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Establishment of an Efficient Somatic Embryogenesis Protocol for Giant Reed (*Arundo donax* L.) and Multiplication of Obtained Shoots via Semi-Solid or Liquid Culture

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Abstract: This study developed an efficient protocol for the in vitro propagation of giant reed (*Arundo donax* L.) biomass, defining a complete cycle of the induction of somatic embryogenesis from immature inflorescences, followed by the maturation of somatic embryos and the subsequent multiplication of the derived shoots in liquid culture in a temporary immersion system (TIS). The best explants were found to be 30 cm long immature inflorescences, preferably collected in spring. Such an explant type was easy to decontaminate, and the spikelets isolated from it provided over 100 embryogenic callus lines. Among the callus induction media tested, gelled MS medium supplemented with 1.1 mg/L 2,4-D provided the highest percentage of responsive spikelets and the highest density of embryogenic callus. Maturation of the embryogenic callus was easily triggered on gelled MS medium devoid of plant growth regulators. The obtained shoots could be further multiplied on previously optimized gelled DKW medium supplemented with 30 g/L sucrose, 5 mg/L BA, 0.1 mg/L IBA, and 6.8 g/L plant agar. Subsequent high multiplication of the developed shoots was achieved in liquid culture in TIS using a Plantform™ bioreactor, with an immersion cycle of 12 min every 8 h.

Keywords: embryogenic callus; giant reed; immature inflorescences; indirect somatic embryogenesis; perennial grass

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1. Introduction

Arundo donax L., commonly called giant reed, is a tall, non-food perennial rhizomatous C3 grass species belonging to the Poaceae family [1,2]. Worldwide, *A. donax* L. is widespread in tropical and sub-tropical regions, including Africa, the USA, Mexico, the Caribbean, South America, and the Pacific Islands [3,4]. The species is currently considered one of the most aggressive invasive species; however, as the plant has many uses, recent studies have also often speculated on its introduction to the Mediterranean region. According to this literature, human activities have disrupted the distribution ranges and dispersal barriers of many organisms and introduced them around the globe, and as a result, some species have become invasive and constitute a major threat to global biodiversity by competing with native species for ecosystem resources [5,6]. Among these, *A. donax* L. is extremely tolerant of different conditions; it grows in a wide variety of soil types ranging from coarse sands to heavy clays, and survives under a wide range of temperatures and rainfall [7,8].

As a result of increasing energy demand today, climate change has become one of the most pressing problems. Indeed, the energy sector is the source of around 3/4 of global greenhouse gas emissions, and if we fail to switch to environmentally friendly forms of

energy generation and improve energy efficiency, global energy security will be at huge risk. Regarding environmentally friendly sources of energy, second-generation biofuels (biomass) are a particular hotspot as a renewable energy source, and are mainly produced by using perennial herbaceous plants and fast-growing trees via different biological and physical processes [9–11]. Biomass production is also important because of its peculiarity, in that it can be converted into a variety of products, including solid fuels, liquid fuels, heat, electricity, and hydrogen [10,11]. *A. donax* L. is one of the most promising bioenergy crops among the rhizomatous grasses for biomass production [12]. Accordingly, in addition to being used widely in the making of woodwind musical instruments, paper pulp production, and construction, *A. donax* L. is also highly desirable for next-generation feedstock due to its low soil management requirements, its growth on marginal lands, and its capacity to reliably produce biomass [12,13]. The thermochemical conversion of cell wall composition into biofuel has been characterized for *A. donax* L. [14]. The lignin in the cell wall, a complex organic polymer which makes plants rigid and woody, is highly heterogeneous for hemicellulose structure in terms of molecular weight and sugar composition [15]. In addition to its high biomass capacity, it requires low irrigation and nitrogen inputs, and shows high tolerance to abiotic stress, including salinity, heavy metals, and herbicide treatments [16,17].

Although the plant is propagated traditionally from rhizomes and stem nodes, the increasing demand for its cultivation has stimulated interest in exploring more efficient (both in terms of the number of plants obtained and the time required to propagate them) in vitro propagation approaches. Among these, the establishment of embryogenic callus cultures, in particular, allows for speeding up of the propagation of selected, true-to-type genotypes in large quantities within a short period of time [18]. Propagation speed can be enhanced even further using bioreactors for liquid culture in a temporary immersion system (TIS), which can consistently improve the mass propagation of in vitro shoots and plantlets. TIS is a semi-automatic liquid culture technique for biomass production that causes explants to interact with the medium intermittently, thus renewing the atmosphere and supplying nutrients to fulfill the growth needs of the shoots [19]. There are several options for TIS available on the market. Among these, Plantform™ was designed by Margareta Welander in 2014 for large-scale in vitro plant propagation and has been used frequently to improve large-scale multiplication of economically important plants, such as date palm (*Phoenix dactylifera* [20]), cannabis (*Cannabis sativa*, [21]), and banana (*Musa* spp., [22]). Its advantages include having light, transparent, autoclavable, and easy-to-manipulate polycarbonate containers (180 × 150 × 150 mm), as well as allowing for air exchange controlled by a separate pump and timer (for further information, readers can visit https://www.plantform.se/pub/, accessed on 17 June 2023). In this study, the TIS Plantform™ system is adapted to improve the biomass production of *A. donax* L.

