



## Article

# Establishment of Direct Organogenesis Protocol for *Arachis hypogaea* cv. Virginia in Liquid Medium by Temporary Immersion System (TIS)

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**Abstract:** Peanuts (*Arachis hypogaea* L.) are a rich source of herbal oil, proteins, minerals, vitamins, fibers, essential amino acids, as well as bioactive compounds, and are thus widely used for human nutrition and animal feed, and for prevention from certain diseases. However, the in vitro regeneration response of the species is generally low, and it also displays a significant variability among its varieties. Thus, the development of advanced protocols and approaches for the in vitro propagation of peanut is still of immense importance. A recently developed in vitro propagation technique, TIS; Temporary Immersion Bioreactor System, provides a new approach for the mass propagation of plants. Accordingly, the present study provides an efficient de novo regeneration protocol for *Arachis hypogaea* L. cv. Virginia by using a TIS. Different concentrations of cytokinins, i.e., benzyladenine (BA) or thidiazuron (TDZ), were tested with several combinations of dry and medium immersion periods of TIS, corresponding to a total of 8, 12, 16, 24, 32, 36, 48, 64, 72, and 96 min daily immersions for the induction of direct organogenesis. The study exhibited that an MS medium added to 110 µM BA or 10 µM TDZ are the most appropriate medium formulations in TIS, when applied for 16 min every 16 h. The application of optimized procedures to cv. NC7 and two valuable Turkish autochthonous varieties, 7 × 77 and Com74, is also reported. To the best of our knowledge, the present study draws attention also for being the first study in which a TIS was used for peanuts.

**Keywords:** de-embryonated cotyledons; de novo regeneration; direct shoot proliferation; Platform™; temporary immersion system; peanut



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## 1. Introduction

Peanut, also known as groundnut (*Arachis hypogaea* L.; family Fabaceae), is the fourth most important leguminous oil crop in the world after soybean, oilseed rape, and cotton [1,2]. It is cultivated in tropical and sub-tropical areas throughout North and South America, Africa, Europe, and Asia [3]. Globally, 41% of peanuts are used as food and 49% are for the extraction of edible oil. Besides oil content, the peanut seed contains protein (22–30% dry weight), many minerals (P, Ca, Mg, K, Se, Mn, and Cu), vitamins (niacin, folate, thiamin, riboflavin, pantothenic acid, pyridoxine, and vitamin E), fiber, and essential amino acids [4–7]. The peanut is also a rich source of bioactive compounds such as resveratrol, caffeic acid, polyphenols, flavonoids, phytosterols, phenolic acids, stilbene, chlorogenic acid, ferulic acid, and coumaric acid that could be used for disease prevention, including metabolic syndrome [6–9].

Peanut production is highly affected by various biotic and abiotic stress factors, including insect pest infestation, drought, salinity, and temperature fluctuations, causing a reduction in yield and quality [1,10]. As an alternative, the in vitro propagation of the

peanut may provide efficient and rapid systems for commercial applications. However, the peanut is highly recalcitrant to in vitro manipulations, and thus the regeneration success obtained so far in tissue culture conditions is very low [11,12]. Moreover, storage through drying or freezing of peanut seeds is a major problem that affects seed viability and reduces in vitro seed germination [13,14]. The recalcitrant nature of seeds depends on the peanut genotypes, specific protocols being required for each type [12,15]. Accordingly, it possesses a recalcitrant nature to in vitro regeneration procedures, just like the other legumes. Indeed, although several protocols of somatic embryogenesis, organogenesis, and micropropagation of peanuts have been developed up to date, plant regeneration remained relatively low. Moreover, the current in vitro protocols for peanuts are labor-intensive and costly, and an automation for mass production is extremely difficult. However, recently developed innovative bioreactors allow for liquid culture in a temporary immersion system (TIS), leading to the explants interacting with the medium intermittently, thus renewing the atmosphere and supplying nutrients to fulfill the growth needs of the plants [16]. In fact, a TIS not only combines the advantages of liquid and semi-solid cultures, but also decreases to a minimum the difficulties (such as low regeneration rate, limited uptake of the nutrients from semi-solid media, hyperhydricity, asphyxia) of such classic propagation techniques. Presently, the TIS exhibits better performance to increase plant biomass and bioactive compounds for several plant species [17,18]. Among several options of TIS available in the market, the present study tested the performance of the Plantform™ bioreactor for the de novo regeneration of peanuts. Plantform™ was designed for large-scale in vitro plant propagation. Its advantages include having light, transparent, autoclavable, and easy to manipulate polycarbonate containers (180 mm × 150 mm × 150 mm), as well as allowing air exchange controlled by a separate pump and timer [19]. Recently, the TIS Plantform™ has been used to improve the micropropagation of the date palm (*Phoenix dactylifera* [20]) and marijuana (*Cannabis sativa* [21]) and provided new perspectives and opportunities for the mass propagation of these challenging species. Plantform™ has also been adapted for the large-scale multiplication of bananas (*Musa* spp. [22]).

Peanut cv. Virginia is a major cash crop with desirable agronomic characteristics and yield stability, especially in the Virginia–Carolina region of the United States. The desirability of the cv. Virginia peanut is associated with its large pod and kernel size, as well as its inshell for gourmet industries. However, the greater productivity of the large-kernelled type such as Virginia requires additional costs [23,24]. For instance, it requires more calcium and soil moisture for germination, which increases the costs [23,25,26]. The main aim of this study was to test the efficiency of liquid culture in TIS, using the Plantform™ bioreactor, for the large-scale propagation of the peanut germplasm. In order to achieve this goal, a range of genotypes were used in two separate experiments, one testing the cv. Virginia, and a second one the cv. NC7 (cv of American origin) as well as two valuable Turkish autochthonous cultivars, namely 7 × 77 and Com74. To the best of our knowledge, the present study is the first that addresses the use of a TIS in peanuts.

