In vitro physical mutagenesis of giant reed (Arundo donax L.)

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Abstract

Giant reed (Arundo donax L.) is a C₃ perennial, warm-season, rhizomatous grass of emerging interest for bioenergy and biomass derivatives production, and for phytoremediation. It only propagates vegetatively and very little genetic variation is found among ecotypes, basically precluding breeding efforts. With the objective to increase the genetic variation in this species, we developed and applied a mutagenesis protocol based on γ-irradiation of in vitro cell cultures from which regenerants were obtained. Based on a radiosensitivity test, the irradiation dose reducing to 50% the number of regenerants per callus (RD₅₀) was estimated at 35 Gy. A large mutagenic experiment was carried out by irradiating a total of 3120 calli with approx. 1×, 1.5× and 2× RD₅₀. A total of 1004 regenerants from irradiated calli were hardened in pots and transplanted to the field. Initial phenotypic characterization of the collection showed correlated responses of biomass-related quantitative traits to irradiation doses. Approx. 10% of field-grown clones showed remarkable morphological aberrations including dwarfism, altered tillering, abnormal inflorescence, leaf variegation and others, which were tested for stability over generations. Clone lethality reached 0.4%. Our results show for the first time that physical mutagenesis can efficiently induce new genetic and phenotypic variation of agronomic and prospective industrial value in giant reed. The methodology and the plant materials described here may contribute to the domestication and the genetic improvement of this important biomass species.

Keywords: bioenergy crop, biomass, feedstock, gamma ray, genetic diversity, marginal land, perennial grass, polyploidy

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Introduction

Giant reed (Arundo donax L.) is a wild, perennial, rhizomatous C₃ Poaceae species of emerging interest for bioenergy production. It is characterized by very low requirements in terms of cultivation inputs; it adapts to a large range of soil and/or climatic conditions (e.g., from semiarid to waterlogging-prone environments) and has unusually high photosynthetic and carbon accumulation capacity, making it one of the most promising biomass crops for the Mediterranean area (Corno et al., 2014; Ge et al., 2016; Monti & Zegada-Lizarazu, 2016; Webster et al., 2016). Biomass produced by giant reed has been used in industrial cellulose production and for thermochemical conversion processes and more recently has attracted interest for production of bioethanol and other liquid biofuels, biomethane (by anaerobic digestion) and added-value bioproducts (Pilu et al., 2014; Ge et al., 2016). Giant reed was also used for riverbank consolidation and shows potential for soils and water phytoremediation (Nsanganwimana et al., 2013).

Giant reed is traditionally considered as native of subtropical Eurasia (including the Mediterranean, Middle East and northern India regions (Hardion et al., 2014). However, a relatively recent colonization of the Mediterranean region by one or very few invasive clones of Asian origin was also suggested (Mariani et al., 2010; Hardion et al., 2014). Different studies (reviewed in Bucci et al., 2013) reported a range of chromosome numbers (2n from 40 to 110), although two cytotypes seemed to prevail: 2n = 108 in both Europe and Asia, and 2n = 72 in Asia only (Hardion et al., 2014). The most likely base numbers appear to be x = 6 or 12, making giant reed highly polyploid (Saltonstall et al., 2010; Hardion et al., 2015). With the exception of few reports of seed reproduction (Perdue, 1958; Bor, 1970; Brach & Song, 2006), the species appears sterile and only propagates vegetatively by rhizome and shoot fragmentation during flooding events, and by shoot layering (Boland, 2006; Ceotto & Di Candilo, 2010; Saltonstall et al., 2010; Zegada-Lizarazu et al., 2010). It is...
unclear whether its sterility is caused by polyploidy (Bucci et al., 2013) or self-incompatibility (Hardion et al., 2015). Besides two recent transcriptome analyses (Sablok et al., 2014; Barrero et al., 2015), no sequencing-based genome investigation has been attempted in giant reed.

Likely as a consequence of both its propagation mechanism and history of geographical diffusion, giant reed is a species with reduced diversity as amply shown by molecular marker-based investigations (Khudamrong-sawat et al., 2004; Ahmad et al., 2008; Mariani et al., 2010; Hardion et al., 2012; Pilu et al., 2014) and by field experiments comparing ecotypes for traits of agronomic importance (Cosentino et al., 2006). However, stable phenotypic differences among ecotypes were more recently detected for traits such as phenology (Cantaluppi et al., 2016), cold, drought or salinity tolerance (Pompeiano et al., 2013; Sanchez et al., 2015; Haworth et al., 2017) and biomass and bioenergy production (Pilu et al., 2014; Amaducci & Perego, 2015).

