horticulturae

MDPI

Article

Establishment of an efficient somatic embryogenesis protocol for giant reed (*Arundo donax* L.) and multiplication of obtained shoots via semi-solid or liquid culture

Elif Aylin Ozudogru[1]*, Elif Karlik[2], Doaa Elazab[3] and Maurizio Lambardi[3]

[1] Istinye University Istanbul, Faculty of Engineering and Natural Sciences, Department of Molecular Biology and Genetics, Istanbul, Turkey; aylin.ozudogru@istinye.edu.tr
[2] İstanbul University, Institution of Science, Department of Biotechnology, Istanbul University; elifkarlik@gmail.com
[3] IBE/Institute of BioEconomy, National Research Council (CNR), 50019 Sesto Fiorentino (Florence), Italy; doaa.elkassas@ibe.cnr.it; maurizio.lambardi@ibe.cnr.it
* Correspondence: aylin.ozudogru@istinye.edu.tr

Abstract: The study developed an efficient protocol for the in vitro propagation of giant reed (*Arundo donax* L.) biomass, defining a complete cycle of induction of somatic embryogenesis from immature inflorescences, followed by maturation of somatic embryos and subsequent multiplication of the derived shoots in liquid culture in a temporary immersion system (TIS). The best explant was found to be 30 cm-long immature inflorescences, preferably collected in spring. Such 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. A subsequent high multiplication of the developed shoots was obtained in liquid culture in TIS, using the Plantform™ bioreactor, with an immersion cycle of 12 min every 8 h.

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

Citation: Ozudogru, E.A.; Karlik, E.; Elazab, D.; Lambardi, M. Establishment of an efficient somatic embryogenesis protocol for giant reed (*Arundo donax* L.) and multiplication of obtained shoots via semi-solid or liquid culture. *Horticulturae* **2023**, *9*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date
Revised: date
Accepted: date
Published: date



Copyright: © 2023 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Arundo donax L., commonly called as giant reed, is a tall, non-food perennial rhizomatous C3 grass species belonging to Poaceae family [1, 2]. In the 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]. *A. donax* L. is extremely tolerant to 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 temperature and rainfalls [5, 6].

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 environment-friendly forms of energy generation and improve the energy efficiency, global energy security will be under a huge risk. Regarding environment-friendly sources of energy, especially second-generation biofuels (biomass) are a hotspot as renewable energy source that are mainly produced by using perennial herbaceous plants and fast-growing trees via different biological and physical processes [7-9]. Biomass production is also important because of its peculiarity to be converted into a variety of products, including solid fuels, liquid fuels, heat,

electricity, and hydrogen [8, 9]. *A. donax* L. is one of the most promising bioenergy crops among rhizomatous grasses for biomass production [10]. 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 [10, 11]. The thermochemical conversion of cell wall composition into biofuel has been characterized for *A. donax* L. [12]. The lignin in the cell wall has HGS-type structure composed of G subunits. Especially, stems are highly heterogeneous for hemicelluloses structure in terms of molecular weight and sugar composition [13]. In addition to high biomass capacity, it requires low irrigation and nitrogen inputs, and shows high tolerance to abiotic stress, including salinity, heavy metals, and herbicide treatments [14, 15].

Although the plant is propagated traditionally from rhizomes and stem nodes, the increasing demand for its cultivation stimulated the interest to explore more efficient (both in terms of number of plants obtained and the time required to propagate) in vitro propagation approaches. Among these, especially the establishment of embryogenic callus cultures allows speeding up the propagation of selected, true-to-type genotypes in large quantities within a short period of time [16]. Propagation speed can be enhanced even further using bioreactors for the liquid culture in 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, leading to the explants interacting with the medium intermittently, thus renewing the atmosphere and supplying nutrients to fulfill the growth needs of the shoots [17]. There are several options of TIS available in the market. Among these, Plantform™ have been used frequently to improve large scale multiplication of economically important plants, such as date palm (*Phoenix dactylifera* [18]), cannabis (*Cannabis sativa*, [19]), and banana (Musa spp., [20]). In this study, TIS Plantform™ system has been adapted to improve 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, then immature inflorescences grow in size and their color fade to yellowish beige as they mature. In the present study, inflorescences at different level 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 Sesto Fiorentino area.