## 2. Materials and Methods

### 2.1. Plant Material

Certified mature seeds of *Arachis hypogaea* L. cv. Virginia were provided by Florsilva Ansaloni (Italy). The oblong pods of about 3–7 cm long contained 2 seeds about 1–1.5 cm long, covered with yellowish-brown shells. In the seed, a 0.5–0.7 cm long embryo was located at the edge of the cotyledons, with the plumule (embryonic shoot) located toward the inner part of the seed and the radicle (embryonic root) toward the bottom of the cotyledon (Figure 1). In a second experiment, optimized protocols were tested with the cvs. NC7, 7 × 77 and Com74, of which the mature seeds were provided by Akdeniz University (Antalya, Turkey). Among these, cv. NC7 was structurally the most similar to the cv. Virginia, while the pods, as well as rotund seeds of cvs. 7 × 77 and Com74, were slightly smaller in size and were covered with a brownish shell.



**Figure 1.** Peanut seeds used as plant material of the study. The figure shows pods, seeds, and embryos of *Arachis hypogaea* L. cv. Virginia, respectively.

For conservation, pods were removed, bare seeds were checked for apparent symptoms of mold. Clean seeds were transferred to tightly closed glass jars and maintained at 4 °C in darkness until they were used. Maximum conservation time of the seeds never exceeded 2 years.

### 2.2. Decontamination of the Seeds and Explant Preparation

The seeds were soaked in 70% (*v/v*) ethanol solution for 5 min for surface sterilization. Then, the seeds were subjected decontamination with 10% (*w/v*) H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) for 5 min followed by 20% (*v/v*) NaOCl for 20 min with 2–3 drops of Tween 20, both were followed by minimum 5-min rinse in sterile dH<sub>2</sub>O, repeated at least twice. Both cotyledons of the de-embryonated seeds served as explant source.

### 2.3. Determination of Seed Viability and Germination Rate

Decontaminated seeds were evaluated for viability, immediately after their supply as well as following their conservation, by TTC (2,3,5 tryphenyl tetrazolium chloride) test [27]. Randomly selected seed samples of all varieties were soaked in water and maintained in darkness for 24 h. They were then soaked in 1% TTC solution (pH 7.4) and maintained at 30 °C for 24 h. Afterward, the seeds were washed with dH<sub>2</sub>O, and the cotyledons were separated longitudinally to observe both the cotyledons and the embryo for the coloring. Accordingly, the viability of the tissues can be determined rapidly, within 48 h, by observing their color. In the present study, seeds (both the cotyledons and the embryos) that reflected red color (totally or partially) upon TTC test were recorded as ‘alive’. The percentage (%) of seed viability was calculated accordingly.

As different typologies of seed/embryo coloring were observed with TTC test of cv. Virginia, germination trials were conducted to confirm and support the results of the test. Therefore, decontaminated seeds, as well as excised embryos, were germinated *in vitro* on hormone-free MS medium [28] at 22 ± 1 °C. They were maintained in darkness during the first 3 days and then under a 16/8 h photoperiod, with a light intensity of 60 μmol m<sup>-2</sup> s<sup>-1</sup>, for the rest of the germination period. Both the seeds and the excised embryos were recorded as ‘germinated’ upon the emergence of the root and shoot primordia, respectively. De-embryonated cotyledons were then transferred to TIS. As in accordance with the TTC test, the germination trials were conducted upon the arrival of the seeds and at the end of their conservation.

### 2.4. *In Vitro* Propagation via Temporary Immersion System (TIS)

De-embryonated cotyledons of cv. Virginia were transferred into liquid media contained in Plantform<sup>TM</sup> bioreactor for the culture in TIS. To the MS media tested for the induction of direct organogenesis were added of 30 g/L sucrose, and one of two cytokinins, i.e., benzyladenine (BA) at 27.5, 55 or 100 μM, or thidiazuron (TDZ) at 5, 7.5 or 10 μM. Semi-solid MS media (solidified by the inclusion of 7 g/L agar) of the same compositions served as controls.

Several combinations of dry (in every 6, 8, 16, or 24 h) and medium immersion periods (for 8, 16, or 24 min) of TIS, corresponding to a total of 8, 12, 16, 24, 32, 36, 48, 64, 72,

and 96 min daily immersions, were tested for the regeneration of de novo shoots on de-embryonated cotyledons. In addition to periodic immersions in liquid medium, the cultures were ventilated for 10 min every 4 h. For rooting, 4 weeks after the culture initiation, the shoots were detached from the cotyledons, and multiple shoots were separated and transferred to hormone-free semi-solid MS medium. After 3 weeks, rooted shoots were washed gently under running tap water and were transferred to commercial soil for outdoor plants (which was autoclaved previously at 121 °C for 20 min). Plastic containers were maintained in the climatic chamber at  $22 \pm 1$  °C, under 16/8 h photoperiod for a week, during which the relative humidity was maintained by covering the plastic containers with a plastic bag and irrigating the plants once in every two days. Plastic bags were then removed gradually for the acclimatization of the plants, which were then transferred to greenhouse and irrigated regularly.

Optimized protocols of TIS culture with cv. Virginia were subsequently tested with the de-embryonated cotyledons of the other three varieties. All in vitro cultures were maintained in climatic chamber at  $22 \pm 1$  °C, under 16/8 h photoperiod ( $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) provided by cool, daylight fluorescent lights.

### 2.5. Data Collection and Statistical Analysis

A total of 20 randomly selected seeds of each variety were used for TTC test upon arrival and after conservation. Similarly, 40 randomly selected decontaminated seeds of cv. Virginia and 20 decontaminated seeds of NC7 and Turkish autochthonous varieties were used for germination trials upon arrival and after conservation.

Two replicates of 25 de-embryonated cotyledons of cv. Virginia were used for each cycle of TIS, and the experiment was repeated twice. Three replicates of 12 de-embryonated cotyledons of cv. Virginia were used as the control on semi-solid media, and the experiment was repeated twice. As for the other three varieties, the optimized protocols were tested using a sample of 30 explants both in TIS and semi-solid media.