Mutagenesis is one of the most effective tools available for the genetic improvement of annual and perennial crop species and has contributed to the development of thousands of cultivars worldwide (Bado et al., 2015; Bradshaw, 2016). Among the different types of mutagenic treatments, irradiation has been the most frequently used, with a predominance of γ-rays (Mba, 2013). Seeds are the preferred target for mutagenic treatments; however, buds, shoot apices and in vitro-cultured tissues can be treated in vegetatively propagated species (Predieri, 2001; Bado et al., 2015). Mutagenesis has already been applied to biomass species such as miscanthus (Lee et al., 2012; Perera et al., 2015) and poplar (Douglas, 1986), and in vitro physical mutagenesis has been applied to sugarcane (Nikam et al., 2014). Thus, in vitro physical mutagenesis appears to be a promising strategy for increasing genetic variability for breeding purposes, although, to the best of our knowledge, it has never been attempted in giant reed.

The objective of this work was to establish an efficient mutagenesis protocol of giant reed based on γ-ray irradiation of in vitro tissues (calli), with the final aim to produce novel and useful genetic variation for the genetic improvement of the species. A first description of field-grown giant reed morphological mutants is presented.

Materials and methods

Plant material and micropropagation protocol

A single wild plant of giant reed characterized by vigorous growth was collected in the Po Valley region (Italy) and used as source of in vitro-propagated meristemtic tissues. A giant reed-specific micropropagation protocol was developed to induce and establish stable multiplication of undifferentiated calli before preparing the tissue for the irradiation treatment. Calli were induced from sterile 2–5 cm long immature inflorescences after a 2-month period incubation on an induction medium (Table S1). Subsequently, induced calli were grown and multiplied on multiplication medium and subcultured every 4 weeks for a period of at least 4 months before the mutagenetic treatment. Organs differentiation of mutagenized calli took place in a differentiation medium (Table S1).

Radiosensitivity test experiment and identification of the 50% reduction dose (RD50)

A test experiment was carried out to assess the radiosensitivity of the plant materials and identify the ideal irradiation dose for large-scale mutagenesis. The optimal dose should be characterized by an acceptable compromise between high frequency of induced mutations and high calli regeneration rate, expected to increase and decrease, respectively, by increasing irradiation dose. Viable 5 mm diameter calli were transferred to multiplication media in Petri dishes (20 calli/dish) and immediately irradiated at different doses using a 60Co source Gammacell 220 apparatus (MDS Nordion International Inc., Ottawa, ON, Canada). Six dishes were irradiated per dose, corresponding to a total of 120 treated calli/dose. Irradiated calli were cultured onto the multiplication medium for 4 weeks before transferring them onto the differentiation medium for the regeneration of rooted plantlets via organogenesis. Petri dishes were kept at 25°C with a 16/8 hours light/dark cycle. Calli were subcultured onto a fresh differentiation medium every 4 weeks for a period of 10 months.

An early visual estimate of radiosensitivity was obtained by periodically scoring calli for their tissue-browning index (TBI), which was defined as the degree of callus browning as compared to a colored reference ladder (Fig. 1a). TBI scores ranged from 0 (perfectly green) to 100 (fully browned). Each callus was scored and the average TBI score per dish and per treatment calculated. TBI scores were estimated at 10, 30, 50, 180 and 300 days after irradiation treatment (DAT).

The rate of viable regenerants (VR) per callus was utilized as the final index of radiosensitivity. The concept of 50% reduction dose (RD50), as described in Kodym et al. (2012), was utilized in place of the more traditional 50% lethal dose (LD50). RD50 indicates the irradiation dose causing a 50% reduction in a biological index (in our case, the number of regenerants per callus) as compared with control (untreated) dose. RD50 was estimated after collecting and graphing VR values from the radiosensitivity test experiment across the seven doses, and fitting a logistic regression curve (Fig. 1b). Observations on TBI and VR stopped at 300 DAT.

After roots and shoots differentiation, plantlets (1–2 cm height) were transferred to 48-well trays filled with sandy soil for a 4-week period, at 25°C, 16/8 h light/dark and 95% relative humidity. This time period enabled plantlets to adapt to reduced air humidity and a nonaseptic environment. When plants reached 5 cm height, they were transferred into 1 liter pots for the final hardening phase at greenhouse conditions.
Based on the results of the radiosensitivity test experiment, a larger \(\gamma\)-irradiation experiment was carried out. In vitro culture, irradiation procedure, plant regeneration and hardening were performed as described for the test experiment, with the exception that hardened plants were transferred and kept in 121 pots for 8–14 months at greenhouse conditions.

**Field evaluation and definition of mutant classes**

Mutagenized, independently regenerated hardened plants were randomly (irrespectively of the irradiation dose) transplanted in the field (at Ca’ Bosco, near Ravenna, Italy) during spring (April 2015). Eleven control plants derived from untreated calli and subjected to the same regeneration and hardening processes were transplanted along with the irradiated plants. Plants were spaced at 2.5 \(\times\) 2.5 m between and within row and cultivated following standard agronomic practice. At the end of the first growing season (November 2015), all shoots were mechanically shredded.

For each of the 1011 field-grown giant reed clones, three biomass-related quantitative traits were collected in October 2015: maximum plant height, number of shoots (or tillering) and stem diameter. Maximum plant height was expressed in m and collected by manually measuring the highest shoot for each plot. Number of shoots was obtained by directly (visually) counting all shoots taller than 0.1 m per plot. Stem diameter (expressed in mm) was the average value from 5 to 10 representative shoot stems per plot (measurements were taken with a handheld caliper by considering the external stem diameter, between two stem nodes, at 0.5 m height from the soil level).

