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High Frequency Regeneration of *Arabidopsis thaliana* L. from Leaves Callus Cultures

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Abstract

Arabidopsis thaliana represents a model plant, and different in vitro techniques were developed for this important plant. This study aimed at presenting an efficient plant regeneration approach from leaves derived callus. For callus initiation and regeneration leaves of 20 days- aged axenic Arabidopsis seedlings were excised, cut and cultured on both agar solidified B5 and Murashige and Skoog medium (MS) supplemented with various types and concentrations of plant growth regulators. The Results indicated that high regeneration frequency of green plantlets was observed (more than 120 shoots) from callus on B5 medium, eighty of these shoots were produced on B5 medium containing 5mgL^{-1}BA and 0.9mgL^{-1}IAA. The number of shoots regenerated on MS medium was 21. Regenerated shoots were easily and similarly rooted using full or 1\2 strength MS0 medium. The rooted plantlets were adapted and successfully transplanted in field conditions. In this study, an efficient protocol for regeneration of A. thaliana was developed and optimal concentrations of phytohormones were determined.

Key words: *Arabidopsis thaliana,* Regeneration, Callus Cultures, Leaves, Cytokinin

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INTRODUCTION

Arabidopsis thaliana L. (thale cress) or mouse-ear, a member of Brassicaceae family, known as a model organism in plant biology and genetics (Hoffman, 2002, Johnson and Bouchez, 2014). It is widely used to study different aspects of plant development and molecular biology as it has many desirable features which made it an attractive model, such as a small genome, the total size of chromosomes together equals 125 mega base pairs (Wilson, 2002) and possesses haploid chromosome number unusually n=5 (Lysak *et al*., 2006, Johnson and Bouchez, 2014). A short life cycle, production of numerous quantities of seeds and genetic variations contributed to adaptation and growth in different environments (Wilson, 2002).

Efficient regeneration protocol of plants through organogenesis is an important prerequisite for crop improvement. Organogenesis is an *in vitro* process which produces unipolar structure of either shoot or root primordium. The success of organized development through organogenesis is highly dependent on the manipulation of plant hormones mainly auxin and cytokinin (Loyola-Vargas and Ochoa-Alejo, 2012). When auxin and cytokinin were used at high concentration a mass of growing cells (callus) was induced in explants. One of the studies reported the possibility of regeneration of *A. thaliana* from callus (Acks, 1977).

In the 1980s several of *Arabidopsis* regeneration methods were developed simultaneously with *Agrobacterium*–mediated transformation (Sugimoto and Meyerowitz, 2013). Other study referred that hypocotyl and root explants of *Arabidopsis* represent good substances for callus initiation and regeneration (Mather and Koncz, 2014). Che and his colleagues (2006) identified set of genes that function as a molecular signature of the various processes during shoot regeneration in *Arabidopsis*. In addition, different concentrations of auxin were used for rooting the produced shoots (Cheng *et al*., 2010). Since plant regeneration is a key technology in plant molecular biology and genetic transformation (Sugimoto and Meyerowitz, 2013), the present work aims to promote regeneration capability from leaf callus of this plant species.

MATERIALS AND METHODS

Seeds Sterilization and Germination

Seeds of *A. thaliana* Col-0 were provided from Nottingham *Arabidopsis* Stoke Centre UK. Seeds were surface sterilized by soaking in ethyl alcohol 97%for 3 min, rinsed thoroughly in sterile water three times. Sterilized seeds were grown on surface of 25ml of agar-solidified MS (Murashige and Skoog, 1962) medium. Specimens maintained in dark for 0, 24 and 48 h at 4 ºC then transferred to culture room conditions (25±2ºC, 16 h photoperiod provided by cool white fluorescent lamps).