2.2. Decontamination of the plant material and explant preparation

The leaves and inflorescences were first washed under cool running tap water for minimum 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 few drops of Tween 10, for 15 min. Both steps were performed vis agitation under laminar flow hood and were followed by 5-min rinse in sterile dH_2O, at least three times. Leaf



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** and **C**) and Sesto Fiorentino (**D** and **E**), Tuscany Region, Italy. The size of the inflorescences is 20 (**B**), 30 (**C**), 40 (**D**) and 50 cm (**E**), respectively.

segments were prepared by cutting off the leaf border on every side and dissecting the leaves into ~ 10 x 10 mm fragments. They were then placed on gelled medium horizontally (by putting in contact either the apical or the basal leaf surface in separate trials) or vertically. About 10 mm-long spikelets were isolated from inflorescences and placed horizontally on gelled medium (Figure 2).

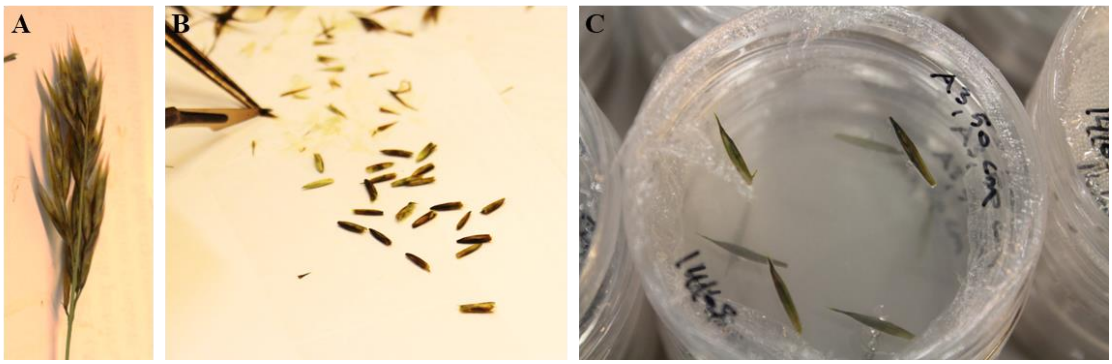


Figure 2. Culture initiation of *A. donax* L. (**A**) Decontaminated 4-5 cm-long fragments of inflorescences that served as explant source, (**B**) ~ 10 mm-long spikelets, isolated from decontaminated inflorescences and used as explant of the study, (**C**) spikelets horizontally placed on gelled medium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

2.3. Induction of somatic embryogenesis

Leaf fragments and spikelets were first transferred on gelled MS medium [21], devoid of plant growth regulators, and evaluated at 23±1°C in darkness for evidence of contamination. After one week, the explants not showing 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 to fresh medium in 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. Callus was proliferated by periodic subcultures in 8-week intervals to 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 induction of plantlet formation. They were maintained at 23±1°C, 16 h photoperiod, provided by cool white fluorescent lamps with a light intensity of 36 μmol m^{-2} s^{-1}, and subcultured to fresh medium in 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 a 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 [22] 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 in every 8 hours (equal to 36 min/day) or 8 min immersion in every 16 hours (equal to 12 min/day). In addition, the cultures were ventilated for 10 min in every 4 hours 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 in 4-week intervals to fresh medium of the same composition.

. Experiment 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, each Petri dish containing 3 explants. Callus induction was performed 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); experiments were repeated twice. First three subcultures were performed in 8-week intervals by transferring the explants (displaying or not 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 proliferate by periodic subcultures to fresh medium of the same composition in 8-week intervals. The collected data consisted of: (i) % of regenerating explants following 1st, 2nd and 3rd subculture, (ii) observation of the callus development after the 4th subculture, in which the obtained callus density was visually categorised as average (+), moderate (++) or high (+++) (Figure 3).

Embryogenic callus maturation was performed 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).

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 accordingly with the formula below [23]:

