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Physiological responses of *Arundo donax* ecotypes to drought: a common garden study

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Abstract

Genetic analyses have suggested that the clonal reproduction of *Arundo donax* has resulted in low genetic diversity. However, an earlier common garden phenotyping experiment identified specimens of *A. donax* with contrasting biomass yields (ecotypes 6 and 20). We utilized the same well-established stands to investigate the photosynthetic and stress physiology of the *A. donax* ecotypes under irrigated and drought conditions. Ecotype 6 produced the largest yields in both treatments. The *A. donax* ecotypes exhibited identical high leaf-level rates of photosynthesis (P_N) and stomatal conductance (G_s) in the well-watered treatment. Soil drying induced reductions in P_N and G_s, decreased use of light energy for photochemistry, impaired function of photosystem II and increased heat dissipation similarly in the two ecotypes. Levels of biologically active free-abscisic acid (ABA) and fixed glycosylated-ABA increased earlier in response to the onset of water deficit in ecotype 6; however, as drought progressed, the ecotypes showed similar increases in both forms of ABA. This may suggest that because of the low genetic variability in *A. donax* the genes responding to drought might have been activated similarly in the two ecotypes, resulting in identical physiological responses to water deficit. Despite the lack of physiological ecotypic differences that could be associated with yield, *A. donax* retained a high degree of P_N and biomass gain under water deficit stress conditions. This may enable utilization of *A. donax* as a fast growing biomass crop in rain-fed marginal lands in hot drought prone climates.

Keywords: abscisic acid, biomass, bioenergy, isoprene, photosynthesis, stomatal conductance, water deficit

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Introduction

The development of giant reed (*Arundo donax*) as a perennial biomass crop in drought-prone marginal lands requires physiological analysis of its responses to water deficit and identification of those traits that confer resistance to drought. The rapid growth of *A. donax* is sustained by high leaf-level rates of photosynthesis (P_N) and accompanied by significant transpirative water-loss due to concurrent stomatal opening (Rossa *et al.*, 1998; Haworth *et al.*, 2016). However, the clonal reproduction of *A. donax* has resulted in a low genetic diversity (Ahmad *et al.*, 2008; Mariani *et al.*, 2010; Saltonstall *et al.*, 2010; Pilu *et al.*, 2014) that may account for observations of similarities in productivity and drought tolerance in plants collected from different regions (Cosentino *et al.*, 2006; Sánchez *et al.*, 2015). Low genetic variability may also constrain the development of more productive and/or drought tolerant *A. donax* varieties. Indeed, the genetic background of *A. donax* is much less explored than other biomass crops (e.g. Chen *et al.*, 1997; Souch & Stephens, 1998; Zhang *et al.*, 2004). For example, it is currently unclear whether sufficient genetic variation occurs in *A. donax* to warrant the use of genotype or ecotype as a descriptive term (e.g. Khudamrongsawat *et al.*, 2004; Ahmad *et al.*, 2008). Here, we will use the term ecotype in respect of the ecological differences in habitat and any associated genetic variation represented by the *A. donax* accessions. Analysis of the photosynthetic and stress physiology of *A. donax* ecotypes under field conditions is crucial to the characterization of the species and understanding its potential as a biomass crop.

Soil drying induces an increase in the flow of abscisic acid (ABA) from the roots to the leaves (Davies &

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Zhang, 1991), and increased conversion of biologically inactive 'fixed' glucose-conjugated ABA (GE-ABA) in the vacuole to biologically active free ABA within the cytosol of the leaves (Dietz *et al*., 2000; Seiler *et al*., 2011). This increased foliar concentration of [ABA] causes stomatal closure (Zhang & Davies, 1990) reducing transpirative water-loss, but also leading to a reduction in the uptake of CO_2 for photosynthesis (Pinheiro & Chaves, 2011; Lauteri *et al*., 2014). *Arundo donax* grown in 5 L pots exhibited declines in P_N from ~20–30 to ~1–2 μmol m^{-2} s^{-1} that corresponded to reductions in G_s of 0.35–0.45 to ~0.01 mol m^{-2} s^{-1} after constant water deficit was maintained for 60 days (Sánchez *et al*., 2015). *Arundo donax* ecotypes from an arid and warm-humid environment grown in 50 L pots in a common garden experiment exhibited identical declines in P_N and G_s after water was withheld over a three-week period. Despite the contrasting nature of their habitats no difference was observed in leaf gas-exchange, but a difference in xylem traits was recorded, suggesting that despite the low genetic diversity of *A. donax*, some ecotypic differences in response to water deficit do occur (Haworth *et al*., 2016). These studies indicate that *A. donax* possesses highly effective physiological stomatal control to prevent desiccation (Haworth *et al*., 2015, 2016). However, at present there is little information regarding the possible role of ABA in the drought response of *A. donax*.

As P_N decreases under drought, the available photosynthetic sinks for intercepted light energy decline. Consequently, energy dissipation as heat via non-photochemical quenching (NPQ) increases (Demmig-Adams & Adams, 2000). Pot grown *A. donax* exhibited concomitant reductions in foliar relative water content (RWC) and the performance of photosystem II (Nackley *et al*., 2014). Extended drought may degrade the protective systems leading to damage to the photosynthetic apparatus (Pinheiro & Chaves, 2011). During drought, *A. donax* undergoes morphological changes such as leaf-roll and loss to reduce transpiration by decreasing the area of exposed leaf surface (Cosentino *et al*., 2014). If the severity of drought increases, *A. donax* loses the above ground parts of the plant and sequesters carbohydrates and nutrients in the rhizome below-ground until growth conditions improve (Mann *et al*., 2013).

The emission of isoprenoid biogenic volatile organic compounds reduces the deleterious impact of heat and oxidative stress (Sharkey *et al*., 2008). Not all plants emit isoprenoids, but emitters possess greater tolerance of oxidative stress associated with excess excitation energy (Centritto *et al*., 2014) and especially characterizes fast-growing plants (Loreto & Fineschi, 2015). Analysis of ^{13}C labelling, in leaves fumigated with ^{13}C, indicates that isoprene is derived from recently synthesized photosynthate in well-watered plants (Brilli *et al*., 2007). Isoprene emission may also be linked to the production of foliar ABA via the methyl-erythritol phosphate pathway (Barta & Loreto, 2006). The rate of emission of isoprene is positively related to the level of P_N (Loreto & Sharkey, 1990), and this relationship is observed not only in *A. donax* (Hewitt *et al*., 1990) but across the *Arundineae* tribe of the *Poaceae* (Ahrar *et al*., 2015). *Arundo donax* occupies habitats that receive high levels of photosynthetically active radiation and also exhibits high rates of P_N (Rossa *et al*., 1998). However, rates of isoprene emission in pot-grown *A. donax* in a glasshouse (Ahrar *et al*., 2015) were not significantly greater than other fast growing species such as *Populus nigra* with similar rates of P_N (Centritto *et al*., 2011). The role of isoprene emission in the water deficit response of *A. donax* and any protective function is currently unclear.