During in vitro multiplication, the collected data consisted of (i) percentage of regenerating explants, (ii) average shoot number per regenerating explant, and (iii) average shoot length (cm). In addition, the Shoot Forming Capacity Index (SFC), formulated as  $\text{SFC} = (\text{average shoot number per regenerating explant}) \times (\% \text{ of regenerating explants}) / 100$  [29], was calculated to highlight the overall multiplication potential of each experiment. For rooting, the shoots were assembled in two separate groups according to their origin of multiplication; the ones that were propagated on semi-solid media and the ones that were propagated in TIS. Rooting data consisted of (i) percentage of rooting shoots and (ii) average root number. Rooted shoots were then transferred to soil and their acclimatization rate (%) was recorded after 1 month.

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 [30], to compare multiple percentages. Discrete data were subjected to ANOVA, followed by the least significant difference (LSD) test at  $p \leq 0.05$  to compare means.

## 3. Results

### 3.1. Decontamination of the Seeds

The protocol for peanut seed decontamination that was used in the present study was previously optimized [31] and tested on numerous other plants as well, with satisfactory results. Accordingly, a decontamination rate of over 83% was always obtained in the present study.

### 3.2. Evaluation of Seed Viability via TTC Test and Its Confirmation by Germination Trials

After separating the cotyledons of the cv. Virginia longitudinally and exposing the embryos, four different typologies of coloring were observed, as follows: (T1) cotyledons and the embryo were colored entirely; (T2) cotyledons were colored entirely, while the

embryo was colored only at the plumule; (T3) cotyledons were colored partially, while the embryo was colored entirely; and (T4) cotyledons were colored partially and the embryo was colored only at the plumule (Table 1). All these typologies were considered as ‘alive’, when these results were confirmed with the following germination trials, which gave comparable results in terms of germination rate (i.e., 100%), both for the entire seeds and the excised embryos of the cv. Virginia (Table 2). The increase in the percentage of partially colored cotyledons (T3 and T4), following seed storage at 4 °C in darkness, can be attributed to the degradation of lipid and oil components of the seeds.

**Table 1.** Different typologies of red coloring observed with the *Arachis hypogaea* L. cv. Virginia seeds following application of TTC test for seed viability.

	T1 * (%)	T2 (%)	T3 (%)	T4 (%)
Upon seed arrival	40	28	20	12
After conservation at 4 °C in darkness	36	16	32	16

\* T1—cotyledons and the embryo are colored entirely; T2—cotyledons are colored entirely, embryo is colored only at the plumule; T3—cotyledons are colored partially, embryo is colored entirely; T4—cotyledons are colored partially, embryo is colored only at the plumule.

**Table 2.** Seed viability and germination rates of entire seeds and excised embryos of *Arachis hypogaea* L. varieties.

Variety	Upon Seed Arrival			After Conservation at 4 °C in Darkness		
	Viability According to TTC Test (%)	Germination of the Entire Seed (%)	Germination of the Excised Embryo (%)	Viability According to TTC Test (%)	Germination of the Entire Seed (%)	Germination of the Excised Embryo (%)
Virginia	100 a *	100 a	100 a	100 a	100 a	100 a
NC7	100 a	100 a	100 a	100 a	100 a	100 a
7 × 77	85 b *	75 b	80 b	80 b	70 b	65 b
Com74	85 b	85 b	85 b	85 b	90 ab	80 b

\* Percentages followed by the same letter are not significantly different at  $p \leq 0.05$  by the post hoc multiple comparisons test. Different letters in each column represent significance at  $p \leq 0.05$ .

Similar to cv. Virginia, cv. NC7 always demonstrated a maximum germination rate; however, the two other Turkish varieties showed relatively lower germination rates (70–90% for the entire seeds and 65–85% for the excised embryos), both upon seed arrival and after their conservation, yet this never went below 65%. The obtained germination rates were in correlation with the TTC test results as well (Table 2), showing the validity of the TTC test for a fast determination of peanut seed viability and germinability.

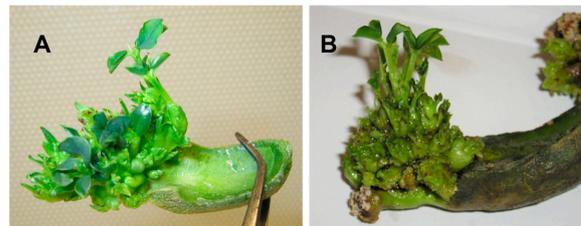
Average germination time for cv. Virginia was 13.2 days for the entire seeds and 9.2 days for the excised embryo. The difference between the germination rates of the seeds and the excised embryos that were germinated upon arrival or after conservation was not statistically significant. A similar trend was observed with the three other varieties as well (data not reported).

### 3.3. Direct Organogenesis of the De-Embryonated Seeds

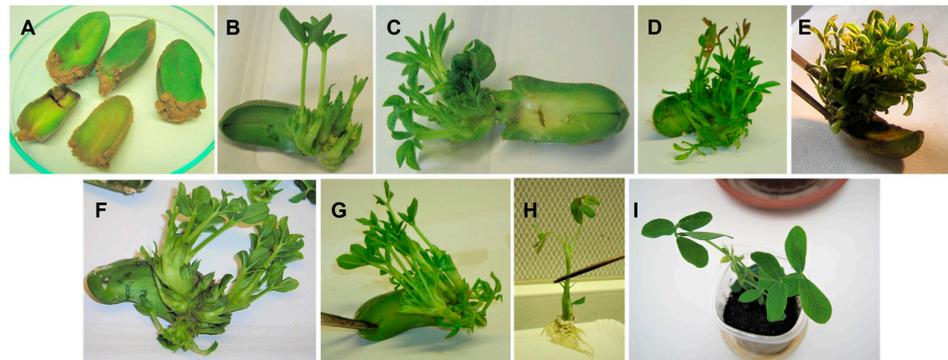
In order to investigate the effect of the TIS bioreactor system on the de novo regeneration of *Arachis hypogaea* L., de-embryonated cotyledons of cv. Virginia were transferred to the containers of Plantform™ (25 cotyledons per container) and subjected to several medium immersion regimes.

