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The effect of summer drought on the yield of *Arundo donax* is reduced by the retention of photosynthetic capacity and leaf growth later in the growing season

Matthew Haworth^{1,*}, Giovanni Marino¹, Ezio Riggi¹, Giovanni Avola¹, Cecilia Brunetti^{1,2}, Danilo Scordia³, Giorgio Testa³, Marcos Thiago Gaudio Gomes^{1,4}, Francesco Loreto⁵, Salvatore Luciano Cosentino³ and Mauro Centritto¹

¹Tree and Timber Institute, National Research Council of Italy (CNR-IVALSA), Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze, Italy, ²Department of Agrifood Production and Environmental Sciences (DiSPAA), University of Florence, Viale delle Idee 30, 50019 Sesto Fiorentino, Firenze, Italy, ³Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania, via Valdisavoia 5, 95123 Catania, Italy, ⁴Department of Biological Sciences, Center for Human and Natural Sciences, Federal University of Espírito Santo, Avenida Fernando Ferrari, 514, Goiabeiras, CEP 29075-910, Vitória, Espírito Santo, Brazil and ⁵Department of Biology, Agriculture and Food Sciences, National Research Council of Italy (CNR-DiSBA), Rome, Italy

* For correspondence. E-mail haworth@ivalsa.cnr.it

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- **Background and Aims** The development of *Arundo donax* as a biomass crop for use on drought-prone marginal lands in areas with warm to hot climates is constrained by the lack of variation within this species. We investigated the effect of morphological and physiological variation on growth and tolerance to drought under field conditions in three ecotypes of *A. donax* collected from habitats representing a climate gradient: a pre-desert in Morocco, a semi-arid Mediterranean climate in southern Italy and a warm sub-humid region of central Italy.
- **Methods** The three *A. donax* ecotypes were grown under irrigated and rain-fed conditions in a common garden field trial in a region with a semi-arid Mediterranean climate. Physiological and morphological characteristics, and carbohydrate metabolism of the ecotypes were recorded to establish which traits were associated with yield and/or drought tolerance.
- **Key Results** Variation was observed between the *A. donax* ecotypes. The ecotype from the most arid habitat produced the highest biomass yield. Stem height and the retention of photosynthetic capacity later in the year were key traits associated with differences in biomass yield. The downregulation of photosynthetic capacity was not associated with changes in foliar concentrations of sugars or starch. Rain-fed plants maintained photosynthesis and growth later in the year compared with irrigated plants that began to senesce earlier, thus minimizing the difference in yield. Effective stomatal control prevented excessive water loss, and the emission of isoprene stabilized photosynthetic membranes under drought and heat stress in *A. donax* plants grown under rain-fed conditions without supplementary irrigation.
- **Conclusions** *Arundo donax* is well adapted to cultivation in drought-prone areas with warm to hot climates. None of the *A. donax* ecotypes exhibited all of the desired traits consistent with an 'ideotype'. Breeding or genetic (identification of quantitative trait loci) improvement of *A. donax* should select ecotypes on the basis of stem morphology and the retention of photosynthetic capacity.

Key words: Photosynthesis, bioenergy, drought, biomass, chlorophyll fluorescence, common garden.

INTRODUCTION

The rapid growth of biomass crops is associated with high rates of photosynthesis (P_N) and transpirative water loss (Karp and Shield, 2008; Angelini *et al.*, 2009). Supplying the large volumes of water needed to sustain growth is a major challenge for the cultivation of biomass crops in drought-prone marginal lands in areas with warm to hot climates (Cosentino *et al.*, 2006; Tuck *et al.*, 2006). Field trials in the Mediterranean have shown that the giant reed (*Arundo donax*) outperforms other biomass crops in terms of primary metabolism, growth and biomass yield under both irrigated and rain-fed conditions (Angelini *et al.*, 2009; Mantineo *et al.*, 2009). The clonal reproduction of *A. donax* may have impaired its

development as a biomass crop due to a lack of genetic diversity that can be exploited in breeding more productive and/or drought-tolerant varieties (Pilu *et al.*, 2014; Valli *et al.*, 2017). However, previous studies have reported variation in the biomass yield of *A. donax* ecotypes collected from different habitats (Cosentino *et al.*, 2006; Sánchez *et al.*, 2015; Haworth *et al.*, 2017c). We established a common garden field trial in a semi-arid region with a strong Mediterranean climate involving three ecotypes of *A. donax* that have demonstrated contrasting responses to water availability in order to investigate whether these ecotypic differences in yield might be associated with variation in physiological and/or morphological traits.

The three ecotypes of *A. donax* were collected from habitats representing a climate gradient: a warm-humid sub-Mediterranean region of Central Italy [the ‘Italian’ ecotype from Haworth *et al.* (2017b), hereafter referred to as the ‘Tuscan’ ecotype]; a semi-arid Mediterranean region of Sicily, southern Italy [‘ecotype 6’ from the slopes of Mount Etna (Cosentino *et al.*, 2006), hereafter referred to as the ‘Sicilian’ ecotype]; and a strongly arid pre-desert area of Marrakech, Morocco (hereafter referred to as the ‘Moroccan’ ecotype). *Arundo donax* is frequently found adjacent to water bodies growing in soils with high water availability (Danin, 2004; Watts and Moore, 2011), suggesting that the ‘yield penalty’ imposed by drought may be too severe for a fast-growing species such as *A. donax* to be successfully cultivated in drought-prone marginal lands. Species or varieties with lower rates of P_N and stomatal conductance (G_s) generally show smaller proportional reductions in yield after drought stress than their faster growing counterparts with greater rates of photosynthetic gas exchange (Lauteri *et al.*, 2014). *Arundo donax* does show pronounced reduced biomass gain in the short term during severe drought (Haworth *et al.*, 2017b). However, while rain-fed plants do produce lower biomass yield than irrigated plants over the whole growing season, these reductions are often not statistically significant (Cosentino *et al.*, 2014; Haworth *et al.*, 2017c; Curt *et al.*, 2018). Maintenance of the productive performance of *A. donax* under drought-stressed conditions may be related to an extensive root system enabling access to water deep within the soil profile (Mann *et al.*, 2013); adjustment of stomatal physiological behaviour to optimize water use efficiency (WUE) under a range of growth conditions (Haworth *et al.*, 2018a, c); and modification of the emission of protective antioxidants such as volatile organic compounds (Ahrar *et al.*, 2015; Haworth *et al.*, 2017a).

Previous studies have observed remarkably little variation between *A. donax* ecotypes in gas exchange measurements of P_N and G_s under well-watered and drought-stressed conditions (Sánchez *et al.*, 2015; Haworth *et al.*, 2017b, c; Zegada-Lizarazu *et al.*, 2018) consistent with evidence indicating a lack of genetic variability within the species (Khudamrongsawat *et al.*, 2004; Ahmad *et al.*, 2008; Mariani *et al.*, 2010; Pilu *et al.*, 2014). However, to the best of our knowledge, more in-depth analyses of the photosynthetic capacities of contrasting *A. donax* ecotypes have not been reported in the literature. Physiological differences in the emission of isoprene have been observed between the Tuscan and Moroccan ecotypes under moderate drought stress. The increased emission of isoprene in the Moroccan ecotype may reflect the selective pressures induced by multigenerational growth in an environment characterized by high temperatures and the rapid onset of persistent drought events (Haworth *et al.*, 2017a), due to the role played by isoprene as a mobile antioxidant in stabilizing photosynthetic membranes (Velikova and Loreto, 2005).