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

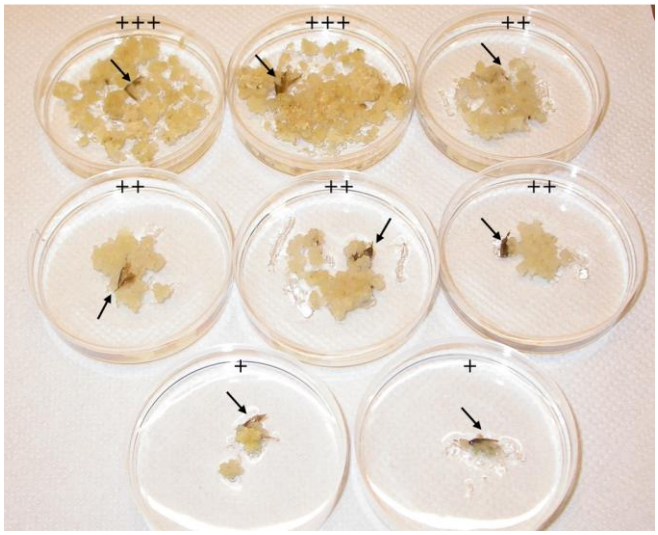


Figure 3. Categorization of *A. donax* L. callus density as average (+), moderate (++) or high (+++) according to visual observations at the end of the 4th subculture period. Arrows indicate the original explant (spikelet).

Statistical analysis of percentages was carried out by means of the test for homogeneity of proportions, and significant treatment differences selected by a non-parametric statistical test, the Post hoc multiple comparisons test [24], to compare multiple percentages.

3. Results

3.1. Callus induction on gelled medium

Contamination incidence of the leaf fragments was only 8.3% at the end of one-week culture on hormone-free MS medium. However, 4 weeks after the transfer of visibly clean leaf fragments to culture induction media, further 20.8% of them were contaminated and discarded. Remaining leaf fragments were subcultured to fresh medium for other two consecutive subculture period, 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 least. Indeed, 30 cm-long inflorescences collected in May remained clean during the first week of culture, then showed only 11.3% of contamination and the one collected in October showed 13.5% of 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% of contamination, respectively, at the end of first subculture on callus induction medium. These results suggest a linear correlation between the size of the inflorescences and the rate of contamination. However, the season of sample collection or the province of the mother plant did not seem to influence contamination incidence.

Differently from the leaf fragments, spikelet isolated from inflorescences gave rise to callus 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), then increased in density (Figure 4B) and eventually spread on the medium (Figure 4C) later in culture. The time required for callus induction on responsive spikelets ranged between 3-10 weeks, however numerous spikelets remained non-responsive even after three subculture periods (Figure 4D, Table 1).

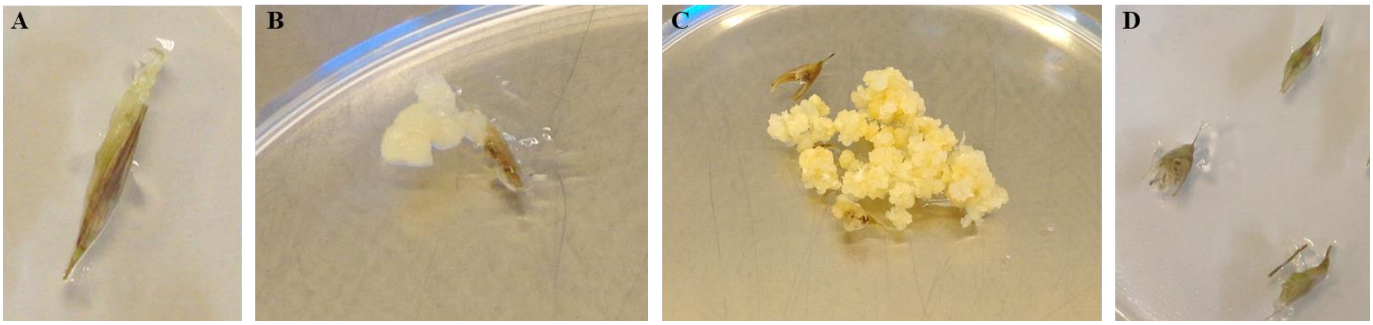


Figure 4. Callus proliferation on spikelets of *A. donax* L. (**A**) Callus induction at the distal end of the spikelet, (**B**) proliferation of the callus and increase in its density, (**C**) spread of obtained callus on gelled-medium, (**D**) non-responsive spikelets.

Spikelets isolated from 30 cm-long inflorescences, collected in May 2019 from Sesto Fiorentino showed to be more responsive than the others, and 6.7-20% of them readily gave rise to callus 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 4 of the 5 medium compositions and eventually gave rise to callus on all the tested media. However, although the callus proliferation was satisfactory also here, the percentage of the responsive explants was lower than the equivalent spikelets of 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. 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, the latter providing a callus mass that was categorized as average. The other medium formulations, especially the ones that were enriched with NAA, showed 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 amount of callus. Among these, especially 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 provided callus proliferation at high or moderate level.