2. Materials and Methods

2.1. Plant Material

Leaves and immature inflorescences of *Arundo donax* L. were tested as the plant material for the induction of embryogenic callus proliferation. *A. donax* L. produces purplish-colored inflorescences two times a year (one in the spring and one in the fall), and then, immature inflorescences grow in size and their color fades to yellowish beige as they mature. In the present study, inflorescences at different levels of maturity and size (i.e., 20, 30, 40, and 50 cm) were collected in spring (1st, 2nd, and 3rd weeks of May) and fall (end of September–beginning of October) in the 2019 from two different areas of Italy: Sesto Fiorentino (Florence, Tuscany region; 43°49′54.95″ N, 11°11′57.26″ E), and Piombino (Livorno, Tuscany region; 42°56′0.49″ N, 10°31′39.25″ E) (Figure 1). Leaves were collected in spring from the Sesto Fiorentino area.

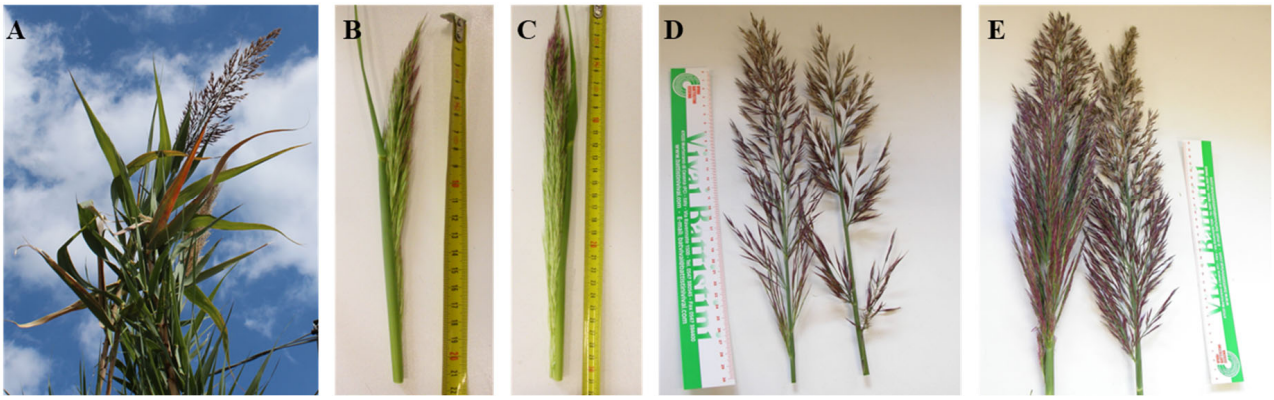

Figure 1. Mother plant of *A. donax* L. located in Sesto Fiorentino, with its immature inflorescences of spring (**A**), and immature inflorescences of *A. donax* L. collected in Piombino (**B**,**C**) and Sesto Fiorentino (**D**,**E**), Tuscany Region, Italy. The sizes of the inflorescences are 20 (**B**), 30 (**C**), 40 (**D**), and 50 cm (**E**), respectively.

2.2. Decontamination of the Plant Material and Explant Preparation

The leaves and inflorescences were first washed under cool running tap water for a minimum of 30 min, brushed gently with a commercial detergent, and then, rinsed well. They were then cut into 4–5 cm long segments and decontaminated by immersing them in 70% (*v*/*v*) EtOH solution for 2 min, followed by 10% (*v*/*v*) NaOCl solution containing a few drops of Tween 10 for 15 min. Both steps were performed with agitation under a laminar flow hood and were followed by a 5 min rinse in sterile dH_2O, at least three times. Leaf segments were prepared by cutting off the leaf border on every side and dissecting the leaves into ~10 × 10 mm fragments. They were then placed on gelled medium horizontally (by putting either the apical or the basal leaf surface in contact with the medium in separate trials) or vertically. Spikelets about 10 mm long were isolated from the inflorescences and placed horizontally on gelled medium (Figure 2).

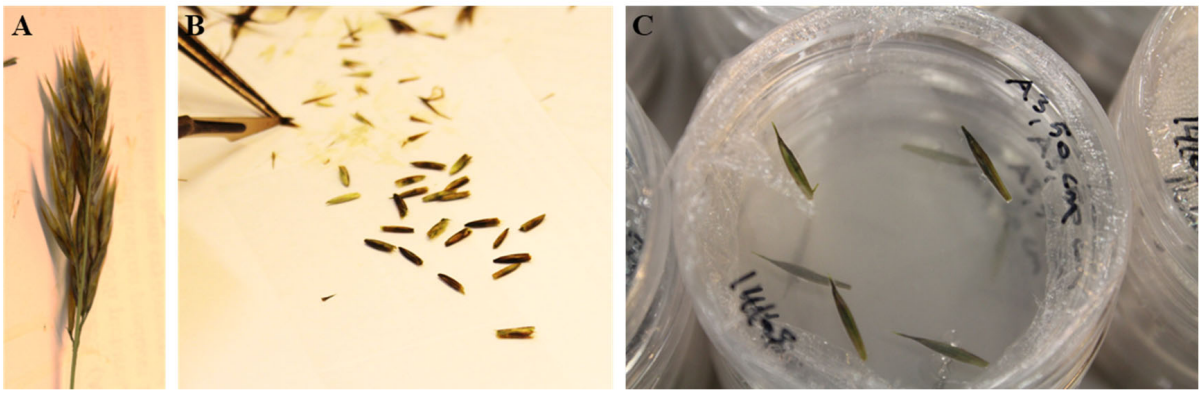

Figure 2. Culture initiation of *A. donax* L. (**A**) Decontaminated 4–5 cm long fragments of inflorescences that served as explant source; (**B**) ~10 mm-long spikelets, isolated from decontaminated inflorescences and used as explants in the study; (**C**) spikelets horizontally placed on gelled medium for callus induction.