De-embryonated cotyledons that were cultured on semi-solid MS media of the same compositions served as control samples. Independently of the culture type, de-embryonated cotyledons expanded significantly in size (almost to double their original size) and turned green within the first week of their transfer to the media. Responsive explants started

to display shoot primordia by the beginning of the second week of culture (Figure 2), while the non-responsive ones remained green and occasionally gave rise only to soft and brown, non-regenerative callus tissue of a neglectable amount (Figure 3A). Regeneration rates of the cotyledons cultured on semi-solid media were unfavorable at all the tested concentrations of the two cytokinins (Figure 3B,C), with a maximum of 26.6%, with an average of 3.2 shoots, when they were cultured on medium containing 27.5  $\mu\text{M}$  BA (Table 3). Increasing the BA concentration had a significant detrimental effect on the percentage of regenerating explants (which dropped to 6%), although it did not significantly change the average shoot number per explant. The only positive effect of increasing the BA concentration was observed with the shoot lengths, which increased slightly (from 0.56 cm to 0.72 cm) with 55  $\mu\text{M}$  BA and significantly with 110  $\mu\text{M}$  BA (0.97 cm). The regeneration rates obtained with TDZ ranged between 10 and 14%. Although this was still unfavorable, the average shoot numbers (max. 7.8 shoots with 10  $\mu\text{M}$  TDZ) and the shoot lengths (max. 1.21 cm with 5  $\mu\text{M}$  TDZ) obtained here were slightly better. According to the SFC index, the lowest concentration of BA and the highest concentration of TDZ provided relatively higher multiplication rates, the latter being considered as the most effective growth regulator type and concentration for cv. Virginia.



**Figure 2.** Adventitious shoot primordia induction in the proximal region of the de-embryonated cotyledons of *Arachis hypogaea* L. cv. Virginia, when cultured in liquid MS medium containing (A) 110  $\mu\text{M}$  BA or (B) 10  $\mu\text{M}$  TDZ for two weeks.



**Figure 3.** De novo regeneration of *Arachis hypogaea* L. cv. Virginia through direct organogenesis. (A) Non-responsive de-embryonated cotyledons, giving rise or not to soft, brown callus tissue. (B) Shoot regeneration when de-embryonated cotyledons are cultured on semi-solid MS medium, supplemented with 110  $\mu\text{M}$  BA or (C) 10  $\mu\text{M}$  TDZ. (D) Shoot regeneration in TIS under 16 min/16 h medium immersion, in liquid MS medium supplemented with 110  $\mu\text{M}$  BA or (E) 10  $\mu\text{M}$  TDZ. (F) shoot regeneration in TIS under 16 min/24 h medium immersion, in liquid MS medium, supplemented with 110  $\mu\text{M}$  BA or (G) 10  $\mu\text{M}$  TDZ. (H) Rooting of the obtained shoots on semi-solid MS.0 and (I) acclimatized plantlets under greenhouse conditions.

**Table 3.** De novo regeneration of *Arachis hypogaea* L. cv. Virginia shoots on de-embryonated cotyledons, when cultured on semi-solid MS media.

Medium Composition	% of Regenerating Explants *	Number of Shoots per Regenerating Explant **	SFC ***	Average Shoot Length (cm) **
MS + 27.5 $\mu$ M BA	26.6 a	3.2 $\pm$ 0.9 b	0.85	0.56 $\pm$ 0.04 b
MS + 55 $\mu$ M BA	6.0 c	4.3 $\pm$ 2.4 b	0.26	0.72 $\pm$ 0.14 b
MS + 110 $\mu$ M BA	8.5 c	4.2 $\pm$ 2.3 b	0.36	0.97 $\pm$ 0.25 a
MS + 5 $\mu$ M TDZ	10.0 bc	5.8 $\pm$ 2.3 a	0.58	1.21 $\pm$ 0.33 a
MS + 7.5 $\mu$ M TDZ	13.3 b	4.2 $\pm$ 0.8 b	0.56	1.00 $\pm$ 0.01 a
MS + 10 $\mu$ M TDZ	14.0 b	7.8 $\pm$ 4.8 a	1.09	0.96 $\pm$ 0.18 a

\* Percentages followed by the same letter are not significantly different at  $p \leq 0.05$  by the post hoc multiple comparisons test. Different letters in each column represent significance at  $p \leq 0.05$ . \*\* Means followed by the same letter are not significantly different at  $p \leq 0.05$  by ANOVA followed by the LSD test. Average values  $\pm$  standard errors. \*\*\* SFC (shoot forming capacity) index = (average shoot number per regenerating explant)  $\times$  (% of regenerating explants)/100 [29].

Performing the multiplication of cv. Virginia in a TIS significantly improved all three parameters of the de novo regeneration. Indeed, the highest percentage of regenerating explants (71.7% vs. 26.6% on semi-solid medium) and the highest number of shoots per regenerating explants (10.2 vs. 7.8 on semi-solid medium) were recorded when de-embryonated cotyledons were subjected to 16 min of medium immersion every 16 h (corresponding to a total of 24 min of daily immersion) (Table 4, Figure 3D,E). The highest shoot length (4.41 cm vs. 1.21 cm on semi-solid medium) was recorded when the de-embryonated cotyledons were subjected to 24 min of medium immersion every 8 h (corresponding to 72 min of daily immersion). The SFC index indicated three medium immersion regimes in particular as the most effective ones: 16 min of immersion every 16 h (corresponding to 24 min of daily immersion), 16 min of immersion every 24 h (corresponding to 16 min of daily immersion, Figure 3F,G), and 24 min of immersion every 16 h (corresponding to 36 min of daily immersion). The least effective ones were 8 min of immersion every 16 h (corresponding to 12 min of daily immersion) and 8 min of immersion every 24 h (corresponding to 8 min of daily immersion). In addition, there were five other combinations which never provided regeneration: 8 min of immersion every 6 h (32 min of daily immersion), 8 min of immersion every 8 h (24 min of daily immersion), 16 min of immersion every 6 h (64 min of daily immersion), 24 min of immersion every 6 h (96 min of daily immersion), and 24 min of immersion every 24 h (24 min of daily immersion). These results show that (i) 8 min of medium immersion has always been unsatisfactory or unproductive, both when applied in a wide manner every 16 or 24 h and closely every 6 or 8 h, indicating that the shortest medium immersion period tested in the study was not suitable for cv. Virginia, independently of the elongation of dry period in between the medium immersions; (ii) 16 min of immersion was too intense when applied every 6 h (64 min of daily immersion); however, less frequent applications provided the most efficient multiplication results; (iii) similarly, 24 min of immersion every 6 h (96 min of daily immersion) proved to be detrimental due to the immersion intensity; however, less frequent applications (every 8 or 16 h) showed to provide a satisfactory percentage of regenerating explant and average shoot number. However, 24 h of dry period between the immersions proved to be detrimental again. These results are rather remarkable as they demonstrate that not only the medium immersions or daily medium immersion times are determinative, but also that the mode of application of these immersions is very effective. For instance, among the three tested immersion periods, 16 min was shown to be the most appropriate one; however, when this immersion time was applied every 6 h, no regeneration was obtained. Similarly, 16 and 24 min of daily immersions showed to be the most appropriate ones; however, when, for instance a 24 min of daily immersion time was applied as 8 min