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.

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	-
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, Oct)				
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, Sep)				
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, Sep)				
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 selected by a non-parametric statistical test, the Post hoc multiple comparisons test [24].

**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.

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 microscope, and eventually 12 lines were selected based on their excellent proliferation ability (based on visual observation regarding callus density obtained) and promising embryogenic potential (based on observations under microscope). Majority of those selected lines (7 out of 12) were induced on MS medium supplemented with 1.1 mg/L 2,4-D, while other 3 callus lines were induced on MS medium supplemented with 1.1 mg/L 2,4-D and 0.9 mg/L NAA, and 2 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) maturated into somatic embryos only on hormone-free MS medium devoid of AC (MS0). The other 8 lines responded to both media at variable levels, ranging between 10.5% to 97.1%, however in general MS0 showed to be more favourable for embryo maturation. Of these 9 callus lines, 8 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). Especially three lines (i.e., IBE/SE-10, IBE/SE-11 and IBE/SE-12) have been very 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, D and E). Following steps of the study continued by using plantlets obtained from those 3 callus lines on MS0.

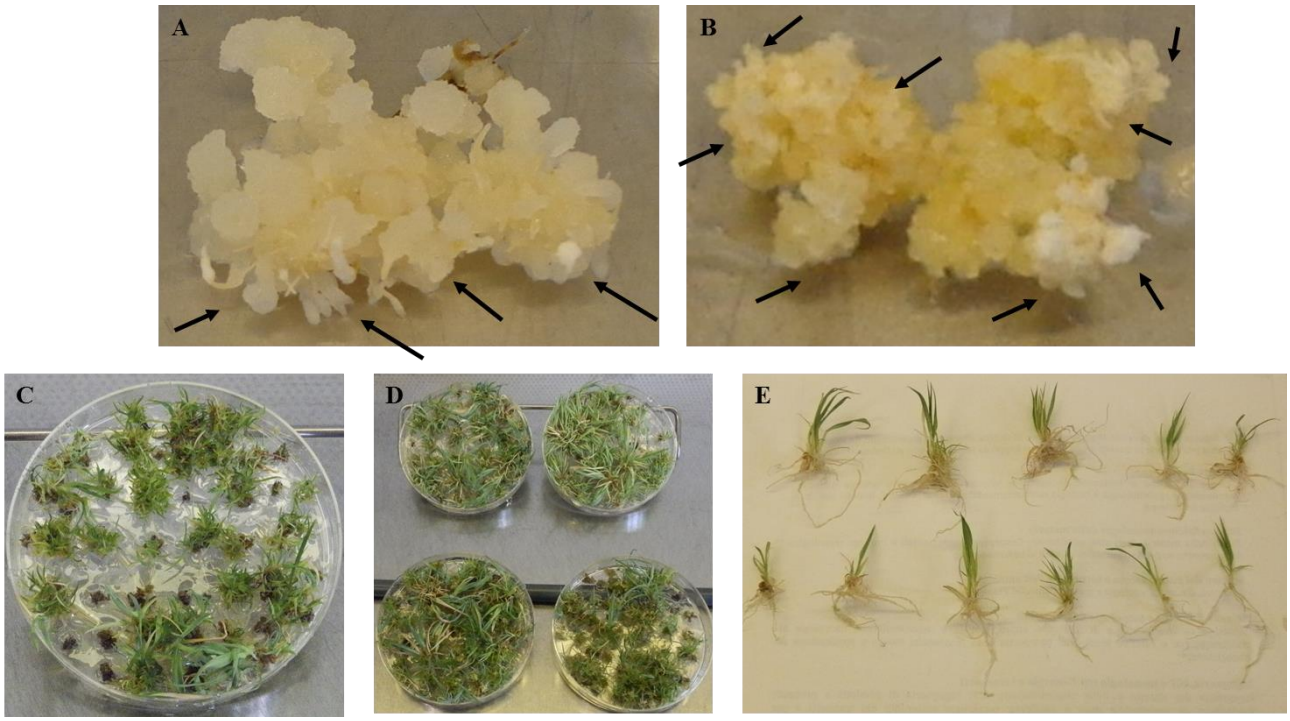


Figure 5. Emergence of *A. donax* L. somatic embryos on MS0, from callus originated from spikelets isolated from 30 cm-long (**A**) and 50 cm-long inflorescences (**B**), and maturation of the embryos into whole plantlet (**C-E**). Arrows indicate the callus zones where embryo emergence is clearly evident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

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, Oct / A1	10.5 c	16.7 c
IBE/SE-4	P, 30 cm, Sep / 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, Oct / A1	25.0 c	20.0 c
IBE/SE-8	P, 30 cm, Sep / 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, the Post hoc multiple comparisons test [24].