2.3. Induction of Somatic Embryogenesis

Leaf fragments and spikelets were first transferred to gelled MS medium [23] devoid of plant growth regulators and evaluated at 23 ± 1 °C in darkness for evidence of contamination. After one week, the explants that did not show any signs of contamination were transferred to callus induction medium, i.e., gelled MS medium supplemented with 100 mg/L myo-inositol, 30 g/L sucrose, 3 g/L gelrite, and several concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 1.1, 2.2 or 4.4 mg/L) and/or 1-Naphthaleneacetic

acid (NAA; 0.9 or 1.9 mg/L). They were then cultured at 23 ± 1 °C, in darkness, by periodic subcultures in fresh medium at 8-week intervals. After 3 subcultures (corresponding to 24 weeks), the callus induced on the spikelet was separated from the initial explant and put in direct contact with the medium. Each spikelet that gave rise to an embryogenic callus mass represented a callus line, and each callus line was always treated separately in the following steps. The callus was proliferated by periodic subcultures at 8-week intervals in fresh medium.

2.4. Somatic Embryo Maturation and Plantlet Formation

After three subcultures on callus induction medium, twelve callus lines (i.e., IBE/SE/1—IBE/SE/12) were selected (depending on their superior proliferation performance and embryogenic potential) and were transferred to gelled hormone-free MS medium, supplemented or not with 0.5 g/L activated charcoal (AC) for embryo maturation and the induction of plantlet formation. They were maintained at 23 ± 1 °C, under a 16 h photoperiod, provided with cool white fluorescent lamps with a light intensity of 36 µmol m^{-2} s^{-1}, and subcultured in fresh medium at 4-week intervals.

2.5. Multiplication of Shoots Derived from Somatic Embryogenesis

After 2 subcultures on callus maturation medium, plantlets derived from somatic embryos of three selected callus lines (i.e., IBE/SE/10, IBE/SE/11, and IBE/SE/12, characterized by superior regeneration potential) were isolated from the callus tissue, the shoots were cut off of the roots and transferred to multiplication medium, which was applied in three different ways: (i) 100 mL of gelled DKW medium [24] contained in 500 cc glass jars, (ii) 40 mL of stationary liquid DKW medium contained in 500 cc glass jars, and (iii) 500 mL of liquid DKW medium in TIS, using the Plantform™ bioreactor. The medium immersion regimes applied were 12 min immersion every 8 h (equal to 36 min/day) or 8 min immersion every 16 h (equal to 12 min/day). In addition, the cultures were ventilated for 10 min every 4 h for the renewal of the gases accumulated in the culture container. The multiplication medium was supplemented with 30 g/L sucrose, 5 mg/L benzyladenine (BA), 0.1 mg/L indol-3-butyric acid (IBA), and 6.8 g/L plant agar in the case of the semi-solid medium. The shoots were multiplied by periodic subcultures at 4-week intervals in fresh medium of the same composition.

2.6. Experimental Design, Data Collection, and Statistical Analysis

About 75–100 spikelets and about 50–60 leaf fragments were tested in each decontamination trial. The explants were contained in Ø 55 cm Petri dishes, with each Petri dish containing 3 explants. Callus induction was performed on gelled MS medium supplemented with 100 mg/L myo-inositol, 30 g/L sucrose, 3 g/L Gelrite, and 1.1, 2.2 or 4.4 mg/L 2,4-D, in combination with 0, 0.9, or 1.9 mg/L NAA, contained in Ø 90 cm Petri dishes (3 leaf fragments or 3 spikelets per Petri dish), and all trials were performed with 25 replications (i.e., 25 Petri dishes per trial); the experiments were repeated twice. First, three subcultures were performed at 8-week intervals by transferring the explants (displaying or not displaying callus induction) to fresh medium of the same composition. After three subcultures, the obtained callus was isolated from the initial explant and put in direct contact with the medium, where it continued to be proliferated by periodic subcultures in fresh medium of the same composition at 8-week intervals. The collected data consisted of: (i) % of regenerating explants following 1st, 2nd, and 3rd subculture, and (ii) observation of the callus development after the 4th subculture, in which the obtained callus density was visually categorized as average (+), moderate (++), or high (+++) (Figure 3).

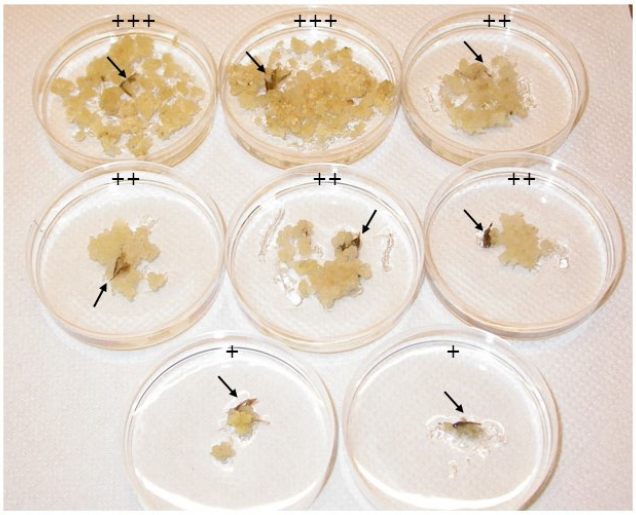

Figure 3. Categorization of *A. donax* L. callus density as average (+), moderate (++), or high (+++) according to visual observations at the end of the 4th subculture period. Arrows indicate the original explant (spikelet).

