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

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Abstract

Giant reed (*Arundo donax* L.) is a C_3 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 (RD_{50}) 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× RD_{50}. 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 C_3 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

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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 (Khudamrongsawat *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 meristematic 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 mutagenic treatment. Organs differentiation of mutagenized calli took place in a differentiation medium (Table S1).

Radiosensitivity test experiment and identification of the 50% reduction dose (RD_{50})

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 ^{60}Co 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 (RD_{50}), as described in Kodym *et al.* (2012), was utilized in place of the more traditional 50% lethal dose (LD_{50}). RD_{50} 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. RD_{50} 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 γ-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 12 l 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 × 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.

Phenotype stability of five putative morphological mutants (along with an untreated control, for comparison) was further tested by clonal propagation (by rhizome subdivision, five subclones per clone) in pots. Propagation was carried out in October 2015, and subclones were cultivated in pots in greenhouse during the winter and transplanted in the field in spring 2016. Observations were collected in pots (winter) and in the field during the second growing season (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 *in vitro* physical mutagenesis of giant reed was searched in a test experiment. Calli treated at ≤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

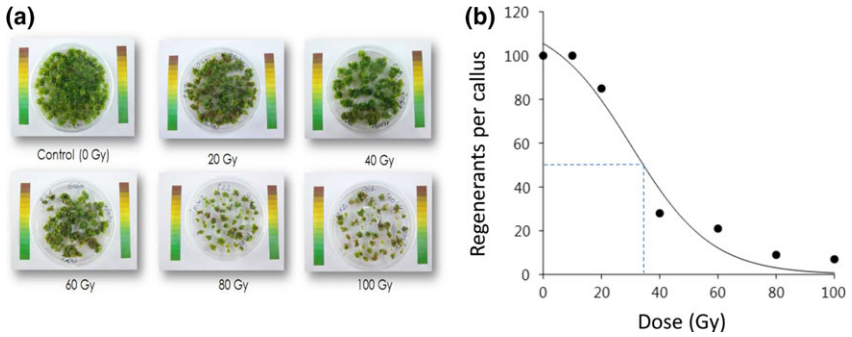

Fig. 1 (a) Representation of γ-irradiation sensitivity of giant reed calli at 120 days after treatment (DAT) as utilized to derive the tissue-browning index (TBI). Effect of 10 Gy dose is not shown; (b) Reduction–dose curve indicating the radiosensitivity response of giant reed calli. Curve indicates the reduction in the number of regenerated plantlets per callus (expressed as percentage of control untreated samples), based on seven different γ-irradiation doses (0–100 Gy). Dots represent mean values of six replicates. Interpolate curve was fitted using logistic regression. Dashed lines indicate the reduction dose 50% (RD_{50}), namely the γ-irradiation dose that resulted in 50% reduction in number of regenerated plantlets per callus.

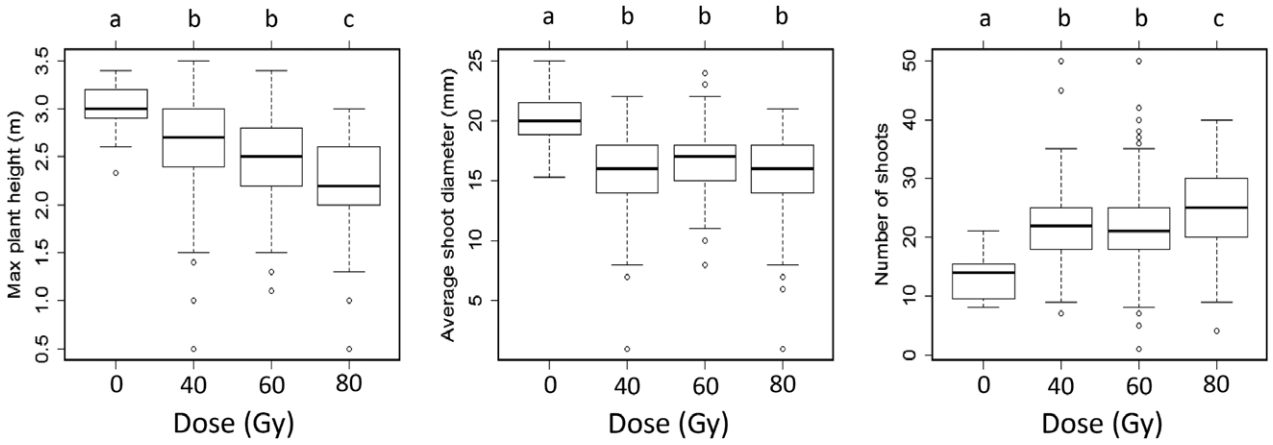

Fig. 2 Effects of different γ-irradiation doses on giant reed biomass-related traits measured on clones obtained from the large-scale mutagenesis experiment, 6 months after field transplantation. Box plots report median, first and third quartile and 95% confidence interval of the median. Different letters indicate different mean values ($P < 0.05$, Tukey's test).

($P < 0.01$, Tukey's test). 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×, 1.5× and 2× 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).

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

Dose (Gy)	Treated calli	TBI (%) at different DAT					VR*	
		10	30	50	180	300	(No.)	%†
0	120	1	1	1	1	1	101	84 (a)
10	120	1	3	6	3	1	103	86 (a)
20	120	3	3	9	7	3	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).

