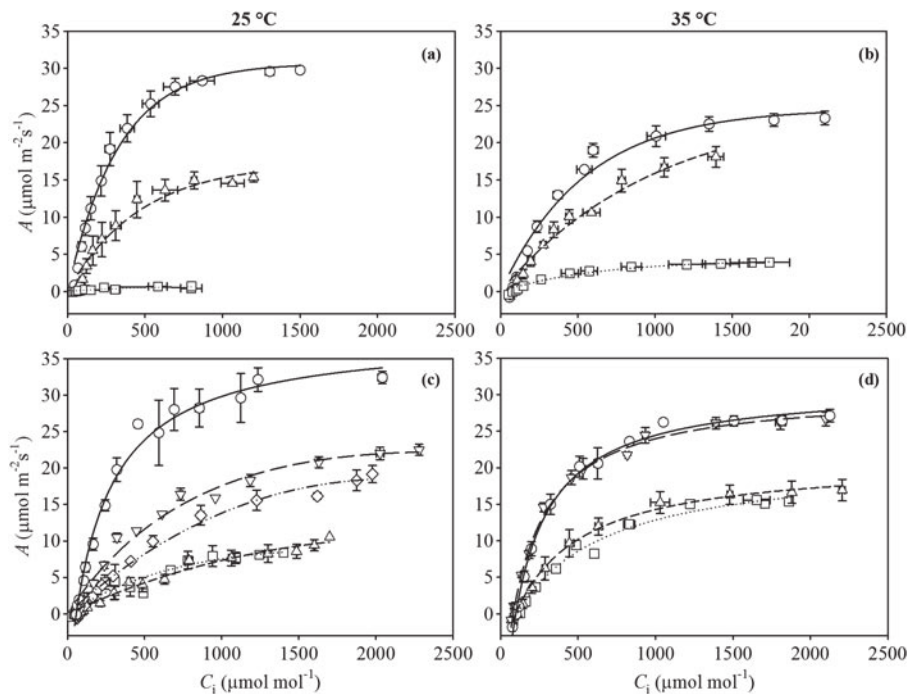


Figure 1. Average responses of photosynthesis (A) to internal CO_2 concentration (C_i), and relative best-fit curves plotted from the respective J_{\max} , V_{cmax} and A_{\max} average values (Table 2), in *P. nigra* saplings grown at 25 °C (a, c) and at 35 °C (b, d). Values are means of four plants ± 1 SE. During the drying cycle (a, b) data are shown at FTSW₁₀₀ (circle, solid line), FTSW₅₀ (triangle, short dash line) and FTSW₁₀ (square, dot line). During the recovery period (c, d) data are shown at 1 (square, dot line), 2 (triangle, short dash line), 3 (diamond, dash-dot-dot line), 6 (inverted triangle, long dash line) and 8 (circle, solid line) DAR.