The biomass yield of *A. donax* is associated with the density, weight and height of stems (Cosentino *et al.*, 2006; Angelini *et al.*, 2009). Indeed, the greater yield of the Sicilian ecotype in trials was related to increased stem height (Haworth *et al.*, 2017c). Observations of the Moroccan and Tuscan ecotypes grown in pots found differences in the morphology of xylem vessels (but not stem height), with the Moroccan ecotype possessing larger vessels and increasing the proportion of larger

vessels under drought stress. In contrast, the Tuscan ecotype reduced xylem vessel diameter, thus lowering hydraulic conductivity and potentially increasing resistance to xylem embolism during episodes of reduced water availability or high evapotranspirative demand (Haworth *et al.*, 2017b). Stem morphological parameters have been shown to be heritable traits in *A. donax* (Pilu *et al.*, 2014), suggesting that selection based upon stem morphology may enable the development of *A. donax* varieties on the basis of yield and drought tolerance characteristics.

We conducted a common garden experiment in Catania, Sicily, Italy, to assess the morphological and physiological characteristics of the Moroccan, Sicilian and Tuscan *A. donax* ecotypes and whether any variation in traits could be utilized in the development of more productive and/or drought-tolerant genotypes (a so-called ‘ideotype’: Donald, 1968). The aims of this study were to: (1) monitor leaf gas exchange, isoprene emission and photosynthetic capacity in the three ecotypes under rain-fed and irrigated conditions at key intervals related to soil water availability throughout the growing season; (2) investigate whether any variation in morphological traits influenced biomass yield in irrigated and rain-fed plots of the three *A. donax* ecotypes; and (3) assess whether any physiological and/or morphological characteristics of the *A. donax* ecotypes may be desirable in terms of enhanced growth and biomass yield and/or drought tolerance in the context of the development of *A. donax* as a viable biomass crop for cultivation in drought-prone rain-fed marginal lands.

MATERIALS AND METHODS

Plant material and field site

Rhizomes were collected from single clonal stands in Marrakesh, Morocco (30 °C mean summer temperature, approx. 200 mm precipitation per annum), the slopes of Mount Etna in Sicily, Italy (26 °C mean summer temperature, approx. 400 mm precipitation) and Sesto Fiorentino, Florence, Italy (mean summer temperature of 23 °C, approx. 800 mm precipitation), during March 2015. After collection, the rhizomes were cut into portions 20 cm in length (approx. 200 g in weight) with at least one visible growing bud per rhizome. The rhizomes were planted in six 4.0 × 3.0 m sized plots for each *A. donax* ecotype at a distance of 50 cm between plants within rows 0.8 m apart with six plants per row (a total of 30 plants per plot at a density of 2.5 plants m⁻²) at the agricultural research station of the University of Catania (37°25’N, 15°03’E; 10 m a.s.l.). To minimize edge effects, a 2.4 m thick border of three rows of *A. donax* was planted around the experimental field. To allow the plants to establish, irrigation equivalent to 100 % of evapotranspiration (ET_c) was applied to all plants in 2015. In February 2016, on day 45 of the study (day 1 being 1 January 2016) the stems were cut at a height of 10 cm and new stems were allowed to develop for the 2016 growing season. In the 2016 growing season, irrigation of half of the experimental field was stopped, while the remainder of the field continued to receive supplementary irrigation (i.e. three irrigated plots and three rain-fed plots for each ecotype) equivalent to 100 % of ET_c determined as:

$$ET_C = E_o \times K_p \times K_c$$

where E_o is the evaporation of water from a class-A pan (mm); K_p is the pan coefficient; and K_c is the crop growth stage (ranging from 0.5 to 1.6). Daily rainfall was subtracted from the daily calculation of water to be supplied as irrigation (Allen et al., 1998). Stem height, the total number of stems per rhizome and the number of green leaves on five stems per plot were recorded on days 96, 119, 152, 181, 211, 255, 305 and 415. To be consistent with standard commercial farming practices used in the cultivation of *A. donax*, the final harvest of the *A. donax* plants was conducted on day 415 (February 2017) as this is the period of the year when the moisture content of the stems is lowest and drying of the plants would require the least energy (Cosentino et al., 2006). Yield as dry matter was calculated by collecting 1 m² sub-samples of the stems and leaves from the plots of each ecotype and treatment, and then oven drying them at 105 °C until their weight remained constant. Soil samples were collected from a depth of 0–90 cm, and the soil water content was determined gravimetrically by drying the soil in a ventilated oven at 105 °C (Killi et al., 2014). The soil at the site is a clay-rich xerofluent soil. Full details of the location are provided in Cosentino et al. (2006).

Leaf gas exchange measurements

Leaf gas exchange analyses were performed in four measurement periods during the growing season: first (days 123–134), second (days 200–211), third (days 249–260) and fourth (days 298–09) (Fig. 1). All measurements were performed *in situ* using a LiCor Li6400XT plant photosynthesis system connected to a 6400-40 leaf chamber fluorimeter cuvette (LiCor, Lincoln, NE, USA). Simultaneous point measurements of P_N , G_s , the internal sub-stomatal concentration of CO₂ (C_i) and the actual quantum efficiency of PSII (Φ_{PSII}) (Genty et al., 1989) were performed on the second uppermost fully expanded leaf on two plants from the centre of each plot. The following environmental conditions were set in the cuvette: 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR; 10 % blue and 90 % red light), 400 ppm [CO₂], leaf temperature set to 30 °C (tight control of leaf temperature under field conditions is difficult: leaf temperature varied from 25.3 to 36.1 °C with a mean value of 29.9 °C and s.d. of 2.3 °C) and a relative humidity (RH) of 60 %. To reduce diffusive leaks through the chamber gasket, a supplementary gasket was added and the IRGA (infrared gas analyser) exhaust air was fed into the space between the chamber and the supplementary external gasket. To determine Φ_{PSII} , the multiphase fluorescence setting was used with an initial saturating pulse of 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. However, it was not possible to estimate mesophyll conductance (G_m) of the *A. donax* plants using the variable J method (Harley et al., 1992), possibly due to underestimation of maximal fluorescence under light-adapted conditions (F_m') (Loriaux et al., 2013) or limitation of the approach associated with species with high G_s and G_m where the difference between the internal sub-stomatal concentration of [CO₂] and that within the chloroplast envelope is less apparent (Loreto et al., 1992).