Putative morphological mutants were identified for the three morphological metric traits described above and were classified into four mutant classes: short stature, thin stem, high-tillering and low-tillering. For these traits, the putative mutants corresponded with the extreme outlier plants as identified by comparison with the box plot distributions of control (0 Gy) plants (Fig. 2). Two additional mutant classes were identified, namely variegate and abnormal shoots. Mutants were classified as variegate when showing at least one shoot with clearly variegated leaves or leaf sectors (white or yellow colored) on multiple leaves of the shoot. Mutants were classified as abnormal shoot when showing obvious aberrant shoot development in terms of stem, leaves or inflorescences shape and/or architecture. For all these putative mutants, their extreme phenotypic values were confirmed in 2016.

**Statistical analysis**

Analysis of variance was performed to test the effect of different irradiation doses (0, 40, 60 and 80 Gy doses; dose as fixed factor) on biomass-related quantitative traits collected during field evaluation. Trait mean comparisons between doses were conducted using Tukey’s test. All statistical analyses were conducted using R (R Core Team, 2015), with Tukey’s HSD function and Tuckey’s test.

**Results**

**Development of the mutagenic protocol**

The optimal irradiation dose for \(\textit{in vitro}\) physical mutagenesis of giant reed was searched in a test experiment. Calli treated at \(\leq\)20 Gy and control (untreated) calli suffered little or no tissue browning and differentiated shoots throughout the whole period of observation (Fig. 1a and Table 1). However, higher irradiation doses (from 40 to 100 Gy) showed a dramatic TBI increase and significantly reduced VR (from 23% to 6%, respectively. Table 1) as compared to untreated control

![Fig. 1](image-url)
Overall, increasing irradiation doses correlated positively with TBI ($r^2 = 0.94$, $P < 0.01$, at 50 days after treatment. Table 1) and negatively with VR ($r^2 = -0.93$, $P < 0.01$), as expected. Based on the above test experiment, the RD$_{50}$, namely the irradiation dose corresponding to a 50% reduction in the number of viable regenerated plantlets per callus as compared with untreated control, was estimated to be 35 Gy (Fig. 1b).

Large-scale mutagenic experiment

Based on the estimated RD$_{50}$, 40, 60 and 80 Gy doses (corresponding to approx. $1 \times$, $1.5 \times$ and $2 \times$ RD$_{50}$) were utilized to treat 1200, 1200 and 720 calli, respectively, which produced a total of 1004 regenerants (Table 2). VR was negatively related to irradiation dose, with values of 34.8, 33.1 and 26.3% for 40, 60 and 80 Gy doses, respectively. As in the test experiment, 40 and 60 Gy doses provided very similar VR, while VR was significantly lower for 80 Gy ($P < 0.05$; Tukey). This notwithstanding, in this experiment VR was generally higher than that observed in the test experiment, so that even the 80 Gy dose provided a sizable number of plantlets (Table 2).

**Effects of irradiation dose on quantitative traits and identification of putative mutants**

A collection of 1015 pot-hardened clones were transplanted in the field (1004 clones regenerated from irradiated calli and 11 regenerated from untreated calli). Four clones (0.4%) died by the end of the first growing season (October 2015). Thus, phenotypic scores were collected on a total of 1011 clones.

![Box plots](image)

**Table 1** Effect of different $\gamma$-irradiation doses on in vitro plant regeneration in giant reed

| Dose (Gy) | Treated calli | TBI (%) at different DAT | VR* (No.) | %† |
|-----------|---------------|--------------------------|-----------|----|
| 0         | 120           | 1 1 1 1 1 1 1 101 84 (a) |
| 10        | 120           | 1 3 6 3 1 103 86 (a)     |
| 20        | 120           | 1 3 6 9 7 85 71 (a)     |
| 40        | 120           | 3 5 18 47 100 28 23 (b) |
| 60        | 120           | 4 8 33 58 100 21 18 (b) |
| 80        | 120           | 4 9 57 100 100 9 8 (c)  |
| 100       | 120           | 4 7 42 100 100 7 6 (c)  |

TBI, tissue-browning index; DAT, days after irradiation treatment; VR, viable regenerants.

*Viable regenerants at 300 days after irradiation treatment.

†Percentage of viable regenerants per callus. Different letters indicate significantly different values ($P < 0.05$, Tukey’s test).