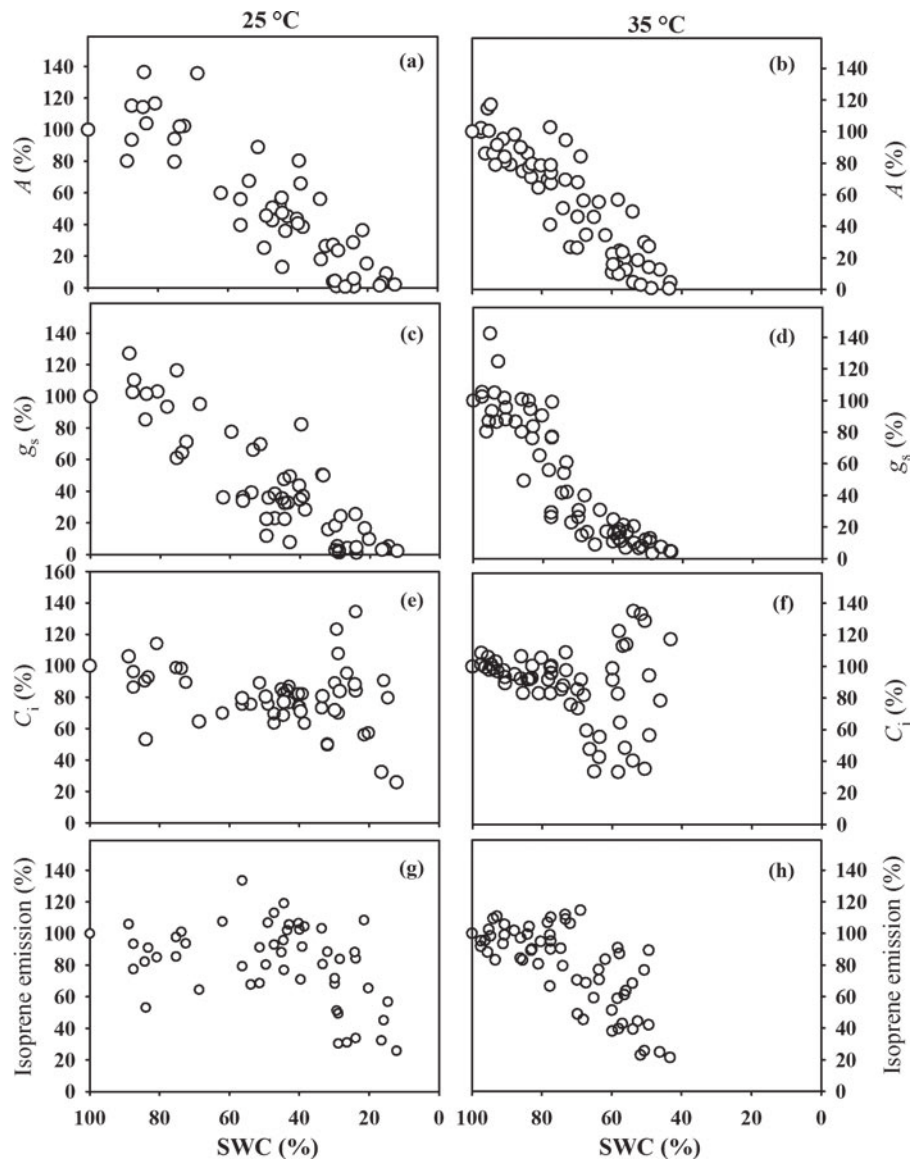


Figure 2. Normalized (%) data of (a, b) photosynthesis (A), (c, d) stomatal conductance (g_s), (e, f) intercellular CO₂ concentration (C_i) and (g, h) isoprene emission shown as a function of SWC in *P. nigra* saplings grown at 25 °C (a, c, e, g) and 35 °C (b, d, f, h).

$\sim 28.22 \pm 2.02\%$ at 25 °C: i.e., carbon assimilation and transpiration declined more quickly and were eventually fully inhibited at significantly higher soil water availability in plants grown at high temperature. C_i showed a declining trend as SWC decreased at both growth temperatures (Figure 2e and f), indicating that A was relatively less inhibited than g_s . However, as SWC approached the endpoint, i.e., when water stress became more severe, C_i showed a much greater variability at both 35 and 25 °C. A few C_i values were actually higher than in control plants towards the SWC endpoint, which indicates the onset of metabolic limitations of photosynthesis. Isoprene emission remained relatively constant until SWC reached a threshold of $\sim 40\%$ at 25 °C (Figure 2g). Then, as SWC was further reduced, there was a continuous trend of decreased isoprene emission.

As for A and g_s , high growth temperature increased the SWC threshold ($\sim 80\%$) at which isoprene emission started to decline (Figure 2h).

Overall, isoprene emission was less affected than A and g_s as SWC declined. R_n showed a trend similar to that of A at 25 °C in response to SWC (Figure 4a), i.e., R_n showed a declining trend after reaching an SWC threshold of $\sim 70\%$. The lower dataset available for R_n at 35 °C (Figure 4b) and for R_d at both growth temperatures (Figure 4c and d) does not allow identification of an SWC threshold after which R_n and R_d started declining as SWC decreased. As for the previous parameters, R_n and R_d also reached their minimum values at a higher SWC at 25 °C than at 35 °C, although they were not fully inhibited by very severe water stress.

