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Published in:
GCB Bioenergy

DOI:
10.1111/gcbb.12351

Publication date:
2016

Citation for published version (APA):
Stavridou, E., Webster, R., Robson, P., & Hastings, A. (2016). The impact of soil salinity on the yield, composition and physiology of the bioenergy grass *Miscanthus x giganteus*. *GCB Bioenergy*, *9*(1), 92-104. https://doi.org/10.1111/gcbb.12351

GLOBAL CHANGE BIOLOGY
BIOENERGY

GCB Bioenergy (2016), doi: 10.1111/gcbb.12351

The impact of soil salinity on the yield, composition and physiology of the bioenergy grass *Miscanthus × giganteus*

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Abstract

High salinity land may provide an alternative resource for the cultivation of dedicated biomass crops for renewable energy and chemicals, thus avoiding competition for land use with food crops. The commercial perennial grass *Miscanthus × giganteus* is a leading biomass crop; however, its response to salt stress is largely unknown. *Miscanthus × giganteus* was grown in pots irrigated with nine different NaCl concentrations (0, 2.86, 5.44, 7.96, 10.65, 14.68, 17.5, 19.97 and 22.4 dS m^{-1}). Biomass yield was reduced by 50% at 10.65 dS m^{-1} NaCl. Root dry matter inhibition occurred at the highest salt concentration tested, while rhizome dry weight and the ratios of root/rhizome and below-/above-ground dry matter were not affected by elevated salinity. The accumulative effect of increasing salinity reduced stem height and elongation, while photosynthesis was reduced to a smaller extent. The duration and strength of salinity exacerbated the reduction. Water use efficiency (WUE) was maintained except at the highest salinity and plants maintained stomatal conductance (g_s) and leaf water content at low to moderate salinity. *Miscanthus × giganteus* showed strong induction of the osmoprotectant, proline and no significant increase in malondialdehyde content under increasing salinity. The ash content in leaves, increased, reducing the biomass quality at high salinity concentrations. The effects of salinity on the yield and the availability of land area in European geographical area for agriculture were investigated. Understanding the potential for growth of the C4 biomass crop Miscanthus on underutilized or abandoned land may offer a new range of targets for improved economics, crop management and breeding.

Keywords: bioenergy, biomass quality, biorefining, *Miscanthus × giganteus*, photosynthesis, plant physiology, salinity tolerance

Received 19 January 2016; revised version received 19 January 2016 and accepted 2 February 2016

Introduction

The environmental stresses resulting from climate change and unsustainable irrigation practices are predicted to impact crop productivity and reduce the area of available land for agriculture by 2–9% globally and by 11–17% within Europe (Zhang & Cai, 2011). The increasing impact of climate change on production of, and demand for, plant-derived products imposes challenges for the use of existing agricultural land. One solution could be the use of marginal land for the cultivation of biomass crops. Second-generation perennial biomass crops that are tolerant to environmental stress conditions would contribute to the reduction in CO_2 emissions, while limiting competition with food production (Popp *et al.*, 2014).

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Soil salinity is a severe abiotic stress caused primarily by an abundance of sodium chloride (NaCl), from both natural accumulations and from irrigation and crop evapotranspiration (Flowers & Flowers, 2005). Saline soils are characterized by having an electrical conductivity higher than 4 dS m^{-1} (where 4 dS m^{-1} ≈40 mM NaCl), which many crops are unable to tolerate (Shannon & Grieve, 1998; Qadir *et al.*, 2000). Salt-affected marginal land may provide an alternative land resource for the cultivation of second-generation biomass crops (Oliver *et al.*, 2009) such as Miscanthus.

Miscanthus × giganteus is a high yielding C4 crop cultivated for biomass in the majority of commercial plantations. Miscanthus has many characteristics that make it a suitable and sustainable source of biomass including a low energy requirement for cultivation and suitability for cultivation on low-grade agricultural land (Lewandowski *et al.*, 2000). Miscanthus biomass has also been reported to have good combustion quality when

compared to other lignocellulose crops (Lewandowski & Kicherer, 1997). The cultivation of perennial rhizomatous crops such as Miscanthus may enhance soil carbon sequestration (Lal, 2004a; Pidlisnyuk *et al.*, 2014), which in the long term could be beneficial for the recovery of marginal land through improving soil structure and fertility (Lal, 2004b).

Miscanthus has good potential for use on underutilized or abandoned marginal land where excessive salinity and low moisture levels limit plant growth. Studies have shown that Miscanthus can grow in coastal areas where salt spray affects plant growth (Ogura & Yura, 2008; Scheiber *et al.*, 2008; Hung *et al.*, 2009). Płażek *et al.* (2014) showed that salt levels in excess of 100 mM reduced *M.* × *giganteus* productivity, while *Miscanthus sinensis* accessions exhibited greater variability for salt tolerance (Sun *et al.*, 2014). However, the pattern of growth and development of *M.* × *giganteus* in a wide range of salt concentrations, the associated responses to salt stress and the potential impact on combustion properties are largely unknown.

Salinity can reduce crop yield with a significant metabolic effort afforded to plant adaptation, growth maintenance and stress responses with a subsequent decrease in yield (Munns & Gilliham, 2015). Salinity has two main components affecting plant growth. Initially, the water potential is lowered, and the plant experiences an osmotic stress similar to drought associated with concentrated solutes in the root zone. The subsequent ionic imbalance as salts perturb the uptake of nutrients and the accumulation of ions over time is the main cause of toxicity (Munns *et al.*, 1995; Flowers & Flowers, 2005; Verslues *et al.*, 2006). Many different traits contribute to salinity tolerance, which are species and developmental stage dependent (Munns, 2002; Flowers & Flowers, 2005; Jones *et al.*, 2015).

Biomass yield is rapidly responsive and negatively correlated to osmotic stress while the intensity of the subsequent membrane injury depends on the rate of salt absorption and the ability to compartmentalize the salts in different tissues (Volkmar *et al.*, 1998). Maintenance of plant yield is directly associated with tolerance to abiotic stresses including salinity. However, considering the complexity of salt tolerance along with the underlying mechanisms controlling yield, alternative stress-related traits should be accounted for (Flowers *et al.*, 1997; Flowers & Flowers, 2005; Ashraf & Akram, 2009). Potentially, a combination of drought and salt tolerance would enable the plant to cope with the different components of salt stress. To describe the response of plants to salinity stress, Maas & Hoffman (1977) introduced the concept of the salt tolerance threshold as the level of salinity over which yield is reduced significantly in a biphasic trend. The threshold level is responsive to environmental factors, and therefore, it is not static for each crop (Volkmar *et al.*, 1998), thus requiring controlled conditions to obtain accurate threshold values (Shannon, 1985).

In second-generation biomass crops such as Miscanthus, the quality of biomass may impact upon combustion properties and lower the ash melting temperature, affecting the degree of fouling, slagging and corrosion (Lewandowski *et al.*, 2003). The basic requirements for increased net energy for biomass production are low moisture and inorganic elemental contents. There are several minerals contributing to ash formation [potassium (K), chlorine (Cl), nitrogen (N), calcium (Ca), sodium (Na), aluminium (Al), iron (Fe), silicon (Si) and sulphur (S)] that have an impact on biomass thermal conversion efficiency (Jørgensen, 1997; Brosse *et al.*, 2012). Low ash melting behaviour can lead to slagging and fouling, two of the main problems due to ash depositions in the boiler and the heat transfer section, respectively (Baxter *et al.*, 2012). The quantity and characteristics of ash and residue products from Miscanthus combustion are important aspects of combustion system design and operation (James *et al.*, 2012).