**Table 2** Number of giant reed viable regenerant plants (VR) obtained from the large-scale mutagenesis experiment per dose level

| Dose (Gy) | Irradiated calli (No.) | VR (No.) | %* |
|-----------|------------------------|----------|----|
| 40        | 1200                   | 418      | 34.8 (a) |
| 60        | 1200                   | 397      | 33.1 (a) |
| 80        | 720                    | 189      | 26.3 (b) |
| Total     | 3120                   | 1004     | 32.2 |

*Percentage of viable regenerants per callus. Different letters indicate significantly different values ($P < 0.05$, Tukey’s test).
To test whether different irradiation doses had any effect on giant reed phenotypic traits, three shoot architecture traits (maximum shoot height, number of shoots per plant and stem diameter) were analyzed. The three traits were significantly influenced by irradiation dose (Fig. 2; Table S2). Specifically, the number of shoots per clone increased with irradiation dose, from 15.3 shoots for control (untreated) to 21.8, 21.9 and 24.1 shoots in the 40-, 60- and 80-Gy-treated plants, respectively (Fig. 2a). Conversely, shoot height was negatively related to the irradiation dose, as the maximum shoot height (2.8 m) was observed in controls and decreased progressively to 2.7, 2.5 and 2.2 m, in the 40-, 60- and 80-Gy-treated plants, respectively (Fig. 2b). Plants obtained from untreated calli showed significantly larger stem diameter (18.2 mm, \( P < 0.05 \), Fig. 2c) compared to the irradiated ones; however, no significant difference was observed for stem diameter among 40, 60 and 80 Gy irradiation doses (Fig. 2c).

Based on shoot trait measurement and/or scoring, 93 clones (corresponding to approx. 10% of all treated clones) were identified as putative morphological mutant clones and were preliminarily classified in six classes (Table 3). The class abnormal shoot included a curly leaves/inflorescences mutant (Fig. 3c), a mutant characterized by patent leaves and a mutant characterized by shortened, deviated internodes. The class variegated included four similar mutants showing different levels and colors of leaf variegation (Fig. 3a); all four variegated mutants were chimeric. High-tillering and low-tillering classes included six and four mutants showing extremely high or low propensity (Fig. 4) to develop additional shoots per plot, respectively. The class short stature included 64 clones. This was a relatively heterogeneous class including both weak, stunted plants (Figs 3b and 4) and plants with shortened internodes. The thin stem class includes 12 putative mutants characterized by stems remarkably thinner than the untreated control (Fig. 4). No mutant was found with shoots taller or stems larger than the untreated control. As expected, the frequency of putative mutants increased with irradiation dose from 4.8% (40 Gy) to 22.8% (80 Gy; Table 3).

To further test the stability of the observed phenotypes, five putative mutant clones which were already clearly identified at the end of the first field growing season were vegetatively propagated by rhizome subdivision and phenotypic traits collected after approx. 10 months (Table 4 and Table S2; Fig. 4). All five mutants confirmed to be stable, supporting a genetic basis of the observed phenotypes. Also in this case, the original five mother plants continued to show the same phenotypes during the second year (checked in September 2016, not shown).

Many additional clones showed strongly modified expression of typical quantitative traits such as flowering time, inflorescence size and architecture, shoot habitus, leaf size and others. However, as variation for these traits is highly quantitative (i.e., affected by multiple genes, environmental factors and uncontrolled managing effects related to the regeneration, hardening and transplantation phases), it will only be possible to confirm these clones as mutants after they will be tested in replicated experiments.

**Discussion**

A mutagenesis program requires handling a high number of individuals. Giant reed is a relatively large plant with neither sexual reproduction nor seed production. Thus, while mutagenesis on pollen or seed was precluded, *in vitro* cell culture mutagenesis followed by regeneration appeared a suitable choice to produce a large number of mutagenized clones. This choice was also supported by the availability of reliable protocols for giant reed *in vitro* culture and regeneration (Takahashi et al., 2010) and by the former successful application of similar approaches in other species (reviewed in Jain et al., 2010 and Suprasanna et al., 2012) including physical \( (y) \) irradiation in the botanically related species sugarcane (Nikam et al., 2014). Based on regeneration rate per callus, irradiation doses in the 40–60 Gy range seemed appropriate to maximize the recovery of mutants in giant reed. Our RD\(_{50}\) estimate appears in the range of RD\(_{50}/LD\(_{50}\) estimates previously obtained in other polyplody species such as sugarcane (Nikam et al., 2014), banana (Sales et al., 2013), cassava (Magalhaes et al., 2015) and rose (Bala & Pal Singh, 2013). Higher irradiation doses (1.5× and 2× RD\(_{50}\)) were also applied in our

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**Table 3** Summary of giant reed putative mutant clones for qualitative traits identified after field observations

| Mutant class   | Clone per irradiation dose (No.) | Total (No.) |
|----------------|----------------------------------|-------------|
|                | 40 Gy   | 60 Gy   | 80 Gy   |                |
| Abnormal shoot | –       | 2       | 1       | 3              |
| Variegate      | 1       | 2       | 1       | 4              |
| Short stature  | 12      | 22      | 30      | 64             |
| High-tillering | 1       | 3       | 2       | 6              |
| Low-tillering  | 1       | 2       | 1       | 4              |
| Thin stem      | 2       | 5       | 5       | 12             |
| Lethal*        | 1       | 2       | 1       | 4              |
| All putative mutants | 18     | 38      | 41      | 97             |
| All clones     | 397     | 418     | 189     | 1004           |
| % mutant clones| 4.5     | 9.1     | 21.7    | 9.7            |

*Number of dead clones in September 2016 (15 months after field transplantation).*
experiment to maximize the recovery of visible mutant plants. Indeed, in polyploid species, the expression of phenotypes upon artificial mutagenesis is expected to be masked by high gene functional redundancy (Comai, 2005). Therefore, higher doses should increase the probability of deleting two or more copies of the same genes, thus increasing the visible mutant recovery rate.