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 shoots were cultured on previously optimized proliferation medium, which was applied in 3 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).

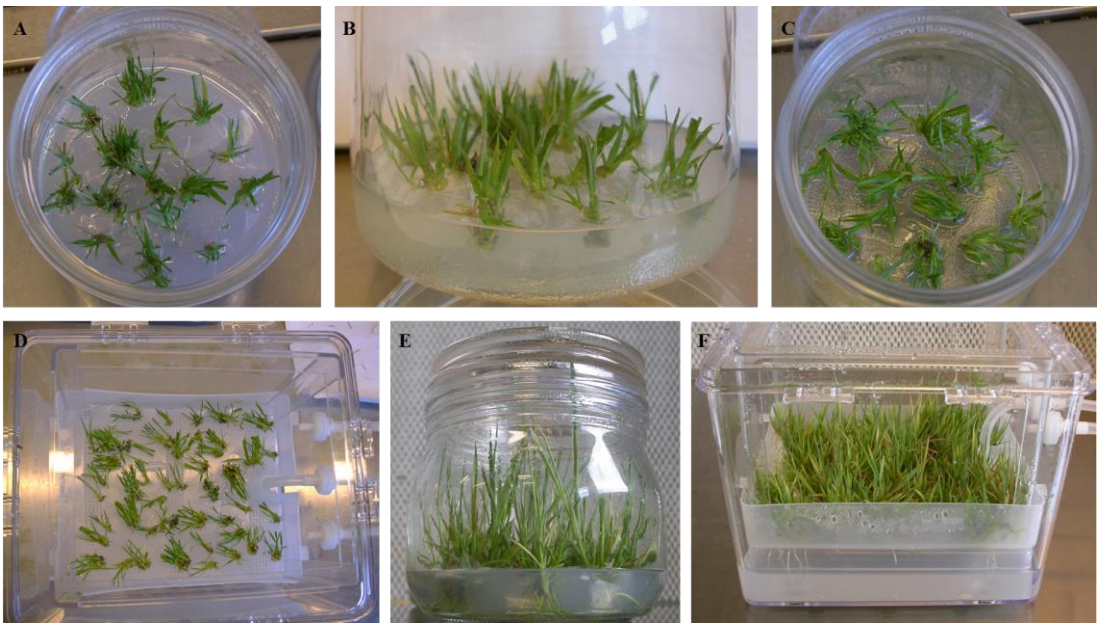


Figure 6. Multiplication of *A. donax* L. shoots, generated by indirect somatic embryogenesis from spikelets isolated from immature inflorescences. Culture initiation on gelled medium (**A** and **B**), stazionary liquid medium (**C**), and TIS (**D**). Multiple shoots obtained at the end of the culture period on gelled medium (**E**), and 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 = (Ln FW_F – Ln FW_I) x 100 / number of days in culture*

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

4. Discussion

Nowadays, increasing energy demands lead to use of non-food plants in both biofuel/biomass and bio-based compound production. Increasing the speed of biomass productionhas great opportunities to convert as biofuels without competing with food production for land, water, and markets, as currently occurs with maize and sugar [10, 25, 26[. As a promising plant, *A. donax* L. has high yield of cellulosic biomass due to its rapid growth, which could be easily converted into biofuel [10, 27]. In this study, we established an efficient protocol to increase 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 of this species. There are, however, some patented information, which suggested a protocol for somatic embryogenesis of the species [28, 29]. 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, 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 minutes, followed by a rinse in sterile water), the inflorescence is cut into cross-sectional pieces, (iii) explants are pretreated with cold temperature (e.g., 14 days at 5°C, in dim light, 30 μmol m^{-2} s^{-1}), (iv) explants are cultured for several weeks on gelled MS basal medium containing 0.2 mg/L 2,4-D and 0.02 mg/L thiadiazuron, in darkness at room temperature to generate totipotent tissue, (v) 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 tissue are then transferred to gelled MS medium containing 0.01-1 mg/L thidiazuron to induce plantlet formation. In contrast to what reported by Márton and Czakó, in the present study emerged immature inflorescences of a particular dimension was proved 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 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 the plantlet formation was different, as well.