Embryogenic callus maturation was performed on gelled hormone-free MS medium, supplemented or not with 0.5 g/L AC, contained in Ø 90 cm Petri dishes, and each Petri dish contained several callus clusters (of about 1 mm in diameter) of the same callus line. All trials were performed with 5–10 replications, depending on the callus density of the line, and the data collection consisted of the % of callus clusters that displayed somatic embryo maturation and conversion to plantlets. For the multiplication of the shoots, 15 shoots were transferred to gelled or stationary liquid medium contained in 500 cc glass jars (with 3 replications), while 45 shoots were transferred to the TIS (with 2 replications). The multiplication medium was DKW medium supplemented with 30 g/L sucrose, 5 mg/L BA, and 0.1 mg/L IBA. Gelled media contained, in addition, 6.8 g/L plant agar.

The fresh weight of the plants was recorded at the beginning (FW_I, initial fresh weight) and at the end (FW_F, final fresh weight) of the culture period, and the relative growth rate (RGR) was calculated according to the formula below [25]:

$$RGR = (\text{Ln } FW_F - \text{Ln } FW_I) \times 100/\text{number of days in culture}$$

Statistical analysis of the percentages was carried out by means of the test for homogeneity of proportions, and significant treatment differences selected using a non-parametric statistical test, followed by a post hoc multiple comparisons test [26], to compare multiple percentages.

3. Results

3.1. Callus Induction on Gelled Medium

The contamination incidence of the leaf fragments was only 8.3% at the end of one of week culture on hormone-free MS medium. However, 4 weeks after the transfer of visibly clean leaf fragments to the culture induction medium, a further 20.8% of them were contaminated and discarded. The remaining leaf fragments were subcultured to fresh medium for another two consecutive subculture periods, never displaying callus induction.

As for the inflorescences, the largest one (50 cm) showed the highest contamination rate (22.2% after 1 week on MS0, 31.4% after 4 weeks on callus induction medium), while the smallest one demonstrated the lowest. Indeed, the 30 cm long inflorescences collected in May remained clean during the first week of culture, and then, showed only 11.3% contamination, and those collected in October showed 13.5% contamination after 4 weeks on callus induction medium. Similarly, 20 and 30 cm long immature inflorescences collected from Piombino in fall displayed only 15.2 and 13.5% contamination, respectively, at the

end of the first subculture on callus induction medium. These results suggest a relationship between the size of the inflorescences and the rate of contamination. However, the season of sample collection and the province of the mother plant did not seem to influence contamination incidence.

Differently from the leaf fragments, spikelets isolated from inflorescences gave rise to calli on almost all the tested medium formulations. Callus formation initiated at the distal end of the spikelet that was in contact with the medium (Figure 4A), and then, increased in density (Figure 4B) and eventually spread on the medium (Figure 4C) later in the culture. The time required for callus induction on responsive spikelets ranged between 3 and 10 weeks; however, numerous spikelets remained non-responsive even after three subculture periods (Figure 4D, Table 1).

Figure 4. Callus proliferation on spikelets of *A. donax* L. (**A**) Callus induction at the distal end of the spikelet, (**B**) proliferation of the callus and increase in its density, (**C**) spread of obtained callus on gelled medium, (**D**) non-responsive spikelets.

Table 1. Effect of medium composition on the callus induction performance and the density of the proliferated callus of the leaf fragments and spikelets of *A. donax* L.

Callus Induction Medium	% of Regenerating Explant			Density of Callus after 4 Subcultures **
	After 1st Subculture	After 2nd Subculture	After 3rd Subculture	
Leaf fragments				
A1—MS + 1.1 mg/L 2,4-D	0.0 d *	0.0 d	0.0 d	-
A2—MS + 2.2 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A3—MS + 4.4 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A4—MS + 1.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
30 cm long inflorescences, collected in May, from Sesto Fiorentino (SF, 30 cm, May)				
A1—MS + 1.1 mg/L 2,4-D	20.0 a	46.7 a	53.3 a	13.3%, +++; 20%, ++; 20%, +
A2—MS + 2.2 mg/L 2,4-D	15.0 a	14.3 b	14.3 b	6.7%, +++; 7.6%, +
A3—MS + 4.4 mg/L 2,4-D	0.0 d	13.3 b	15.4 b	7.7%, ++; 7.7%, +
A4—MS + 1.9 mg/L NAA	6.7 b	6.7 c	6.7 c	6.7%, +++
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	6.7 b	33.3 a	33.3 a	6.7%, +++; 6.7%, ++ 19.9%, +
40 cm long inflorescences, collected in May, from Sesto Fiorentino (SF, 40 cm, May)				
A1—MS + 1.1 mg/L 2,4-D	17.4 a	30.8 a	33.3 a	8.3%, +++; 25%, +
A2—MS + 2.2 mg/L 2,4-D	0.0 d	0.0 d	11.1 bc	11.1%, +
A3—MS + 4.4 mg/L 2,4-D	0.0 d	8.3 b	8.3 c	8.3%, +
A4—MS + 1.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-