every 8 h or as 24 min in 24 h, no regeneration was obtained, while when applied as 16 min every 16 h, one of the best results was obtained.

**Table 4.** De novo regeneration of *Arachis hypogaea* L. cv. Virginia shoots on de-embryonated cotyledons, when cultured in TIS, under the influence of different medium immersion regimes.

Medium Composition	Medium Immersion Regime	% of Regenerating Explants *	Number of Shoots per Regenerating Explant **	SFC ***	Average Shoot Length (cm) **
MS + 27.5 µM BA	8 min/16 h, 12 min daily immersion	8.7 e	4.1 ± 0.7 bc	0.36	N.R. ****
MS + 55 µM BA	8 min/16 h, 12 min daily immersion	13.5 d	3.2 ± 0.5 c	0.43	N.R.
MS + 110 µM BA	8 min/16 h, 12 min daily immersion	11.1 e	2.2 ± 0.4 d	0.24	N.R.
MS + 5 µM TDZ	8 min/16 h, 12 min daily immersion	15.7 d	2.7 ± 0.9 d	0.42	N.R.
MS + 7.5 µM TDZ	8 min/16 h, 12 min daily immersion	18.7 cd	3.1 ± 0.7 c	0.58	N.R.
MS + 10 µM TDZ	8 min/16 h, 12 min daily immersion	23.3 cd	3.8 ± 0.9 c	0.88	N.R.
MS + 27.5 µM BA	8 min/24 h, 8 min daily immersion	-	-	-	-
MS + 55 µM BA	8 min/24 h, 8 min daily immersion	-	-	-	-
MS + 110 µM BA	8 min/24 h, 8 min daily immersion	8.7 e	4.9 ± 0.5 bc	0.43	N.R.
MS + 5 µM TDZ	8 min/24 h, 8 min daily immersion	12.7 d	3.2 ± 0.8 c	0.41	N.R.
MS + 7.5 µM TDZ	8 min/24 h, 8 min daily immersion	10.7 e	3.7 ± 0.2 c	0.40	N.R.
MS + 10 µM TDZ	8 min/24 h, 8 min daily immersion	15.3 d	5.1 ± 0.7 b	0.78	N.R.
MS + 27.5 µM BA	16 min/8 h, 48 min daily immersion	15.5 d	4.1 ± 0.9 bc	0.64	0.52 ± 0.11 d
MS + 55 µM BA	16 min/8 h, 48 min daily immersion	19.8 d	4.2 ± 0.7 bc	0.83	1.07 ± 0.43 c
MS + 110 µM BA	16 min/8 h, 48 min daily immersion	29.8 cd	4.8 ± 0.9 bc	1.43	0.44 ± 0.01 d
MS + 5 µM TDZ	16 min/8 h, 48 min daily immersion	38.6 c	4.6 ± 0.5 bc	1.78	0.84 ± 0.51 d
MS + 7.5 µM TDZ	16 min/8 h, 48 min daily immersion	21.3 cd	5.2 ± 0.8 b	1.11	1.51 ± 0.18 c
MS + 10 µM TDZ	16 min/8 h, 48 min daily immersion	17.0 d	4.4 ± 1.3 bc	0.75	0.67 ± 0.01 d
MS + 27.5 µM BA	16 min/16 h, 24 min daily immersion	37.3 c	4.2 ± 0.6 bc	1.57	2.95 ± 0.46 b
MS + 55 µM BA	16 min/16 h, 24 min daily immersion	43.7 bc	5.6 ± 0.5 b	2.45	2.20 ± 0.16 b
MS + 110 µM BA	16 min/16 h, 24 min daily immersion	71.7 a	5.4 ± 0.8 b	3.87	0.97 ± 0.25 c

Table 4. Cont.