Pigna et al. [11] developed a suspension culture protocol for *A. donax* L. with the aim of establishing protoplast culture to be used in genetic transformation studies. Suspension cultures were established by using callus cultures induced from stem explants according to the protocol of Takashi et al. [30]. 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. 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 callus 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. Increase in tissue mass over the same period (i.e., 14 days) in suspension culture was 5-fold. These results demonstrated 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 5-fold increase was still relatively low when compared to suspension cultures of other biofuel crops and suggested that extensive effort has still to be put in optimization of 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. [31] embraced an approach similar to the one used in the present study for the multiplication of shoots derived from somatic embryogenesis, testing the efficiency of liquid medium, both as stationary culture and TIS in RITA® system, in comparison to semi-solid medium (solidified by agar or Gellan gum).. They detected a sensitivity of *A. donax* L. to gelling agents, Gellan gum being more potent to improve shoot number, as well as FW and DW of the plant material, and concluded that cultivation of the species in liquid medium was cheaper and yet as effective as the medium solidified by Gellam gum. Increasing BA content of the medium to 4 mg/L was effective, as well, for secondary shoot proliferation. In accordance with Antal et al. [32], the use of BA, together with 0.05 mg/L NAA in the shoot induction medium from stem cuttings showed 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 beneficiary effect of MS medium containing 3 mg/L kinetin and 3 mg/L indole-3-acetic acid (IAA) for the multiplication of the shoots.

In classical micropropagation techniques, problematics such as low regeneration rates, limited uptake of the nutrients from semi-solid media, hyperhydricity and asphyxia could limit the growth of plantlets [33]. TIS, on the other hand, overcomes these difficulties by leading to explants interacting with the medium intermittently, but with their full surface [17]. For large-scale propagation, the liquid culture in TIS is a favorable system, especially to increase biomass production of several species such as bananas, cannabis (*Cannabis sativa*), *Schisandra chinensis,* and *Leucojum aestivum* [20, 34, 35].

5. Conclusions

Giant reed *(Arundo donax* L.) is considered today one of the most promising bioenergy crops among rhizomatous grasses for biomass production. 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. In this perspective, the present study provides an efficient indirect somatic embryogenesis protocol for *A. donax* L. and subsequent shoot multiplication, suitable for large-scale production of giant reed biomass. To optimize the biomass production process, the study highlighted the importance of using inflorescences as explants for the induction of embryogenic callus, harvested at a precise moment of immaturity, of developing an appropriate culture medium, and of applying a liquid culture in TIS for the multiplication of shoots originating from somatic embryos. The obtained shoots were vigor and healthy, never demonstrated any abnormality or growth retardance, showing to be an elite material for biomass production.

Author Contributions: EAO and ML designed the research; EAO conducted the experiment. The first draft of the manuscript was written by EAO, EK edited previous versions of the manuscript, reviewed afterwards by ML and DE. All authors read and approved the final manuscript.

Funding: This research was funded by the ''International Treaty on Plant Genetic Resources for Food and Agriculture - RGV-FAO 6, 2020-2022". This work was also supported by the Istinye University (Istanbul, Turkey).