In this study, the effects of salinity on *M.* × *giganteus* across a range of nine different water electrical conductivities are examined. The aim was (i) to estimate how *M.* × *giganteus* biomass production is affected by different concentrations of NaCl, (ii) to identify the tolerance threshold in *M.* × *giganteus*, (iii) to investigate the impact of salinity on biomass quality and (iv) to model the potential yield of *M.* × *giganteus* growing in saline areas from available yield and soil data.

Materials and methods

Plant material and stress treatments

The experiment was performed in a controlled environment glasshouse at IBERS, Aberystwyth University with 16 and 8 h, respectively, day/night photoperiod from supplemental lighting with an average of 500 μmol photons m^2 s^{-1} photosynthetically active radiation and 25 and 15 °C day/night cycle. An excess of *M.* × *giganteus* plantlets, obtained from approximately 20 g rhizome pieces, were established and grown in 6.2 L pots containing John Innes No. 2 compost. A homogeneous population of *M.* × *giganteus* plants was selected for the experiment using measurements of stem length.

The experimental treatments were applied for a 64-day period and comprised of eight NaCl concentrations: 2.86, 5.44, 7.96, 10.65, 14.68, 17.5, 19.97 and 22.4 dS m^{-1} (corresponding approximately to 30, 60, 90, 120, 150, 180, 210 and 240 mM of NaCl), plus a control treatment with no added NaCl. The experimental design was completely randomized with five biological replications for each treatment. To avoid osmotic shock, plants were adapted by incrementally applying NaCl

 doi: 10.1111/gcbb.12351

concentrations by 30 mM per day starting on day 21 (9 October 2013) until all treatments reached the target concentrations on day 27 (15 October 2013). All treatments were watered to maintain a constant soil moisture content of 0.35 $m^3\ m^{-3}$ with the corresponding NaCl solutions. Moisture content was measured as an average of three measurements per pot using a moisture sensor (SM300; Delta-T Devices Ltd., Cambridge, UK) inserted at three roughly equidistant points around the surface of the pot, and readings were recorded by a hand-held moisture meter (HH2 moisture meter; Delta-T Devices Ltd.). Pots were irrigated with ½ strength Hoagland's solution of the corresponding NaCl concentrations on day 49.

All physiological measurements were performed between 09:00 and 14.00 hours. At the beginning of the experiment, the tallest stem of each pot (where multiple stems were present) was selected as the main stem and all the measurements were taken from the youngest leaf with fully expanded ligule from that stem.

Growth measurements

Stem length of the longest stem was measured from the base of the stem at soil level to the fully expanded ligule of the youngest leaf.

Leaf area was assessed by measuring the length and width (at half leaf length) of the youngest fully expanded leaf with a ligule and was calculated as described by Clifton-Brown & Lewandowski (2000) (Eqn 1):

$$\text{Area}\,(\text{cm}^2) = 0.74 \times \text{length}\,(\text{cm}) \times \text{width}\,(\text{cm}) \tag{1}$$

At the end of the experiment, total above-ground biomass was harvested and weighed to give fresh weight (F.W.), and biomass was then dried at 60 °C to constant weight to estimate dry weight (D.W.).

Physiological measurements

Dark-adapted chlorophyll *a* fluorescence measurements were made on the youngest fully expanded leaf with a ligule using a Handy PEA chlorophyll fluorimeter with dark adaptation leaf clips (Hansatech Instruments Ltd., Norfolk, UK) after 30 min dark adaptation. A dark adaptation time of 30 min was established following the equipment guidelines. Fluorescence parameters measured included maximal fluorescence (F_m), minimal fluorescence (F_o), variable fluorescence (F_v), maximal quantum efficiency of PSII photochemistry (F_v/F_m) and the performance index (PI) calculated using the manufacturer's software.

Stomatal conductance (g_s, $\text{mmol}\ m^{-2}\ s^{-1}$) was measured using an AP4 porometer (Delta-T devices Ltd.).

Relative chlorophyll content was measured on three leaves per plant [the youngest leaf with the fully expanded ligule (0), one immediately older leaf (−1) and one immediately younger leaf (+1)] using a SPAD-502 meter (Konica Minolta Optics Inc., Tokyo, Japan). A mean value was calculated from three readings taken at quarterly intervals along each leaf.

Leaf water content (WCl) was evaluated from total above-ground biomass measurement at harvest, and water content was calculated according to Eqn (2):

$$\text{WCl}\,(\text{g L}^{-1}) = \frac{\text{F.W.} - \text{D.W.}}{\text{D.W.}} \tag{2}$$

Water use efficiency (WUE) was calculated as the ratio between above-ground biomass (g) produced and the total amount of water applied (L).

Steppuhn *et al.* (2005a,b) introduced a comparative salinity tolerance index (ST-Index) based on the nonlinear regression parameters of C_{50} and s that indicates crop tolerance to root-zone salinity (Eqn 3b). The relative yield (Y_r) was calculated as the ratio of absolute yield (above-ground biomass) over the biomass in control conditions (Y_m) where salinity has very little or no influence on the yield (Maas, 1990). To describe Y_r as a function of the NaCl concentrations, we fit an exponential response curve with 97.1% variance accounted for:

$$Y_r = A + B(R^x) \tag{3a}$$

In Eqn (3a), the A is the asymptote when parameter R is between 0 and 1, and B is the range parameter.

Salinity tolerance index (ST-Index) was estimated according to Eqn (3b) (Steppuhn *et al.*, 2005a,b) with some modifications:

$$\text{ST-Index} = C_{50}(1 + s) \tag{3b}$$

In Eqn (3b), the index is based on the nonlinear parameters of C_{50} and s (Eqn 3a), where C_{50} is the NaCl concentration, at which Y_r is reduced by 50% and s is the slope (dY_r/dC) of a tangent to the fitted line at C_{50}.

Biochemical analysis

Proline was extracted using a cold extraction procedure according to Carillo *et al.* (2008) by mixing 20 mg of leaf fresh weight aliquots with 400 μL of ethanol: water (40 : 60 v/v). Proline content was measured spectrophotometrically using the method of Carillo & Gibon (2011) from three biological and three technical replicates per treatment.

Lipid peroxidation was estimated by the total content of 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids that has been utilized as a biomarker for lipid peroxidation (Mittler, 2002). TBARS content was estimated from leaf material using the method of Gautier *et al.* (2010). The amount of MDA-TBA complex was calculated from the excitation coefficient, $\varepsilon = 155\ \text{mM cm}^{-1}$ according to Eqn (4) (Yasar *et al.*, 2010):

$$\text{MDA equivalents}\,(\text{nmol g}^{-1}) = \left[\frac{(A_{532} - A_{600})}{\varepsilon} \cdot 1000 \cdot V\right] \Big/ \text{F.W.} \tag{4}$$

In Eqn (4), A_{532} and A_{600} are the absorbance at 532 nm and 600 nm, respectively, V is the volume of the extract, and F.W. is the fresh weight of the sample.

