

Communication

# Characterization of Different *Arundo donax* L. Clones from the Mediterranean Region

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**Abstract:** The present study assessed the behavior of four clones of *Arundo donax* L. (giant reed) as a perennial rhizomatous grass of increasing interest due to its high biomass production and great adaptability to stress conditions. In this study, a molecular, physiological, and biomass characterization was performed in greenhouse conditions on four Mediterranean clones. The majority of physiological and biomass parameters were not significantly different between clones. However, it was possible to observe large differences in the chromosome count for the four clones. In this way, we detected different numbers of chromosomes for each clone (98 to 122), but surprisingly, no correlation was observed between their chromosome numbers and their physiological and biomass responses.

**Keywords:** *Arundo donax* L.; bioenergy crop; chromosomes; physiology; biomass



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## 1. Introduction

Bioenergy generation from biomass crops is one of the solutions that is being proposed to overcome the growing global consumption of fossil fuel resources and the negative consequences that this brings to the environment [1]. The use of so-called second-generation biofuels avoids competition for natural resources and arable land while avoiding food price rises. In this sense, about 20 perennial rhizomatous grasses have been tested in the European Union under extensive research programs, with giant reed being positively evaluated and considered a very promising species for biomass production [1].

Giant reed is a wild fast-growing perennial rhizomatous C<sub>3</sub> grass belonging to the *Poaceae* family [1] and is broadly distributed across warm temperate regions of the world, where it is adapted to a wide variety of soil types, as well as drought and salinity conditions [2–5]. Furthermore, several studies have shown its high photosynthetic potential and biomass yield [1,6].

Only vegetative reproduction has been reported in giant reed, despite it having inflorescences. Although details of giant reed's infertility are not well known, some researchers argue that it may be related to the origin of this species [7]. Another peculiarity of giant reed is the difference in the number of chromosomes reported, from 24 up to 110 chromosomes, depending on the sampling site [8,9].

Despite growing interest in the use of giant reed as a perennial grass for biomass production, little is known about the molecular, physiological, and biomass differences between clones. Therefore, the aim of this work was to perform a physiological, molecular, and biomass characterization of different giant reed clones in controlled conditions.

## 2. Materials and Methods

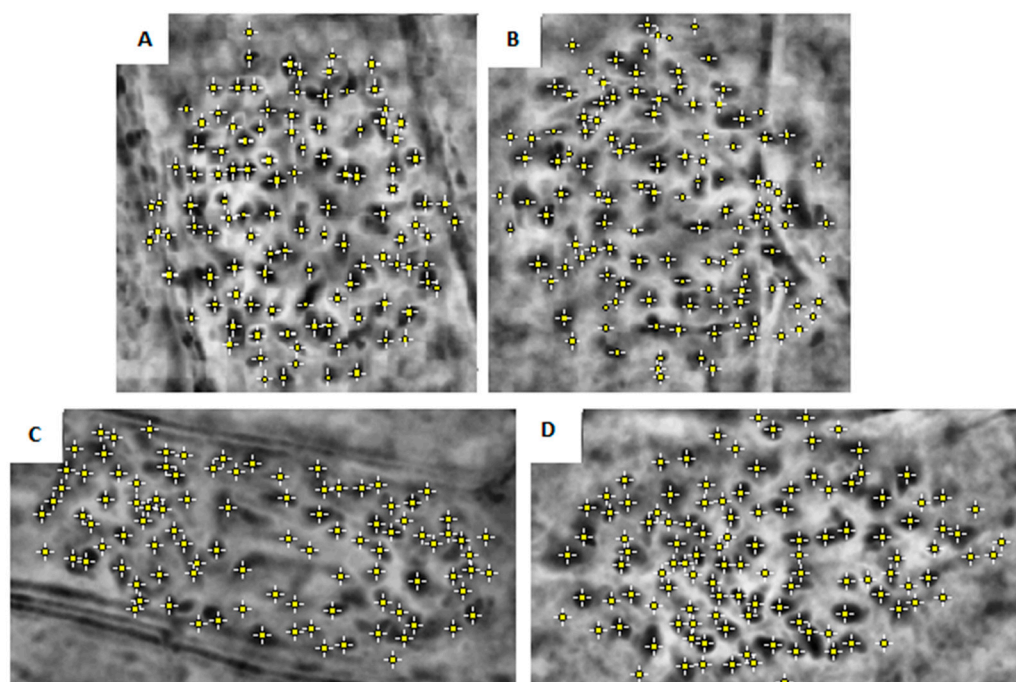
### 2.1. Plant Material

Four different giant reed clones were used in this experiment: ‘Fondachello’ (Italy, 10 ma.s.l.; 37°24′ N, 15°03′ W), ‘Martinensis’ (Spain; 291 ma.s.l.; 41°23′ N, 1°36′ W), ‘Granadensis’ (Spain; 773 ma.s.l.; 37°10′ N, 3°36′ W), and ‘Piccoplant’, with the latter clone provided by a private company (Piccoplant, Oldenburg, Germany).