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.

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 *variegate* included four similar mutants showing different levels and colors of leaf variegation (Fig. 3a); all four variegate 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).

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

	Clone per irradiation dose (No.)			
Mutant class	40 Gy	60 Gy	80 Gy	Total (No.)
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).

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 (γ) 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 polyploid species such as sugarcane (Nikam *et al.*, 2014), banana (Sales *et al.*, 2013), cassava (Magaia *et al.*, 2015) and rose (Bala & Pal Singh, 2013). Higher irradiation doses (1.5× and 2× RD_{50}) were also applied in our

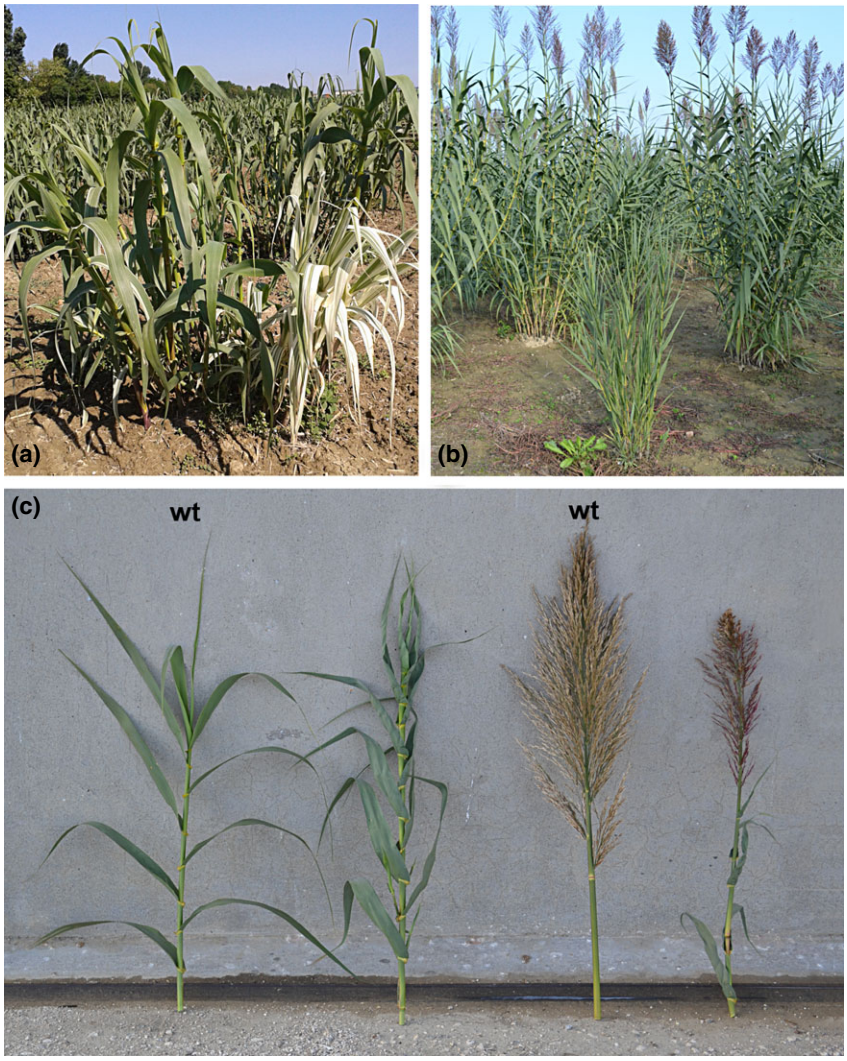

Fig. 3 Examples of giant reed putative mutant clones. (a) *Variegate*, showing example of chimerism; (b) *short stature* (in the foreground, with wild-type-size plants in the background); (c) *abnormal shoot* (wild-type (wt) vegetative shoot and inflorescence are shown as comparison).

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 ($n \approx 2.4$ pg ≈ 2.3 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

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.

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 (Kaeppler *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 homohistons 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

Table 4 Giant reed mutant clones tested for phenotypic stability. Additional data are provided in Table S2

Mutant code	Mutant class and observations*	Observation level
5-60-10	*Variegate*	Pots
22-80-2	*Low-tillering* (and tall shoots)	Pots
157-40-221	*Thin stem* (and patent leaves)	Pots
182-60-17	*Short stature* (and bushy, leafy)	Pots
182-60-60	*Short stature* (and erect leaves, semi dwarf)	Pots

*Mutant classes are fully described in Materials and methods.

be due to chimeras generated by genetic mutations (Marcotrigiano, 1997).

Interestingly, the average number of shoots per plant and shoot height correlated positively and negatively, respectively, with irradiation dose. More specifically, the higher the irradiation dose, the higher the number of buds which were initiated and/or developed in the rhizome of field-grown plants, which resulted in more shoots of smaller size. Previous investigations had already shown that increased shoot and root branching is one of the stress-induced morphogenic responses in plants, which may involve the perception of reactive oxygen species and altered phytohormone physiology (Potters *et al*., 2007). Additionally, moderate stresses are well known to induce bud formation in perennial plant species (Grossnickle, 2012). However, whether the response observed in our case 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 biomethane 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 polyploidy 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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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.