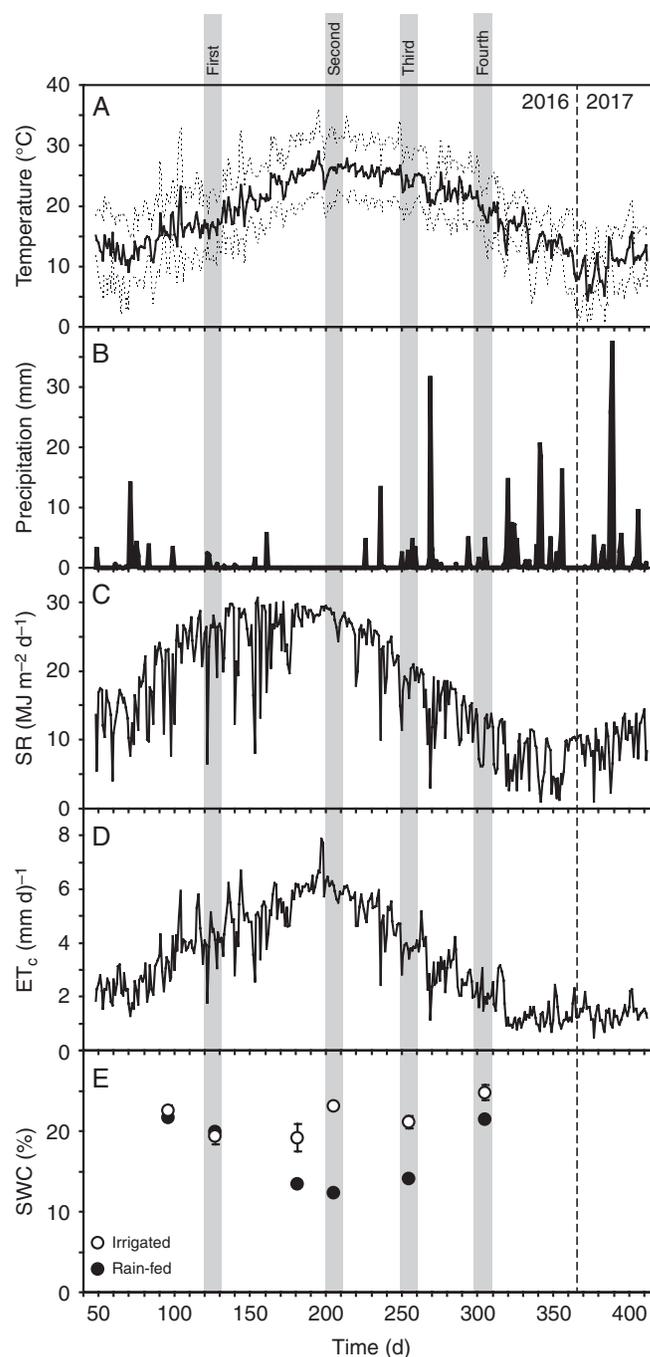


FIG. 1. Growth conditions during the study period: (A) daily temperature at the site – the upper dashed line shows the maximum daily temperature, the solid line shows the mean daily temperature and the lower dashed line shows the minimum daily temperature; (B) daily precipitation; (C) daily solar radiation; (D) evapotranspiration (ET_c); and (E) gravimetric measurements of soil water content taken at the mid-point of each measurement period from the irrigated and rain-fed portions of the experimental field. The four measurement periods are marked by horizontal grey shading. Day 0 was considered to be 1 January 2016. The previous year's (2015 growing season) *A. donax* was harvested on day 45. The dashed vertical line at day 366 marks the end of the 2016 calendar year.

The same leaves used for point measurements of gas exchange and chlorophyll fluorescence were also used in determination of the response of P_N to increasing C_i . The [CO₂] within the leaf

cuvette was lowered to $50 \mu\text{mol mol}^{-1}$ for 30 min to open stomata fully and remove any diffusive limitations to P_N before $[\text{CO}_2]$ was increased in stages every 3–4 min after P_N had stabilized ($[\text{CO}_2]$ steps: 50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and $2000 \mu\text{mol mol}^{-1}$) (Centritto *et al.*, 2003). Leaf temperature remained at 30°C and RH at 60 % throughout the P_N - C_i response curve. The maximum carboxylation rate of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) ($V_{c_{\max}}$), the maximum rate of electron transport for regeneration of ribulose-1,5-bisphosphate (RuBP) (J_{\max}), triose phosphate utilization (TPU) and mesophyll conductance (G_m) were calculated from the P_N - C_i curve corrected to a standard temperature of 25°C following Ethier and Livingston (2004). Due to the extremely rapid stomatal closure (Haworth *et al.*, 2018a) observed in drought-stressed *A. donax*, it was not possible to complete the P_N - C_i response curve above a $[\text{CO}_2]$ of $400 \mu\text{mol mol}^{-1}$; stomatal limitations effectively prevented assessment of the biochemical assimilation of $[\text{CO}_2]$ (e.g. Lovelli and Perniola, 2014). Therefore it was only possible to calculate $V_{c_{\max}}$ from the P_N - C_i response in the rain-fed plants under conditions of low soil water content during the second and third measurement period.

Isoprene emission

The emission of isoprene was measured in the field from the same leaves of *A. donax* used for gas exchange analysis under the same environmental settings, but using a LiCor Li6400 fitted with a 6 cm^2 cuvette and LED light unit. When monitoring isoprene emission, air from the cuvette with the enclosed leaf passed through a biphasic adsorbent trap containing 30 mg of Tenax and 20 mg of Carboxen (Gerstel, Mülheim an der Ruhr, Germany). A pump (Elite 5, A. P. Buck, Orlando, FL, USA) was used to pass 2 L of air through each trap at a rate of 200 mL min^{-1} . Measurements of the concentration of isoprene in the ambient air (blanks) were performed using an empty leaf cuvette before and after each measurement. The traps were then stored at 4°C prior to analysis in the laboratory. Isoprene was first desorbed from traps at high temperature and then measured using a gas chromatograph–mass spectrometer (GC-MS; Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) connected to a thermal desorption unit (Gerstel) with an Agilent HP-INNOWAX ($30 \text{ m} \times 0.32 \text{ mm} \times 0.15 \mu\text{m}$) GC column. A 5977A mass selective detector with electron ionization operating at 70 eV was used for analysis. Isoprene was identified through comparison of the retention time and mass spectrum with an isoprene analytical standard (Sigma Aldrich, St. Louis, MO, USA) injected into the GC-MS at different concentrations. The isoprene analytical standard was also used to construct a calibration curve by injecting known concentrations of isoprene into the GC-MS. The data were analysed using Agilent MassHunter Workstation software.

Leaf sampling, determination of foliar relative water content and analysis of starch and sugars

Leaf samples were collected at the end of each measurement period after completion of the leaf gas exchange and chlorophyll

fluorescence measurements. The first fully most expanded leaf was collected from the same two plants in each plot adjacent to the leaf that had been used for physiological analysis ($n = 6$ for each ecotype and treatment). The lower 4–5 cm of each leaf was removed to be used for determination of foliar relative water content (RWC) following the protocol of Diaz-Pérez *et al.* (1995). The remainder of the leaf was flash-frozen in liquid nitrogen before being stored at -80°C prior to analysis of starch and sugar content. To identify and quantify soluble carbohydrates, $3 \times 5 \text{ mL}$ of a solution of EtOH/ H_2O (75/25) was used to extract carbohydrates from 300 mg of fresh leaf tissue. The ethanol was then reduced under a vacuum at 30°C with a Büchi P12 Multivapor unit equipped with a Büchi V-855 vacuum controller (Büchi, Flawil, Switzerland), and the resulting pellet was then rinsed with 2 mL of Milli-Q water. The aqueous extract was then purified by solid–liquid extraction through -CHX and -SAX pre-packed Bond-Elute cartridges (Varian, Harbor City, CA, USA), and the eluate was completely reduced under a vacuum. The samples were then rinsed with ultrapure water and injected in a Series 250 LC binary pump equipped with an LC 30-R1 detector (Perkin-Elmer). Soluble carbohydrates were then separated using a $7.7 \times 300 \text{ mm}$ Hi-Plex Ca column (Agilent Technologies, CA, USA) maintained at $85 \pm 1^\circ\text{C}$; the eluent used was ultra-pure water at a flow rate of 0.8 mL min^{-1} during a 20 min run. Individual carbohydrates were identified by comparison of retention times with those of carbohydrate standards (Sigma Aldrich). Starch was quantified following Chow and Landhäusser (2004) on the pellet of material that was produced during ethanol extraction for the analysis of soluble carbohydrates. Starch was digested into glucose using a mixture of enzymes consisting of 1000 U of α -amylase and 5 U of amyloglucosidase. The resultant glucose was then quantified using peroxidase–glucose oxidase/*o*-dianisidine reagent (Sigma-Aldrich), reading the absorbance at 525 nm after the addition of sulphuric acid.

Statistical analyses

Statistical analyses were performed using SPSS 24 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) with a least significant difference (l.s.d.) post-hoc test was used to assess differences in variance between samples associated with either ecotype or treatment effects, with homogenous groups denoted in figures by the use of identical letters. A multiple regression was used to identify which factors were significantly associated with the yield of the *A. donax* ecotypes.