Samples of leaves were ground and stored in falcon tubes to measure ash content (%). Beakers (25 mL) were dried in an oven for 30 min at 100 °C, placed in a desiccator to cool and weighed on a balance to an accuracy of four decimal places. Ground sample (1 g) was dried overnight at 100 °C in the weighed beakers. The samples were placed into desiccators to

 doi: 10.1111/gcbb.12351

cool and then reweighed. The beakers with the samples were placed in a Muffle furnace at 550 °C for 16 h and in an oven at 100 °C until the sample temperature decreased to 100 °C, and the samples were weighed after 30 min. The percentage of ash content per sample was calculated according to Eqn (5):

$$\%\text{Ash (dried basis)} = \frac{\text{Mass of the ash sample (g)}}{\text{Original mass of the dried sample (g)}} \cdot 100 \quad (5)$$

Statistical analyses

All the statistical analyses were performed using the computing environment R (R Core Team, 2015). The effects of salinity levels and time (days) on the morphological, physiological and biochemical parameters were assessed using one-way ANOVA (for salinity or time) and two-way ANOVA (for salinity, days and their interactions) with the ez and afex packages (Lawrence, 2015; Singmann *et al*., 2015). All data were tested for normality (Shapiro test) and homogeneity of variances (Levene's test), and if normality failed and homogeneity passed, transformations were attempted (Table S1). For the two-way ANOVA, data were also tested with Mauchly's test for sphericity, and if the assumption of sphericity was violated, the corresponding Greenhouse–Geisser corrections were performed. If significant differences were found among treatments, then the Tukey's HSD *post hoc* test was performed to determine specific treatment differences using the Agricolae package (de Mendiburu, 2015).

Changes in plant height over time at the different levels of NaCl were modelled via fitting logistic curves (LC) (Eqn 6) to individual plants using GenStat© (17th edition):

$$Y_{ij} = A + \frac{C}{1 + e^{(-B(X_i - M))}} \quad (6)$$

In Eqn (6), Y_{ij} is the height of j experimental unit in the ith recording time, A is the minimum asymptote, C is the final or potential height, $A + C$ is the upper asymptote; the curve's maximum value, B is the shape parameter; the steepness of the curve, M is the point of inflexion where the absolute growth rate is maximum, and X_i is the ith recording time.

Salinity response equation and spatial impact on yield

The experimental data were used to determine an equation that could be used to predict the reduction from the maximum yield on nonsaline soils so that the actual yields could be predicted on saline soils.

The relationship between the soil conductivity (C_s) and irrigation water conductivity (C_w) and the soil cation exchange capacity (CEC), soil porosity (θ) and soil material density (ρ_{ma}) was determined using the Waxman–Smits method (Waxman & Smits, 1968) according to Eqn (7):

$$C_s = (BQ_v + C_w)/F^*. \quad (7)$$

In Eqn (7), F^* is the resistivity formation factor = $0.62/\theta^{2.15}$, B is the ionic equivalent conductance of the exchange cation (Mhos M^{-1})/(Eq L^{-1}), B is related to C_w. $B = (1 - 0.8e^{-50\ C_w})$ 3.86, Q_v is the concentration of exchange cations per unit pore volume, Eq L^{-1}. Q_v is related to CEC according to Eqn (8).

$$Q_v = (\text{CEC}(1 - \theta)/\theta)\rho_{ma} \quad (8)$$

From the linear regression of soil conductivity as a function of water conductivity (Fig. S1), the slope represents the reciprocal of the formation resistivity factor (0.336) (F^*) and the intercept with y axis is the soil conductivity due to the clay content of the soil $BQ_v/F^*(1.88)$. The soil conductivity, $C_{sw,}$ due to the water salinity is C_w/F^*. Using Eqn (7), the CEC of the soil (John Innes No 2) was calculated to be 0.484 mEq g^{-1}.

The above-ground dry matter yields of all replicates were normalized as a proportion of the nonsaline mean maximum yield which was 24.8 g per plant (Y_{sal}). Using Minitab©, a nonlinear regression was made of the response variable of the Y_{sal} using the explanatory variable soil conductivity, C_{sw}. The nonlinear regression of the proportion of reduction compared to nonsaline dry matter yield (Y_{dm}) using soil conductivity due to water conductivity (C_{sw}) as an explanatory variable suggested Eqn (9):

$$Y_{dm} = 1.0(\exp(-0.163894 \times C_{sw})) \quad (9)$$

The standard error of the regression is 0.14, and the lowest of all forms was tried and respects both initial point and final point (Fig. S2). Using this relationship, the areas in Europe with degraded soils due to salinity were investigated to see which areas could be used to grow *M.* × *giganteus* and what is the salinity induced yield reduction.

The MiscanFor model (Hastings *et al*., 2009) was used to predict the mean harvest yield for the period of 1990–2008 of *M.* × *giganteus* for the European area using the CRU 3.1 (Mitchell *et al*., 2004) climate data and the Harmonized World Soil Data (HSWD) (FAO/IIASA/ISRIC/ISSCAS/JRC, 2012) as input on a 0.00833 degree grid. The topsoil salinity from the HSWD expressed in units of dS m^{-1} was extracted from the HSWD on the same 0.00833 degree grid and used in Eqn (9) to calculate the proportion of the Miscanthus yield resulting from the salinity stress of the soil. The results are presented spatially for the European geographical area (Fig. 5).

Results

Effects of salinity on growth and yield

Final plant height was negatively associated with salt concentration (Fig. 1). The upper asymptote of the fitted logistic curves decreased linearly ($P = 0.03$) with increasing salinity at the rate of approximately −0.96 cm per unit increase in salinity. The shape parameter increased linearly ($P < 0.001$) with increasing salinity, with steeper curves for 22.4 dS m^{-1} NaCl than the 2.86 dS m^{-1} NaCl-treated plants ($P < 0.05$). However, the time at which maximum absolute growth occurred was not affected ($P = 0.174$) by salinity with an overall mean value of 19.6 days. Height changed significantly with time ($P < 0.001$) with the greatest