Table 1. *Cont.*

Callus Induction Medium	% of Regenerating Explant			Density of Callus after 4 Subcultures **
	After 1st Subculture	After 2nd Subculture	After 3rd Subculture	
50 cm long inflorescences, collected in May, from Sesto Fiorentino (SF, 50 cm, May)				
A1—MS + 1.1 mg/L 2,4-D	0.0 d	0.0 d	25.0 ab	25%, +
A2—MS + 2.2 mg/L 2,4-D	0.0 d	8.3 c	8.3 c	8.3%, +++
A3—MS + 4.4 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A4—MS + 1.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	0.0 d	0.0 d	6.7 c	6.7%, +
30 cm long inflorescences, collected in October, from Sesto Fiorentino (SF, 30 cm, October)				
A1—MS + 1.1 mg/L 2,4-D	5.5 b	13.7 b	15.3 b	10.3%, +++; 5%, ++
A2—MS + 2.2 mg/L 2,4-D	2.7 c	3.5 c	5.0 c	5%, +
A3—MS + 4.4 mg/L 2,4-D	3.2 c	5.0 c	5.0 c	5%, +
A4—MS + 1.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	3.5 c	5.0 c	6.7 c	6.7%, ++
20 cm long inflorescences, collected in September, from Piombino (P, 20 cm, September)				
A1—MS + 1.1 mg/L 2,4-D	0.0 d	10.0 b	10.0 bc	5%, +++; 5%, ++
A2—MS + 2.2 mg/L 2,4-D	5.0 b	5.0 c	5.0 c	5%, +++
A3—MS + 4.4 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A4—MS + 1.9 mg/L NAA	5.3 b	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	5.0 b	5.0 c	5.0 c	5%, +++
30 cm long inflorescences, collected in September, from Piombino (P, 30 cm, September)				
A1—MS + 1.1 mg/L 2,4-D	4.5 bc	9.1 b	9.7 bc	5% +++; 4.7%, +
A2—MS + 2.2 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A3—MS + 4.4 mg/L 2,4-D	0.0 d	0.0 d	0.0 d	-
A4—MS + 1.9 mg/L NAA	0.0 d	0.0 d	0.0 d	-
A5—MS + 1.1 mg/L 2,4-D + 0.9 mg/L NAA	2.3 c	4.5 c	4.5 c	2.3%, +++; 2.2%, ++

* Different letters in each column represent a significant statistical difference determined by a non-parametric statistical test according to the post hoc multiple comparisons test [26]. ** Callus masses obtained from each spikelet were recorded as a callus line, and each line was observed visually and categorized as average (+), moderate (++), or high (+++), depending on the total density of the proliferated callus after a total of 4 subcultures.

Spikelets isolated from 30 cm long inflorescences, collected in May 2019 from Sesto Fiorentino, were shown to be more responsive than the others, and 6.7–20% of them readily gave rise to calli during the first subculture period on 4 of the tested medium compositions. Among these, 53.3% of the spikelets that were cultured on MS medium supplemented with 1.1 mg/L 2,4-D (medium A1) and 33.3% of the spikelets that were cultured on MS medium supplemented with 1.1 mg/L 2,4-D and 0.9 mg/L NAA (medium A5) provided callus induction, most of which proliferated at the moderate/high level following three consecutive subcultures. The spikelets that provided callus induction and proliferation on the other three media ranged between 6.7% and 15.4%, and the callus obtained was, again, generally at the moderate/high level. Similarly, spikelets isolated from inflorescences of the same size, collected from the same province but in fall, responded instantly to four of the five medium compositions and eventually gave rise to calli on all the tested media. However, although the callus proliferation was also satisfactory here, the percentage of the responsive explants was lower than the equivalent spikelets in the spring.

Callus induction was less efficient and much slower in spikelets isolated from larger inflorescences of the same season and same province, and usually started during the 2nd or 3rd subculture period. The best results (33.3% and 25.0%, respectively) were obtained with the spikelets isolated from 40 and 50 cm long inflorescences on MS medium supplemented with 1.1 mg/L 2,4-D, with the latter providing a callus mass that was categorized as average. The other medium formulations, especially the ones that were enriched with NAA, were shown to be unfavorable for these explant types.

Very few spikelets of the inflorescences collected from Piombino in fall responded to callus induction media; however, the ones that responded gave rise to significant amounts of calli. Among these, the ones cultured on MS media supplemented with 1.1 mg/L 2,4-D or 1.1 mg/L 2,4-D plus 0.9 mg/L NAA, in particular, provided callus proliferation at high or moderate levels.

These results indicated that 30 cm long immature inflorescences of spring provided more suitable explants for embryogenic callus induction. These explants were relatively easier to decontaminate, as well. Among the tested media, MS medium supplemented with 1.1 mg/L 2,4-D triggered callus induction on spikelets from inflorescences of all sizes, and always provided the most favorable results, both for the % of responsive explants and the density of the proliferated callus. Indeed, 7 of the 12 selected callus lines were induced on this medium composition.

3.2. Maturation of the Embryogenic Callus and Emergence of the Plantlets

More than 100 callus lines were obtained from decontaminated spikelets cultured on callus induction media. They were examined visually and under a microscope, and eventually, 12 lines were selected based on their excellent proliferation ability (based on visual observation regarding the callus density obtained) and promising embryogenic potential (based on observations under the microscope). The majority of those selected lines (seven out of twelve) were induced on MS medium supplemented with 1.1 mg/L 2,4-D, while the other three callus lines were induced on MS medium supplemented with 1.1 mg/L 2,4-D and 0.9 mg/L NAA, and two callus lines were induced on MS medium supplemented with 2.2 mg/L 2,4-D (medium A2, Table 2). Three of the selected lines (IBE/SE/1, IBE/SE/2, and IBE/SE/4) did not respond to the media tested for maturation. One line (IBE/SE/9) matured into somatic embryos only on hormone-free MS medium devoid of AC (MS0). The other eight lines responded to both media at variable levels, ranging between 10.5% to 97.1%; however, in general, MS0 was shown to be more favorable for embryo maturation. Of these nine callus lines, eight were induced on spikelets that were isolated from 30 cm long inflorescences (Figure 5A), while the other was from a 50 cm long one (Figure 5B). Three lines (i.e., IBE/SE-10, IBE/SE-11 and IBE/SE-12) were especially responsive to maturation media. In general, more than 80% of them developed into mature somatic embryos on MS.0, and more than 46% developed into embryos on medium supplemented with AC, all of which eventually developed into plantlets (Figure 5C–E). The following steps of the study continued using plantlets obtained from those three callus lines on MS0.