RESULTS

Over the course of the growing season as temperature (Fig. 1A) and ET_c (Fig. 1D) rose and levels of precipitation fell, soil water content (SWC) in the upper 90 cm soil layer declined from about 23 % to a low of 11 % (Fig. 1E). This reduction in soil water availability corresponded to declines in P_N and G_s in all three *A. donax* ecotypes, but foliar RWC was unaffected by SWC (Fig. 2A–I). When water availability did not limit gas exchange early in the growing season, the *A. donax* ecotypes exhibited similar high rates

of P_N of 25–30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figs 2D–F and 3). Rates of G_s were highest in the Sicilian ecotype during the first measurement period (0.6–0.8 $\text{mol m}^{-2} \text{s}^{-1}$) (one-way ANOVA: $F_{1,34} = 6.688$; $P = 0.015$). However, the G_s values of the irrigated Sicilian plants declined by 38 % over the growing season (Fig. 2H). In contrast, the G_s values of the irrigated

Moroccan and Tuscan ecotypes remained constant during the first three measurement periods. Notably, in the fourth measurement period after the recovery of soil water levels, the rain-fed Moroccan and Tuscan ecotypes exhibited higher G_s , P_N and ΦPSII than their irrigated counterparts (although G_s was higher in the rain-fed than in the irrigated

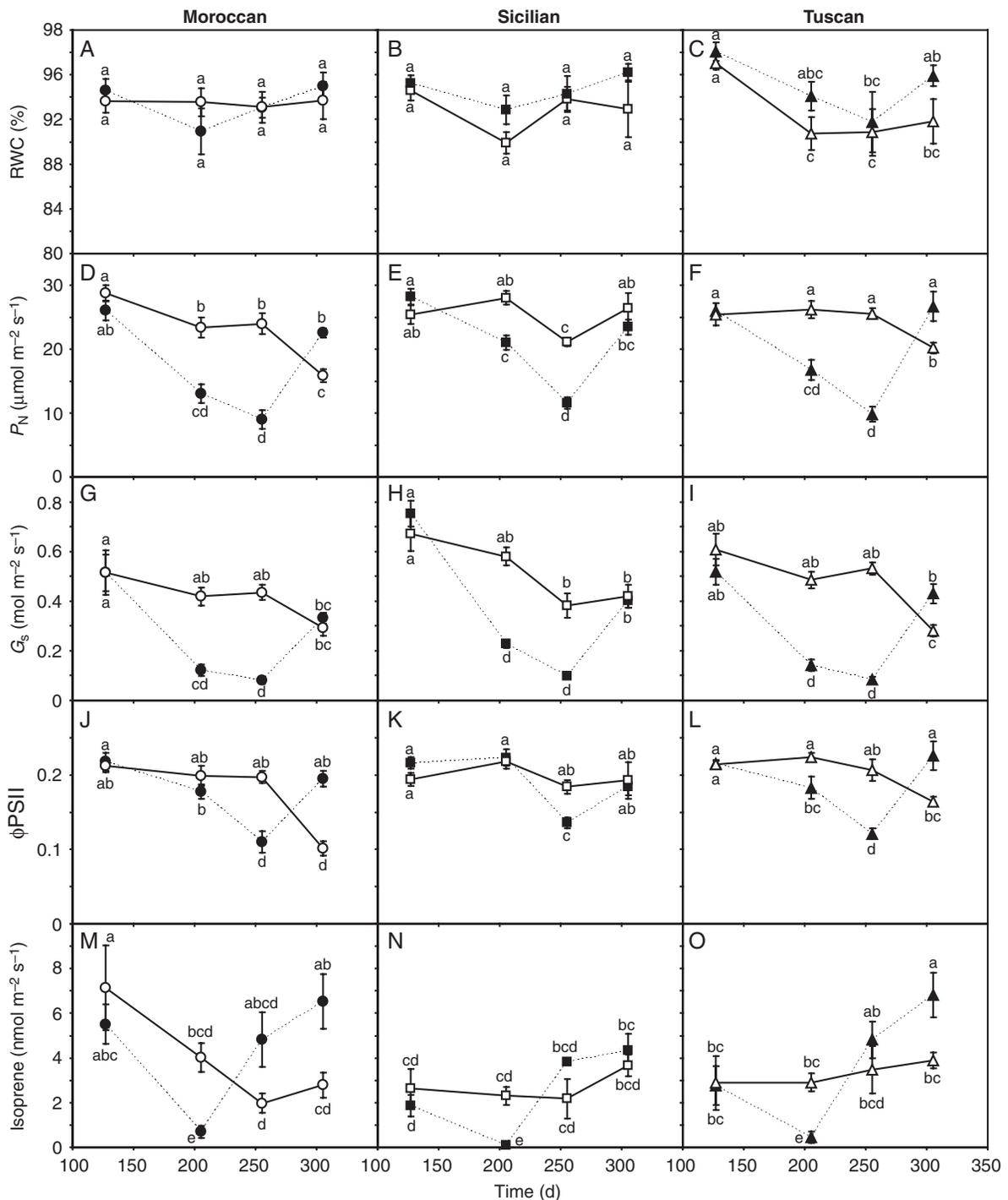


FIG. 2. Foliar relative water content (RWC) (A–C), photosynthesis (P_N) (D–F), stomatal conductance to water vapour (G_s) (G–I), the actual quantum efficiency of photosystem II (ΦPSII) (J–L) and isoprene emission (M–O) of Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated (solid line, open symbols) and rain-fed (dashed line, filled symbols) conditions. Error bars indicate 1 s.e. standard error either side of the mean. Letters indicate homogenous groups for each parameter using a one-way ANOVA with an l.s.d. post-hoc test.

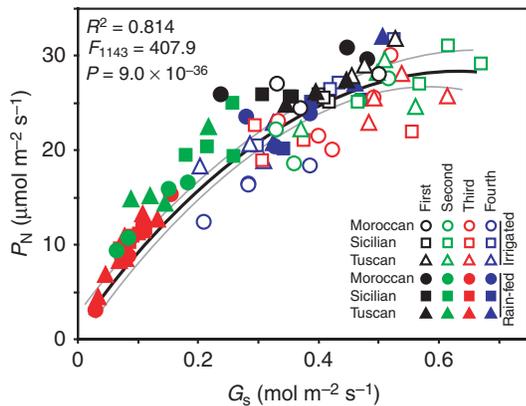


FIG. 3. The relationship of photosynthesis (P_N) to stomatal conductance (G_s) in Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated and rain-fed conditions during the four measurement periods. Non-linear regression was used to assess the relationship. The solid black line represents the line of best fit and the grey lines either side of the linear regression indicate 95 % confidence intervals of the regression line.

Moroccan ecotype, this was not significant). In contrast, the irrigated and rain-fed Sicilian plants exhibited similar G_s , P_N and Φ PSII values during the final measurement period (Fig. 2D–H). The rain-fed Moroccan and Tuscan plants showed an increase in isoprene emission beginning in the third period and continuing into the fourth, where they were statistically greater than the emission rates of their irrigated counterparts (one-way ANOVA: $F_{1,22} = 10.283$; $P = 0.018$). In contrast, the rain-fed Sicilian ecotype, while showing a similar pattern of increased isoprene emission, generally exhibited lower rates of isoprene emission in the third and fourth measurement periods that did not differ from values observed in irrigated plants (Fig. 2M–O) (one-way ANOVA: $F_{1,10} = 3.851$; $P = 0.097$). Photosynthesis was strongly related to G_s in the *A. donax* ecotypes at all of the measurement points throughout the growing season (Fig. 3) (linear regression: $R^2 = 0.814$; $F_{1,143} = 407.9$; $P = 9.0 \times 10^{-36}$).