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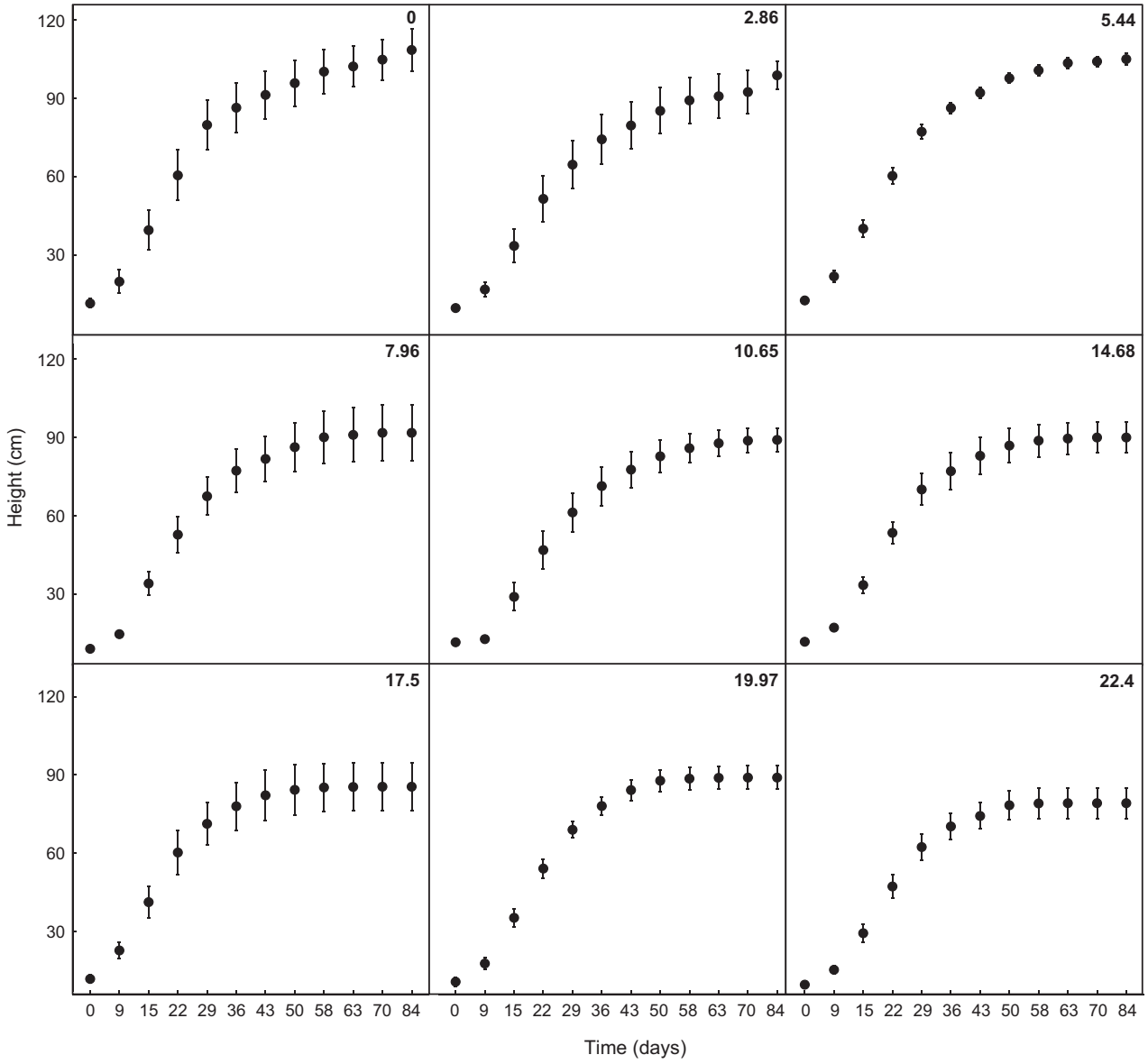

Fig. 1 Changes in height (cm) over time (days) of *Miscanthus* × *giganteus* plants in response to changes in salinity concentrations ranging from 0 to 22.4 dS m^{-1} NaCl; data are mean ± SE (n = 5).

inhibition of height being observed on day 43 for plants treated with 22.4 dS m^{-1} NaCl (highest NaCl treatment) while there was a delayed inhibition of height for control plants and plants treated with 2.86 (lowest NaCl treatment) (day 58) and 5.44 (day 63) dS m^{-1} NaCl (Fig. 1).

The rate of stem elongation (cm day^{-1}) showed a significant change with time (days), main effect ($P < 0.05$), reaching the maximum rate between 15 and 22 days when stem elongation started to decline until day 58 and remained constant between days 63 and 84. Salinity had a significant main effect on rate of elongation on days 29 and 50–84 ($P < 0.05$). On day 84, the control and 2.84 dS m^{-1}-treated plants had significantly higher rates of elongation compared to the other seven NaCl concentrations ($P < 005$).

Salinity had a significant main effect ($P < 0.05$) on the above-ground D.W., which was reduced in NaCl concentrations over 7.96 dS m^{-1} NaCl with the greatest decrease occurring at 19.97 and 22.4 dS m^{-1} NaCl ($P < 0.05$) (Fig. 2a). Salinity depressed the root weight D.W. ($P < 0.009$) mainly affecting the 22.4 dS m^{-1} NaCl-treated plants that differ significantly from the control and 2.86 dS m^{-1} NaCl-treated plants ($P < 0.05$) (Table 1). However, no significant differences between salinity levels were detected when comparing rhizome

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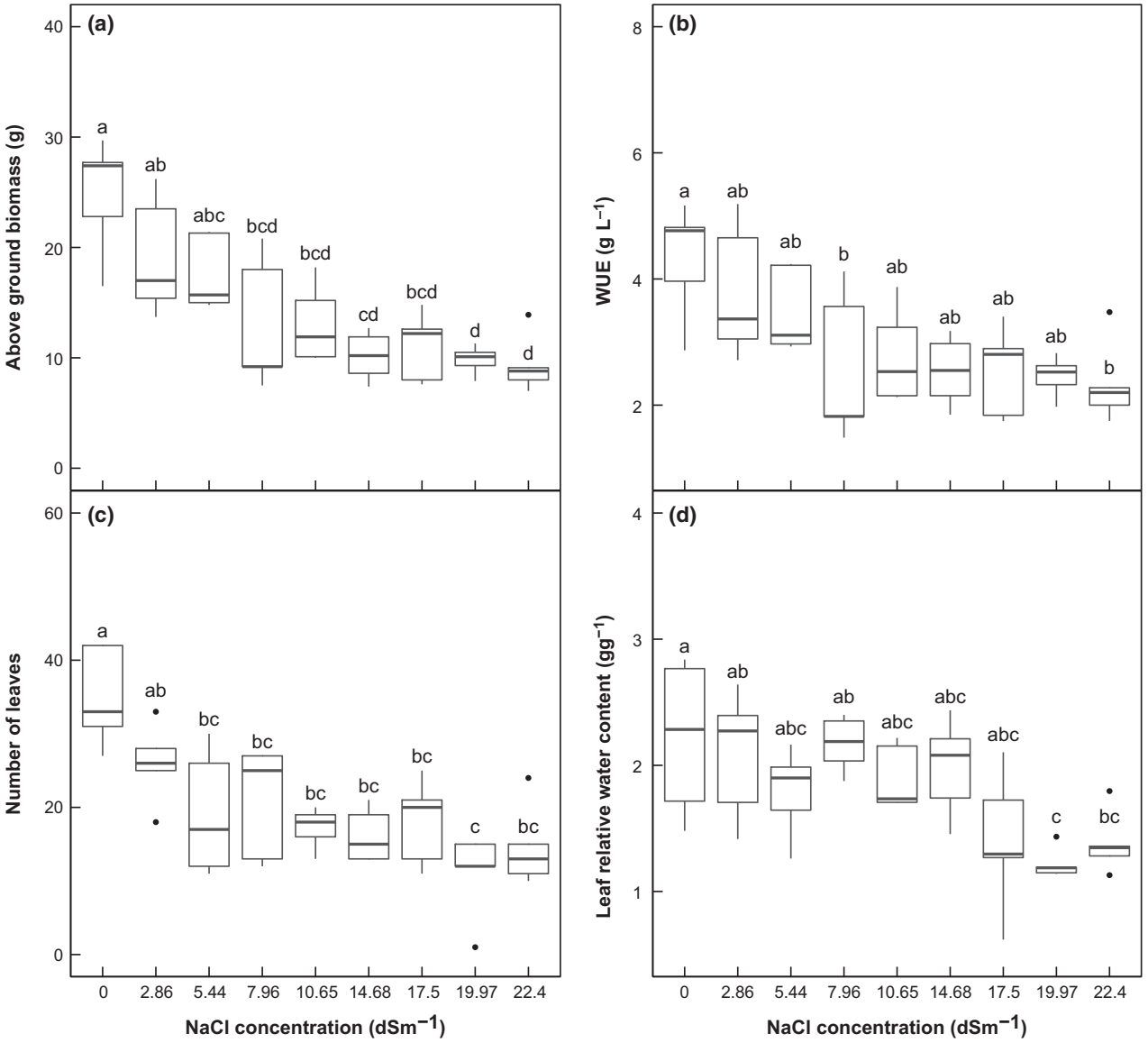

Fig. 2 Response of the above-ground dry biomass (a, g), the cumulative WUE (b) (g D.W. L^{-1} water applied), the number of leaves (c) and the leaf relative water content (d) of *Miscanthus* × *giganteus* plants to salinity gradients ranging from 0 to 22.4 dS m^{-1} NaCl; data show the median (2nd quartile; horizontal line) and the 1st and 3rd quartiles of the data (n = 5). Dots indicate outliers.

