**Arabidopsis thaliana** L. Seeds are A novel source of somatic embryos

Rasha Fawzi Al-Jirjees1*, Shifa Mahdi Salih1 and Mozahim Kasim AL- Mallah1

1Department of Biology, Biotechnology Lab, College of Education for Pure Sciences, University of Mosul, Iraq.

*E-mail: rasha.fawzi2016@uomosul.edu.iq

**Abstract.** Somatic embryogenesis is a valuable tool for investigating the totipotency of plant cells. A simple and efficient protocol for inducing somatic embryogenesis from seeds of *Arabidopsis thaliana* were established. Surface sterilized seeds were placed on agar-solidified Murashige and Skoog (MS) medium free from growth regulators. Callus initiation began7 days after seeds culture and became visible with the naked eye within 10-14 days. It was friable and yellowish white in color. Within 20 days, callus was transferred to Gamborg's B5 medium containing 1.0 mgL^-1 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 0.05 mgL^-1 Kin for multiplication. The results indicated that somatic embryos had been recorded only in B5 medium supplemented with 0.4 mgL^-1 TDZ (N-phenyl-N’-1,2, 3-thiazol-5-y lurea) and it was the best one. Through our observation, different stages of somatic embryos have been found. The results revealed that the continuous transfer of small masses containing several embryos at different stages to the same induction medium subsequently formed a large cluster of shoots, which were rooted in MS medium free from growth regulators and MS hormone-free medium with 0.2 activated charcoal. The percentages of rooting were 63% and 51% respectively. This study proved that Arabidopsis thaliana seeds are a novel source for somatic embryos.

**Keywords.** *Arabidopsis thaliana*, Seeds, Somatic Embryos, TDZ, Plant regeneration.

1. Introduction

Somatic embryogenesis (SE) is a method of differentiating cells to bipolar structures similar to zygotic embryos in plants [1]. The (SE) process shows that plants' specific development capabilities have already evolved to move to embryogenic development program in somatic cells [2]. Plant cells have been recognized for their ability to be reprogrammed into embryogenesis [3]. Somatic embryos (SEs) formation has been demonstrated as evidence of plant cell totipotency since the development of SEs was observed from colonies of single cell origin [4]. In addition to being a tool for studying and understanding early embryonic growth, SE is also a key plant biotechnology tool used for the asexual propagation of (hybrid) plants and it is also important to rapidly reproduce, preserve, or regenerate genetically modified plants during transformation processes [5]. SEs may grow into whole plants and since the 1960s; numerous protocols have been developed that allow the efficient regeneration, through *in vitro* cultivation conditions, of dozens of plant species through SEs [6]. Although hormones and stress are common treatments that can encourage SE [7, 8], there is little understanding as to how
these causes contribute to SEs formation, including mechanisms interacting to align the SEs with regulatory networks of transcription factors [9]. The detection of exogenous and endogenous factors facilitating embryogenic transfer to in vitro cultured somatic cells contributes to a deeper understanding of the totipotency of plant cells. Unlike advanced tissue culture experience which encourages the SEs induction [10]. Much less are known the endogenous factors that determine the capability of the tissue to an embryogenic response. However, in the last decade there were strong progress in deciphering the molecular mechanism regulating the embryogenic transformation of somatic plant cells and extensive molecular analysis of the functioning of auxins in SEs induction [2]. The Somatic embryogenesis process is usually divided into two major phases: one phase of the induction and one development. During the induction process, isolated somatic cells undergo conditions that facilitate cell proliferation and dedifferentiation and are assumed to have the capacity to undergo SE and some of the cultivated cells begin to differentiate in somatic embryos during the developmental phase [11]. A host of factors, including cell-to-cell signaling, have been involved in SE induction [12], modification of the wall composition [13]. The alteration in hormones [14,15] and the epigenetic shifters [16,17]. In plant biology, due to lack of early cytological or morphological markers for SE the key experimental factor was the impossibility to precisely isolate and study the cells reacting to SE-induction. As a result, the molecular processes that affect exactly the fate and embryogenicity of such cells in the callus are not known yet. Many plant species like Arabidopsis thaliana (Arabidopsis), well respond to somatic embryogenesis [18,19]. Arabidopsis has been instrumental in disclosing fundamental molecular pathways for regulating in vitro plant somatic cell developmental plasticity. Recently it has built a rather good framework for the study, among others, of functional genomics, biology systems, synthetic biology and in vitro embryogenesis [20]. SE has earned the most recognition, due to its cognitive as well as functional importance in the field of plant biotechnology, among the various morphogenic pathways induced by cultivated plant explants [21]. The goal of this study was to obtain plants from somatic embryos of Arabidopsis thaliana seeds which represent a novel source of SEs.