Early in the growing season when SWC was high (Fig. 1E), the photosynthetic capacity of the *A. donax* ecotypes was remarkably similar when assessed using P_N-C_i response curves (Fig. 4A–D). Drought stress did not strongly affect $V_{c_{max}}$ with the exception of the third measurement period (Fig. 4A–C), which may be considered the maximum extent of stress, where the $V_{c_{max}}$ values of the rain-fed plants were significantly lower than their irrigated counterparts in the Moroccan (one-way ANOVA: $F_{1,10} = 27.793$; $P = 0.0004$) and Tuscan (one-way ANOVA: $F_{1,10} = 21.723$; $P = 0.0034$) ecotypes. However, $V_{c_{max}}$ values of the rain-fed Moroccan and Tuscan plants were 53 and 27 % greater, respectively, than those of their irrigated counterparts during the fourth measurement period (Fig. 5A–C). Due to the rapid stomatal closure of *A. donax*, it was not possible to complete the P_N-C_i curves of the rain-fed plants during the second and third periods where SWC was at its lowest (Fig. 1E). The J_{max} of irrigated Moroccan plants showed a progressive decline throughout the growing season, and by the final measurement period J_{max} values of the rain-fed plants were 46 % higher than in the irrigated *A. donax* (Fig. 4D) (one-way ANOVA: $F_{1,10} = 14.889$; $P = 0.012$). In contrast, levels of J_{max} in the irrigated Sicilian and Tuscan ecotypes were relatively constant

throughout the growing season, with non-statistically significant differences between irrigated and rain-fed plants during the final fourth measurements (Fig. 4E, F). The TPU of irrigated plants was greatest in the Moroccan ecotype during the third measurement period, while the highest TPU values occurred in the second measurement period in the Sicilian and Tuscan ecotypes. Rain-fed Sicilian and Tuscan plants showed 102 and 83 % higher TPU values, respectively, than irrigated plants in the fourth period, whereas there was no difference in TPU values of the rain-fed and irrigated Moroccan plants (Fig. 4G–I) (one-way ANOVA: Sicilian, $F_{1,10} = 46.115$; $P = 0.001$; Tuscan, $F_{1,10} = 13.168$; $P = 0.015$; Moroccan, $F_{1,10} = 0.004$; $P = 0.954$). Mesophyll conductance of the irrigated plants ranged from 0.196 to 0.273 mol m⁻² s⁻¹ bar⁻¹ during the growing season. Rates of G_m were higher, but not significantly, in the rain-fed Tuscan and Moroccan ecotypes during the final measurement period (Fig. 4J–L).

Analysis of foliar levels of carbohydrates suggested a general trend of declining concentrations of starch and sugars during the growing season in the irrigated *A. donax* plants (Fig. 5). The concentration of starch in the leaves of the rain-fed *A. donax* declined more rapidly than that of their irrigated counterparts, but all were statistically identical by the fourth measurement period (Fig. 5J–L). No differences were observed in the concentration of sucrose between leaves of irrigated and rain-fed *A. donax* plants (Fig. 5D–F). In rain-fed plants, levels of fructose generally declined over the first three measurement periods before increasing in the fourth period (Fig. 5G–I). The concentration of glucose also fell from the first to the second period before rising in the third and fourth measurement periods in the rain-fed *A. donax* ecotypes (Fig. 5A–C).

Stem height (Fig. 6A–C), the total number of leaves (Fig. 6D–F) and the number of green leaves (Fig. 6G–I) of the irrigated and rain-fed plants were broadly consistent from day 96 to 181 in the three *A. donax* ecotypes. After day 181, the irrigated plants showed increased growth relative to their rain-fed counterparts. However, it is noteworthy that from day 255 to 415, the greatest increases in stem height (39.3 cm) and leaf number (11.2) occurred in the rain-fed Moroccan plants, while growth had slowed in the irrigated *A. donax* (Fig. 6A). At the end of the growing season, rain-fed plants from all three ecotypes had green leaves, while the leaves of their irrigated counterparts were entirely senesced (Fig. 6G–I). At the final harvest, there was no significant difference in the number of leaves (Fig. 7A) or stem height (Fig. 7C) between irrigated and rain-fed plants of the same ecotype. Irrigation did not significantly affect density of stems; the Tuscan ecotype exhibited the highest density of stems and the Moroccan the lowest (Fig. 7B). Stem height was greatest in the Moroccan ecotype and lowest in the Tuscan (Fig. 7C), and these ecotypic differences corresponded to biomass yield, with the Moroccan *A. donax* being the most productive (Fig. 7D). Irrigation induced increased yield in all three ecotypes. However, this stimulation of growth was non-significant in all three ecotypes, ranging from 7.7 to 19.4 %. A multiple regression was used to predict biomass yield of *A. donax* from ecotype, irrigation regime, morphological characteristics (shown in Fig. 7), Φ PSII and gas exchange parameters (shown in Figs 2 and 4). Yield was significantly predicted ($R^2 = 0.991$; $F_{4,26} = 34.638$; $P = 0.002$) by irrigation ($P = 0.006$), stem height ($P = 0.033$), P_N in the third measurement period