The frequency of clones (9.7%) showing aberrant phenotypes was in our experiment relatively high (for a highly polyploid species). Different mechanisms likely contributed to this result. First, gene functional redundancy is probably less than that predicted based on genome duplications, given the high grade of genome re-arrangements, rapid sequence loss (of homeolog genes) and genome downsizing occurring after most polyploidization events (Parry et al., 2009; Tayalé & Parisod, 2013; Soltis et al., 2015). Indeed, genome downsizing mechanisms have likely acted in giant reed given its relatively low genome size \( n \approx 2.4 \text{ pg} \approx 2.3 \text{ Gb} \) as compared to its presumed high ploidy (9 or 18x). This is also exemplified by the relatively reduced number (six) of 5S, 45S loci and nucleolus, which are usually maintained as single locus in a genome (Hardion et al., 2015). Second, the multiple genomes which originated extant giant reed were likely heterogeneous (Hardion et al., 2015); therefore, mutational events (e.g., deletions) where one dominant allele would turn to a recessive one at a heterozygous locus (and thus inducing a phenotype) are expected to be relatively frequent (Suprasanna et al., 2012). Third, dominant mutations are also expected to occur, albeit with drastically reduced frequency (<1% of recessive mutations), as previously
empirically shown in other systems (Gottschalk & Wolff, 1983). The molecular nature of the induced variants remains to be investigated. Some of the variants might have resulted from somaclonal variation as in vitro cultures were involved in our protocol. Somaclonal variation is known to include several types of molecular events including change in chromosome number and structure, transposon activation and movement, point mutations and/or methylation changes (Kaeppeler et al., 2000; Bairu et al., 2011; Ong-Abdullah et al., 2015). As γ-irradiation is thought to mostly induce deletions, a molecular analysis disclosing deletions across the mutant collection should indirectly enable us to quantify the impact of somaclonal variation in our materials. Applications of next-generation sequencing to characterize genome features of this collection are currently in progress.

Because multicellular tissues were irradiated, it is likely that some regenerated plants were chimeric (i.e., carrying sectorial genetic differences of cells and tissues in the same individual). However, protocols suitable to gradually separate chimeras and produce solid homo-histons by several generations of vegetative propagation have been described and can be applied when needed (Predieri, 2001; Mba, 2013). Notably, the four ‘variegate’ mutants that were identified within our collection all turned out to be not solid (i.e., the variegation was clearly of different grade among different shoots of the same plant). This notwithstanding, ‘variegate’ clones require further investigations as they might not simply

Fig. 4 Representative images of phenotypic stability of giant reed mutant clones. Plants shown here were obtained by vegetative propagation (by rhizome subdivision) from putative mutant clones identified in the field based on visual observations. Photographs were taken at approx. 6 months of cultivation in greenhouse after rhizome subdivision. (a) Clone #22-80-2, low-tillering, tall; (b) clone #157-40-221, thin stem, patent leaves; (c) clone #182-60-17, short stature, bushy; (d) clone #182-60-60, erect, semidwarf. wt = wild-type (untreated control) plants.
High- or low-tillering erect leaves) to typical dominant dwarf maize mutants showed similarities (including shorter internodes and instance, some giant reed short stature mutants lar mutants described in other grass species. For putative mutants bear obvious resemblance with simi-
confirmed their abnormal phenotypes in vegetatively were completely mechanically shredded at the end of observations during two successive years of cultivation, showed phenotypic stability based on field plot obser-
real mutants. First, all highlighted clones (Table 3) received early by the calli/early regenerants, or was a injury caused by the irradiation treatment and per-
was directly triggered by the primary cellular injury caused by the irradiation treatment and perceived early by the calli/early regenerants, or was a consequence of a secondary type of stress due to the mutation load is currently unclear.
Although all clones showing aberrant phenotypes should in principle be considered putative mutants until further investigations will be carried out, three observations suggest that these clones may indeed be real mutants. First, all highlighted clones (Table 3) showed phenotypic stability based on field plot observations during two successive years of cultivation, where shoots (i.e., any aboveground plant structures) were completely mechanically shredded at the end of the first year. Second, five randomly chosen clones confirmed their abnormal phenotypes in vegetatively propagated subclones. Third, several of our giant reed putative mutants bear obvious resemblance with similar mutants described in other grass species. For instance, some giant reed short stature mutants showed similarities (including shorter internodes and erect leaves) to typical dominant dwarf maize mutants (Sheridan, 1988). Similarly, high- or low-tillering mutants are well known in maize, barley and rice (Neuffer et al., 1997; Chuck et al., 2007; Hussien et al., 2014). Many additional clones showed more subtle, quantitative differences in comparison with untreated control plots, for traits such as flowering time, shoot habitus (erect vs. prostrate), leaf size and others. Replicate experiments will be required to test the stability of these phenotypes.