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jiménez-Ruiz, J.; Hardion, L.; Pablo, J.; Vila, B.; Santín-Montanyá, M.I. Monographs on Invasive Plants in Europe N° 4: *Arundo donax* L.. *Bot. Lett.* **2021,** *168*, 131–151.
2. Lewandowski, I.; Scurlock, J.M.O.; Lindvall, E.; Christou, M. The Development and Current Status of Perennial Rhizomatous Grasses as Energy Crops in the US and Europe. *Biomass Bioenerg.* **2003,** *25*, 335–361.
3. Häfliger, E.; Scholz, H. (1981) Grass Weeds (2 Bde): weeds of the subfamilies Chloridoideae, Pooideae, Oryzoideae. Giba-Geigy: Basel, Switzerland, **1981,** pp. 223.
4. Bell, C.E., DiTomaso, J.M. and Brooks, M.L. 2006. Wildfires and invasive plants in southern California. *Proc. Calif. Weed Sci. Soc.* 58: 40-42.
5. Ceotto, E.; Di Candilo, M. Shoot Cuttings Propagation of Giant Reed (*Arundo donax* L.) in Water and Moist Soil: The Path Forward? *Biomass Bioenerg.* **2010**, *34*, 1614–1623.
6. Mariani, C.; Cabrini, R.; Danin, A.; Piffanelli, P.; Fricano, A.; Gomarasca, S.; Dicandilo, M.; Grassi, F.; Soave, C. Origin, Diffusion and Reproduction of the Giant Reed (*Arundo donax* L.): A Promising Weedy Energy Crop. *Ann. Appl. Biol.* **2010**, *157*, 191-202.
7. Jahirul, M.; Rasul, M.; Chowdhury, A.; Ashwath, N. Biofuels Production through Biomass Pyrolysis —a Technological Review. *Energies* **2012**, *5*, 4952–5001.
8. Muench, S.; Guenther, E. A Systematic Review of Bioenergy Life Cycle Assessments. *Appl. Energy* **2013**, *112*, 257–273.
9. Roos, A.; Ahlgren, S. Consequential Life Cycle Assessment of Bioenergy Systems – a Literature Review. *J. Clean. Prod.* **2018**, *189*, 358–373.
10. Angelini, L.G.; Ceccarini, L.; Nassi o Di Nasso, N.; Bonari, E. Comparison of Arundo Donax L. And Miscanthus X Giganteus in a Long-Term Field Experiment in Central Italy: Analysis of Productive Characteristics and Energy Balance. *Biomass Bioenerg.* **2009**, *33*, 635–643.
11. Pigna, G.; Dhillon, T.; Dlugosz, E.M.; Yuan, J.S.; Gorman, C.; Morandini, P.; Lenaghan, S.C.; Stewart, C.N. Methods for Suspension Culture, Protoplast Extraction, and Transformation of High-Biomass Yielding Perennial Grass *Arundo donax*. *Biotechnol. J.* **2016**, *11*, 1657–1666.
12. Basso, M.C.; Cerrella, E.G.; Buonomo, E.L.; Bonelli, P.R.; Cukierman, A.L. Thermochemical Conversion of *Arundo donax* into Useful Solid Products. *Energy Sources* **2005**, *27*, 1429–1438.
13. Peng, F.; Bian, J.; Peng, P.; Xiao, H.; Ren, J.; Xu, F.; Sun, R.-C. Separation and Characterization of Acetyl and Non-Acetyl Hemicelluloses of *Arundo donax* by Ammonium Sulfate Precipitation. *J. Agric. Food Chem.* **2012**, *60*, 4039–4047.
14. Bajguz, A.; Hayat, S. Effects of Brassinosteroids on the Plant Responses to Environmental Stresses. *Plant Physiol. Biochem.* **2009**, *47*, 1–8.
15. Zhang, J.; Lu, Y.; Zhang, S.; Lu, F.; Yang, H. Identification of Transcriptome Involved in Atrazine Detoxification and Degradation in Alfalfa (*Medicago Sativa*) Exposed to Realistic Environmental Contamination. *Ecotoxicol. Environ. Saf.* **2016**, *130*, 103–112.
16. Etienne, H. 2005. Somatic embryogenesis protocol: coffee (*Coffea arabica* L. and *C. canephora* P.). In *Protocols for Somatic Embryogenesis in Woody Plants*, Jain S.M., Gupta P.K., Eds., Springer, Dordrecht, The Netherlands, pp. 167-179.