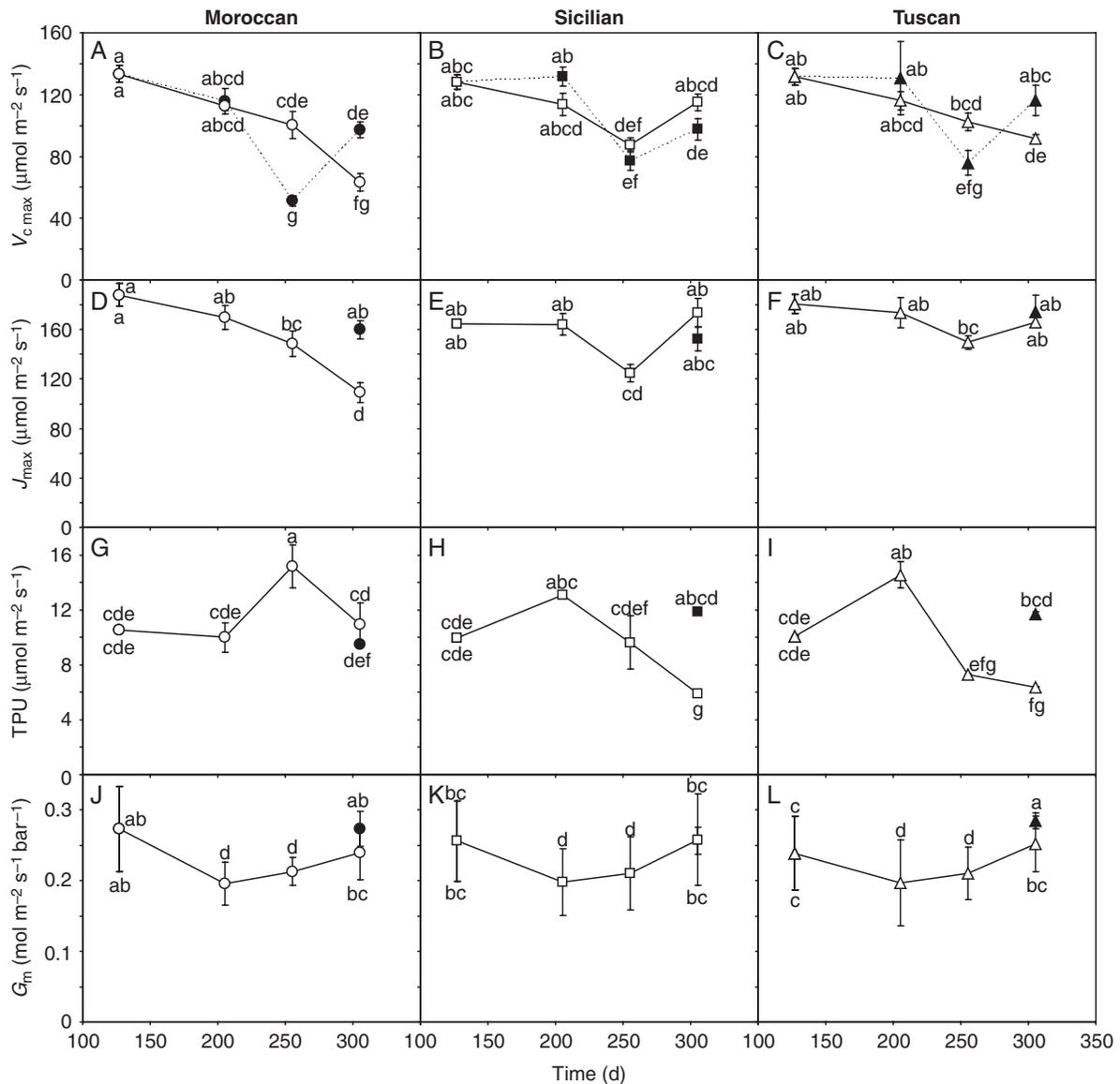


FIG. 4. Parameters calculated from the P_N-C_i response curves of Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated (solid line, open symbols) and rain-fed (dashed line, filled symbols) conditions: (A–C) the maximum carboxylation rate of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) ($V_{c\max}$); (D–F) the maximum rate of electron transport for regeneration of ribulose-1,5-bisphosphate (RuBP) (J_{\max}); (G–I) triose phosphate utilization (TPU); and (J–L) mesophyll conductance to CO_2 (G_m). The P_N-C_i response curves are available in [Supplementary Data Fig. S1](#). Error bars indicate 1 s.e. either side of the mean. Letters indicate homogenous groups for each parameter using a one-way ANOVA with an l.s.d. post-hoc test.

($P = 0.032$), and both P_N ($P = 0.011$) and G_s ($P = 0.018$) during the fourth period.

DISCUSSION

Low genetic variability in *A. donax* has constrained efforts to characterize ecotypic differences that may be utilized in the development of commercially viable productive and/or drought-tolerant ideotype varieties (Valli et al., 2017). This study has confirmed previous observations of remarkable similarity in photosynthetic gas exchange parameters between ecotypes (e.g. Sánchez et al., 2015; Haworth et al., 2017c; Zegada-Lizarazu et al., 2018) and identified differences in the pattern of changes in photosynthetic capacity throughout the growing season.

Morphological variation that may account for the differences in yield observed between *A. donax* ecotypes in this (Fig. 7D) and other studies (e.g. Cosentino et al., 2006; Sánchez et al., 2015; Haworth et al., 2017c) was identified.

The three *A. donax* ecotypes examined in this study showed high rates of P_N and G_s similar to previous gas exchange measurements on field-grown (e.g. Cosentino et al., 2016; Haworth et al., 2017a) and naturally occurring (Rossa et al., 1998) stands of *A. donax*. Early in the growing season, when soil water availability was not limiting to growth, the *A. donax* ecotypes showed very similar levels of P_N , G_s and Φ_{PSII} when determined on a leaf area basis (Fig. 2). This lack of variability in gas exchange and chlorophyll fluorescence parameters confirms the findings of previous analyses of the same (Cosentino

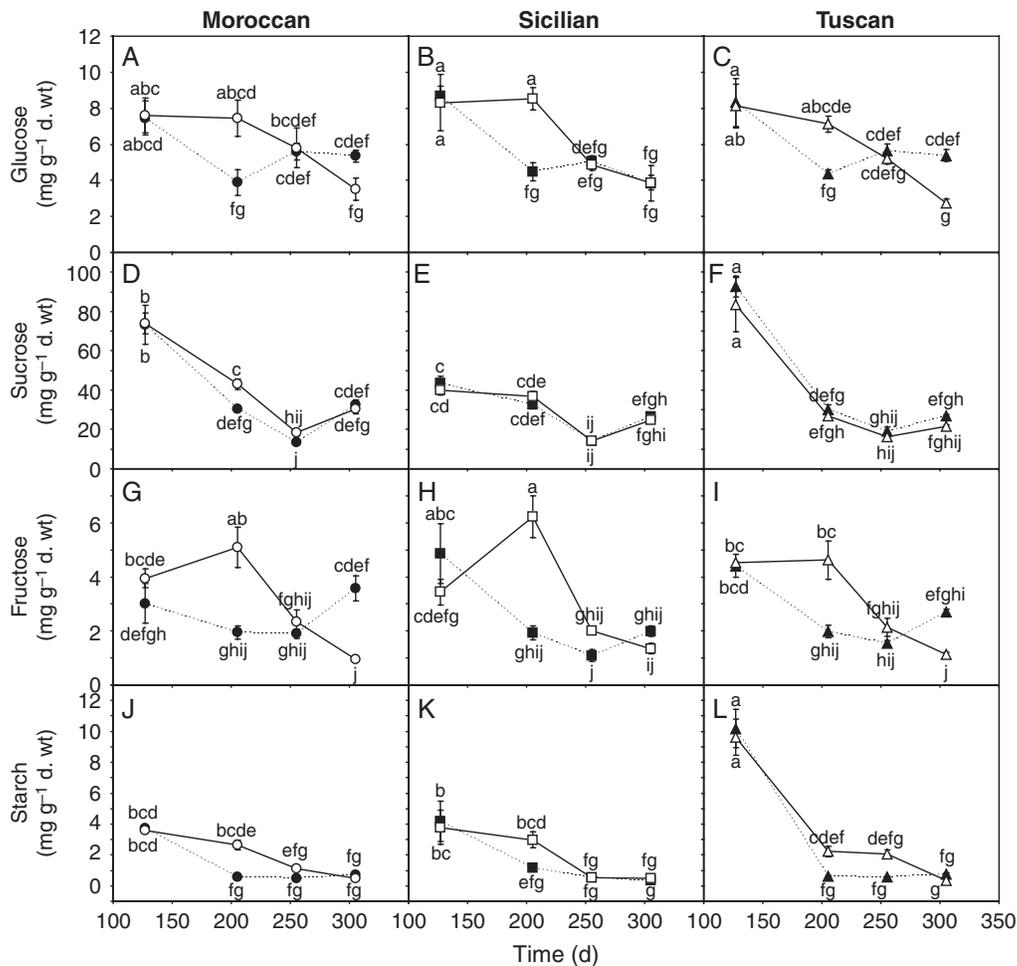


FIG. 5. Concentrations of glucose (A–C), sucrose (D–F), fructose (G–I) and starch (J–L) in the leaves of the Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated (solid line, open symbols) and rain-fed (dashed line, filled symbols) conditions. Error bars indicate 1 s.e. either side of the mean. Letters indicate homogenous groups for each parameter using a one-way ANOVA with an l.s.d. post-hoc test.

et al., 2016; Haworth et al., 2017b, c; Zegada-Lizarazu et al., 2018) and different (Sánchez et al., 2015) *A. donax* ecotypes. The photosynthetic capacity observed in the three *A. donax* ecotypes during the same initial measurement period (Fig. 4) was very similar, accounting for the similarity in P_N rates under the same growing conditions (Fig. 2D–F) and indicative of the lack of genetic variability found in the species (Khudamrongsawat et al., 2004; Mariani et al., 2010; Pilu et al., 2014). Notably, after the first measurements on irrigated *A. donax*, we observed variation between the ecotypes in photosynthetic capacity (Fig. 4) which corresponded to point measurements of P_N (Fig. 2D–F). The retention of photosynthetic capacity throughout the growing season, as observed in the Sicilian ecotype (Figs 2E and 4B), may be a feature to be exploited in achieving higher P_N and growth in *A. donax*. The decline in values of $V_{c_{max}}$ and J_{max} throughout the year in the irrigated Moroccan *A. donax* are indicative of the downregulation of photosynthetic capacity (e.g. Van Oosten et al., 1994; Wingler and Roitsch, 2008). However, foliar concentrations of sugars and starch in the Moroccan *A. donax* also fell over the course of the growing season, and similar patterns were observed in the carbohydrate metabolism of the Sicilian and Tuscan ecotypes.