In order to reduce heterogeneity, careful multiplication was done to a similar fresh weight at the beginning of the experiment. Plantlets were grown in a greenhouse at the University of Barcelona (Spain) in 5 L plastic pots containing peat:perlite:vermiculite (3:1:1) and well irrigated with a complete Hoagland solution [10]. The growth conditions were 25/15 °C day/night, 0.7 kPa of vapor pressure deficit, 40–60% RH, and ~1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. Measurements were done six months after planting.

### 2.2. Measurements

Nodes (10 cm) were planted in 300 mL plastic pots with coarse sand and Hoagland solution. Stems were grown for three weeks in a controlled chamber (conditions the same as the previous stage) to obtain new fresh roots. After the growth period, root meristematic zones (tips) were cut to look for the greatest cell division activity, following the technique of Haddadchi et al. and Bucci et al. for dyeing [9,11]. Chromosomes of 50 metaphasic cells (five cells/five roots/two plants) were counted for each clone using a Leitz DMIRB microscope (Chicago, IL, USA) and Fiji software (Image J, Wisconsin, USA); pictures were taken with a Leica DFC 360 FX camera (Chicago, IL, USA) (Figure 1).



**Figure 1.** Chromosome counting in four giant reed clones: (A), Piccoplant; (B), Fondachello; (C), Martinensis; (D), Granadensis.

Measurements of carbon assimilation ( $A_{\text{sat}}$ ;  $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ), stomatal conductance ( $g_s$ ;  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ ), maximum velocity of Rubisco carboxylation ( $V_{c, \text{max}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), maximum electron transport rate contributing to RuBP regeneration ( $J_{\text{max}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), transpiration ( $T$ ;  $\text{mmol m}^{-2} \text{s}^{-1} \text{H}_2\text{O}$ ) and maximum quantum efficiency ( $F_v/F_m$ ), photochemical efficiency of PSII ( $F_v'/F_m'$ ), and non-photochemical quenching (NPQ) were carried out in each clone ( $n = 3$ ) using a portable photosynthesis system Li6400 (Li-Cor Inc., Lincoln, NE, USA) [2]. Instantaneous water use efficiency ( $\text{WUE}_i$ ) was calculated as  $A_{\text{sat}}/T$ .

Chlorophyll content and relative water content (RWC, %) were also measured according to Sánchez et al. [2].

Whole plants were harvested, weighed and oven dried at 60 °C until constant weight was reached. Subsequently, the dry biomass of shoots (leaves and stems) and roots (roots and rhizomes) was determined. Total fresh weight and plant leaf area were estimated using a flat-bed scanner (Hewlett-Packard ScanJet model Iicx, San Diego, CA, USA) prior to drying and analyzed with image processing software (Image, University of Sheffield, 2003). Parameters such as height along the longest stem from the base to the latest totally expanded leaf (H; cm) and leaf area (LA; m<sup>2</sup>) were measured. The shoot dry weight (SDW; g), shoot/root ratio (S/R, g·g<sup>-1</sup>), leaf area index (LAI), and leaf mass area (LMA; g·m<sup>-2</sup>) were calculated.

### 2.3. Statistical Analysis

A statistical study of a one-factor ANOVA was performed with the SPSS 21.0 software package. Means ± standard errors of each replicate were calculated for each measured parameter. When a particular F-test was significant, the means were compared using a Tukey multiple comparison ( $p < 0.05$ ).

## 3. Results and Discussion

The number of chromosomes in each clone ranged from 98 to 122, indicating significant differences between clones (Table 1). To date, the number of giant reed chromosomes has not been precisely determined due to the small size and the high number of chromosomes present. Indeed, a wide range of different numbers of chromosomes have been reported for the species: 24, 48, and 96 [12]; 40 [13]; 84 [11]; 100 [14]; 108 [15]; and 110 chromosomes [8,9]. In our case, we found a maximum of 122 chromosomes; this variability was due more to the difficulties related to the technique used. Using other techniques, such as flow cytometry, could more accurately reveal the true amount of DNA in the clones.

**Table 1.** Chromosome numbers in each clone. Values represent the mean ± SE of fifty replicates ( $n = 50$ ). Values with different letters are significantly different ( $p < 0.05$ ) according to the Tukey test.