Gas-exchange parameters of leaves at decreasing FTSW

Measuring physiological parameters as a function of FTSW makes it possible to resolve the critical issue of whether there is any difference in plant responses at equivalent levels of water available to support transpiration irrespective of the total amount of soil water (i.e., SWC). Overall, similar trends of the measured parameters were seen as FTSW declined, indicating that inherent drought kinetics were not affected by high growth temperatures (Figures 3 and 4e and h). Photosynthesis (Figure 3a and b), g_s (Figure 3c and d) and C_i (Figure 3e and f) started declining significantly at FTSW₅₀ at both 25 and 35 °C (Table 1). However, when water stress became severe, C_i increased significantly (Figure 3e and f). This increase in C_i was

significantly higher at 35 °C, where it started occurring at a higher FTSW (i.e., FTSW₁₀) than at 25 °C, where it occurred at FTSW₅ only (Table 1). R_n and R_d of plants grown at 25 °C (Figure 4e and g) and 35 °C (Figure 4f and h) also showed a similar declining trend in response to FTSW. At the FTSW endpoint, R_n and R_d were inhibited similarly in both temperature treatments (Table 1).

Plants grown at 25 °C showed a sustained isoprene emission even at FTSW₃₀ (Figure 3g), when A was ~42% lower than in control plants (Table 1). Similarly, in plants grown at 35 °C isoprene emission was still unchanged at FTSW₅₀ (Figure 3h), while values of A were on average ~73% lower than in control (Table 1). Severe drought stress conditions