2. Materials and Methods

2.1. Plant material and callus formation

Arabidopsis thaliana col-0 seeds were supplied from Nottingham Arabidopsis Stock Center (NASC), UK. These seeds were surface sterilized by submerging in ethanol alcohol 96% for 3 min, then rinsed three times with sterilized distilled water under a laminar flow hood. Dried seeds were germinated on agar-solidified MS medium [22] free from growth regulators and incubated in the culture room in the dark at 25± 2°C. Once callus was induced from the intact specimens seeds, they were transferred to 16 h light/8 h dark photoperiod provided by cool-white fluorescent tubes. The callus induction frequency was recorded after 3 weeks of culturing. For calculating the callus induction frequency, the following equation has been used:

\[
\text{Callus induction frequency} = \left( \frac{\text{Number of seed with calli}}{\text{Number of incubated seeds}} \right) \times 100 \% \quad \text{[1]}
\]

2.2. Somatic embryogenesis and plant regeneration

During 20 days, callus was transferred to Gamborg's B5 medium [24] containing 1.0 mgL⁻¹ 2,4-D and 0.05 mgL⁻¹ Kin for multiplication. Sub culturing of this callus was carried out at 3-4 week intervals at the same medium. To enhance proliferation and plant regeneration, clumps of about 0.5 gm. of callus were cultivated on B5 medium supplemented with four different combinations of plant growth regulators as shown below:
1-5.0 mgL⁻¹ BA
2-5.0 mgL⁻¹ BA + 0.9mgL⁻¹ IAA
3-0.4 mgL⁻¹ TDZ
4-0.4 mgL⁻¹ TDZ+ 0.1mgL⁻¹ IBA

All the samples were kept in the growth chamber at 25±2°C (16h Light/8h dark). A binocular dissecting microscope was used to investigate different phases of somatic embryos.

2.3. Rooting and acclimatization

Shoots originating from germinated embryos were individually removed, and transplanted in 100 ml volume glass jars containing agar solidified hormone-free MS0 medium which was also used with 0.2% activated charcoal for rooting. Subsequently, rooted shoots were removed from the medium washed from the agar carefully, and each plantlet was transferred to small pot with a diameter of 6.5cm and 5cm height containing peat-moss for adaptation.

3. Results and Discussion

The results of this study showed that Arabidopsis thaliana seeds which were placed on MS hormonal-free (MS0) germination media, seven days after cultivation, can form callus rather than seedlings. Recent transcriptomic data confirm that calli can form through different initial routes, whereby a coordinating stress, hormone and evolved responses converge into a single gene regulatory network [25]. This callus became visible with the naked eye within 10-14 days and the callus induction frequency was 100%. In general, It was yellowish white in color with friable texture (Figure 1, A). It was recognized that calli are highly diverse and can be grouped according to their macroscopic properties calli without obvious organ regeneration, for example, are usually known as friable or compact callus [26]. In one hand, we don't find any previous study referred to callus induction from Arabidopsis thaliana seeds on MS medium free from growth regulators. On the other hand, [27] have suggested a potential callus induction from Arabidopsis thaliana seeds on Gamborg's B5 media with 20 g/l sucrose, 0.5 mgL⁻¹ (2,4-D ) and 0.05 mgL⁻¹ kinetine. Also callus formation from seeds, stems and leaf pieces of Arabidopsis thaliana was observed on B5 medium and modified B5 medium [28]. Addition of auxin and cytokinin promotes callus in various plant species. In general, an intermediate proportion of auxin and cytokinin facilitates induction of callus [26]. Since the seeds of Arabidopsis are very small and delicate, forceps may have damaged them when they are being carried to the cultivated medium, so callus induction occurred. Iwase and his colleagues [29] pointed out that wounding promotes callus formation in various parts of Arabidopsis seedlings and they showed that an AP2/ERF transcription factor, WOUND INDUCED DEDIFFERENTIATION 1 (WIND1), is participates in the control of Arabidopsis cell differentiation. At the wound site WIND1 is immediately induced and facilitates cell de-differentiation and subsequent cell proliferation to form a mass of pluripotent callus cells. As a consequence, cell dedifferentiation is genetically regulated mechanism. The results obtained showed that somatic embryos were recovered only in B5 medium supplemented with 0.4mgL⁻¹TDZ, and it was the best one, and through our careful observation, different stages of somatic embryos have been found (Table1).
Table 1. Number of somatic embryos stages produced from seeds callus of Arabidopsis thaliana.

| Media                      | *No. embryos stage |
|----------------------------|--------------------|
|                            | Globular | Heart | Torpedo | Cotyledonary |
| B5 + 0.4 mgL⁻¹ TDZ         | 117      | 87    | 73      | 72           |
| B5+0.4 mgL⁻¹ TDZ+ 0.2 mgL⁻¹ IBA | 100      | 0     | 0       | 0            |
| B5+1.0 mgL⁻¹ 2,4D+0.05mgL⁻¹ Kin | 0        | 0     | 0       | 0            |
| B5 + 5 mgL⁻¹ BA + 0.9 mgL⁻¹ IAA | 0        | 0     | 0       | 0            |
| B5 + 5 mgL⁻¹ BA            | 0        | 0     | 0       | 0            |