Establishment of Callus

Leaves were excised from 20days old axenic *Arabidopsis* seedlings and cut into two pieces. Each portion was placed horizontally on the surface of 20 ml of agar solidified B5medium (Gamborg *et al*., 1968) contained in glass jars of 100ml capacity. The callus inducing media were as follows: B5 media was fortified with 2,4-D of concentration 1.0, 2.5 mgL^{-1} interacted with each concentration of Kin 0.05 mgL^{-1}.In some cases sucrose was decreased to $20gL^{-1}$. Also B5 media were used but with other addition of growth regulators .They were $1.0mgL^{-1}$ NAA with each of 0.05, 2.0 mgL^{-1} BA and 0.4 mgL^{-1} TDZ with $0.1mgL^{-1}$ IBA. The basal MS medium supplemented with different interactions of growth regulators were used for callus induction:

MS+$0.05mgL^{-1}$Kin+$0.5mgL^{-1}$NAA (Noor and Ferda, 2015).
MS+$1.0mgL^{-1}$NAA+$2.0mgL^{-1}$BA
MS+$0.05mgL^{-1}$Kin+$1.0mgL^{-1}$2,4-D
MS+$0.5mgL^{-1}$2,4-D+$1.0mgL^{-1}$BA
MS+$0.4mgL^{-1}$TDZ+$0.1mgL^{-1}$IBA

The produced calli were subcultured every four weeks before the death of the peripheral cells.

Shoot Regeneration

One gram samples of leave-derived callus was transferred aseptically to the surface of 25ml aliquots of the regeneration media contained in 100ml glass capped jars. Regeneration media used were B5 and MS.

Each salts provided with the suitable growth regulators as mentioned below:

B5 (control)
B5+5.0mgL^{-1}BA+0.9mgL^{-1}IAA
B5+2.0mgL^{-1}BA+1.0mgL^{-1}NAA
B5+0.4mgL^{-1}TDZ+0.1mgL^{-1}IBA
MS (control)
MS+1.0mgL^{-1}TDZ+0.2mgL^{-1}IBA
MS+0.4mgL^{-1}TDZ+0.1mgL^{-1}IBA
MS+0.01mgL^{-1}IAA+2.0mgL^{-1}Zeatin

Shoots Rooting

Single shoots (lengths approx. 4-5cm) were excised and transplanted vertically into 25ml of agar-solidified full–strength MS0 medium. Other groups of shoots also plunged vertically in 25ml of agar solidified half strength MS medium. They were kept in the same condition of culture room.

Acclimatization

Regenerated plantlets were transferred to pots containing mixture of sterile soil and peat moss (v/v) and kept in the same conditions of culture room.

RESULTS AND DISCUSSION

Axenic seedlings production

Seeds start germination after 3-7 days on agar solidified MS medium. Data indicated that keeping seeds at 4ºC condition enhanced germination (Table 1) since *Arabidopsis* has seed dormancy, and it is 'non-deep physiological dormancy' (Baskin and Baskin, 2014). It is under genes control and it demands definite environmental conditions to be overcome (Dekkers *et al.*, 2016). This type of dormancy could be broken by short periods of cold (Dave *et al.*, 2016).The germinated seed is continuing their growth and producing intact seedlings within 6-8 weeks.

Table 1- Germination of *A. thaliana* L.Col-0 seeds pre-exposed to 4ºC on solid MS medium

Exposure to 4ºC (h)	Germination (%)	No. of seedlings
0	28	17
24	40	24
48	70	42

Sixty seeds/ treatment

Callus Cultures Production

The results indicated that callus was induced from leaf explants, and both B5and MS media promoted callus induction. Different studies detected the importance of auxin- cytokinine in plant growth and development (Cheng *et al.*, 2010, Shimizn-Sato *et al.*, 2009; Tanaka *et al.*, 2006). Therefore, both cytokinin and auxin at high concentrations induced callus formation (Sugimoto and Meyerowitz, 2013). Generally there are no clear differences between the two media. Callus induction percent ranged between75-100% (Table 2).

The best callus induction occurred in B5 medium containing 0.05mgL^{-1} Kinetin and 1mgL^{-1} 2,4-D(Fig.1-a).The callus was friable and yellowish-green color. One of the studies referred that, the existence of 2,4-D in the medium induced formation and growth of undifferentiated callus of *Arabidopsis* (Corcose, 1976).