Since April 1997, the University of Catania has hosted a collection of 39 ecotypes of *A. donax* collected from contrasting habitats in Southern Italy. These *A. donax* plants have grown in a common garden phenotyping experiment for 18 years in a semi-arid Mediterranean climate. The results from the first 2-years of growth indicated that ecotype 20 was one of the two most productive *A. donax* specimens, while ecotype 6 was amongst the least productive (Cosentino *et al*., 2006). Thus, we investigated the gas-exchange responses and yield of ecotypes 6 and 20 under irrigated and water deficit conditions. The identification of crop varieties with high leaf-level rates of P_N is important to the development of more productive cultivars (Hubbart *et al*., 2007; Lauteri *et al*., 2014). We hypothesized that the more productive ecotype 20 might exhibit greater leaf-level rates of P_N and stomatal conductance (G_s), while the less productive ecotype 6 might be less susceptible to water stress, possibly due to lower G_s and growth rate. The aim of this study was to: i) assess physiological and morphological response to drought in ecotypes 6 and 20; ii) quantify isoprene emission rates and changes in amounts of foliar free-ABA and GE-ABA during water deficit, and iii) identify any physiological traits in ecotypes 6 and 20 that may be related to biomass production under well-watered and water deficit conditions.

Materials and methods

Plant material and growth conditions

In 1997, rhizomes of 39 ecotypes of *A. donax* were collected from a range of sites (lakes, rivers and uncultivated areas) in Sicily and Calabria, Southern Italy. These rhizomes were planted in a common garden experiment at the experimental farm of the University of Catania (37°25′N 15°03′E). The rhizomes were

planted directly into the soil at a depth of 30 cm in 2.5 × 3.0 m plots at a density of 2.67 plants m^{-2} with plants placed at a distance of 0.75 m between rows and 0.5 m in each row. In the first year the plots were fertilized with 100 kg ha^{-1} of phosphate, and 80 of kg ha^{-1} nitrogen were supplied during the first and second years. Supplementary irrigation was also provided during the first two years to enable the rhizomes to more fully establish. Irrigation and fertilization were not applied for the next 15 years. Biomass production and morphology of the *A. donax* specimens was assessed from 1997 to 1998. Full details of the site, soil and experimental set-up are given in Cosentino *et al.* (2006).

In the first two years of the study, ecotype 20 produced the greatest biomass yield, while ecotype 6 was amongst the least productive. The two ecotypes were collected from contrasting environments: ecotype 20 from a coastal habitat and ecotype 6 from the slopes of Mount Etna (Fig. 1). In the summer of 2014 all plots received supplementary irrigation equivalent to 100% of potential evapotranspiration during July to August. Evapotranspiration (ETc.) was calculated each day as:

$$\text{ETc.} = E_o * K_p * K_c$$

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 (between 0.7 and 1.1). Daily rain-fall was subtracted from the daily calculation of water to be supplied as irrigation (Allen *et al.*, 1998; Cosentino *et al.*, 2015). On average 4.494 m^3 ha^{-1} of water was supplied each day. On the 20th of September (day 20 in Fig. 2) irrigation was ceased in one of the two plots for each ecotype and allowed to continue in the remainder. Measurements were conducted prior to the cessation of irrigation (time 0) and over the next 40 days as the soil dried (time 1 and 2). Soil samples were collected from three depths (0–30 cm; 30–60 cm, and; 60–90 cm) at the mid-point of each time period, the soil water content determined gravimetrically by drying in a ventilated oven at 105 °C. The available soil water content for plant growth was then determined following Klute (1986) and Killi *et al.* (2014) (Fig. 2).

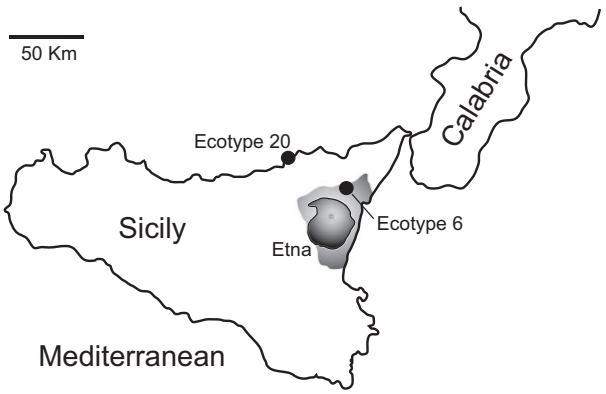


Fig. 1 Map showing the original location where the *Arundo donax* clonal populations used in this study were collected. Ecotype 6 was collected from Piedimonte Etneo (37°48N 15°10E; 348 m a.s.l.), and ecotype 20 was collected from Capo D'Orlando (38°08N 14°43E; 8 m a.s.l.). Re-drawn from Cosentino *et al.* (2006).

Leaf gas-exchange and chlorophyll-fluorescence

Measurements of P_N, G_s, the internal sub-stomatal concentration of $[CO_2]$ (C_i) and electron transport rate (ETR) were performed on the mid-section of the second fully expanded leaf from the flag leaf using a LiCor Li6400XT fitted with a 2 cm^2 leaf cuvette (Li-Cor, Inc., Nebraska, USA). Four replicate plants from the centre of each plot were analysed for each ecotype and treatment. Cuvette settings of 400 ppm $[CO_2]$, 2000 μmol m^{-2} s^{-1} of photosynthetically active radiation (10% blue and 90% red light) and a leaf temperature of 30 °C were used. To reduce diffusive leaks through the chamber gasket, a supplementary gasket was added and the IRGA exhaust air was fed into the interspace between the chamber and the supplementary gaskets (Rodeghiero *et al.*, 2007). Gas-exchange measurements were performed between 10.00 and 12.00 each day when the plants exhibited the highest levels of P_N and G_s. The maximum (F_v/F_m) and the actual quantum yield of photosystem II (ΦPSII: ΔF/F′m), and the dissipation of light energy as non-photochemical quenching (NPQ) were recorded using a Hansatech FMS-2 (saturating pulse of 10,000 μmol m^{-2} s^{-2}) and dark adaptation clips (Hansatech, King's Lynn, UK) after 30 min of dark adaptation and exposure to actinic light of 2000 μmol m^{-2} s^{-2} for a minimum of 10 min after the first saturating pulse (Genty *et al.*, 1989; Maxwell & Johnson, 2000).