*Average of 6 replicate \ treatment

Cell differentiation and initiation of embryogenesis may be regulated by hormones [30, 31, 32]. In the induction of somatic embryos in Paeonia Ostii [33] a combination 0.5 mgL⁻¹ thidiazuron (TDZ) and 0.5 mgL⁻¹ 2,4-D was found to be successful. Thidiazuron has received considerable attention over recent decades because of its prominent significance in in vitro culture with Auxin as well as cytokinin like effects in various plant species [34]. There are some available theories that describe the mode of action of TDZ, but it is still unclear. The TDZ action mechanism can be described as helping in accumulate and/or synthesize endogenous growth hormones [35]. Our study showed that the callus is easily divided and formed abundant green nodules. These nodules developed and emerge repeatedly in this callus to embryogenic masses after 15 days (Figure 1.B) which produced globular stage after 45 days (Figure 1.C), then developed after 60 days to heart stage (Figure 1.D). High numbers of these heart embryos were started elongation and transformed to torpedo stage (Figure 1.E). Subsequently after 3rd sub cultures majority of them transform to the differentiated cotyledonary stage (Figure 1.F).

Figure 1. Somatic embryo developmental stages from nodule-like structure produced in callus of Arabidopsis thaliana L: (A) Callus initiation from seeds on MS0 medium. (B) Embryogenic masses formed on B5 medium + 0.4 mgL⁻¹ TDZ after 15 days of culture (arrows) (C) Development of globular stage embryos in (a) (arrows). (D)Heart stage embryos (arrows). (E)Torpedo stage (arrows).
Additionally, subculture of the embryogenic masses at the same medium (B5 + 0.4 mgL\(^{-1}\) TDZ) led to the emergence of a new variety of nodules (Figure 2.A). It was followed by its development into secondary embryo: globular, heart and torpedo that have basic roots (Figure 2.B). When these structures were left at the same medium, leaf-like structures appeared within 4 weeks (Figure 2.C) easy to separate and subsequently transferred to the MS0 medium for additional 3 weeks that allowed the complete development of the leaves (Figure 2.D) which were finally formed plants. In addition, to increasing root system growth.

Figure 2. Secondary embryos developmental stages from subculture of *Arabidopsis thaliana* L. callus on B5 + 0.4 mgL\(^{-1}\) TDZ: (A) Formation of abundant green nodules and development of globular stage embryos. (B) Embryo-genic masses development and shine Heart, Torpedo stages embryos. (C)After 4 weeks cotyledonary stage embryos (arrows). (D) Separation and subsequently transferring of shoot to MS0 medium.

When the cell lines reach the point of moderation, somatic embryogenesis can be formed, and this will involve the addition of Cytokinins, which prevent the polar transfer of auxin while raising hormone levels within the cell because keeping the auxin in the medium prevents the development of embryos that is, the spherical phase does not produce auxin during the formation of primitive embryogenesis masses, after which embryos continue to develop and function like somatic embryogenesis masses [27]. *Arabidopsis thaliana* is known by its high capacity to form somatic embryos directly and indirectly, and this is depending on the type and age of the explant [36, 37, 38]. Indirect somatic embryos formation is more efficient and less stable as mutant plants may form [39]. The results indicated that the continuous transfer of a small masses containing several embryos and secondary embryos at different stages to the same induction medium subsequently formed a large cluster of young and small shoots (Fig 3.A) and upon transfer100 branch, some of them had roots (Figure 3.B) separately to the MS0 media. These branches had the ability to grow and configure them for the root
system (Figure 3.C) after 15 days (Table 2). After that the regenerated plants adapted to the soil conditions (Figure 3.D).

Although TDZ is grouped under cytokinin because of its natural cytokinetic response in \textit{in vitro} culture, there are evidence in different plant species such as soybeans which refer that TDZ has both auxin and cytokinin-like activities [40].

| Media            | No. Shoot regeneration/ No. Shoot regeneration from secondary embryos | Rooting% |
|------------------|------------------------------------------------------------------------|----------|
| MS0              | 100/20/36/5                                                           | 36/25    |
| MS with 0.2 charcoal | 100/20/51/10                                                         | 51/50    |

Generally TDZ at low concentrations induces somatic embryogenesis in different plant species such as olive [41] blume orchid [42], the geranium [43]. TDZ induced the synthesis and accumulation of purines [44], moreover it alters cytokinin metabolism [45]. Somatic embryogenesis is characterized by their capability to grow simultaneously and to give a large number of foetuses, and by their capacity to repeat the production of embryos until a full year after the transference to new media [46]. Finally, the protocol of this paper is easy and very efficient to produce large quantities of SEs which considered a very attractive system for studying plants development. Also this method could be used as helpful tool to explain various biochemical and physiological embryogenesis events.

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