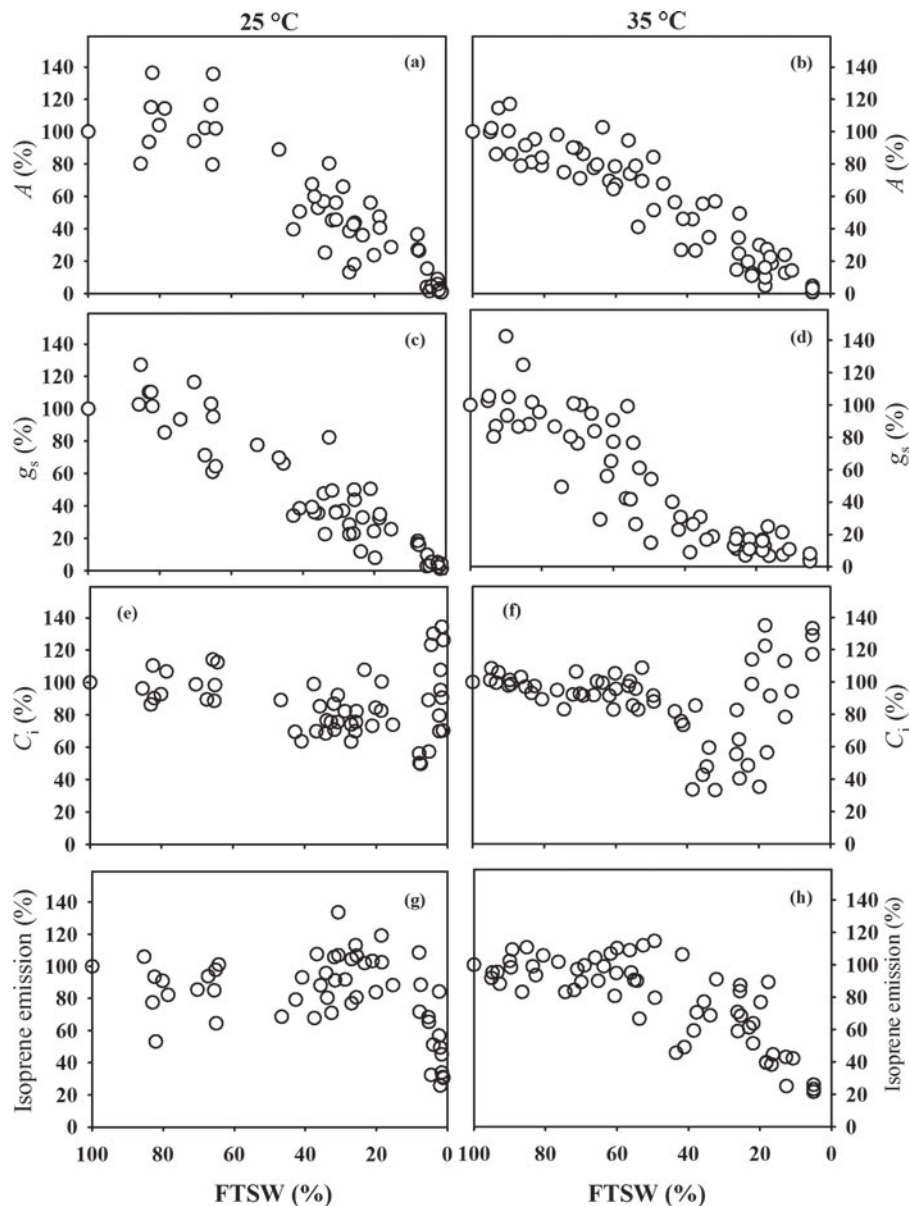


Figure 3. Normalized (%) data of (a, b) photosynthesis (A), (c, d) stomatal conductance (g_s), (e, f) intercellular CO₂ concentration (C_i) and (g, h) isoprene emission shown as a function of FTSW in *P. nigra* saplings grown at 25 °C (a, c, e, g) and 35 °C (b, d, f, h).

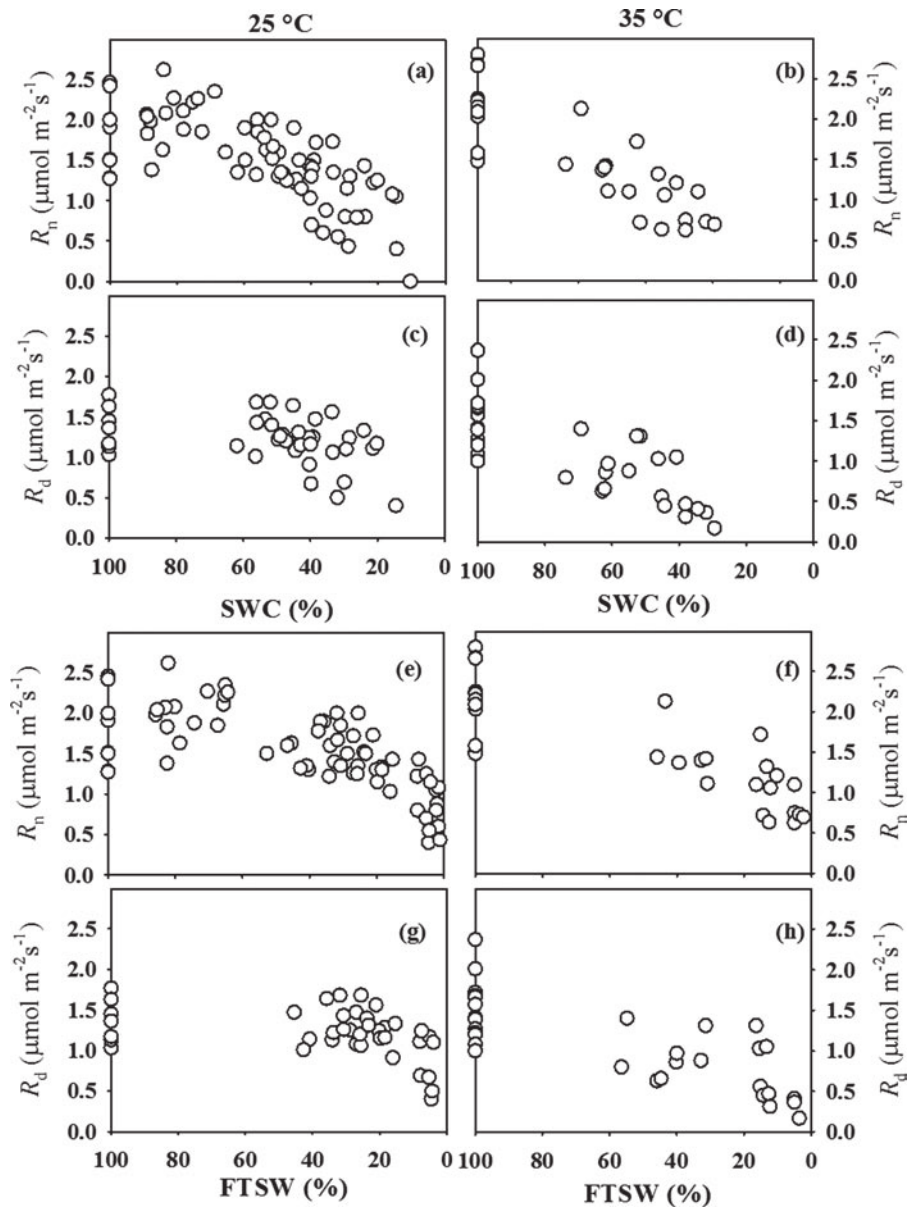


Figure 4. Dark (R_n) (a, b, e, f) and light (R_d) (c, d, g, h) respiration shown as a function of SWC (a–d) and as FTSW (e–h) in *P. nigra* saplings grown at 25 °C (a, c, e, g) and 35 °C (b, d, f, h).