Isoprene emission

The emission of isoprene was measured in the field from the same leaves of *A. donax* used for gas-exchange analysis, but a LiCor Li6400 fitted with a 6 cm^2 cuvette and LED light unit was used. Four replicate plants from the centre of each plot were analysed for each ecotype and treatment. Cuvette settings of 400 ppm $[CO_2]$, 2000 μmol m^{-2} s^{-1} of photosynthetically active radiation (10% blue and 90% red light) and a leaf temperature of 30 °C were used. The match tube on the LiCor cuvette was detached, and air from the leaf passed through a biphasic adsorbent trap containing 30 mg of Tenax and 20 mg of Carboxen (GERSTEL GmbH & Co.KG, 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. The volatiles collected in the traps were analysed using a gas chromatographer – mass spectrometer (GC-MS) with an Agilent HP-INNOWAX (30 m × 0.32 mm × 0.15 μm) GC column. A 5977A mass selective detector with electron ionization operating at 70 eV was used for analysis. Isoprene was identified by matching the spectrum peak with a library spectral database (NIST 11.L) and 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 data were analysed using Agilent MassHunter Workstation

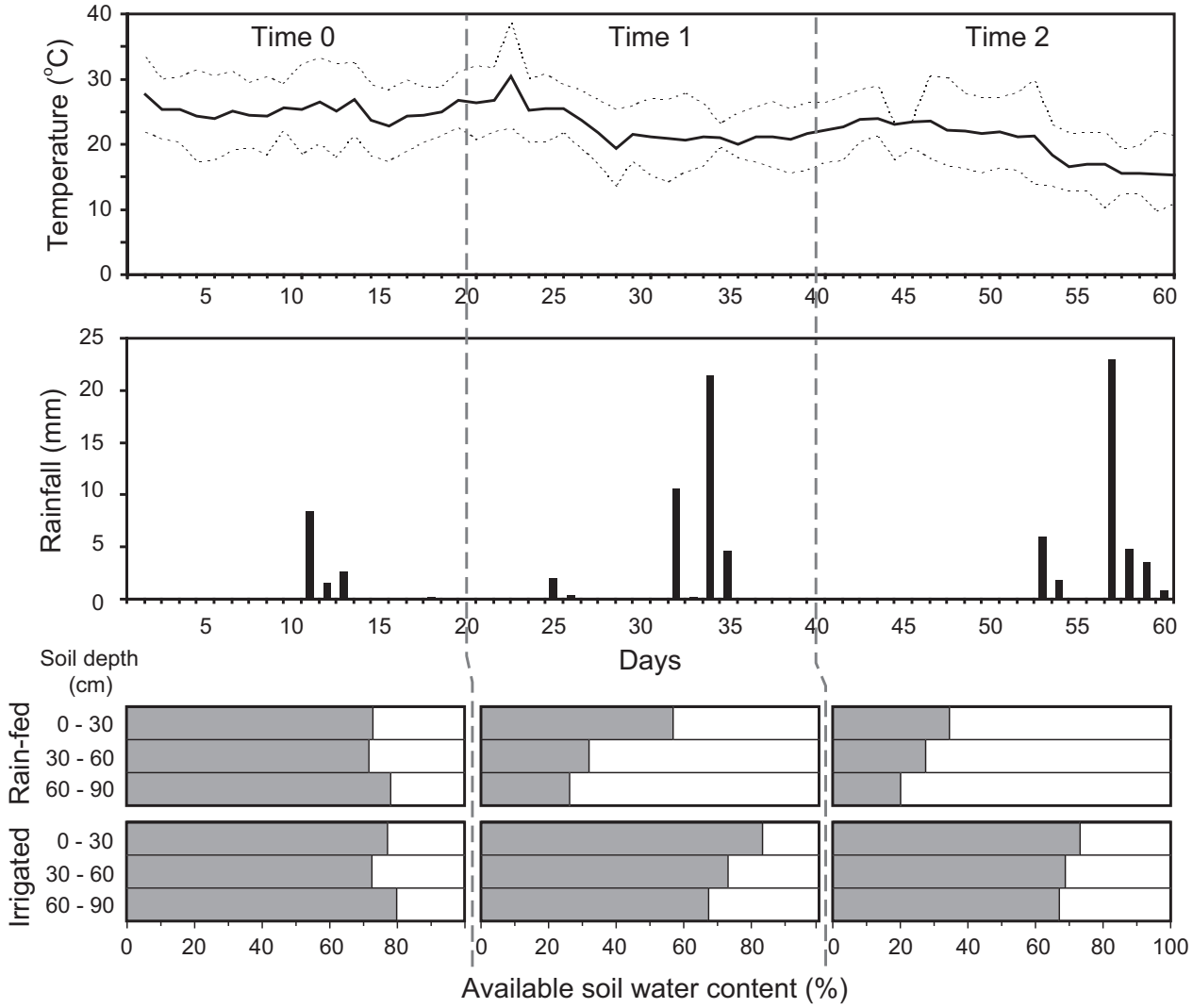


Fig. 2 Growth conditions during the study period: (a) daily temperature at the site - the upper dashed line shows the maximum daily temperature, the solid line shows the mean daily temperature and the lower dashed line shows the minimum daily temperature; (b) daily rainfall, and; (c) gravimetric measurements of soil water content taken at the mid-point of each measurement period. The measurement period began on the 1st September (day 1), irrigation ceased on the 20th September (end of time 0) and analysis of the physiological response to soil drying of the *Arundo donax* ecotypes was performed during time 1 (24th September to 9th October) and time 2 (10th to 30th October).

software (Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA).