Table 2. Somatic embryo maturation and plantlet formation from *A. donax* L. embryogenic callus on hormone-free MS medium, supplemented or not with activated charcoal (AC). SF: Sesto Fiorentino, P: Piombino. Month refers to the collection time of inflorescences (year 2019).

Selected Callus Lines	Province of Origin/Callus Induction Medium	% of Plantlet Induction on *	
		MS.0	MS + AC
IBE/SE-1	SF, 30 cm, May/A5	0.0 d	0.0 d
IBE/SE-2	SF, 40 cm, May/A1	0.0 d	0.0 d
IBE/SE-3	SF, 30 cm, October/A1	10.5 c	16.7 c

Table 2. *Cont.*

Selected Callus Lines	Province of Origin/Callus Induction Medium	% of Plantlet Induction on *	
		MS.0	MS + AC
IBE/SE-4	P, 30 cm, September/A1	0.0 d	0.0 d
IBE/SE-5	SF, 30 cm, May/A1	16.7 c	25.7 c
IBE/SE-6	SF, 30 cm, May/A1	75.5 a	40.7 b
IBE/SE-7	SF, 30 cm, October/A1	25.0 c	20.0 c
IBE/SE-8	P, 30 cm, September/A5	52.7 b	50.0 b
IBE/SE-9	SF, 30 cm, May/A2	20.0 c	0.0 d
IBE/SE-10	SF, 30 cm, May/A5	97.1 a	46.0 b
IBE/SE-11	SF, 30 cm, May/A1	93.1 a	86.1 a
IBE/SE-12	SF, 50 cm, May/A2	81.2 a	75.0 a

* Different letters in each column represent a significant statistical difference selected by a non-parametric statistical test, followed by the post hoc multiple comparisons test [26].

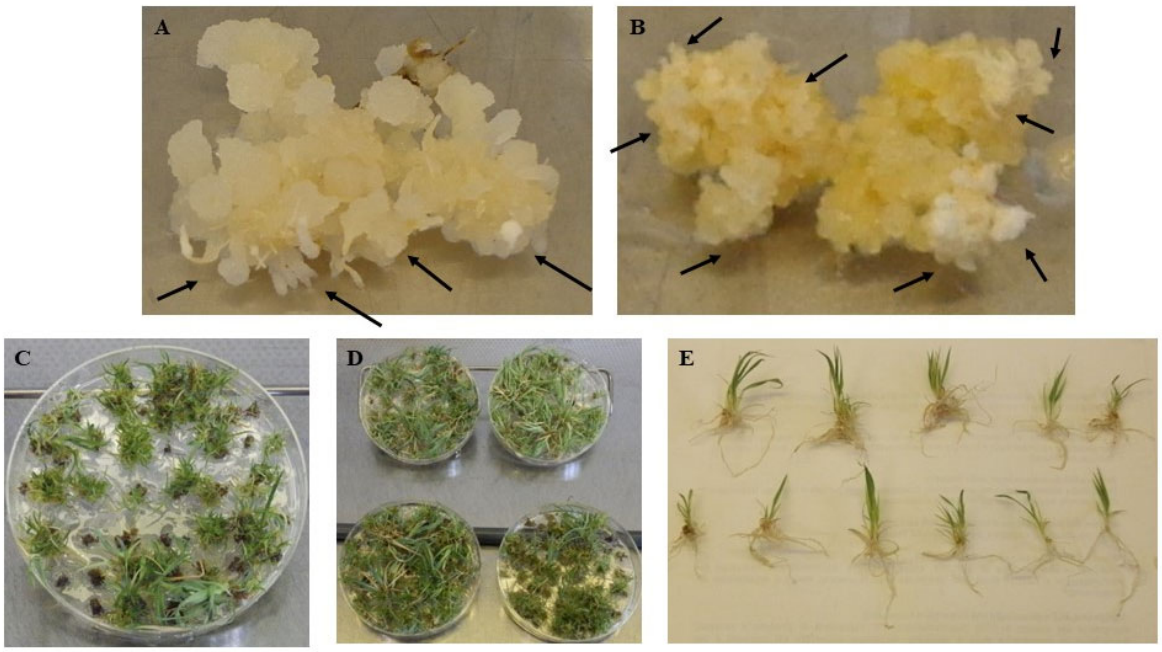

Figure 5. Emergence of *A. donax* L. somatic embryos on MS0, from callused originating from spikelets isolated from 30 cm long (**A**) and 50 cm long inflorescences (**B**), and maturation of the embryos into whole plantlets (**C**–**E**). Arrows indicate the callus zones where embryo emergence is clearly evident even upon direct observation.

For validation of the in vitro multiplication potential of the plantlets obtained through indirect somatic embryogenesis, seedlings developed from somatic embryos were cut off of the roots, and the shoots were cultured on previously optimized proliferation medium, which was applied in three different procedures: gelled, stationary liquid, or liquid in TIS (Figure 6). Multiplication of the shoots was achieved in all three different culture conditions. However, the liquid culture in TIS, using the Plantform™ bioreactor with an immersion cycle of 12 min every 8 h, produced a superior multiplication rate (Table 3). Stationary liquid culture provided the lowest multiplication rate (RGR ranging from 5.00 to 5.93).