Medium Composition	Medium Immersion Regime	% of Regenerating Explants *	Number of Shoots per Regenerating Explant **	SFC ***	Average Shoot Length (cm) **
MS + 5 $\mu$ M TDZ	16 min/16 h, 24 min daily immersion	52.5 b	4.8 $\pm$ 0.7 bc	2.52	1.63 $\pm$ 0.15 c
MS + 7.5 $\mu$ M TDZ	16 min/16 h, 24 min daily immersion	42.8 bc	9.2 $\pm$ 1.8 a	3.94	1.84 $\pm$ 0.11 c
MS + 10 $\mu$ M TDZ	16 min/16 h, 24 min daily immersion	43.2 bc	10.2 $\pm$ 1.5 a	4.41	1.49 $\pm$ 0.08 c
MS + 27.5 $\mu$ M BA	16 min/24 h, 16 min daily immersion	33.7 c	7.5 $\pm$ 0.9 ab	2.53	0.97 $\pm$ 0.07 c
MS + 55 $\mu$ M BA	16 min/24 h, 16 min daily immersion	40.2 bc	6.2 $\pm$ 0.6 b	2.49	1.46 $\pm$ 0.13 c
MS + 110 $\mu$ M BA	16 min/24 h, 16 min daily immersion	43.3 bc	6.7 $\pm$ 0.9 b	2.90	1.07 $\pm$ 0.07 c
MS + 5 $\mu$ M TDZ	16 min/24 h, 16 min daily immersion	41.2 bc	5.9 $\pm$ 0.9 b	2.43	1.13 $\pm$ 0.11 c
MS + 7.5 $\mu$ M TDZ	16 min/24 h, 16 min daily immersion	38.3 c	6.4 $\pm$ 0.5 b	2.45	1.21 $\pm$ 0.31 c
MS + 10 $\mu$ M TDZ	16 min/24 h, 16 min daily immersion	35.5 c	8.9 $\pm$ 2.9 a	3.16	1.01 $\pm$ 0.04 c
MS + 27.5 $\mu$ M BA	24 min/8 h, 72 min daily immersion	18.7 cd	3.8 $\pm$ 0.4 c	0.71	3.02 $\pm$ 1.07 ab
MS + 55 $\mu$ M BA	24 min/8 h, 72 min daily immersion	31.3 c	4.2 $\pm$ 0.4 bc	1.31	2.51 $\pm$ 0.37 b
MS + 110 $\mu$ M BA	24 min/8 h, 72 min daily immersion	38.3 c	2.8 $\pm$ 0.7 d	1.07	2.07 $\pm$ 0.18 b
MS + 5 $\mu$ M TDZ	24 min/8 h, 72 min daily immersion	44.7 bc	3.6 $\pm$ 0.5 c	1.61	4.03 $\pm$ 1.11 a
MS + 7.5 $\mu$ M TDZ	24 min/8 h, 72 min daily immersion	40.3 bc	5.2 $\pm$ 0.8 b	2.10	4.41 $\pm$ 0.91 a
MS + 10 $\mu$ M TDZ	24 min/8 h, 72 min daily immersion	40.7 bc	5.4 $\pm$ 0.7 b	2.20	3.71 $\pm$ 1.06 a
MS + 27.5 $\mu$ M BA	24 min/16 h, 36 min daily immersion	31.2 c	3.5 $\pm$ 0.9 c	1.09	1.82 $\pm$ 0.27 c
MS 55 $\mu$ M BA	24 min/16 h, 36 min daily immersion	38.7 c	3.2 $\pm$ 0.6 c	1.24	1.71 $\pm$ 0.17 c
MS + 110 $\mu$ M BA	24 min/16 h, 36 min daily immersion	59.7 b	4.7 $\pm$ 0.9 bc	2.81	3.01 $\pm$ 0.09 ab
MS + 5 $\mu$ M TDZ	24 min/16 h, 36 min daily immersion	52.3 b	4.9 $\pm$ 0.9 bc	2.56	3.14 $\pm$ 0.07 a
MS + 7.5 $\mu$ M TDZ	24 min/16 h, 36 min daily immersion	35.8 c	4.4 $\pm$ 0.5 bc	1.58	2.03 $\pm$ 0.41 b
MS + 10 $\mu$ M TDZ	24 min/16 h, 36 min daily immersion	41.7 bc	5.9 $\pm$ 2.9 b	2.46	3.10 $\pm$ 0.61 a

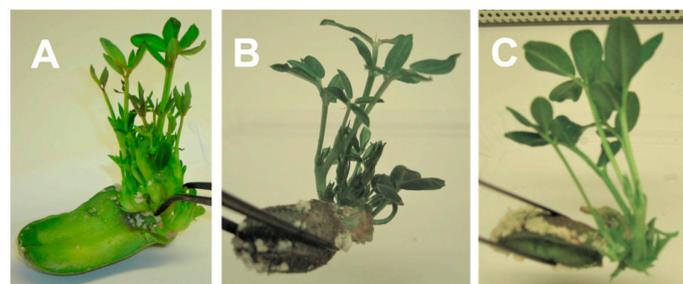
\* Percentages followed by the same letter are not significantly different at  $p \leq 0.05$  by the post hoc multiple comparisons test. Different letters in each column represent significance at  $p \leq 0.05$ . \*\* Means followed by the same letter are not significantly different at  $p \leq 0.05$  by ANOVA followed by the LSD test. Average values  $\pm$  standard errors. \*\*\* SFC (shoot forming capacity) index = (average shoot number per regenerating explant)  $\times$  (% of regenerating explants)/100 [29]. \*\*\*\* NR; not reported.

As for the cv. NC7 and the two Turkish autochthonous varieties, the TIS provided a significantly higher percentage of regenerating explants, average number of shoots, and SFC indices when selecting two immersion regimes, i.e., 16 min of medium immersion every 16 h, corresponding to 24 min daily immersion, and 16 min medium immersion every 24 h, corresponding to 16 min daily immersion, were applied (Table 5). In particular, the 16 min/24 h immersion cycle provided a significant increase in those parameters for the cv. NC7 (Figure 4A), with an SFC index of 2.80 (vs. 0.33 on semi-solid medium). The two other varieties demonstrated a similar trend when they were cultured under a 16 min/16 h immersion regime, providing an SFC index of 4.52 (vs. 0.22 on semi-solid medium) and 2.28 (vs. 0.51 on semi-solid medium), for cvs. 7 × 77 (Figure 4B) and Com74 (Figure 4C), respectively. The only parameter that was not affected or affected negatively, although not significantly, in the TIS was the shoot length. It did not change for 7 × 77 when cultured under a 16 min/16 h immersion regime, but all the other theses of all three varieties resulted with slightly shorter shoots.

**Table 5.** De novo regeneration of *Arachis hypogaea* L. varieties, NC7, 7 × 77, and Com74, from de-embryonated cotyledons cultured on semi-solid or liquid MS medium, added of 10 µM TDZ.