Mutants for several agronomically and industrially important phenotypes could be searched in our collection. Ample variation for quantitative traits linked with yield/biomass, such as the number of shoots, shoot height and others, was clearly observed and could be verified in replicated trials. Additionally, mutants could be searched for improved response to multiple cuts per year and the extension of leaf juvenility, which are growth-related traits recently shown to improve bio-
methane yield (Di Girolamo et al., 2013; Ragaglini et al., 2014), and for enhanced cold and drought tolerance as little variation exists between giant reed ecotypes for these traits (Pompeiano et al., 2013; Haworth et al., 2017). Extreme variants could be searched for (lower) ash content, which is an important quality parameter in several energy-generating processes and is known to vary between giant reed ecotypes (Amaducci & Perego, 2015), or for cell wall properties, including cellulose, hemicellulose and lignin, or their chemical modifications, which may strongly impact energy transformation or be important as independent bioproducts (Chen & Dixon, 2007; Marriott et al., 2014; Wang et al., 2016). Based on similar hypotheses, mutants characterized by improved saccharification properties were identified in a mutagenized population of Brachypodium (Marriott et al., 2014). Finally, clones characterized by reduced stem fragmentation and rooting propensity at shoot nodes, and/or reduced rhizome diffusion could help to mitigate the supposed giant reed invasiveness, a perceived threat in some environments (Saltonstall et al., 2010).

The paucity in giant reed genetic diversity, the lack of organized breeding efforts and the increasing interest in multiple industrial applications make the identification of novel giant reed genotypes and the release of genetically improved giant reed cultivars two important priorities in the field of plant feedstock research. In this work, we showed that it is possible to generate remarkable genetic and phenotypic variation for agronomically relevant traits in this highly polyploid species by means of in vitro physical mutagenesis. The protocol and the materials described here could represent the beginning of giant reed genetic improvement and could be of interest in breeding programs of other vegetatively propagated species suffering of low genetic variability.
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References

Ahmad R, Liew P, Spencer D, Jasieniuk M (2008) Molecular evidence for a single genetic clone of invasive Arundin donax in the United States. Aquatic Botany, 88, 113–120.

Amaducci S, Perego A (2015) Field evaluation of Arundo donax clones for bioenergy production. Industrial Crops and Products, 75, 122–128.

Bado S, Forster BP, Nielsen S, Ali AM, Lagoda PJ, Till BJ, Laimer M (2015) Plant mutation breeding: current progress and future assessment. Plant Breeding Reviews, 39, 23–87.

Bairu MW, Arena AO, Van Staden J (2011) Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation, 63, 147–173.

Bala M, Pal Singh K (2013) Genetic manipulation of giant reed (Arundo donax L.) grown under variable nitrogen fertilization. Annals of Applied Biology, 163, 793–802.

Barrero RA, Guerrero FD, Moolhuijzen P, Goolsby JA, Tidwell J, Bellgard MI (2015) Shoot transcriptome of the giant reed, Arundo donax: Data in Brief, 3, 1–6.

Boland JM (2006) The importance of layering in the rapid spread of Arundo donax (giant reed). Madrono, 53, 303–312.

Bor NL (1970) Gramineae. In: Florajaponica, Vol. 70 (ed. Rechinger KH), pp. 147–244. Akademische Druck und Verlaganstalt, Graz, Austria.

Brach AR, Song H (2008) eFloras: new directions for on line florae exemplified by the Flora of China Project. Taxon, 55, 188–192.

Bradshaw JE (2016) Plant Breeding: Past, Present and Future. Springer International.

Buccu A, Cassani E, Landoni M, Cantaluppi E, Pilu R (2013) Analysis of chromosome number and speculations on the origin of Arundo donax L. (Giant Reed). Cytologia and Genetics, 47, 237–241.

Cantaluppi E, Cassani E, Puglisi D, Corno L, Munaro M, Landoni M, Pilu R (2016) Study on the inflorescences of Arundo donax L. clones sampled in Italy. Brazilian Journal of Botany, 39, 275–285.

Ceotto E, Di Candilo M (2010) Shoot cuttings propagation of giant reed (Arundo donax L.) in water and moist soil: the path forward? Biomass and Bioenergy, 34, 1614–1623.

Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. Nature Biotechnology, 25, 759–761.

Chung C, Cigan MA, Saetem K, Hake S (2007) The heterochronic maize mutant Correged results from overexpression of a tandem microRNA. Nature Genetics, 39, 544–549.

Comai L (2009) The advantages and disadvantages of being polyploid. Nature Reviews Genetics, 6, 836–846.

Corno L, Pilu R, Adam F (2014) Arundo donax L.: a non-food crop for bioenergy and bio-compound production. Biotechnology Advances, 32, 1533–1549.