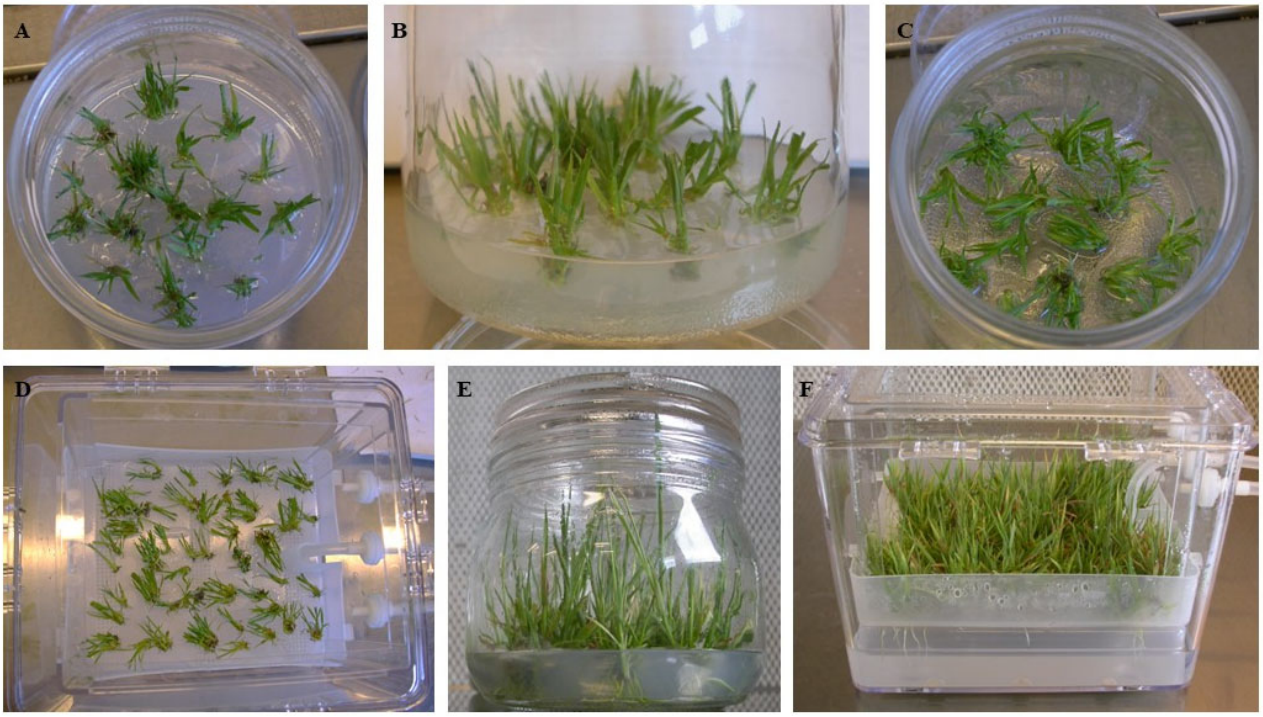

Figure 6. Multiplication of *A. donax* L. shoots, generated by indirect somatic embryogenesis from spikelets isolated from immature inflorescences. Culture initiation on gelled medium (**A**,**B**), stationary liquid medium (**C**), and TIS (**D**). Multiple shoots obtained at the end of the culture period on gelled medium (**E**), and TIS (**F**).

Table 3. Multiplication of the shoots of *A. donax* L. obtained from selected callus lines proliferated by indirect somatic embryogenesis from spikelets.

Selected Callus Lines	Initial Plant Weight (gr)	Final Plant Weight (gr)	RGR *
Gelled DKW multiplication medium			
IBE/SE-10	2.74	22.21	6.77
IBE/SE-11	2.03	17.86	7.00
IBE/SE-12	2.42	19.83	6.80
Stationary liquid DKW multiplication medium			
IBE/SE-10	2.78	17.52	5.93
IBE/SE-11	2.28	13.52	5.74
IBE/SE-12	3.43	16.15	5.00
Liquid DKW multiplication medium in TIS (12 min/8 h)			
IBE/SE-10	7.21	86.90	8.03
IBE/SE-11	7.16	86.92	8.06
IBE/SE-12	7.05	87.75	8.12
Liquid DKW multiplication medium in TIS (8 min/16 h)			
IBE/SE-10	8.51	45.35	5.38
IBE/SE-11	7.24	43.16	5.74
IBE/SE-12	8.06	44.08	5.48

* RGR = $(\text{Ln } FW_F - \text{Ln } FW_I) \times 100/\text{number of days in culture}$.

Independently of the culture conditions, all the obtained shoots were vigorous and healthy and never demonstrated any abnormality or growth retardance.

4. Discussion

Nowadays, increasing energy demands lead to the use of non-food plants in both biofuel/biomass and bio-based compound production. Increasing the speed of biomass production has great potential to convert it to biofuel without competing with food production for land, water, and markets, as currently occurs with maize and sugar [12,27,28]. As a promising plant, *A. donax* L. has a high yield of cellulosic biomass due to its rapid growth, which could be easily converted into biofuel [12,29]. In this study, we established an efficient protocol to increase the biomass production of *A. donax* L. for future biofuel applications.