(i.e., FTSW₅) determined a much higher inhibition of isoprene emission at 35 °C (~70%) than at 25 °C (~44%) compared with the respective control plants (Table 1). However, independently of growth temperature, similar rates of emission were detected at the end of the drought treatment (Table 1). The percentage of photosynthetic carbon that was lost as isoprene increased exponentially after exceeding an FTSW threshold of ~80% at both growth temperatures (Table 1). However, because of the uncoupled trends of isoprene emission and A in response to FTSW, the amount of photosynthetic fixed carbon lost as isoprene was always significantly higher at 35 °C than at 25 °C, and was increased by ~96% at the FTSW endpoint.

Discussion

Respiration and photosynthesis responses to water stress and high temperature

Drought is of paramount importance in determining ecosystem respiration (Reichstein et al. 2006). However, the response of R_n to a combination of high growth temperature and drought stress in plants has never been thoroughly investigated until now (Lawlor and Cornic 2002, Atkin and Macherel 2009). Furthermore, R_d responses to the interaction between high growth temperature and water stress are virtually absent. Therefore, we assessed leaf respiration in plant growth at 25 °C and at 35 °C subjected to limited soil water availability

through direct measurement of CO_2 release by *P. nigra* leaves in both dark and light conditions. Although respiration is very sensitive to short-term temperature increases, growth at high temperature often results in a downward acclimation in respiration rates. Our results highlight that elevated temperature did not significantly alter either R_n or R_d (Table 1). This is consistent with other results demonstrating the thermal acclimation of the respiration process (Campbell et al. 2007, Ow et al. 2008a, 2008b, Way and Sage 2008, Tjoelker et al. 2009, Fares et al. 2011). Water stress progression induced a sharp decline in both R_n and R_d , but these two parameters were never fully inhibited by very severe water stress (Figure 4). This is in keeping with the findings reported in a survey by Atkin and Macherel (2009), which showed that the impact of water stress on R_d at leaf level was reduced in two-thirds of the reviewed studies. We also show that irrespective of the growth temperature, and independent of drought stress level, R_d is ~30% lower than R_n . Two mechanisms probably contribute to make the R_d/R_n ratio stable. In unstressed or moderately stressed leaves, a fraction of respiratory CO_2 is re-fixed by photosynthesis (Pinelli and Loreto 2003), whereas in drought-stressed leaves photosynthesis no longer acts as a strong sink for respiratory CO_2 but R_d is strongly inhibited (Loreto et al. 2001).

Differently from respiration, we expected lower photosynthetic rates in well-watered plants grown at 35 °C rather than at 25 °C (Table 1) in accordance with the C_3 metabolism of *P. nigra* plants (Berry and Björkman 1980, Hikosaka et al. 2006). This result was further confirmed by the significantly lower A_{max} and J_{max} at 35 °C than at 25 °C (Table 2). Similar results were also found by Way and Sage (2008) in a study on thermal acclimation of photosynthesis and respiration in black spruce seedlings. They found that in seedlings grown at high temperatures A was 19–35% lower than at low temperatures, and, as in our study, photosynthetic capacity was significantly down-regulated in high-temperature plants.

Photosynthesis and respiration are generally intimately linked (Dutilleul et al. 2003, Atkin et al. 2005), but water limitation and high temperature may change the balance between respiration and photosynthesis and, consequently, the leaf carbon balance (Atkin and Macherel 2009). Increasing air temperatures by exceeding temperatures optimal for carbon gain may have the potential to alter plant function. Our results show that, despite R_d acclimation, lower A (–33%) at high growth temperatures (Table 1) resulted in a ratio of R_d to A almost doubled in plants grown at 35 °C compared with those grown at 25 °C, which may imply that a larger proportion of daily fixed carbon is respired at high temperatures. Similar results were found in thermal acclimation studies conducted by Way and Sage (2008) on black spruce and by Fares et al. (2011) on hybrid poplar. Way and Oren (2010) also confirmed, in a recent survey of 63 studies on the effects of temperature on tree growth,

that respiration is less sensitive than photosynthesis to increased temperature. Water stress can typically increase the R_d to A ratio (Galmes et al. 2007, Atkin and Macherel 2009). Similarly, the much lower sensitivity of R_n and R_d (Figure 4) compared with A (Figure 2) as water stress progressed indicates that temperature interactions with water stress may dominate poplar acclimatory capability in maintaining carbon homeostasis with the future scenario of climate change.