Cosentino SL, Copani V, D’agostina GM, Sanzone E, Mantinone M (2006) First results on evaluation of Arundo donax L. clones collected in Southern Italy. Industrial Crops and Products, 23, 212–222.

Di Girolamo G, Grigatti M, Barbanti L, Angelidaki I (2013) Effects of hydrothermal pretreatments on Giant reed (Arundo donax) methane yield. Bioreource Technology, 147, 152–159.

Douglas GC (1986) Effects of Gamma Radiation on Morphogenesis and Maturation in Cultured Stem Explants of Poplar. Nuclear Techniques and in vitro Culture for Plant Improvement. Procedure International Symposium, pp. 121–128. IAEA, Vienna.

Ge X, Xu F, Vasco-Correa J, Li Y (2016) Giant reed: a competitive energy crop in comparison with miscanthus. Renewable and Sustainable Energy Reviews, 54, 350–362.

Gottschalk W, Wolff G (1983) Monographs on Theoretical and Applied Genetics 7: Induced mutations in plant breeding. Springer-Verlag, New York.

Grossnickle SC (2012) Why seedlings survive: influence of plant attributes. New Forests, 43, 711–738.

Hardion L, Verlaque R, Saltonstall K, Leriche A, Vila B (2012) Revised systemsatics of Mediterranean Arundo (Poaceae) based on AFLP fingerprints and morphology. Taxon, 61, 1217–1226.

Hardion L, Verlaque R, Saltonstall K, Leriche A, Vila B (2014) Origin of the invasive Arundo donax (Poaceae): a trans-Asian expedition in herbaria. Annals of Botany, 113, 455–462.

Hardion L, Verlaque R, Rosasco M, Rossello JA, Vila B (2015) Impact of polyploidy on fertility variation of Mediterranean Arundo L. (Poaceae). Comptesrendus Biologies, 338, 298–306.

Haworth M, Centritto M, Giovannelli A, Marino G, Proietti N, Capitini D, Loreto F (2017) Xylem morphology determines the drought response of two Arundo donax ecotypes from contrasting habitats. Global Change Biology, 9, 119–131.

Hussain A, Tavakoli E, Horner DS, Muftor-Amatirain M, Muelhibauer GJ, Rossini L (2014) Genetics of tallering in rice and barley. The Plant Genome, 7, 1.

Jain SM, Ochatt SJ, Kulkarni VM, Predieri S (2010) In vitro culture for mutant development. Acta Horticulturae, 865, 59–68.

Kaeppler SM, Kaeppler HF, Rhee YW (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology, 43, 179–188.

Khidamomrongswat J, Tayyar R, Holt JS (2004) Genetic diversity of giant reed (Arundo donax) in the Santa Ana River, California. Weed Science, 52, 395–405.

Kodym A, Afza R, Forster B, Ukiy Y, Nakagawa H, Mba C (2012) Methodology for physical and chemical mutagenic treatments. In: Plant mutation breeding and biotechnology (eds Shu QY, Forster BP, Nakagawa H), pp. 169–181. CAB International, Wallingford.

Lee GJ, Zhang L, Choi YL, Chang SJ, Yoo YK, Kim DS, Kim SH (2012) Biomass and molecular characteristics of multi-tilling Miscanthus mutants. Korean Journal of Plant Resources, 25, 745–752.

Magaià HE, Joseph J, Elsy CR, Francis RM, Bastian D (2015) Creation of variability by in vitro mutagenesis in cassava (Manihot esculenta, Crantz). Journal of Tropical Agriculture, 53, 123–130.

Martoččijano M (1997) Chimeras and variegation: patterns of deceit. Horticultural Science, 32, 773–784.

Mariani C, Cabrini R, Danin A, Piffanelli F, Fricono A, Gomarasca S, Soave C (2010) Origin, diffusion and reproduction of the giant reed (Arundo donax L.): a promising woody energy crop. Annals of Applied Biology, 157, 191–202.

Marniott PE, Sibout R, Catherine Lapierre C et al. (2014) Range of cell-wall alterations enhance saccharification in Brachypodium distachyon mutants. PNAS, 111, 14601–14606.

Mba C (2013) Induced mutations unleash the potentials of plant genetic resources for food and agriculture. Agronomy, 3, 200–231.

Monti A, Zegada-Lizarazu W (2016) Sixteen-year biomass yield and soil carbon storage of giant reed (Arundo donax L.) grown under variable nitrogen fertilization rates. BioEnergy Research, 9, 248–256.

Neuffer MG, Cole EH, Wessler SR (1997) Mutants of Maize. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Nikam AA, Devarumath RM, Shitole MG, Ghole VS, Tawar PN, Suprasanna P et al. (2012) Methodology for identification of mutagenic agents using gamma-radiation to induce novel flower colour mutations. Molecular Breeding, 30, 165–177.