Table 2- Formation of callus from leaf explant of *A. thaliana* L**.**

Media (mgL^{-1})		Callus Induction	
		(%)	**(Day)**
B5	Control	0	**0**
	2,4-D 1.0 + Kin 0.05	100	**8**
	2,4-D 2.5 + Kin 0.05	90	**14**
	NAA 1.0 + BA 0.05	85	**13**
	NAA 1.0 + BA 2.0	100	**10**
	TDZ 0.4 + IBA 0.1	100	**15**
MS	Control	0	**0**
	2,4-D 0.5 + BA 1.0	90	**10**
	Kin 0.05 + NAA0.5	85	**13**
	Kin 0.05 + 2.4-D 1.0	85	**13**
	TDZ 0.4 + IBA 0.1	75	**15**
	NAA 1.0 + BA 2.0	100	**11**

20 samples\treatment

Regeneration of shoots

The results pointed out the ease of shoot regeneration from callus .The obtained data indicated that all regeneration media enhanced shoot formation. Callus fragments varied in number of shoot produced and all tested media supported shoot formation (Table 3).

Table 3- High regeneration of *Arabidopsis thaliana* L. plants from leaf callus.

Regeneration Medium (mgL^{-1})	No. of callus fragments	No. of Shoots
B5 + BA 5.0 + IAA 0.9	35	**80**
B5 + BA 2.0 + NAA 1.0	11	**23**
B5 + TDZ 0.4 + IBA0.1	11	**20**
MS + IAA 0.01+Zeatin 2.0	2	**8**
MS + TDZ 0.4 + IBA 0.1	3	**8**
MS + TDZ 1.0 + IBA 0.2	3	**5**

No. of replicates 10 sample each treatment

Obviously, the transfer of callus to various regeneration media promoted differentiation of shoots (Fig.1-b).Subsequently numerous shoot primordia were developed from the same fragment of callus(Fig.1-c). It is well known that auxins and cytokinins considered key factors that control organogenesis. In *Arabidopsis*, protocol for de novo organogenesis was established (Che *et al*., 2006; Sugimoto and Meyerowitz, 2013). The high concentrations of cytokinin in the medium promots shoot regeneration (Atta *et al.,* 2006, Kareem *et al*., 2015). Recently, actual progress has been done tounderstand the molecular mechanisms related to ability of plant to form organs, there is a role of endogenous cytokinin biosynthesis in addition to AHK4 receptor which mediated cytokinin signaling in the process of organogenesis (Pernisova *et al*., 2018). Many experiments for shoot regeneration were carried out and the results indicated differentiation of hundreds of shoots. Several jars containing regeneration media were fully occupied with regenerates (Fig.1- d).The high regeneration of *A. thaliana* plants from callus is probably due to one or more reasons. The first concern is the availability of *iaaH* of functional genes in genome of cells forming callus. These genes are controlling auxin content (Gohlke and Deeken, 2014). Similar results were obtained with this plant species (Mathur and Koncz, 2014).The second reason is that callus of this species seems to be rich with meristematic

cells of high cellular division capability because of their high content of hormones. In such case, their interaction with the exogenous growth regulator save the typical hormones balance enough to produce such calli (Barkla *et al*., 2014).The other reason might be explained by overcoming problems arise in plant regeneration from certain phenotype or genotype of callus such as in *Lotus corniculatus* (Acks, 1977). Single fragment of callus produced numerous shoots at different developmental stages (Fig.1-e). Interestingly about 144 shoots were differentiated from callus in this work.

Rooting of produced shoots

Data (Table 4) showed that agar-solidified half strength MS0 medium was less supported shoots rooting compared with full strength MS0 medium. Large number of shoots was rooted in MS0 medium. Generally, rooting of the regenerated shoots produced from various experiment was feasible (Fig.1-f). This coincides with other studies (Li *et al*, 2011; Mathur and Koncz, 2014).