D.W., nor the ratios of root/rhizome and below-/above-ground D.W. (Table 1). From the exponential response curve (Eqn 3a), the Y_r decreased by 13.67% for each unit increase in dS m^{-1} NaCl. The salt concentration associated with 50% reduction in Y_r was estimated at 10.86 dS m^{-1} NaCl (approximately 120 mM), and the steepness parameter (s) was equal to −0.01997. Therefore, the ST-Index calculated using Eqn (3b) was 10.64.

Salinity-affected leaf number ($P < 0.05$) (Fig. 2c), control and 2.86 dS m^{-1}-treated plants had the highest number of leaves at the end of the experiment. Leaf area was significantly reduced with salinity ($P = 0.0032$), although the reduction was not linear. The largest leaf area was measured in plants treated with 5.44 dS m^{-1} NaCl ($P < 0.05$), whereas the 17.5 and 19.97 dS m^{-1} NaCl-treated plants had the lowest leaf area values ($P < 0.05$) (data not shown). Leaf relative water content was effected by salinity ($P = 0.0008$), and plants treated with 17.5 and 19.97 dS m^{-1} NaCl were significantly different compared to the control ($P < 0.05$) (Fig. 2d).

Effects of salinity on physiology and photosynthesis

The cumulative WUE significantly decreased with increasing salinity ($P = 0.0047$). Plants treated with 7.96 and 22.4 dS m^{-1} NaCl had significantly lower WUE

Table 1 Tukey's HSD (THSD) *post hoc* test for the main effect of treatment (NaCl) on root D.W., rhizome D.W., and the ratios root/rhizome and below/above D.W. in nine different NaCl concentrations

	Root D.W. (g)			Rhizome D.W. (g)			Root/rhizome			Below/above D.W. (g)		
NaCl (mM)	Mean	±SE	THSD	Mean	±SE	THSD	Mean	±SE	THSD	Mean	±SE	THSD
0	7.54	1.22	a	20.22	5.32	a	0.43	0.06	a	1.06	0.17	a
2.86	8.66	3.20	a	11.62	2.69	a	0.86	0.33	a	1.03	0.10	a
5.44	5.84	1.08	ab	15.76	3.51	a	0.40	0.06	a	1.22	0.25	a
7.96	4.02	0.62	ab	9.82	0.75	a	0.41	0.06	a	1.23	0.24	a
10.65	5.30	0.91	ab	11.00	1.06	a	0.49	0.07	a	1.27	0.12	a
14.68	3.82	0.14	ab	14.98	2.96	a	0.29	0.05	a	1.90	0.31	a
17.5	3.98	0.64	ab	14.84	4.12	a	0.37	0.12	a	1.61	0.26	a
19.97	4.06	0.50	ab	11.30	2.30	a	0.44	0.12	a	1.54	0.19	a
22.4	2.94	0.26	b	9.28	2.38	a	0.42	0.11	a	1.42	0.39	a

Different letters indicate significant differences at $P < 0.05$. Data are mean ± SE.

($P < 0.05$) (Fig. 2b). Both salinity and time (days) had significant main effects on stomatal conductance (g_s) ($P < 0.05$) (Tables 2 and 3). Stomatal conductance was significantly higher on day 65 ($P < 0.05$) (Table 2). Plants treated with 22.4 dS m^{-1} NaCl (highest NaCl treatment) had the lowest g_s ($P < 0.05$), while concentrations at 5.44 and from 14.68 to 19.97 dS m^{-1} NaCl were moderately effected (Table 3). The mean g_s for all time points of each individual treatment was reduced by 40.3% at 22.4 dS m^{-1} in response to increased NaCl concentrations (Table S4).

Salinity induced leaf senescence over a period of 55 days. The changes in relative leaf chlorophyll content showed significant salinity by day interactions ($P < 0.05$). The relative chlorophyll content remained constant in control plants throughout the experiment and changed significantly over time in concentrations at 2.86, 10.65 and over 17.5 dS m^{-1} NaCl (Fig. 3). The variance in the leaf relative chlorophyll content increased after day 57, and salinity induced senescence only on day 85 (end time point) when significant changes were observed between the different levels of NaCl ($P < 0.05$). Plants treated with concentrations 5.44, 7.96 and from 17.5 to 22.4 dS m^{-1} NaCl showed significant reductions in chlorophyll content when compared to control plants.

Salinity by day interactions affected significantly ($P = 0.024$) the quantum yield of dark-adapted leaves (F_v/F_m). While F_v/F_m was negatively associated with increasing salinity per day, especially from plants treated with concentrations over 17.5 dS m^{-1}, it varied significantly across days, at 5.44 and 19.97 dS m^{-1} NaCl, showing a decline on days 66 and 87, respectively ($P < 0.05$) (Table 4). The PI was also reduced over time with time (days) main effect being significant at $P < 0.001$ (Table 4).

Ash, proline and MDA content

Ash and proline content of leaves changed significantly in response to salinity ($P < 0.05$). The ash content was

Table 2 Tukey's HSD (THSD) *post hoc* test for the main effect of days on the variable stomatal conductance (g_s, mmol m^{-2} s^{-1}) in nine different NaCl concentrations

	g_s (mmol m^{-2} s^{-1})		
Days	Mean	±SE	THSD
65	43.1	2.23	a
73	33.5	3.34	b
77	33.7	2.52	b
85	27.1	1.71	b

Different letters indicate significant differences at $P < 0.05$. Data are mean ± SE.

Table 3 Tukey's HSD (THSD) *post hoc* test for the main effect of treatment on the variable stomatal conductance (g_s, mmol m^{-2} s^{-1}) in nine different NaCl concentrations

	g_s (mmol m^{-2} s^{-1})		
NaCl (mM)	Mean	±SE	THSD
0	45.4	3.46	a
2.86	41.7	3.15	a
5.44	33.9	3.50	ab
7.96	45.5	6.12	a
10.65	38.6	5.89	a
14.68	30.4	2.31	ab
17.5	28.1	2.26	ab
19.97	27.5	2.79	ab
22.4	22.6	2.91	b

Different letters indicate significant differences at $P < 0.05$. Data are mean ± SE.