The stability of P_N values at high $[CO_2]$ in the P_N-C_i response curves indicated the absence of any feedback limitation to P_N (Sharkey et al., 2007). Furthermore, in the irrigated *A. donax* plants, TPU did not follow variations in $V_{c_{max}}$ and J_{max} (Fig. 4) (cf. Yang et al., 2016). These findings suggest that downregulation of photosynthetic capacity in the leaves of the *A. donax* ecotypes was not associated with the availability of phosphorylated compounds (Sharkey and Vanderveer, 1989) or with the inhibition of the expression of photosynthetic enzymes (Sheen, 1990; Van Oosten et al., 1994) related to increased abundance of sugars (e.g. Wingler and Roitsch, 2008). One possible explanation for the decline in sugar and starch concentrations, despite high P_N , in the leaves of *A. donax* over the course of the year (Fig. 5) may be the high availability of sinks for photosynthate (e.g. Paul and Driscoll, 1997) in the form of rapid stem elongation (Fig. 6) (Cosentino et al., 2016) or storage within the rhizome (Proietti et al., 2017). The higher levels of foliar starch found earlier in the year may reflect the impact of carbohydrates translocated from the rhizome to the stem to fuel rapid growth alongside high P_N leading to a build-up of carbohydrates stored as starch (e.g. Asaeda et al., 2006). Future work should focus on the concentration of sugars in leaves along the stem to

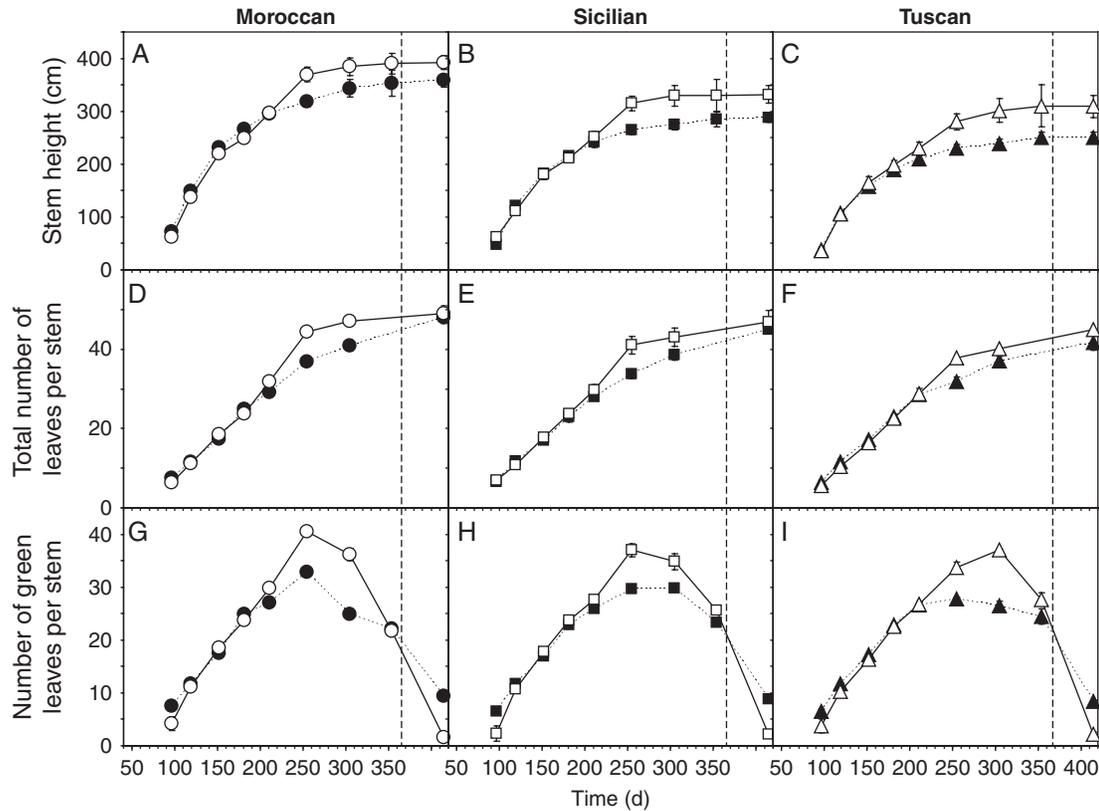


FIG. 6. Stem height (A–C), the total number of leaves per stem (D–F) and the number of green leaves per stem (G–I) of the Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated (solid line, open symbols) and rain-fed (dashed line, filled symbols) conditions during the 2016 growing season. Error bars indicate 1 s.e. either side of the mean. The dashed vertical line at day 366 marks the end of the 2016 calendar year.

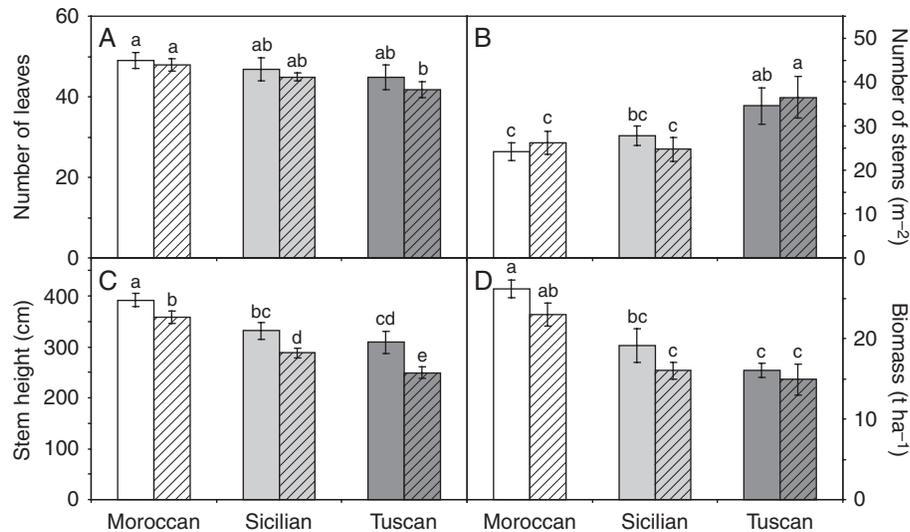


FIG. 7. Morphological parameters of the Moroccan, Sicilian and Tuscan ecotypes of *A. donax* grown under irrigated (open fill) and rain-fed (hatched fill) conditions at the final harvest on day 415: (A) number of leaves; (B) density of stems; (C) stem height; and (D) biomass. Error bars indicate 1 s.e. either side of the mean. Letters indicate homogenous groups for each parameter using a one-way ANOVA with an l.s.d. post-hoc test.

allow development of a more representative analysis of photosynthetic capacity and carbohydrate metabolism in *A. donax*.