Impact of drought on photosynthesis limitations

The A/C_i responses (Figure 1) showed an apparent metabolic impairment of photosynthesis already when the FTSW was 60%. In particular, a reduced Rubisco activity, as represented by the flatter slope of the A/C_i response at low C_i (Farquhar and Sharkey 1982, Wullschlegel 1993, Centritto et al. 1999b), was detected. However, the use of the FTSW parameter allowed us to detect in a very sensitive way the C_i trend as water stress progressed (Figure 3e and f). C_i steadily declined until drought stress became severe, indicating that g_s dropped more than A . This is a clear indication that A was limited by CO_2 transport (Cornic 2000). Only towards the FTSW endpoint (i.e., at about FTSW_{10} and FTSW_{20} at 25 and 35 °C, respectively) was it possible to see C_i values higher than those in control plants, indicating the occurrence of metabolic limitations that restrain CO_2 fixation by photosynthesis (Lawlor and Cornic 2002). These results confirm that metabolic limitations follow the CO_2 transport limitations, taking place only when abiotic stress becomes severe (Cornic 2000, Centritto et al. 2003). Furthermore, it should be said that a reduction in Rubisco activity may not be real when mesophyll limitations to CO_2 transport occur and increase under stress conditions. By reducing the CO_2 transport between the intercellular spaces and the chloroplasts, mesophyll limitations make the $[\text{CO}_2]$ lower at the carboxylation sites (C_c), which in turn make the slope of the A/C_c response steeper (Loreto et al. 1994, Centritto et al. 2009). We are not able to say whether a drop in C_c also occurred in the mesophyll of our drought-stressed poplars. However, this seems to be a common occurrence in drought-stressed plants and therefore makes it likely that the reduction in Rubisco activity, as inferred from A/C_i responses (Figure 1) and from the calculated V_{cmax} (Table 2), is mostly artefactual.

A_{max} (Table 2) and the overall A/C_i curves (Figure 1) showed higher photosynthetic rates at 35 °C than at 25 °C as FTSW decreased, and this may be explained by the higher SWC at 35 °C than at 25 °C at each given FTSW as water stress progressed. Moreover, leaves grown at 35 °C completely recovered the photosynthetic properties of pre-stressed leaves already after 6 DAR, whereas such a complete recovery was only visible after 8 DAR in leaves of plants grown at 25 °C. These observations reveal a strong interactive effect of temperature and drought. Furthermore, the rapid recovery of photosynthetic capacity also indicates that water stress did not

cause irreversible biochemical damage and that, consequently, CO₂ transport factors played a dominant role in limiting photosynthesis.

Acclimatory responses of isoprene to water stress and high temperature

It is well known that, in the short term, rising temperature increases isoprene emission exponentially by enhancing its vapor pressure (consequently decreasing its diffusion pathway resistance) and above all by increasing ISPS activity, the optimum temperature range of which is 40–45 °C (Loreto and Sharkey 1990, Sharkey and Yeh 2001). Furthermore, as pointed out in our previous investigations, isoprene emission is resistant to drought stress (Brilli et al. 2007). However, there is much less information about the acclimatory responses of isoprene to warming and to its interaction with water stress. Our results confirmed the early observation of Fortunati et al. (2008) that isoprene emission was stimulated by high growth temperature and that the effect of drought occurred independently of growth temperatures (Table 1). Our results now show that the kinetics of isoprene emission in response to FTSW was similar at the two growth temperatures. Furthermore, because isoprene emission decline was only evident towards the FTSW endpoint, our findings also confirm that isoprene emission is not inhibited by water-stress-induced stomatal closure (Monson and Fall 1989) and that it is uncoupled from *A* in water stress conditions (Tingey et al. 1981, Sharkey and Loreto 1993, Brilli et al. 2007, Loreto and Schnitzler 2010). However, these results also show that severe water stress removes the temperature dependence of isoprene emission, as indicated by the similar emission rates at the two growth temperatures at FTSW 5–10% (Figure 3), and that this overriding limitation occurs at a significantly higher SWC endpoint of extractable soil water (Figure 2). Thus, these findings rule out the hypothesis that rising temperature can offset the inhibition of isoprene under water stress conditions (Peñuelas and Staudt 2010). This is somehow surprising because isoprene can protect plants against heat (Sharkey and Singaas 1995, Behnke et al. 2007, Velikova et al. 2009, Loreto and Schnitzler 2010). Despite this overriding effect of water stress, elevated temperatures significantly increased the amount of photosynthetic carbon lost as isoprene in drought-stressed leaves, which was as high as ~17% of the assimilated carbon at low FTSW (Table 1). This high percentage of daily fixed carbon released back into the atmosphere as isoprene affects, together with respiration (see above), the leaf carbon balance and may play a major role in determining the productivity of isoprene-emitting plants subjected to rising temperatures and drought (Peñuelas and Staudt 2010).