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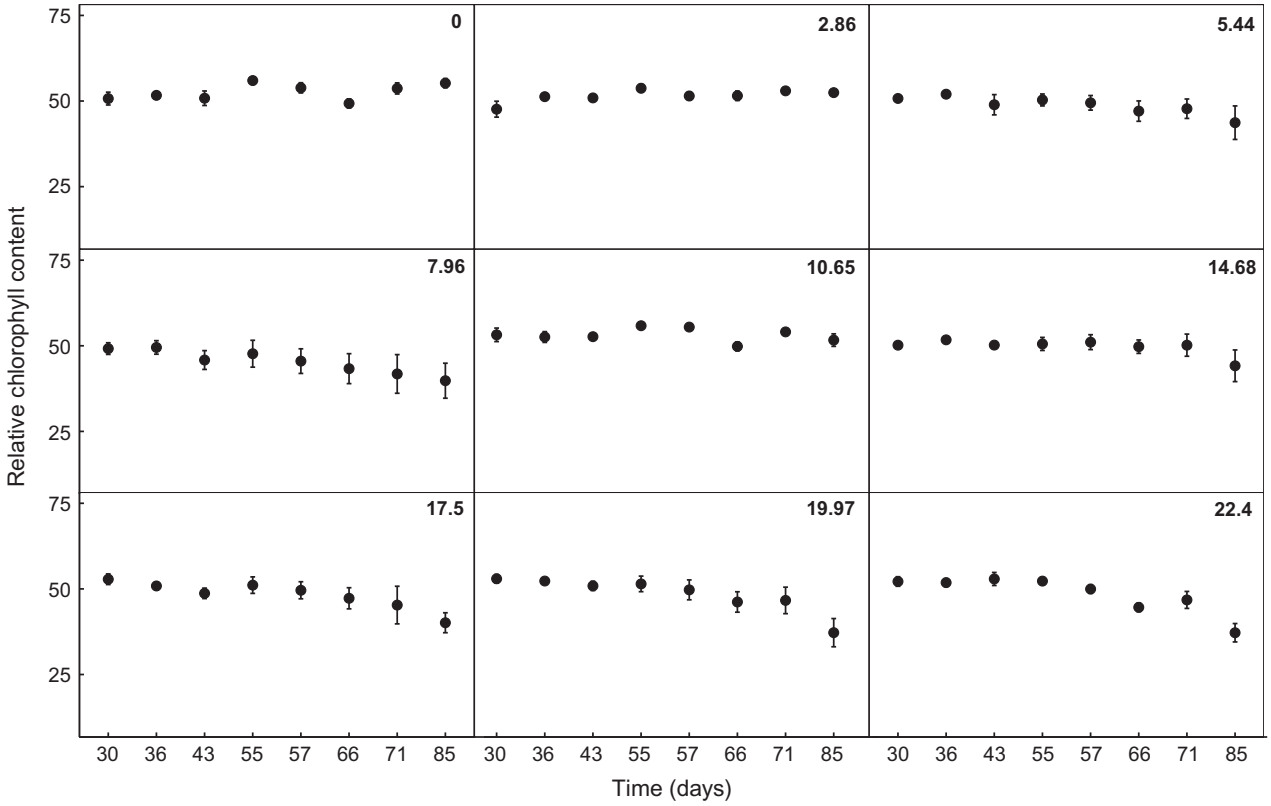

Fig. 3 Response of relative chlorophyll content (SPAD) of *Miscanthus* × *giganteus* leaves over a period of 54 days to salinity gradients ranging from 0 to 22.4 dS m^{-1} NaCl; data are mean ± SE (n = 5).

Table 4 Tukey's HSD (THSD) *post hoc* test for the main effect of days on the variables F_v/F_m and Photosynthesis Index (PI) in nine different NaCl concentrations

	F_v/F_m			PI		
Days	Mean	±SE	THSD	Mean	±SE	THSD
30	0.769	0.002	ab	3.03	0.11	a
37	0.773	0.001	ab	2.82	0.10	ab
44	0.770	0.002	ab	2.85	0.08	a
50	0.770	0.002	ab	2.41	0.10	c
57	0.773	0.002	a	2.44	0.07	bc
73	0.765	0.003	b	2.39	0.11	c

Different letters indicate significant differences at $P < 0.05$. Data are mean ± SE.

significantly greater in concentrations over 14.68 dS m^{-1} NaCl, showing a 42% increase up to 84% over 17.5 dS m^{-1} NaCl (Fig. 4a). There was a similar trend for proline accumulation which increased twofold at 7.96 dS m^{-1} and 48-fold at 10.65 dS m^{-1} and showed a significant increase of 142-fold at 17.5 dS m^{-1} NaCl (Fig. 4b). MDA content showed an 11.6% increase at 14.68 dS m^{-1} NaCl, but was not significant, and was weakly correlated with proline content (r = 0.39, P = 0.01).

Effects of salinity on availability of land area for M. × giganteus agriculture

The total areas of soils of different salinity within on a 0.00833 degree grid were extracted using ArcGis (Table 5), and the potential impact of saline soils on Miscanthus yields was calculated and presented spatially on a map of the European geographical area (Fig. 5). Most saline soils covering 539 567 km^2 in the European geographical area can be used to grow Miscanthus with up to an estimated 11% reduction in yield; a further 2717 km^2 can be used with an estimated 28% reduction in yield, and only, 3607 km^2 will produce a yield reduction greater that 50%.

Discussion

The use of saline land provides an opportunity for growing biomass crops in areas that do not compete with staple food crops; however, there is the potential that a yield penalty may prevent the utilization of such areas for biomass production. We investigated the

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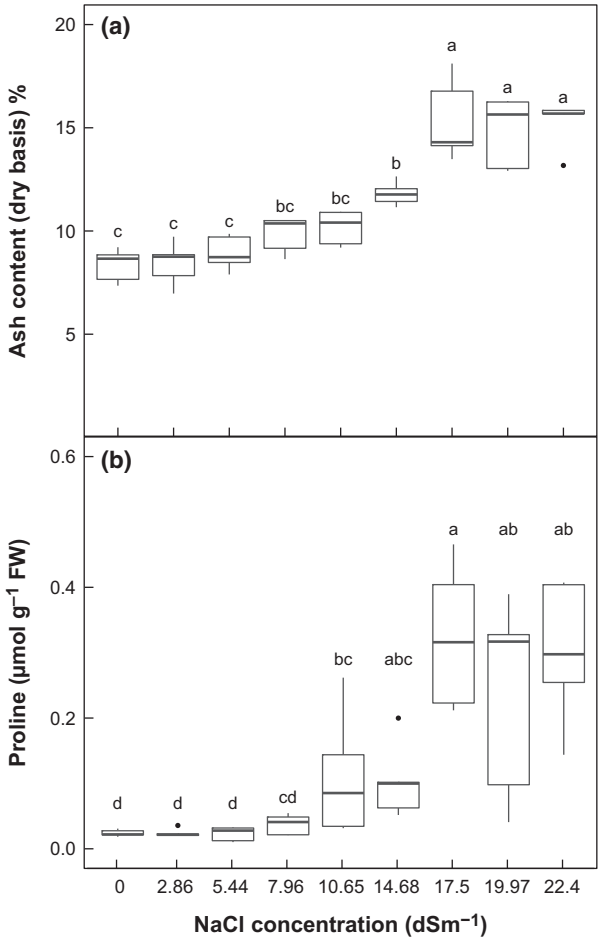

Fig. 4 Change in ash content (a) and proline content (b) (µmol g^{-1} F.W.) of *Miscanthus* × *giganteus* leaves in response to salinity gradients ranging from 0 to 22.4 dS m^{-1} NaCl; data show the median (2nd quartile; horizontal line) and the 1st and 3rd quartiles of the data (n = 5). Dots indicate outliers.