Table 4- Rooting of *Arabidopsis thaliana* shoots produced from callus**.**

Rooting medium	**Number of shoots**		**Rooting (%)**
	Cultured	**Rooted**	
Full strength MSO	23	10	43.4
Half strength MSO	23	5	21.7

The data is of one experiment.

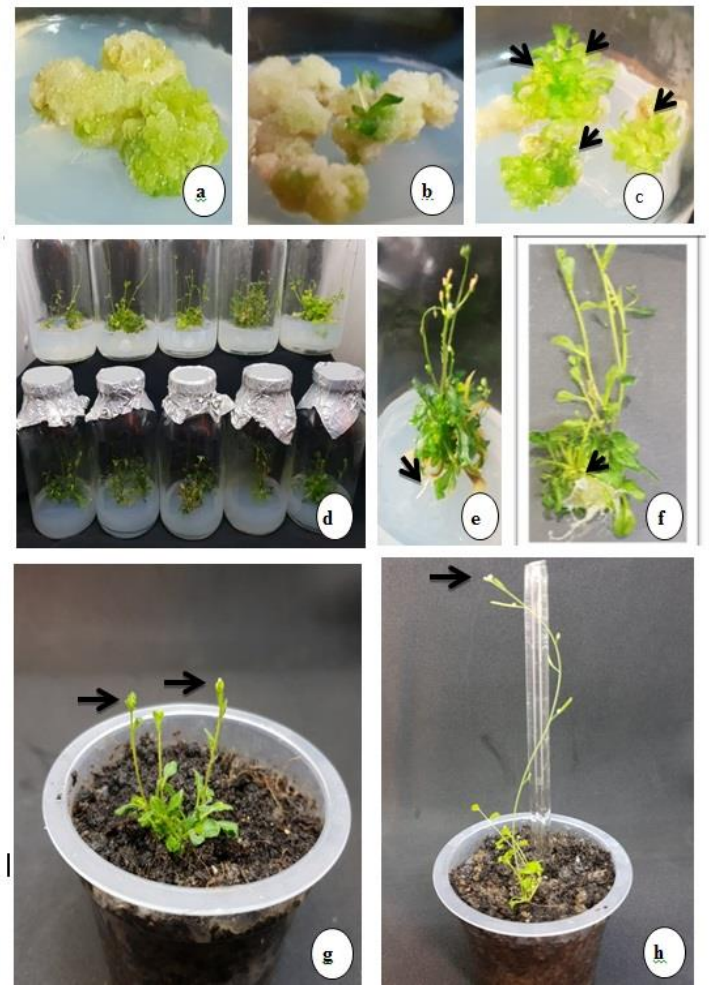

Figure 1- Regeneration of *Arabidopsis thaliana* L. plants from leaf derived callus.

a: Culture of leaf callus grown on B5 + 1.0 mgL^{-1} 2,4-D + 0.05 mgL^{-1} Kin. **b:**Young regenerant (arrowed) developed from callus in (a) placed on the regeneration medium B5+5.0mgL^{-1} BA+0.9mgL^{-1}IAA.**c:** Numerous shoots formation (arrows) in (b) after 4 weeks. **d:**High regeneration of shoots in(c).**e:** Rooting of many shoots (arrowed)in full strength MS0 medium. **f :** close up of shoot rooting (arrowed) in (e).**g:** flower bud (arrows) of one of the regenerated plant after 4days from transferring to soil. **h**: The regenerated plant after 10 days of transferring to soil.

Acclimatization of Plantlets

The number of transferred plantlets was 112, all of them successfully adapted to the natural environment. The flowering buds appeared after 4 days of transferring to soil (Fig.1-g). The plants continue in growth and produced flowers (Fig.1-h).

CONCLUSION

Since *Arabidopsis thaliana* represents a model important plant, in this study an efficient protocol for regeneration of this plant from leaves callus is achieved. This protocol is easy to perform and high number fertile plants were produced.

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