FTSW versus SWC

Expressing plant responses to water stress as a function of both volumetric SWC and equivalent levels of soil water

available to support transpiration allows isolation of the responses of specific traits to the interaction between high growth temperature and equivalent levels of soil water deficit. The results of this experiment showed that there was no major significant effect of high temperature on the relative responses of *A*, *g_s*, isoprene emission and respiration to soil water available to support transpiration (Figures 3 and 4). The pattern of *A* and *g_s* to FTSW, as well as that of isoprene, was similar to the results obtained with other plant species (Sinclair and Ludlow 1986, Ray and Sinclair 1998, Sinclair et al. 1998, Brilli et al. 2007). However, the overall responses of these physiological parameters to decreased SWC were markedly affected by high growth temperature, which resulted in a significant increase in the SWC endpoint (Figures 2 and 4). This implies that the predicted temperature increases in arid or semi-arid environments such as the Mediterranean may reduce the amount of soil water that can be extracted before plant gas exchange decreases, hence exacerbating the effects of drought on tree growth even if soil water availability was not affected by climate change.

Conclusion

Low water availability represents the main environmental constraint for plant growth and productivity worldwide and, especially when coupled with rising temperatures, may strongly influence the flux of carbon assimilated by plants through photosynthesis or released by mitochondrial respiration and as isoprene (Way and Oren 2010, Centritto et al. 2011). There have been, however, only few investigations that have considered the acclimatory response kinetics to the interaction between high growth temperature and water stress. An important feature of this study was to subject plants of similar size to soil drying at equivalent levels of SWC and FTSW. This drought kinetics approach makes it possible to resolve the critical issue of whether there is any difference in plant responses to high growth temperature at equivalent levels of water available to support transpiration irrespective of the total amount of volumetric soil water. Our experimental results highlight the following. (i) Isoprene emission and photosynthesis did not acclimate in response to elevated temperature, whereas light and dark respiration underwent thermal acclimation. These different acclimatory responses led to higher ratios of respiration to photosynthesis as well as to higher amounts of carbon loss as isoprene, which reached ~1.44% of net photosynthesis. (ii) Photosynthetic capacity was mainly limited by CO₂ transport factors in response to water stress as impaired carbon metabolism became apparent only at very low FTSW and was rather transient because full photosynthetic capacity was restored in about a week upon relief of water stress. (iii) The interaction between elevated temperature and water stress anticipated the threshold of soil water availability at which plant gas exchange starts to be limited and increased the SWC

endpoint of extracted soil water. Taken together our results show that a forecasted future warmer and drier climate change will strongly impact on plant growth and productivity by directly reducing the carbon assimilation to respiration ratio, anticipating the threshold of soil water availability at which photosynthesis starts to be limited. The cost of climate change in terms of assimilated carbon will be further aggravated in isoprene-emitting species due to the strong temperature dependence of isoprene biosynthesis and its resistance to soil water scarcity, but when the two stresses act together the temperature dependence will be suppressed. Isoprene dynamical responses to global environmental changes will be crucial for atmospheric chemistry. The physiological observations of acclimation highlighted in this study should now be integrated in a coupled global climate vegetation model that accounts for water stress and high-temperature effects in order to predict the isoprenoid impact on air chemistry and quality at regional and global level as well as the biosphere's response to climate change.

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