Drought stress imposed by growth under rain-fed conditions induced statistically similar declines in both P_N and G_s in the

three *A. donax* ecotypes (Fig. 2D–I). Reduced P_N under rain-fed conditions appeared to be largely the result of diffusive constraints associated with stomatal closure (Fig. 3) (Romero-Munar *et al.*, 2017), illustrating the highly functional and

effective stomatal control found in *A. donax* (Haworth et al., 2018a). This stomatal control probably accounted for the ‘isohydric’ behaviour of the rain-fed plants (Sade et al., 2012), as foliar RWC did not decline as drought progressed (Fig. 2A–C). Drought stress did not significantly affect the $V_{c_{max}}$ of the rain-fed plants in the second measurement period, despite the lowest SWC values being observed during this interval (Fig. 1E). The maximum rate of carboxylation of RubisCO did significantly decline in the rain-fed Moroccan and Tuscan ecotypes by the third measurement period; moreover, $V_{c_{max}}$ in both the rain-fed and irrigated Sicilian plants was lower in this period (Fig. 4A–C). This suggests that higher temperature (the third measurement period occurred after approx. 50 d of sustained high temperatures: Fig. 1A) was a contributory factor in reduced $V_{c_{max}}$ due to lower specificity of RubisCO for CO_2 (Jordan and Ogren, 1984), reduced activity of RubisCO activase (Feller et al., 1998) and lower solubility of CO_2 (Berry and Björkman, 1980). Nonetheless, a combination of drought and high temperatures may have also negatively affected other processes. The thylakoid membranes within the chloroplast envelope are sensitive to damage induced by heat stress (Crafts-Brandner and Salvucci, 2002) and the excess energy associated with drought as photochemistry declines (Kalaji et al., 2016). The reduction in Φ_{PSII} values of the rain-fed plants at the third measurement period may be consistent with reduced PSII performance due to thermal and photo-oxidative damage to the thylakoid membranes associated with high temperatures and drought (Flexas and Medrano, 2002; Killi et al., 2016; Haworth et al., 2018b). This fall in Φ_{PSII} coincided with a rise in the emission of isoprene (Fig. 2M–O), possibly related to the function of isoprene as a mobile antioxidant stabilizing and protecting photosynthetic membranes (Velikova and Loreto, 2005). Analysis of *A. donax* suggests that the methylerythritol phosphate (MEP) pathway regulating isoprene synthesis is co-ordinated with the methionine pathway under drought stress, with increased formation of compounds such as dimethylsulphoniopropionate (Haworth et al., 2017a) which also serves as a protective antioxidant (Sunda et al., 2002; Husband and Kiene, 2007; Husband et al., 2012). The rain-fed Sicilian *A. donax* exhibited a less pronounced reduction in Φ_{PSII} (Fig. 2K) and lower isoprene emission than the Moroccan and Tuscan ecotypes. This may suggest that the Sicilian ecotype possessed enhanced capacity in terms of protective heat-shock proteins (e.g. Heckathorn et al., 1998) and/or antioxidants (e.g. Sekmen et al., 2014) to mitigate the impact of heat and drought stress (e.g. Killi et al., 2016). The Moroccan *A. donax* has also been shown to emit more isoprene, but dissipate less energy as heat via non-photochemical quenching, than the Tuscan ecotype under less severe moderate drought stress (Haworth et al., 2017a). An inverse relationship between isoprene emission and non-photochemical quenching is found in many stressed and non-stressed plant species (Pollastri et al., 2014), suggesting that isoprene effectively regulates the electron transport flow in the photosynthetic membranes. This may suggest that further analysis of the protective physiologies of different *A. donax* ecotypes may enable identification of attributes to develop increased tolerance to drought and heat stress.

An inverse relationship was observed between stem height and density in the three *A. donax* ecotypes (Fig. 7). The largest biomass yield was observed in the Moroccan ecotype

which exhibited the tallest but also the lowest density of stems. Moreover, stem height was the only morphological parameter that significantly contributed to yield and, as one of the few heritable traits found in *A. donax* (Pilu et al., 2014), may represent a key attribute for exploitation in attempts to increase biomass yield. The Moroccan ecotype has been shown to have more xylem vessels with a higher diameter than the Tuscan *A. donax* under both well-watered and drought-stressed conditions (Haworth et al., 2017b). The greater cross-sectional area of the xylem vessels in the Moroccan ecotype may reflect the influence of selective pressures induced by growth in an arid environment where water is available for growth for relatively short periods. In fact, the higher hydraulic conductivity of the Moroccan *A. donax* (Haworth et al., 2017b) may favour rapid growth (Fan et al., 2012) to exploit the brief intervals where conditions are suitable for growth (Grime, 1977). The density of wood is inversely related to the diameter of xylem vessels (Preston et al., 2006). Indeed, the Moroccan stems have been shown to possess a lower structural density than their Tuscan counterpart (Haworth et al., 2017b). Moreover, the differences in water transport structures may also relate to the chemical composition of the stems in terms of the proportions of lignin, cellulose and hemicellulose (e.g. Novaes et al., 2010), a consideration for future work if the *A. donax* material is to be used as a source for bio-compounds (Yoshida et al., 2008; Saikia et al., 2015). It is noteworthy that while irrigation increased biomass (Fig. 7D) and the rate of growth (Fig. 6) of the *A. donax* ecotypes over the course of the growing season, irrigation did not result in a statistically significant increase in overall biomass production. The use of supplementary irrigation in the cultivation of *A. donax* is therefore difficult to justify. A key finding of this study is that in the irrigated *A. donax*, rates of P_N began to decline (Fig. 2D–F) as downregulation of photosynthetic capacity (Fig. 4) occurred, and the plants no longer increased in height (Fig. 6). In contrast, after drought stress as soil water availability increased, the rain-fed *A. donax* recovered P_N with photosynthetic capacity, and extended their growth later into the year, allowing the rain-fed plants to minimize the growth benefit of irrigation. This is particularly notable in terms of the total number of leaves and the number of photosynthetically active green leaves in the rain-fed plants (Fig. 6D–I), allowing increased potential for ‘whole-plant’ light harvesting and growth later in the year (e.g. Koyama and Kikuzawa, 2009). This study replicates the harvesting regime in late February to early March that would most probably be utilized in commercial cultivation of *A. donax* for biomass purposes (Cosentino et al., 2006; Monti et al., 2015); this would suggest that supplementary irrigation of *A. donax* is unnecessary and that *A. donax* is suitable for growth in drought-prone rain-fed marginal lands in regions with a warm to hot climate.

We observed an average above-ground biomass yield of 22.3 t ha⁻¹. This is consistent with values reported for *A. donax* established for 2 years (Hidalgo and Fernandez, 2001; Cosentino et al., 2006; Angelini et al., 2009), although lower than yield values of 38–40 t ha⁻¹ observed in more established plots (>4 years old) (Angelini et al., 2009; Dragoni et al., 2015). The results of this study have shown that while the *A. donax* ecotypes from contrasting habitats are largely similar in terms of maximum photosynthetic capacity (Figs 2 and 4) and have highly effective

stomatal control (Figs 2 and 3), it is possible to unravel physiological and morphological variation between the ecotypes that may enable the development of more productive and/or drought-tolerant commercial varieties of *A. donax*. Stem height and maintenance of P_N rates through the retention of photosynthetic capacity late into the growing season appear to be key attributes towards more productive *A. donax*. None of the ecotypes examined exemplified such an ideotype for *A. donax* (an ideotype being an ideal plant form in line with the concept proposed by Donald, 1968). While the Moroccan ecotype produced the longest stems and largest yield, it also showed the most pronounced downregulation of photosynthetic capacity (Fig. 4) and reduction in leaf gas exchange (Fig. 2D) later in the year. The Sicilian ecotype retained photosynthetic capacity (Fig. 4H) and P_N (Fig. 2E) later in the year, and also emitted less previously fixed carbon as isoprene (Fig. 2A) (e.g. Brilli et al., 2007), but developed shorter stems than its Moroccan counterpart. Stem elongation in *A. donax*, as in many grasses, continues until flowering (Pollock and Cairns, 1991; Cosentino et al., 2007). Given that the flowers of *A. donax* are sterile (Boland, 2006), selection on the basis of delayed or absence of flowering (variation in flowering was observed in Cosentino et al., 2006) may be a viable strategy to increase stem height by prolonging growth later into the year (Clifton-Brown and Lewandowski, 2002). Genetic (Fu et al., 2016) and mutagenic (Valli et al., 2017) approaches utilizing the transcriptome of *A. donax* may offer the best way forward in terms of the development of a highly productive and drought-tolerant *A. donax* ideotype for cultivation in drought-prone rain-fed marginal lands.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of Figure S1: response curves of photosynthesis to the sub-stomatal concentration of $[CO_2]$ (C_i) of Moroccan, Sicilian and Tuscan ecotypes of *Arundo donax*.

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