Clone	Chromosome Numbers Mean ± SE		
Fondachello	122	±1.51	a
Martinensis	116	±2.05	b
Piccoplant	110	±0.8	c
Granadensis	98	±10.88	d

Bucci et al. [9] explained that giant reed infertility may be related to its phylogenetic origin and, in turn, might be closely related to the number of chromosomes. Two hypotheses have been proposed: (i) a fertile tetraploid of *A. plinii* (144 chromosomes) was crossed with a diploid of the same species (72 chromosomes), resulting in a sterile triploid (108 chromosomes); or (ii) a fertile tetraploid of *A. plinii* (144 chromosomes) was crossed with *Phragmites australis* (96 chromosomes). In the end, these hypotheses may help to explain the chromosomal variability, the origin, and the cause of the sterility of giant reed, although this remains unresolved.

No significant differences were found between clones in any of the photosynthetic or fluorescence parameters (Table 2), except for Fv/Fm.  $A_{\text{sat}}$  values were similar to other previously reported values in control conditions using the Martinensis clone [2] or were slightly lower [16]. However, our data were very dissimilar from the  $A_{\text{sat}}$  values reported by Webster et al. [6] and Haworth et al. [17]. As mentioned above, this lack of variability between clones was also observed in other parameters, such as  $g_s$ ,  $V_{\text{c,max}}$ ,  $J_{\text{max}}$ ,  $T_i$  and  $WUE_i$ . In turn, our  $g_s$  and  $WUE_i$  values were lower than those observed in other studies [16]. Nevertheless, the  $V_{\text{c,max}}$  and  $J_{\text{max}}$  values were remarkably high and similar to those reported by Webster et al. [6]. These  $V_{\text{c,max}}$  and  $J_{\text{max}}$  values were almost twice the C3-species average found in natural and unfertilized stands (64  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and

134  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively, according to Wullschlegler et al. [18]), which also confirms the high photosynthetic capacity of *A. donax* [6].

**Table 2.** Means and standard errors ( $n = 3$ ) of carbon assimilation ( $A_{\text{sat}}$ ;  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ ;  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), maximum velocity of Rubisco carboxylation ( $V_{c, \text{max}}$ ;  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), maximum electron transport rate contributing to RuBP regeneration ( $J_{\text{max}}$ ;  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), transpiration ( $T$ ;  $\text{mmol m}^{-2} \text{ s}^{-1} \text{ H}_2\text{O}$ ), instantaneous water use efficiency ( $\text{WUE}_i$ ), maximum quantum efficiency ( $F_v/F_m$ ), photochemical efficiency of PSII ( $F_v'/F_m'$ ), non-photochemical quenching (NPQ), relative water content (RWC, %), and relative chlorophyll content (SPAD, %) of the four giant reed clones. n.s. non-significant differences in the same parameter between clones. Different lowercases letters mean significant differences in the same parameter between clones.

	Piccoplant			Fondachello			Martinensis			Granadensis		
$A_{\text{sat}}$	23.1	$\pm 0.3$	n.s.	22.1	$\pm 0.5$	n.s.	22.8	$\pm 0.9$	n.s.	26.2	$\pm 2.3$	n.s.
$g_s$	0.30	$\pm 0.04$	n.s.	0.35	$\pm 0.01$	n.s.	0.35	$\pm 0.05$	n.s.	0.29	$\pm 0.05$	n.s.
$V_{c, \text{max}}$	113.2	$\pm 3.1$	n.s.	108.2	$\pm 5.3$	n.s.	106.7	$\pm 1.5$	n.s.	120.8	$\pm 3.8$	n.s.
$J_{\text{max}}$	243.3	$\pm 6.0$	n.s.	229.5	$\pm 7.5$	n.s.	226.9	$\pm 24.7$	n.s.	261.0	$\pm 13.5$	n.s.
$T$	5.46	$\pm 0.79$	n.s.	5.99	$\pm 0.67$	n.s.	8.43	$\pm 0.94$	n.s.	6.64	$\pm 1.02$	n.s.
$\text{WUE}_i$	4.43	$\pm 0.64$	n.s.	2.76	$\pm 0.28$	n.s.	3.79	$\pm 0.49$	n.s.	4.02	$\pm 0.28$	n.s.
$F_v/F_m$	0.795	$\pm 0.002$	a	0.781	$\pm 0.007$	ab	0.772	$\pm 0.001$	b	0.784	$\pm 0.002$	ab
$F_v'/F_m'$	0.497	$\pm 0.004$	n.s.	0.505	$\pm 0.008$	n.s.	0.501	$\pm 0.003$	n.s.	0.518	$\pm 0.017$	n.s.
NPQ	1.507	$\pm 0.015$	n.s.	1.372	$\pm 0.108$	n.s.	1.397	$\pm 0.018$	n.s.	1.337	$\pm 0.096$	n.s.
RWC	97.64	$\pm 1.05$	ab	98.48	$\pm 0.76$	a	97.73	$\pm 0.84$	Ab	93.40	$\pm 1.62$	b
SPAD	44.5	$\pm 0.9$	b	43.8	$\pm 0.7$	b	46.9	$\pm 0.3$	Ab	48.6	$\pm 0.7$	a