Table 5 Salinity impact (% D.W. yield) and European geographical area (EU area) (km × km) in different salinity ranges. The table describes the percentage of reduction in D.M. yield for each area (km × km) in the European area affected by the specific salinity range (dS m^{-1})

Salinity range (dS m^{-1})	Salinity impact % reduction in D.W. yield (%)	EU area (km × km)
0.1–0.29	3	182 630
0.3–0.49	6	247 603
0.5–0.99	11	109 334
1–2.99	28	2717
3–4.99	48	6
5–15	56	3601

morphological, physiological and biochemical responses of *M.* × *giganteus* to different treatments of water salinity over time. The research showed that there are different salt tolerance thresholds across diverse physiological responses.

Changes in growth rate or yield are often the only visual response to salt tolerance at moderate to low salinities (Shannon, 1985). The observed gradual decline in biomass D.W. with increasing salinity (Fig. 2a) agrees with the results of Płażek *et al.* (2014), where plants treated with 150 and 200 mM NaCl differ from controls. However, in our study, a significant decline in the above-ground D.W. was observed at lower concentrations (7.96 dS m^{-1} or approximately 90 mM NaCl). Sun *et al.* (2014) showed that in *M. sinensis* seedlings the shoot D.W. was more sensitive to salinity at 30 mM NaCl. While in this research, the salt tolerance threshold of *M.* × *giganteus* for relative yield (reduction of 50%) was at 10.86 dS m^{-1} NaCl (approximately 120 mM).

The loss of older leaves, possibly occurring as a result of the accumulated ionic effect of salt stress over time, contributes to a reduction in above-ground D.W. At the end of this experiment, the number of leaves declined at salinity concentrations greater than 2.84 dS m^{-1} NaCl (Fig. 2c) and is consistent with observations in 50 mM NaCl-treated rice plants, resulting from excess accumulation of salt in the expanded leaf (Yeo *et al.*, 1991).

Rhizome D.W. and the ratios of root/rhizome and below-/above-ground D.W. were not affected by increased salinity, and only, the root D.W. was significantly reduced at the highest salt concentration (22.4 dS m^{-1} NaCl) (Table 1). Płażek *et al.* (2014) showed a similar response in *M.* × *giganteus*, with reduction only in roots D.W. at 200 mM NaCl and no changes in rhizomes D.W. below 200 mM NaCl. This ability of perennial grasses to maintain below-ground biomass under stress conditions could preserve sufficient reserves for the following growing season (Karp & Shield, 2008); while this may be physiologically relevant for transitory stresses like drought, it remains to be seen how this response affects year on year yield under the accumulative stress effect of salinity.

The tolerance of crops to salinity can be compared using a single-value salt tolerance index (ST-Index) (Maas & Hoffman, 1977; Munns, 2002; Steppuhn *et al.*, 2010). These indices are well suited for biomass crops and are not distorted by the ratio of above to below-ground biomass which in this experiment is consistent throughout. The ST-Index for *M.* × *giganteus* was estimated to be 10.68, which ranks it as more tolerant than the closely related forage maize (9.13) and less tolerant than barley (14.00) (adjusted from Steppuhn *et al.*, 2005a,b).

Salinity at 22.4 dS m^{-1} NaCl induced early height inhibition (Fig. 1); however, the growth responses of *M.* × *giganteus* under different salt concentrations are largely explained by the strong interactions between

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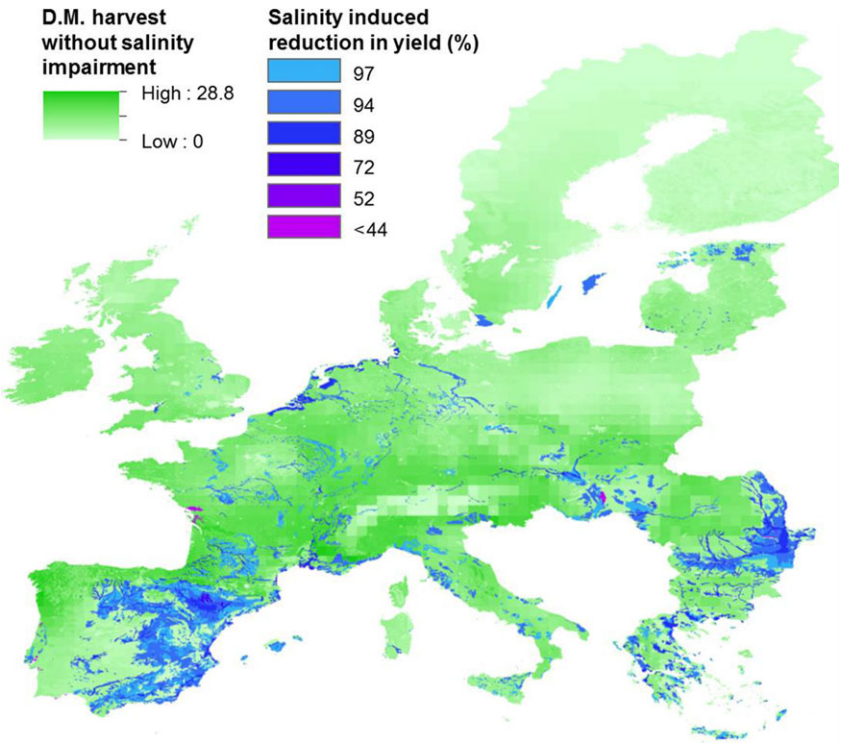

Fig. 5 Map of *Miscanthus* × *giganteus* dry weight (D.W.) harvest yield without salinity impairment with the per cent of potential yield reduction due to the salinity of the soil superimposed. The calculations are made on a 0.00833 degree grid.

salinity and the duration of stress. Scheiber *et al.* (2008) showed that the height of *M. sinensis* showed a quadratic response at 0%, 25% and 50% of foliar-applied seawater spray, but in the highest treatment, the decrease in height was linear (Scheiber *et al.*, 2008). These responses may be induced largely by the osmotic effect of salinity but also due to the possible Na^+ specific effect associated with growth response (Munns & Tester, 2008). Increasing salinity had a nonlinear impact on leaf area, with the greatest leaf area occurring at 5.44 and lowest at 17.5 and 19.97 dS m^{-1} NaCl (ca. 60, 180 and 210 mM). A linear reduction in leaf area was seen in Sorghum (Netondo *et al.*, 2004) and *M. sinensis* (Sun *et al.*, 2014) in all salinity treatments.

The maximum quantum yield of PSII (F_v/F_m) was unaffected by increased salinity, implying stable photochemical conversion efficiency of PSII at 5.44 dS m^{-1} NaCl (day 66) and 19.97 dS m^{-1} NaCl (day 87) (Table 4). This is consistent with studies on *M. sinensis* (Sun *et al.*, 2014), while previous studies of *M.* × *giganteus* showed F_v/F_m decreased significantly above 150 mM NaCl (Płażek *et al.*, 2014). The F_v/F_m of maize declined above 102 mM NaCl (Hichem *et al.*, 2009), whereas F_v/F_m in sorghum was not affected by salt concentrations below 200 mM (Netondo *et al.*, 2004) or 150 mM (Yan *et al.*, 2012). The PI was not affected by salinity at any salt concentration in this study (Table 4), which is consistent with observations in sorghum (Yan *et al.*, 2012).