Abscisic acid analysis

At the end of each measurement period, leaf samples were collected from four replicates for each treatment and ecotype using the second and third fully expanded leaves from the flag leaf of stems from the rhizomes adjacent to those used for gas-exchange and isoprene analyses. Leaf samples for time 0 were collected on the 20th September, time 1 on the 9th October and time 2 on the 31st October between 10:00 and 12:00 h. The leaves were frozen in liquid nitrogen and then stored at −80 °C. To determine concentrations of free-ABA and GE-ABA, 300–350 mg of leaf tissue was added to 40 ng of d6-ABA and 40 ng of d5-ABA-GE (ABA and derivatives provided by Prof. Zaharia, National Research Council of Canada) and ground in liquid nitrogen using a pestle and mortar. Once ground, 3 mL of CH_3OH:H_2O (50:50; pH 2.5 with HCOOH) was added to the leaf homogenate and left at 2 °C for 30 min. The extract was then purified using Sep-Pak C18 cartridges (Waters, Milford, MA, USA), and the eluate dried and rinsed with 500 μL CH_3OH:H_2O (50:50) pH 2.5 (modified protocol of Xiong *et al.*, 2014). To quantify ABA and ABA-metabolites, 3 μL of the eluate was then injected into a LC-ESI-MS/MS, using UPLC (Nexera UPLC Shimadzu Corporation) coupled with an MS/MS detector (TQ 8030) equipped with an ESI source (all from Shimadzu Corporation, Kyoto, Japan). Analyses were performed in the nega-

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tive ion mode. The compounds were separated in a Poroshell C_{18} column (3.0 × 100 mm, 2.7 μm i.d.; Agilent) using a binary solvent system consisting of water with 0.1% of HCOOH (solvent A) and acetonitrile/methanol (1/1) (added with 0.1% of HCOOH, solvent B). The solvent gradient was programmed to change linearly from 95% A to 100% B during a 30-min run at a flow-rate of 0.3 mL min^{-1}. The GC operated at a He flow rate of 1 mL min^{-1}. The GC was programmed with an initial oven temperature of 40 °C (5 min hold), which was then increased 5 °C min^{-1} up to 250 °C (4 min hold). Quantification was conducted in the multiple reaction mode (MRM) using a calibration curve constructed from analysis of standards following the protocol of López-Carbonell *et al.* (2009).

Foliar water content and biomass harvest

The relative water content (RWC) of leaves from the *A. donax* ecotypes was measured for time 0 on the 20th September, time 1 on the 9th October and time 2 on the 31st October. The second fully expanded leaf from the flag leaf was removed from four plants for each ecotype and treatment (plants adjacent to those used for gas-exchange were sampled) and the RWC determined using the approach of Diaz-Pérez *et al.* (1995). The biomass and morphological parameters of the *A. donax* ecotypes were measured in February 2015 – the period of the year when the moisture content of the stems is lowest and drying of the plants would require the least energy, a critical consideration in the commercial exploitation of *A. donax* (Cosentino *et al.*, 2006). A border of 50 cm was removed from each plot, to eliminate border effects influencing the determination of yield. Three central sub-plots of 1 m^2 were then sampled for each ecotype and treatment. Stem density was determined by counting the total number of stems in each sub-plot. Three stems in each sub-plot were randomly selected and stem height and basal stem diameter were measured. Dry mass yield was calculated by collecting sub-samples of the stems and leaves from sub-plots of each ecotype and treatment and then oven drying them at 105 °C until their weight remained constant. The susceptibility to drought of each ecotype was calculated using the susceptibility index (S) of Fischer & Maurer (1978):

$$S = \frac{1 - \left(\frac{Y}{Y_p}\right)}{1 - \left(\frac{X}{X_p}\right)}$$

where: Y = yield under stress; Y_p = yield without stress, and; X and X_p signify the yield of all ecotypes under stress and non-stress conditions, in this case values of 13.2 and 17.9 t ha^{-1} were utilized for *A. donax* under drought and irrigated conditions, respectively, as reported by Cosentino *et al.* (2014) in a study performed at the same site over three growing seasons.

Statistical analyses

Statistical analyses were performed using SPSS 20 (IBM, Armonk, NY, USA). To test the effect of water deficit on *A. donax* ecotypes 6 and 20 we used one-way ANOVAs with an LSD *post-hoc* test to assess differences in variance between samples associated with either ecotype or treatment effects.

Results

Arundo donax ecotypes 6 and 20 exhibited high levels of P_N (~33–38 μmol m^{-2} s^{-1}) and G_s (0.548–0.770 mol m^{-2} s^{-1}) at time 0 prior to the cessation of irrigation in the drought treatment; no statistical difference in P_N or G_s values was observed between the two well-watered ecotypes. These rates of P_N and G_s were maintained under well-watered conditions, with the exception of the latter stages of time 2 where temperatures were lower (Fig. 3). The soil water content available for plant growth in all layers declined by

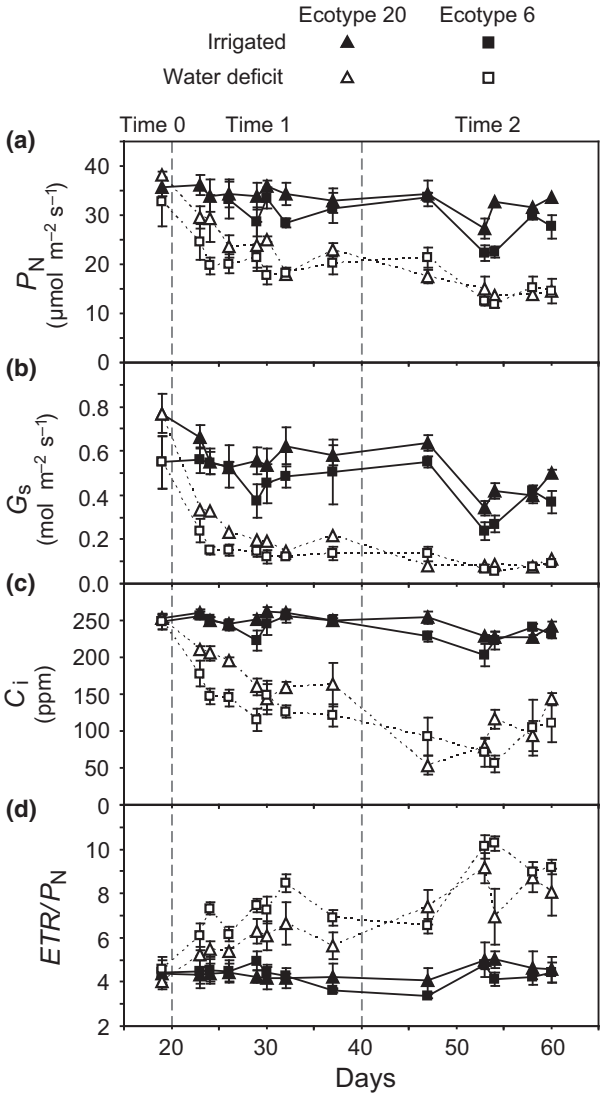


Fig. 3 Photosynthesis (P_N) (a), stomatal conductance (G_s) (b), internal sub-stomatal [CO_2] (C_i) (c) and the ratio of the electron transfer rate to photosynthesis (ETR/P_N) (d) of *Arundo donax* ecotypes 6 (square symbols) and 20 (triangle symbols) under irrigated (solid symbols, solid line) and drought conditions (open symbols, dashed line). Error bars indicate one standard error either side of the mean.