Despite the potential of *A. donax* L. as a bioenergy feedstock, there are very few publications on tissue culture in this species. There is, however, some patented information, which suggests a protocol for somatic embryogenesis of the species [30,31]. As reported by these authors, callus cultures triggered by 2,4-D on nodal segments and segments of immature inflorescences failed to produce sustainable embryogenic cultures. Accordingly, the protocol suggested is as follows: (i) the appropriate explant can be an immature inflorescence, obtained from the tips of pre-flowering shoots with leaf sheaths completely enclosing the developing leaves, but yet unemerged, immature inflorescence; (ii) following sterilization (e.g., diluted commercial bleach containing 10% (*v*/*v*) ethanol and 0.1% Tween 80 for 15 min, followed by rinsing in sterile water), the inflorescence is cut into cross-sectional pieces; (iii) the explants are pretreated with cold temperature (e.g., 14 days at 5 °C, in dim light, 30 μmol m^{-2} s^{-1}); (iv) the explants are cultured for several weeks on gelled MS basal medium containing 0.2 mg/L 2,4-D and 0.02 mg/L thidiazuron, in darkness, at room temperature, to generate totipotent tissue; (v) the obtained totipotent embryogenic cell cultures are cultured on gelled MS medium supplemented with 0.2 mg/L 2,4-D and 0.01 μM thidiazuron; (vi) the totipotent tissues are then transferred to gelled MS medium containing 0.01–1 mg/L thidiazuron to induce plantlet formation. In contrast to what is reported by Márton and Czakó, in the present study, immature inflorescences of a particular dimension were proven to be an appropriate explant source to trigger sustainable embryogenic callus cultures, and cold pretreatment was not required to achieve this. The selection of basal medium formulation (i.e., MS medium) for the production of embryogenic cultures was in correlation with what was reported by these authors; however, the concentration of the 2,4-D or the auxin combination used in the study were slightly different. Similarly, the medium formulation used for plantlet formation was different, as well.

Pigna et al. [13] developed a suspension culture protocol for *A. donax* L., with the aim of establishing a protoplast culture to be used in genetic transformation studies. Suspension cultures were established using callus cultures induced from stem explants according to the protocol of Takahashi et al. [32]. Accordingly, excised internodes were transferred onto MS medium supplemented with 3% sucrose, 0.7 g/L casein hydrolysate, 0.5 g/L L-glutamine, 1 g/L L-proline, 0.4% Gelzan, and 9 μM 2,4-D. The explants were placed in a growth chamber in the dark at 25 °C for a month to induce calli. Once induced, the calli were maintained on the same basal medium, with 13 μM 2,4-D, with a proliferation rate of a 1.4-fold increase in tissue mass over 14 days. However, as callus induction and maintenance were relatively slow, and thus, time-consuming on that semi-solid medium, the authors aimed to develop cell suspension cultures for the species in order to obtain enough tissue for subsequent genetic transformation. Cell suspension cultures were initiated from 10-month-old embryogenic calli by transferring 500 mg of calli into a liquid medium supplemented with 3% sucrose, MS salts, B5 vitamins, 9 μM 2,4-D, and 4.4 μM BA. The increase in tissue mass over the same period (i.e., 14 days) in suspension culture was five-fold. These results demonstrate the superiority of suspension cultures to previously described semi-solid media for tissue mass increase in *A. donax* L. However, the authors also stated that a five-fold increase was still relatively low when compared to the suspension cultures of other biofuel crops, and suggested that extensive effort must still to be made to optimize culture media, as well as to screen different accessions in order to identify genotypes more suitable to in vitro cultivation.

As for the large-scale propagation of *A. donax* L., Cavallaro et al. [33] embraced an approach similar to the one used in the present study for the multiplication of shoots derived from somatic embryogenesis; they tested the efficiency of a liquid medium, both as a stationary culture and TIS in a RITA® system, in comparison to a semi-solid medium (solidified by agar or Gellan gum). They detected sensitivity of *A. donax* L. to gelling agents, with Gellan gum being more effective at improving shoot number, as well as the FW and DW of the plant material, and concluded that cultivation of the species in liquid medium was cheaper, and yet, as effective as in the medium solidified using Gellam gum. Increasing the BA content of the medium to 4 mg/L was effective, as well, for secondary shoot proliferation. In accordance with Antal et al. [34], the use of BA, together with 0.05 mg/L NAA in the shoot induction medium from stem cuttings, was shown to be very effective. However, the concentration that these authors reported was much lower than in this study (0.3 mg/L). Moreover, they determined a beneficial effect of MS medium containing 3 mg/L kinetin and 3 mg/L indole-3-acetic acid (IAA) for the multiplication of the shoots.

As highlighted by Cavallaro et al. [33], which also supports the outcomings of the present study, numerous difficulties in classical micropropagation techniques on semi-solid medium (such as low regeneration rate, the limited uptake of the nutrients, hyperhydricity, and asphyxia), which limit the growth of plantlets [35], can be overcome by the use of liquid culture, and this positive effect can go even further when explants interact with the medium intermittently in TIS [35]. Thus, it would be highly valuable to test the system with many other economically important species for their fast, large-scale propagation.

5. Conclusions

Giant reed *(Arundo donax* L.) is today considered one of the most promising bioenergy crops among the rhizomatous grasses for biomass production. From this perspective, the present study provides an efficient indirect somatic embryogenesis protocol for *A. donax* L. and subsequent shoot multiplication, suitable for the large-scale production of giant reed biomass. To optimize the biomass production process, this study highlights the importance of using inflorescences of about 30 cm long (collected either in spring or fall) as explants for the induction of embryogenic calli, of developing an appropriate culture medium both for callus induction (i.e., MS medium supplemented with 1.1 mg/L 2,4-D) and embryo maturation (i.e., MS0), and of applying a liquid culture in TIS for the multiplication of shoots originating from somatic embryos. The obtained shoots were vigorous and healthy, never demonstrated any abnormality or growth retardance, and were shown to be an elite material for biomass production.

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