The relative water content of leaves (Fig. 2d) and relative chlorophyll content (SPAD) (Fig. 3) were reduced with stress duration and at concentrations over 17.5 dS m^{-1} NaCl (ca. 180 mM). Pronounced leaf senescence can be primarily induced by the osmotic phase of salinity, when growth inhibition and metabolic changes occur (Munns *et al.*, 1995; Munns, 2002). However, the ability of *M.* × *giganteus* to maintain water content of leaves and leaf chlorophyll after prolonged stress duration may indicate a potential mechanism of osmotic adjustment in low to moderately high salinity. The water relations of foliar cells and the cellular function depend on the ion accumulation in the leaves and the osmotic adjustment, which otherwise would result in dehydration either through underadjustment or water loss from the cell walls (Flowers & Yeo, 1986). The initiation of premature senescence in older leaves of *M.* × *giganteus* at high salinity concentrations, combined with the increase in ash content, could be a result of excessive ion accumulation in shoots and leaves induced by prolonged transpiration as a consequence of maintaining open stomata (Munns & Termaat, 1986).

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Water use efficiency was greatly reduced at the highest salt concentration (22.4 dS m^{-1} NaCl, ca. 240 mM) (Fig. 2d). Similar decreases were observed for barley and wheat at 18 dS m^{-1} (Richards, 1992). The unchanged WUE of *M.* × *giganteus* under moderate and low salinity may occur due to osmotic tolerance exhibited in WUE measurements previously reported for Miscanthus (Clifton-Brown & Lewandowski, 2000). The degree of tolerance to the osmotic effect of salinity is reflected in the ability of plants to maintain g_s (Rajendran *et al*., 2009), which is associated with CO_2 assimilation rate and water loss through transpiration and is positively correlated to the relative growth rate in saline soils (Netondo *et al*., 2004; James *et al*., 2008). In the present study, g_s was reduced significantly at 22.4 dS m^{-1} NaCl with increased stress duration, showing a tolerance threshold to the osmotic effect of salinity at low to moderate salinity (Tables 2 and 3). In *M. Sinensis*, the reduction of g_s occurred at concentrations over 30 mM NaCl (Sun *et al*., 2014), whereas in sorghum the stomata closed at concentrations over 250 mM (Netondo *et al*., 2004). Munns & Tester (2008) attributed this reduction to the effects of ion accumulation in leaves and the induced perturbation of water status and local synthesis of abscisic acid in stomatal guard cells, explaining the high ash content at high salinity found in our study.

The ash content was used to determine the impact of saline soils on combustion. *M.* × *giganteus* ash content in leaves increased with increasing salinity up to 84% over 17.5 dS m^{-1} NaCl (Fig. 4a). High ash contents significantly reduce the energy output derived from a specific biomass source (James *et al*., 2012). In Miscanthus, the leaves contain higher mineral concentrations and double the amount of ash than stems or reproductive organs (Monti *et al*., 2008); in this context, leaf senescence, in respect to translocation of accumulates, and leaf loss induced by salinity may contribute to a better biomass quality by removing a large sink of ion accumulates and thus compensate for the total biomass yield loss. Although high ash content lowers the biomass quality for combustion, there are methods to utilize either the ash for soil conditioning and in building materials (Gómez-Barea *et al*., 2009; Dahl *et al*., 2010) or used in fluidized bed combustion technology for low-quality solid fuels (Saidur *et al*., 2011; Sandberg *et al*., 2011).

The accumulation of proline in response to salt stress has a role in osmotic adjustment and can decrease the water potential to help maintain the water content in leaves. Although in high external salt concentrations this occurs at the expense of plant growth, it may allow the plant to survive salt stress or even to recover (reviewed by Munns & Tester, 2008; Ashraf & Foolad, 2007 and Krasensky & Jonak, 2012). In this study, *M.* × *giganteus* showed a strong induction of proline, under increasing salinity; proline content increased up to 142-fold at 17.5 dS m^{-1} NaCl (Fig. 4b). This suggests that the maintenance of leaf water content below 14.68 dS m^{-1} NaCl could be an effect of the salt-induced accumulation of proline in *M.* × *giganteus*.

Under stress conditions, proline also contributes to the scavenging of free radicals, buffering cellular redox potential and stabilizing cellular structures (Ashraf & Foolad, 2007). This may explain the nonsignificant increase in MDA content and its positive correlation with proline in *M.* × *giganteus* with increasing salinity. According to Lutts *et al*. (1996), in rice, a significant positive correlation was observed between MDA and the electrolyte leakage for both old ($r = 0.71$) and young ($r = 0.63$) leaves at 30 and 50 mM NaCl. In *M.* × *giganteus*, concentrations of 100 and 150 mM NaCl did not increase the electrolyte leakage of the rhizomes, but resulted in a decrease in electrolyte leakage in leaves (Płażek *et al*., 2014).

In addition to bioenergy, biomass crops may provide additional or alternative value from phytoremediation which is a very efficient, inexpensive and environmentally accepted strategy used to remediate salt-impacted soils through phytoextraction and may be another potential use for *M.* × *giganteus* (Pidlisnyuk *et al*., 2014); nevertheless, soil salinity with electrical conductivities greater than 20 dS m^{-1} is not appropriate as plant growth is likely to be restricted (Qadir *et al*., 2005).

In the present study, *M.* × *giganteus* biomass production was reduced only at moderate to high salinity concentrations, while physiological processes were affected at higher concentrations, and was dependant on the stress duration; it also demonstrated enhanced accumulation of osmoprotectants and low lipid peroxidation. Based on the map of potential *M.* × *giganteus* yield, only a small area in the European geographical region analysed would pose restrictive limitations in biomass production of Miscanthus due to salinity. The loss of dry matter biomass in *M.* × *giganteus* is predicted to be higher than 48% over an area of 9601 km^2 where saline soils are between 3 and 15 dS m^{-1} NaCl (Fig. 5, Table 5). Miscanthus could therefore be a potential candidate to be tested for phytoremediation and biomass production in areas with low to moderate salinity.

Acknowledgements

Funding was provided by: OPTIMA project (FP7-KBBE-2011-5), Biotechnology & Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant on Energy Grasses & Biorefining (BBS/E/W/10963A01), Defra GIANT LINK Grant. The authors would like to thank Ruth Sanderson for statistical advice and Susan Youell and the IBERS breeding team for providing the plant material.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linear regression of soil conductivity as a function of water conductivity, slope represents the reciprocal of the formation resisitivty factor (0.336) and the intercept with y axis is the soil conductivity due to the clay content of the soil (1.88).
Figure S2. Non-linear regression of proportion reduction of non-saline dry matter yield (Y_{dm}) using soil conductivity due to water conductivity (C_{sw}) as an explanatory variable.
Table S1. Parameters that were transformed to achieve normality.
Table S2. Fluorescence parameters F_v/F_m and PI in nine different NaCl concentrations and six time points (day). Data are mean ± SE.
Table S3. Stomatal conductance (g_s mmol m^{-2} s^{-1}) of *Miscanthus* × *giganteus* at nine different NaCl concentrations and four time points (day).
Table S4. Mean stomatal conductance (g_s mmol m^{-2} s^{-1}) over seven time points of *Miscanthus* × *giganteus* at eight different NaCl concentrations and the percent of reduction compared to the control conditions (0 mM NaCl).

 doi: 10.1111/gcbb.12351