55.3–75.0% over the course of the study; the lowest reduction was in the upper 30 cm where rain-falls were more effective in restoring higher soil water availability (Fig. 2). As soil drying progressed, the two ecotypes showed identical declines in both P_N and G_s. Photosynthesis reduced by 66.7% (Fig. 3a) and G_s fell by 91.2% by time 2 (Fig. 3b). Stomatal conductance during the entire study was positively related to P_N (Fig. 4). Stomatal closure resulted in reductions in C_i of water-stressed leaves, indicating increasing stomatal limitation of photosynthesis (Fig. 3c). As drought progressed and P_N declined the amount of electrons not utilized in photochemistry of photosynthesis rose, as signified by an increase in the ratio of the ETR to P_N (Fig. 3d); however, ETR/P_N values were comparatively low, possibly due to high cuvette PAR and temperature. Soil drying did not affect the maximal yield of photosystem II as indicated by the F_v/F_m ratio (Fig. 5a). However, the quantum yield of photosystem II in the light (ΦPSII) declined, most significantly in ecotype 20 (Fig. 5b), while NPQ increased in both ecotypes (Fig. 5c).

Levels of ABA during drought were different in the two ecotypes (Fig. 6). In the first stage of soil drying, concentrations of free and GE-ABA were higher in the leaves of ecotype 6. However, as drought stress progressed in time 2, the leaves of ecotype 20 showed greater concentrations of both forms of ABA that correlated with a decline in G_s values but no alteration in P_N (Fig. 3). It is noteworthy, that levels of ABA in the irrigated plants also rose during time 2, possibly due to a reduction in mean daily temperature (Fig. 2) (Lim *et al.*, 2007). However, this higher concentration of ABA also corresponded with reduced G_s in time 2 (Figs 3 and 6). Rates of isoprene emission were relatively variable, preventing observation of any significant differences between treatment and ecotype (Fig. 7); although the levels of isoprene release found in this experiment were consistent with those observed in previous studies (Hewitt *et al.*, 1990; Ahrar *et al.*, 2015). However, during the initial period of soil drying in time 1, ecotype 6 showed a 34.6% increase in mean isoprene emission, while ecotype 20 reduced isoprene emission by 15.1%; while these findings are not statistically significant, they do indicate proportional and directional difference in the response of isoprene emission to drought between

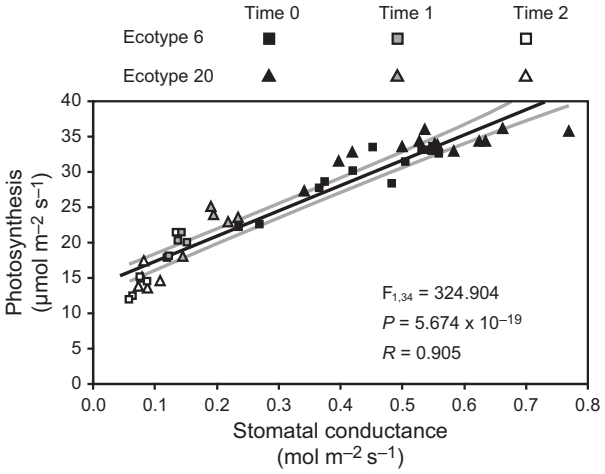


Fig. 4 The relationship between photosynthesis and stomatal conductance in irrigated (solid symbols) and drought (grey symbols at time 1; white symbols at time 2) *Arundo donax* ecotypes 6 and 20 grown in the field. In theory the relationship between P_N and G_s should originate at zero; however, drought stress under field conditions did not progress this far and we have decided to not extrapolate beyond the limits of our observable dataset and therefore a linear regression was applied: the solid black line indicates the linear regression and the grey lines either side of the linear regression indicate 95% confidence intervals of the regression line.

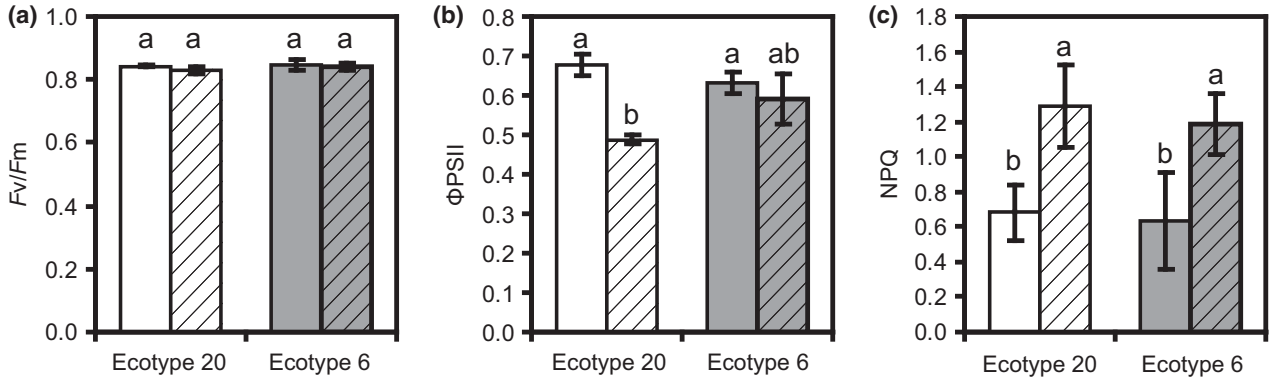


Fig. 5 Chlorophyll fluorescence parameters at time 2 of *Arundo donax* ecotypes 20 (white) and 6 (grey) under drought (hatched) and irrigated (open) conditions: (a) the efficiency of photosystem II (F_v/F_m); (b) quantum yield of photosystem II (ΦPSII: ΔF/F′m), and (c) dissipation of energy as non-photochemical quenching (NPQ). Error bars indicate one standard deviation either side of the mean. Letters indicate significant difference using a one-way ANOVA and LSD *post-hoc* test.

the two ecotypes. In time 2 isoprene emission was broadly similar across treatment and ecotype.