Variety	Medium Immersion Regime	% of Regenerating Explants *	Number of Shoots per Regenerating Explant **	SFC ***	Average Shoot Length (cm) **
Regeneration on semi-solid MS medium					
NC7	Semi-solid medium	13.3 c	2.5 ± 0.7 d	0.33	0.96 ± 0.12 a
7 × 77	Semi-solid medium	10.0 c	2.2 ± 0.9 d	0.22	0.73 ± 0.25 a
Com74	Semi-solid medium	16.6 c	3.1 ± 0.3 c	0.51	0.62 ± 0.09 ab
Regeneration in liquid medium (TIS; 16 min/16 h, corresponding to 24 min daily immersion) *					
NC7	16 min/16 h, 24 min daily immersion	33.3 b	1.3 ± 0.16 e	0.43	0.78 ± 0.06 a
7 × 77	16 min/16 h, 24 min daily immersion	76.6 a	5.9 ± 0.44 b	4.52	0.72 ± 0.06 a
Com74	16 min/16 h, 24 min daily immersion	46.6 b	4.9 ± 0.68 b	2.28	0.35 ± 0.03 b
Regeneration in liquid medium (TIS; 16 min/24 h, corresponding to 16 min daily immersion) *					
NC7	16 min/24 h, 16 min daily immersion	40.0 b	7.0 ± 1.1 a	2.80	0.71 ± 0.12 a
7 × 77	16 min/24 h, 16 min daily immersion	23.3 bc	3.7 ± 0.3 c	0.86	0.40 ± 0.02 b
Com74	16 min/24 h, 16 min daily immersion	30.0 b	3.3 ± 0.2 c	0.99	0.43 ± 0.03 b

\* Percentages followed by the same letter are not significantly different at  $p \leq 0.05$  by the post hoc multiple comparisons test. Different letters in each column represent significance at  $p \leq 0.05$ . \*\* Means followed by the same letter are not significantly different at  $p \leq 0.05$  by ANOVA followed by the LSD test. Average values ± standard errors. \*\*\* SFC (shoot forming capacity) index = (average shoot number per regenerating explant) × (% of regenerating explants)/100 [29].



**Figure 4.** Application of selected TIS immersion regimes three to varieties, cultured in liquid MS medium containing 10 µM TDZ. (A) cv. NC7 (16 min/24 h medium immersion), (B) 7 × 77 (16 min/16 h medium immersion), and (C) Com74 (16 min/16 h medium immersion).

### 3.4. Rooting of the Shoots and Acclimatization of the Plantlets

In a previous preliminary experiment, the rooting of cv. Virginia was tested on MS media, supplemented or not with 5, 10, or 20  $\mu\text{M}$  indole-3-butyric acid (IBA). Among the media supplemented with the auxin, the highest rooting rate (i.e., 29%) was obtained when the MS medium contained 5  $\mu\text{M}$  IBA; however, the medium devoid of auxin provided 60% rooting (with  $6.67 \pm 0.37$  roots of  $1.11 \pm 0.81$  cm long in average), although the root emergence was delayed by 2–3 days (data not reported).

Based on previous observations, in the present study, rooting was only performed on MS medium devoid of growth regulators (MS.0). The maximum rooting rate of the cv. Virginia shoots that were propagated on semi-solid media was recorded as 78%, with  $9.5 \pm 0.67$  roots, on average (Table 6). The rooting performance of the shoots propagated in the TIS was slightly higher (82%), although not significant, with an average root number of  $10.7 \pm 0.37$  (Figure 3H). Rooting performance of the other tested varieties was significantly better (ranging between 71% (Com74) and 84% (NC7)), with an average number of roots of 8.5, 9.2, and 6.0 for NC7, 7  $\times$  77, and Com74, respectively, when the shoots were previously multiplied in the TIS. Their rooting performance on semi-solid media ranged between 58% (7  $\times$  77) and 67% (NC7), and the average numbers of roots recorded for NC7, 7  $\times$  77, and Com74 were 6.4, 9.1, and 6.5, respectively. For acclimatization, the plantlets of each species were bulked, without considering their origin of multiplication. Acclimatization was easily obtained by transferring the plantlets into 250-cc plastic containers and maintaining them under greenhouse conditions. Acclimatization rate recorded after one month ranged between 78 and 87% (Table 6, Figure 3I).

**Table 6.** Rooting performance of *Arachis hypogaea* L. shoots, previously multiplied in vitro either in liquid (in TIS) or on solid media and their acclimatization performance.

Origin of Multiplication	Variety	% of Rooting Shoots *	Average Root Number **	Acclimatization (%) *	
TIS	Virginia	82 a	$10.7 \pm 0.37$ a	Virginia	87 a
	NC7	84 a	$8.5 \pm 0.41$ a		
	7 $\times$ 77	75 ab	$9.2 \pm 0.37$ a	NC7	84 a
	Com74	71 ab	$6.0 \pm 0.21$ b		
Semi-solid media	Virginia	78 a	$9.5 \pm 0.67$ a	7 $\times$ 77	78 a
	NC7	67 ab	$6.4 \pm 0.34$ b		
	7 $\times$ 77	58 b	$9.1 \pm 0.67$ a	Com74	81 a
	Com74	61 b	$6.5 \pm 0.37$ b		

\* Percentages followed by the same letter are not significantly different at  $p \leq 0.05$  by the post hoc multiple comparisons test. \*\* Means followed by the same letter are not significantly different at  $p \leq 0.05$  by ANOVA followed by the LSD test. Average values  $\pm$  standard errors.

## 4. Discussion

The type of the explant used in this study was de-embryonated cotyledons. The TTC test was applied to confirm the viability of the explants, which resulted in four different typologies of coloring: cotyledons and the embryo were colored entirely; cotyledons were colored entirely while the embryo was colored only at the plumule; cotyledons were colored partially while the embryo was colored entirely; and cotyledons were colored partially, and the embryo was colored only at the plumule. The increase in the percentage of partially colored cotyledons following seed storage at 4  $^{\circ}\text{C}$  in darkness can be attributed to the degradation of lipid and oil components of the seeds. This phenomenon was also investigated and discussed earlier by others, e.g., [32,33]. Nevertheless, the results of the present study demonstrate that even if the storage tissues were damaged, this did not interfere or impair the germination ability of peanut seeds and they germinated independently of the presence or integrity of the cotyledons.