The maximum quantum yield of PSII ( $F_v/F_m$ ) is a sensitive indicator of plant photosynthetic performance and lower values may also indicate photoinhibition and/or any type of stress [3]. In this case, although significant differences were observed among *Arundo donax* clones, we considered that the lowest value (Martinensis) was not at the same level as those found in other studies where photosynthetic damage was observed in *Arundo donax* [3]. In addition, we suppose that this decrease in ( $F_v/F_m$ ) in Martinensis was not due to a greater or lesser effect of stress in these clones since no significant differences were observed between other parameters ( $F_v'/F_m'$  and NPQ), and it had the highest water content (RWC) but probably the lowest chlorophyll content (Table 2).

No significant differences were found between clones regarding height (H, cm), although a lot of variability was observed (Table 3). In relation to biomass production measured as SDW, no significant differences were found between clones, although Piccoplant had the greatest production. Moreover, no significant differences between clones were found in S/R and LMA.

**Table 3.** Biomass parameters of the four *Arundo donax* L. clones. H, height (cm); LA, leaf area ( $\text{m}^2$ ); SDW, shoot dry weight (g); S/R, shoot root; LAI, leaf area index; LMA, leaf mass area ( $\text{g m}^{-2}$ ). n.s. non-significant differences in the same parameter between clones. Different lowercases letters mean significant differences in the same parameter between clones.

	Piccoplant			Fondachello			Martinensis			Granadensis		
H	75.8	$\pm 7.2$	n.s.	61.3	$\pm 10.4$	n.s.	57.3	$\pm 3.6$	n.s.	47.1	$\pm 4.9$	n.s.
LA	0.24	$\pm 0.03$	a	0.06	$\pm 0.02$	b	0.14	$\pm 0.02$	ab	0.20	$\pm 0.02$	a
SDW	26.8	$\pm 3.4$	n.s.	16.4	$\pm 2.5$	n.s.	16.8	$\pm 3.6$	n.s.	17.8	$\pm 6.1$	n.s.
S/R	4.3	$\pm 0.2$	n.s.	4.6	$\pm 0.5$	n.s.	3.3	$\pm 0.3$	n.s.	4.1	$\pm 0.8$	n.s.
LAI	21.2	$\pm 2.6$	a	5.7	$\pm 1.6$	b	12.7	$\pm 2.1$	ab	15.7	$\pm 3.4$	ab
LMA	46.0	$\pm 1.2$	n.s.	39.1	$\pm 8.1$	n.s.	51.4	$\pm 5.1$	n.s.	49.0	$\pm 5.3$	n.s.

However, the differences in LAI indicate that Fondachello was the clone with the lowest number of leaves but with a high weight since there were no significant differences in either SDW or LMA (Table 3). It would have been interesting to increase the number of individuals to be studied to improve the statistical results on biomass parameters.



The fact that the experiment was carried out in greenhouse conditions could explain the lack of significant differences in most biomass parameters due to the study's well-watered conditions, a phenomenon that has been observed in other studies [5,17]. The effect of pot cultivation is clearly seen in the low biomass production (understood as SDW), because the root development is limited. However, significant differences among clones related to biomass production have been found by other authors [19,20] when the cultivation of these clones, in well-watered conditions, was carried out in the field.

The study conducted by Hardion et al. [21] did not find any genetic variation in Mediterranean giant reed clones, whereas multiple investigations have shown that the number of chromosomes in giant reed can be very variable. The large difference in the number of chromosomes does not imply that there are large changes at the genetic level [22]. Indeed, a similar genetic plasticity is characteristic of aquatic plants having a predominantly vegetative reproduction system based on the fragmentation of parts of plants, which parallels the giant reed's reproductive strategy.

#### 4. Conclusions

The number of chromosomes in this investigation was highly variable, probably due to the chosen technique. The selection of another methodology would help to have accurate information on the chromosome count. According to our results, differences in the number of chromosomes in the giant reed clones studied under control conditions in a greenhouse were not related to the physiological or yield responses that they presented. It would therefore be interesting to extend this study and examine the same parameters of these clones in a population established under field conditions.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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