Foliar RWC of the two *A. donax* ecotypes also differed at the mid-point of the study. At time 1, no significant effect of drought was observed in ecotype 20 while ecotype 6 exhibited a significant 3.6% reduction in RWC (Fig. 8). At time 2 both *A. donax* ecotypes showed statistically identical leaf RWC values of 87.1–89.9%, which were significantly lower than in well-watered plants. The ecotypes did display differences in biomass and morphology, as ecotype 6 performed better than ecotype 20 under both well-watered and drought conditions (Fig. 9). In ecotype 6, stem height was respectively 17.7 and 33.9% greater in the irrigated and drought treatments than in ecotype 20 (Fig. 9a). The density (Fig. 9c) and diameter (Fig. 9b) of stems were not affected by ecotype or treatment: the greater stem height in ecotype 6 (Fig. 9a) was not associated with any difference in the number or thickness of stems. Dry mass yield was also greater in ecotype 6 than 20 under both irrigated and drought conditions (Fig. 9d). Calculation of the susceptibility index (S) suggests that ecotype 6 (S = 1.067) was more strongly affected by drought than ecotype 20 (S = 0.853). This signifies a greater proportional decline in yield in ecotype 6. However, drought yield in ecotype 6 is greater than that found in irrigated ecotype 20 during the study period; suggesting that the faster growing ecotype 6, while proportionally more vulnerable to water deficit, was the more effective biomass crop during drought experienced in this study period.

Discussion

The two *A. donax* ecotypes analysed in this study exhibited contrasting biomass responses, with ecotype 6 more productive in both the irrigated and drought treatments (Fig. 9d). This result contrasts with the observations of Cosentino *et al.* (2006) in the first two-years of the common garden study involving the *A. donax* ecotypes, where ecotype 20 generated the highest yields. This result may be due to a number of factors such as growth conditions favouring ecotype 6 during this study (Fig. 2), more favourable establishment of ecotype

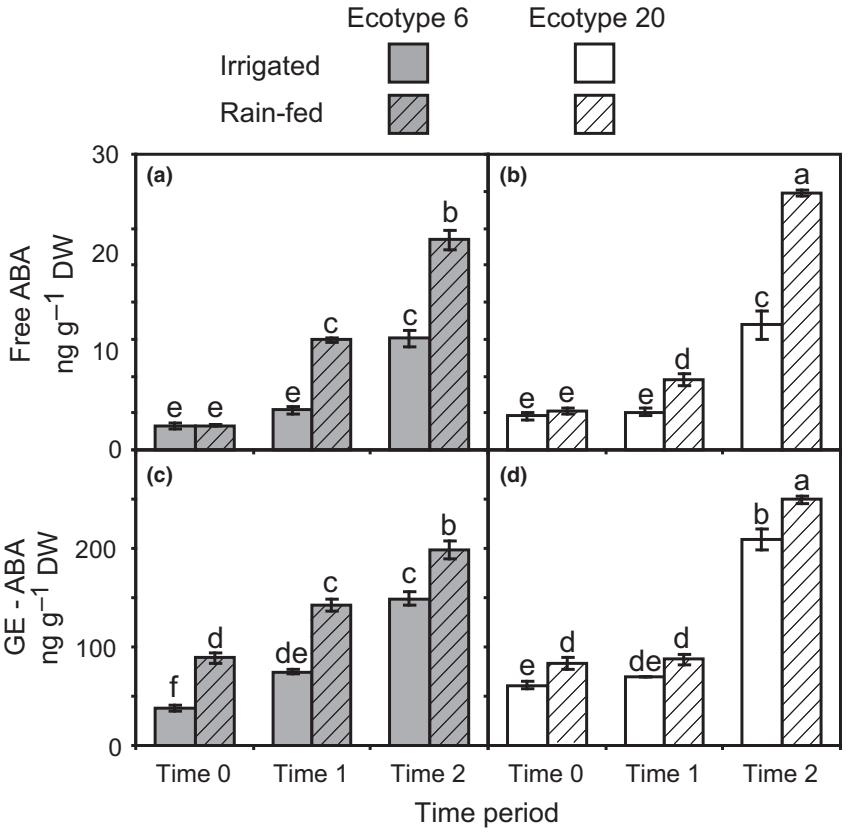


Fig. 6 The amount of free- and GE-ABA in leaves of *Arundo donax* ecotypes 20 (white) and 6 (grey) per unit dry mass (DW) under drought (hatched) and irrigated (open) conditions. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between free- and GE-ABA in all ecotypes/treatment using a one-way ANOVA and LSD *post-hoc* test.

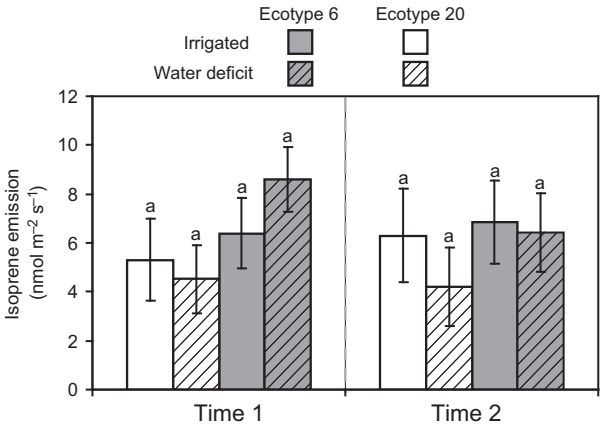


Fig. 7 Rates of isoprene emission in field grown *Arundo donax* ecotypes 20 (white) and 6 (grey) under drought (hatched) and irrigated (open) conditions. Error bars indicate one standard error either side of the mean. Letters indicate significant difference using a one-way ANOVA and LSD *post-hoc* test.

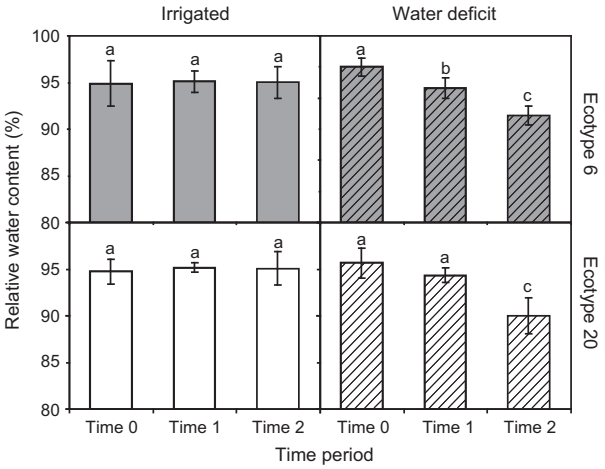


Fig. 8 Relative water content of leaves of *Arundo donax* ecotypes 20 (white) and 6 (grey) under drought (hatched) and irrigated (open) conditions. Error bars indicate one standard deviation either side of the mean. Letters indicate significant difference using a one-way ANOVA and LSD *post-hoc* test.