In order to investigate the effect of the TIS bioreactor system on the de novo regeneration of *Arachis hypogaea* L., de-embryonated cotyledons of cv. Virginia were transferred to the containers of Plantform™ and subjected to several medium immersion regimes. Although one may expect a de-embryonated cotyledon to have minor morphogenic response due to its nutrient-storage nature for the zygotic embryo, as suggested also by [34], it has been indeed reported, for a number of oilseed crops including peanut, that the cotyledons have a significant capacity for an organogenic growth response, see, e.g., [34–45]. However, previous studies with some other species (such as almond and cherry) also demonstrated that this capacity differs according to the location of the cotyledonary tissue and proximal region of the cotyledon (i.e., where the detached embryo was in contact with the cotyledon) displaying the highest regeneration capacity in comparison to the distal region [46–48]. This phenomenon is in correlation with the outcomings of the present study, in which adventitious shoot primordia induction was always obtained in the proximal region. This can be attributed to the persistence of the regeneration capacity of the remaining cells and tissues of the detached embryo that are likely to be able to readily dedifferentiate into meristematic cells. Hence, previous reports and the outcomings of the present study support the idea that, just like the well-known explants such as apical and axillary nodes, internodes, leaf portions and roots, the cotyledon tissues can also be easily manipulated for in vitro regeneration.

The liquid media in the containers of TIS were MS media, added of one of the three concentrations of either BA (27.5, 55 or 110  $\mu$ M) or TDZ (5, 7.5 or 10  $\mu$ M). These concentrations of growth regulators, which are slightly higher than the standard value, were chosen inspired by other studies, e.g., [30,39,43]. For instance, Pestana et al. [39] cultivated cotyledons of cv. Tatu on MS medium supplemented with 110  $\mu$ M BA. Burns et al. [43] tested the effect of 10–80  $\mu$ M BA for cotyledons of cvs. Florida-07, Georgia Green, and VC-2, 160–640  $\mu$ M for cv. New Mexico Valencia A, 160–320  $\mu$ M for cv. Georgia Brown. Similarly, McKently et al. [38] also reported that the optimum concentration of BA for shoot induction from the cotyledons of cv. Forigiant were higher than what is usually used in other plant species. In the present study, although there are some reverse examples, in general TDZ seems to provide a relatively higher percentage of regenerating explants and average shoot numbers; however, this distinction is not so clear for the average shoot length. As for different cytokinin concentrations, there is no linear correlation; increasing concentrations seems to be more efficient when applied in one immersion regime, and less efficient in another one. Yet, it can be concluded that an MS medium added to 110  $\mu$ M BA or 10  $\mu$ M TDZ are the most appropriate medium formulations in TIS.

During the preliminary experiments, liquid culture (in which the cotyledons were continuously and completely immersed in liquid media of the same composition and cultured on orbital shaker) was also tested. However, differently from the results of McKently et al. [38], here no regeneration from those explants was achieved. The explants turned yellowish-brown and deteriorated shortly after the culture initiation. McKently and his co-workers, however, were able to obtain shoot formation both in liquid and solid media of the same composition, although the shoot production on solid media was approximately twice than in liquid and the shoots obtained in liquid media were vitreous.

The present study is the first example of the application of a TIS to peanuts. Thus, comparisons can be conducted on the results of the previous studies, where de-embryonated cotyledons of peanut were cultured on semi-solid MS media for de novo regeneration via direct shoot organogenesis. Among these, McKently et al. [38] obtained 40% of regenerating explants of cv. Forigiant, with a maximum average shoot number of 12. When they applied the optimized protocol to other varieties, the shooting response ranged between 44 and 94%. Pestana et al. [39] tested three different culture temperatures (25, 28, and 35 °C) for cv. Tatu and obtained the highest regeneration rate (56%) and average shoot number (24) when the highest culture temperature was applied. Maina et al. [42] tested the regeneration efficiency of cotyledons of the African varieties and obtained the highest percentage of regeneration (35%) with cv. Chalimba. Radhakrishnan et al. [40] obtained a maximum

of 57% of regeneration from the de-embryonated cotyledons of cv. J11. Hoa et al. [45] obtained 23.3% of regenerating explants with cv. L14, with 6.8 shoots in average for the regenerating explant. All these different results of the previous studies, as well as the results of the present study (which were conducted in a similar manner, using the same type of explant and similar growth regulator type and concentrations), not only reveal how the variety has an influence on shoot induction, but also the superiority of the TIS for de novo regeneration.

Rooting performance of the shoots was slightly higher for cv. Virginia and was significantly better for the other varieties when the shoots were previously multiplied in the TIS. This favorable influence was more evident in cvs. NC7 and 7 × 77. These results suggest that the culture conditions provided in the TIS increase the ability of the shoots to respond to external as well as internal hormonal stimuli, and thus a higher number of shoots readily gave rise to root meristemoids when they were cultured on an appropriate rooting medium. A similar trend was observed with other species as well (e.g., *Anthurium* spp., *Carex oshimensis* cv. Evergold, *Ficus* spp., and *Nandina domestica*; unpublished data).

## 5. Conclusions

Peanuts (*Arachis hypogaea* L.), a valuable source of herbal oil, proteins, minerals, vitamins, fibers, essential amino acids, and bioactive compounds, are an economically important species, consumed worldwide both for nutrition and pharmaceutical/medical uses. However, despite numerous attempts, this species still possesses a recalcitrant nature to in vitro manipulations. The present study which aimed to develop an efficient protocol for the mass propagation of the species, tested the Plantform™ TIS for de novo regeneration using de-embryonated cotyledons of cv. Virginia, and obtained a significant enhancement in the percentage of regenerating explants and average number of shoots. The obtained shoots were healthy, without exhibiting any symptoms of hyperhydricity or any other abnormality associated with liquid culture, and were easily rooted and acclimatized. The selected medium immersion regimes of the TIS were then applied to cvs. NC7, 7 × 77, and Com74, and always provided a satisfactory result. Therefore, it can be concluded that, the present study, in which the use of a TIS was demonstrated for the first time in peanuts with very favorable results, will pave the way to further improve the in vitro propagation of not only peanuts but also other species with recalcitrant in vitro regeneration natures.

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