17. Ramirez-Mosqueda M.A., Iglesias-Andreu L.G., Ramirez-Madero G., Hernandez-Rincon E.U. (2016) Micropropagation of *Steviare baudiana* Bert. in temporary immersion systems and evaluation of genetic fidelity. *S. Afr. J. Bot*. 106: 238–243.
18. Othmani A., Bayoudh C., Sellemi A., Drira N. (2018) Temporary immersion system for date palm micropropagation. In *Date Palm Biotechnology Protocols Volume 1: Tissue Culture Applications, Methods in Molecular Biology*, Jameel M. Al-Khayri et al., Eds., Springer Science+Business Media LLC., Vol. 1637, pp. 239-250, DOI 10.1007/978-1-4939-7156-5_20.
19. Rico S, Garrido J, Sánchez C, Ferreiro-Vera C, Codesido V and Vidal N (2022) A Temporary Immersion System to Improve *Cannabis sativa* Micropropagation. *Front. Plant Sci*. 13:895971. doi: 10.3389/fpls.2022.895971
20. Uma S., Karthic R., Kalpana S., Backiyarani S., Saraswathi M.S. (2021) A novel temporary immersion bioreactor system for large scale multiplication of banana (Rasthali AAB-Silk). *Sci Rep*. 11(1):20371. doi: 10.1038/s41598-021-99923-4.
21. Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 381-385.
22. Driver J.A., Kuniyuki A.H. (1984) In vitro propagation of Paradox walnut Juglans hindsii x Juglans regia rootstocks. *HortScience*, 19: 507-509.
23. Roncasaglia R., Benelli C., De Carlo A., Dradi G., Lambardi M., Ozudogru E.A. (2009) Fattori che influiscono sulla conservazione in crescita rallentata di specie da frutto. *Italus Hortus* 16 (2): 234-238.
24. Marascuilo L.A., McSweeney M. (1977) Post hoc multiple comparisons in sample preparations for test of homogenesity, In *Non-Parametric and Distribution FreeMethods of the Social Sciences*, McSweeney M., Marasculio L.A., Eds., Books/Cole Publ, Belmont, CA., pp. 141-147.
25. Herrera-Alamillo, M.Á.; Robert, M.L. Liquid In Vitro Culture for the Propagation of *Arundo donax*. In *Plant Cell Culture Protocols. Methods in Molecular Biology,* 1st ed.; Loyola-Vargas, V., Ochoa-Alejo, N., Eds.; Humana Press, Totowa, NJ. 2012; Volume 877. pp 153–160.
26. Corno, L.; Pilu, R.; Adani, F. *Arundo donax* L.: A Non-Food Crop for Bioenergy and Bio-Compound Production. *Biotechnol. Adv.* **2014**, *32*, 1535–1549.
27. Jeguirim, M.; Trouvé, G. Pyrolysis Characteristics and Kinetics of *Arundo donax* Using Thermogravimetric Analysis. *Bioresour. Technol.* **2009**, *100*, 4026–4031.
28. Márton L., Czakó, M. (2007a) Method for micropropagation of monocots based on sustained totipotent cell cultures. European Patent Register. https://www.google.hu/patents/EP2150100B1?cl=en&dq=EP+2150100+B1&hl=hu&sa=X&ved=0ahUKEwi6k93Z0MPPAhWJFiwKHXTsCr0Q6AEIHDAA.
29. Márton L., Czakó M. (2007b) Propagating Arundo species in nutrient broth and on teriary medium containing auxin and cytokinin. USA Patent No. US 7863046 B2. 07.05.2007. https://www.google.hu/patents/US7863046?dq=US+7863046+B2&hl=hu&sa=X&ved=0ahUKEwjW04fv0MPPAhVCjywKH RApCu0Q6AEIHDAA.
30. Takahashi, W.; Takamizo, T.; Kobayashi, M.; Ebina, M. Plant Regeneration from Calli in Giant Reed (*Arundo donax* L.). *Grassl. Sci.* **2010**, *56*, 224–229.
31. Cavallaro, V.; Patanè, C.; Salvatore Luciano Cosentino; I. Di Silvestro; Venera Copani Optimizing in Vitro Large Scale Production of Giant Reed (*Arundo donax* L.) by Liquid Medium Culture. *Biomass Bioenerg.* **2014**, *69*, 21–27.
32. Antal, G.; Kurucz, E.; Miklós Fári; Popp, J. Tissue Culture and Agamic Propagation of Winter-Frost Tolerant "Longicaulis". Arundo donax L.. *Environ. Eng. Manag. J.* **2014**, *13*(11), 2709–2715.
33. Ozudogru E.A., Karlik E., Elazab D., Lambardi M. (2022) establishment of direct organogenesis protocol for Arachis hypogaea cv. Virginia in liquid medium by temporary immersion system (TIS). *Horticulturae* 8: 1129-1143.
34. Ptak, A.; Morańska, E.; Skrzypek, E.; Warchoł, M.; Spina, R.; Laurain-Mattar, D.; Simlat, M. Carbohydrates Stimulated Amaryllidaceae Alkaloids Biosynthesis in *Leucojum aestivum* L. Plants Cultured in RITA®Bioreactor. *PeerJ* **2020**, *8*, e8688.
35. Szopa, A.; Kokotkiewicz, A.; Bednarz, M.; Jafernik, K.; Luczkiewicz, M.; Ekiert, H. Bioreactor Type Affects the Accumulation of Phenolic Acids and Flavonoids in Microshoot Cultures of *Schisandra chinensis* (Turcz.) Baill. *PCTOC* **2019**, *139*, 199–206.