20 in the first few years of the common garden experiment (e.g. Mann *et al*., 2013), possible differences in the physiology of the two ecotypes (Figs 6 and 7) or the general lack of genetic diversity present in *A. donax* (e.g. Mariani *et al*., 2010; Pilu *et al*., 2014) associated with high phenotypic plasticity (Ghalambor *et al*., 2007) resulting in a lack of consistency in year-on-year biomass during phenotyping studies (Cosentino *et al*., 2006). Both ecotypes showed identical rates of P_N and G_s on a leaf-area basis under well-watered and drought conditions (Fig. 3). In irrigated conditions, the high rate of carbon assimilation accounts for the high biomass production observed in *A. donax* in comparison to other species (Angelini *et al*., 2009; Mantineo *et al*., 2009; Smith *et al*., 2015). As already observed when addressing the issue of dry mass yield, similar P_N and G_s of the two ecotypes may confirm low genetic diversity in *A. donax* (e.g. Ahmad *et al*., 2008; Mariani *et al*., 2010; Pilu *et al*., 2014). The obtained yields, both in rainfed and irrigated condition, are in accordance to the asymptotic non-linear relationship developed by Cosentino *et al*. (2014) to predict biomass yields of giant reed as function of crop water use in semi-arid Mediterranean environment.

Soil drying induced ~90% reductions in G_s, suggesting that the plants under drought conditions experienced moderate to severe drought (Haworth *et al*., 2016). The internal sub-stomatal concentration of [CO_2] of both *A. donax* ecotypes decreased under drought, consistent with stomatal closure inducing increasing diffusive limitations to CO_2 entry in the leaves, while suggesting no onset of biochemical limitations (known to invert the trend in C_i) (Flexas *et al*., 2002). The reduction in G_s induced by increased [ABA] may be indicative of biochemical protection to maintain foliar water content before soil water availability declines to critical levels (Maseda & Fernandez, 2006). Water deficit did not affect F_v/F_m values of the ecotypes (Fig. 5) despite the observed reductions in RWC and G_s, indicating stability of photochemistry of photosynthesis. In fact, declines in the F_v/F_m ratio of pot grown *A. donax* only occurred at RWCs below 60% (Nackley *et al*., 2014). Nonetheless, the quantum yield of photosystem II in the light and NPQ were more sensitive to reductions in water availability (e.g. Souza *et al*., 2004). This would reflect a decrease in the proportion of energy utilized by photochemical reactions driving photosynthesis and photorespiration (Fig. 3d) and increase in energy dissipated as heat (Fig. 5c), whereas it does not represent permanent damage of the photosynthetic apparatus of water-stressed plants. As rates of P_N are comparatively higher in *A. donax* than other species this may have reduced selective pressures related to carbon assimilation on a leaf-area basis between varieties with contrasting leaf-level rates of P_N (Haworth *et al*., 2011; Galmes *et al*., 2014). A study of 87 *A. donax* specimens from Italy found no geographic genetic variability, but did observe variation and heritability in stem height and diameter (Pilu *et al*., 2014). This may indicate that while the photosynthetic physiology of *A. donax* shows little variability, ecotypes 6 and 20 may vary in terms of stem morphology (Fig. 9a), if not photosynthetic physiology (Fig. 3), thus accounting for the differences observed in yield (Fig. 9d).

The rapid decline in G_s of both *A. donax* ecotypes, with 70.9% of the reduction occurring 5 days after the cessation of irrigation (Fig. 3b), suggests that *A. donax*

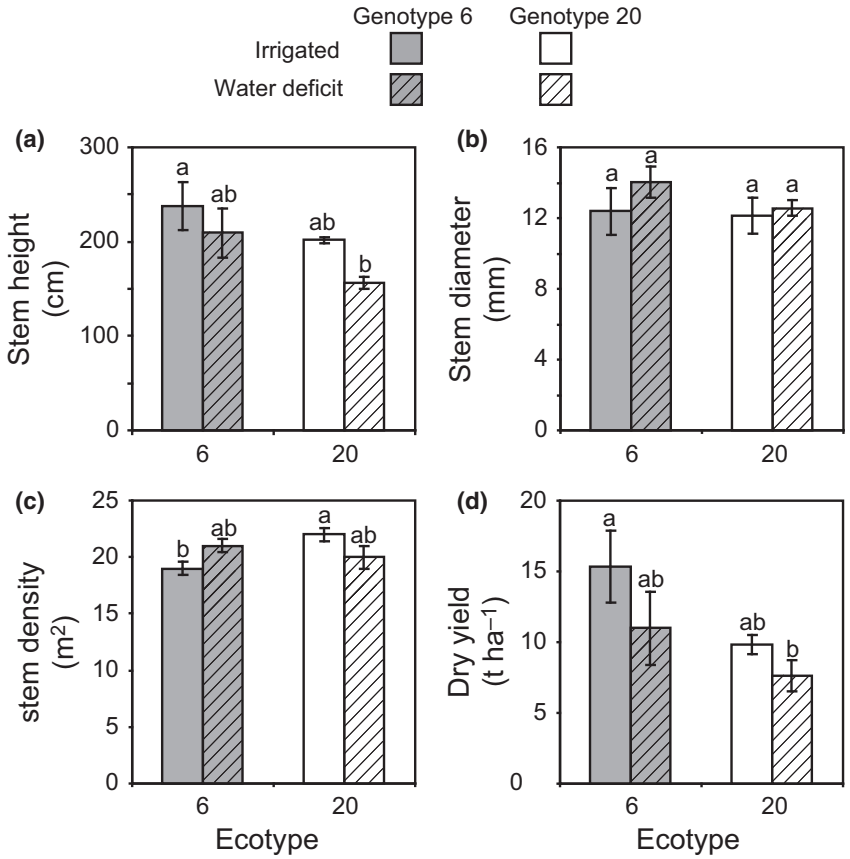


Fig. 9 Morphological characteristics of *Arundo donax* ecotypes 20 (white) and 6 (grey) under drought (hatched) and irrigated (open) field conditions: (a) stem height; (b) stem diameter; (c) the density of stems within the sub-plots, and; (d) dry biomass yield. Error bars indicate one standard error either side of the mean. Letters indicate significant difference using a one-way ANOVA and LSD *post hoc* test.