Noureddine S, Isnard P, Huchard F, Seuret G, Basset G, Angelidaki I (2010) Effects of hydrothermal pretreatments on Giant reed (Arundo donax) methane yield. Bioreource Technology, 101, 710–714.

Ogbo N, Marshall MA, Mba C (2012) The efficacy of chemical mutagenesis in the generation of highly productive, high yield and disease resistant cassava (Manihot esculenta, Crantz). Annals of Applied Biology, 160, 305–317.

Pandey P, Kaur H, Behera AB, Saini SK, Bajaj YPS (2014) Production of biomass from M. giganteus. Nature 518, 30–31.

Parry MAJ, Madgwick PJ, Bayo C (2006) ‘In vitro’ cell culture in the evaluation of a new combination of antifungal agents. Journal of Microbiological Methods, 66, 61–68.

Pereira D, Barnes DJ, Baldwin BS, Reichert NA (2015) Mutagenesis of in vitro cultures of Miscanthus × giganteus cultivar Freedoom and detecting polymorphisms of regenerated plants using ISSR markers. Industrial Crops and Products, 65, 110–116.

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Pilu R, Cassani E, Landoni M et al. (2014) Genetic characterization of an Italian Giant Reed (Arundo donax L.) clones collection: exploiting clonal selection. *Euphytica*, 196, 169–181.
Pompeiano A, Vita F, Miele S, Gaglielmetti L (2013) Freeze tolerance and physiological changes during cold acclimation of giant reed [Arundo donax (L.)]. *Grass Forage Science*, 70, 168–175.
Potters G, Pasternak TP, Guisey Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? *Trends in Plant Science*, 12, 98–105.
Predieri S (2003) Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture*, 64, 185–210.
R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
Ragaglini G, Dragoni F, Simone M, Bonari E (2014) Suitability of giant reed (Arundo donax L.) for anaerobic digestion: effect of harvest time and frequency on the biomethane yield potential. *Bioresource Technology*, 152, 107–115.
Sablok G, Fu Y, Bobbio V, Laura M, Rotino GL, Bagnaresi P, Li M (2014) Fuelling genetic and metabolic exploration of C3 bioenergy crops through the first reference transcriptome of Arundo donax L. *Plant Biotechnology Journal*, 12, 554–567.
Sales EK, Lopez J, Espino RRC, Butardo NG, Gonzalez Diaz L (2013) Improvement of bananas through gamma ray irradiation. *Philippine Journal of Crop Science (PJCS)*, 38, 47–53.
Sales EK, Lopez J, Espino RRC, Butardo NG, Gonzalez Diaz L (2013) Improvement of bananas through gamma ray irradiation. *Philippine Journal of Crop Science (PJCS)*, 38, 47–53.
Saltonstall K, Lambert A, Meyerson LA (2010) Genetics and reproduction of common (Phragmites australis) and giant reed (Arundo donax). *Invasive Plant Science and Management*, 3, 495–505.
Sanchez E, Scordia D, Lino G, Arias C, Cosentino S, Nogues S (2015) Salinity and water stress effects on biomass production in different Arundo donax L. clones. *Bioenergy Research*, 8, 1461–1479.
Sheridan WF (1988) Maize developmental genetics: genes of morphogenesis. *Annual Review of Genetics*, 22, 353–385.
Soltis PS, Marchant DB, Van de Peer Y, Soltis DE (2015) Polyploidy and genome evolution in plants. *Current Opinion in Genetics & Development*, 35, 119–125.
Suprasanna P, Jain SM, Ochatt SJ, Kulkarni VM, Predieri S (2012) Applications of in vitro techniques in mutation breeding of vegetatively propagated crops. In: *Plant Mutation Breeding and Biotechnology* (eds Shu QY, Forster BP, Nakagawa H), pp. 369–383. CABI International, Wallingford.
Takahashi W, Takamizu T, Kobayashi M, Ebina M (2010) Plant regeneration from calli in giant reed (Arundo donax L.). *Grassland Science*, 56, 224–229.
Tayáel A, Parissod C (2013) Natural pathways to polyploidy in plants and consequences for genome reorganization. *Cytogenetic and Genome Research*, 140, 79–96.
Wang Y, Fan C, Hu H, Li Y, Sun D, Wang Y, Peng L (2016) Genetic modification of plant cell walls to enhance biomass yield and biofuel production in bioenergy crops. *Biotechnology Advances*, 34, 997–1017.
Webster RJ, Driever SM, Kromdijk J et al. (2016) High C3 photosynthetic capacity and high intrinsic water use efficiency underlies the high productivity of the bioenergy grass Arundo donax. *Scientific Reports*, 6, 20694.
Zegada-Lizarazu W, Elbersen W, Cosentino SL, Zatta A, Alexopoulos E, Monti A (2010) Agronomic aspects of future energy crops in Europe. *Biofuel Bioproducts and Biorefining*, 4, 674–691.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Media composition for giant reed in-vitro tissue culture. Component amount are expressed as mg l⁻¹.

**Table S2.** Giant reed mutant clones tested for phenotypic stability: mean trait values and statistical comparison with untreated control.

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