does not progressively adjust its water use efficiency to the development of drought (e.g. Mahmood *et al.*, 2015) and instead employs a 'use it or lose it' water use strategy (Bacelar *et al.*, 2012) dependent upon a high degree of stomatal functionality to prevent desiccation (Franks & Farquhar, 2007). This may reflect large changes of ABA, the hormone that is known to control stomatal closure in water stress conditions (Davies & Zhang, 1991). Indeed, stomatal closure occurred alongside increases in the concentration of free- and GE-ABA (Fig. 6). Ecotype 6 exhibited more pronounced rises in free- and GE-ABA than ecotype 20 in the first stage of drought at time 1, indicating a more rapid root-to-shoot signal of soil drying (e.g. Fujita *et al.*, 2005), possibly accounting for increased yield in the drought-stressed plants (e.g. Chen *et al.*, 1997). The rise in conjugated GE-ABA in ecotype 6 may also reflect greater drought tolerance through increased storage of the stress hormone ABA (Seiler *et al.*, 2011). The concentrations of free- and GE-ABA in the drought-stressed plants were higher in both plants in time 2, suggesting a progression of drought (Liu *et al.*, 2005), and/or a reduction in temperature towards the latter stages of the study (Fig. 2) as the amount of ABA also increased in the well-watered plants (Lim *et al.*, 2007). This rise in ABA in the plants grown under irrigated conditions in time 2 also corresponded to reduced G_s (Fig. 3). The higher amounts of free-ABA in ecotype 6 at time 1 may be related to lower foliar RWC developing more rapidly in this ecotype than in ecotype 20 (Fig. 8). Increased leaf [ABA] during drought often occurs prior to any declines in RWC (Liu *et al.*, 2005), but stomatal sensitivity to [ABA] is increased at low water potentials (Tardieu & Davies, 1992); possibly suggesting that the influence of free-ABA on stomatal closure in *A. donax* was more pronounced in time 2. The higher foliar concentrations of free- and GE-ABA in ecotype 6 at time 1 also coincided with a 34.6% increase in isoprene emission of drought-stressed plants, in respect to well-watered plants (Fig. 7). In contrast, levels of ABA remained constant in ecotype 20, whereas isoprene emission declined, although non-significantly, in drought-stressed leaves at

time 1 (Fig. 7). This suggests that part of foliar ABA synthesized under early stress is generated by a labile pool of carbon that is also used to form isoprene within the methyl eritrythol phosphate (MEP) pathway, confirming that isoprene may be used as a proxy of ABA formation under certain circumstances (Barta & Loreto, 2006). However, due to the variable nature of isoprene emissions (Sharkey & Loreto, 1993), further characterization of isoprene emission in *A. donax* in response to stress under more highly controlled conditions would be required before any firm conclusion could be drawn.

The emission of isoprene plays a protective role in the reduction of oxidative and heat stress in many plants (Sharkey *et al*., 2008). As isoprene is derived from recently assimilated carbon, emissions generally do not increase during soil drying (Centritto *et al*., 2011; Brilli *et al*., 2013). However, in the *A. donax* ecotypes isoprene emission remained stable during the development of drought, indicating an increasing proportion of photosynthetic carbon feeding isoprene biosynthesis during the occurrence of stress. Only when water deficit stress is severe, as P_N approaches zero, the emission of isoprene declines before increasing during re-watering (Brilli *et al*., 2007). In our case, increased emission in drought-stressed ecotype 6 at time 1 is not surprising as P_N (17–21 μmol m^{-2} s^{-1}) provided sufficient carbon for isoprene biosynthesis. However, as isoprene increases photoprotection of photosynthetic machinery under stress conditions (Velikova *et al*., 2011), we maintain that ecotype 6 might have developed a better strategy to protect photosynthesis from transient water stress.

The two *A. donax* ecotypes analysed in this study exhibited largely identical photosynthetic and gas-exchange responses to growth under well-watered and drought conditions. Photosynthetic rates in drought-stressed *A. donax* remained comparatively high into time 2 (~11–15 μmol m^{-2} s^{-1}), possibly due to its extensive root system permitting extraction of water from deep within the soil profile (Mann *et al*., 2013). The protective dissipation of heat (Fig. 5c) and emission of isoprene (Fig. 7) permitted the retention of photosynthetic function (Fig. 3a) and reductions in biomass yield of ~30–35% (Fig. 9) that are significantly lower than the 48.2% proportional loss experienced by 5 year-old *Miscanthus* x *giganteus* under similar conditions (Mantineo *et al*., 2009). In general, the yield of irrigated and drought-stressed *A. donax* was greater than that of other fast growing bioenergy crops such as *Cynara cardunculus* and *Miscanthus* x *giganteus* (Cosentino *et al*., 2007; Mantineo *et al*., 2009). This suggests that *A. donax* possesses the physiological adaptation to serve as an effective biomass crop in drought-prone semi-arid areas with a hot Mediterranean climate.

Conclusions

Physiological analysis of the most (20) and least (6) productive *A. donax* ecotypes from the study of Cosentino *et al*. (2006) under rain-fed and irrigated conditions produced an unexpected result. Ecotype 6 generated higher biomass yields than ecotype 20 in both treatments during the study period. This faster growth in ecotype 6 was not associated with significant differences in leaf-area levels of P_N or G_s, function of photosystem II or emission of isoprene. This lack of variation in photosynthetic physiological parameters may reflect low genetic diversity in *A. donax*. The contrast in biomass results from the earlier study may also reflect this lack of genetic variability, resulting in an absence of consistency in yield measurements when phenotyping *A. donax* ecotypes. Future studies should employ a wider geographical collection of specimens and present results over a greater time period to ensure that the most consistently productive ecotypes are identified. Despite the lack of a physiological ecotypic difference in this study, both ecotypes exhibited rapid rates of P_N under well-watered conditions, and as soil dried the signalling of drought via ABA and protective mechanisms ensured that the rain-fed plants retained comparably high rates of P_N and only experienced reductions in yield of 30–35%. The stress physiology of *A. donax* is adaptable to growth in semi-arid hot Mediterranean climates, making it a viable crop species for biomass production in drought-prone marginal lands.

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