

Somatic embryogenesis of hypocotyl derived calli from an eggplant cultivar

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Abstract: Optimization of tissue culture and regeneration conditions of eggplant is necessary for achieving different goals such as gene transformation and the development of somaclonal variations. In this study, hypocotyl explants were used to produce callus in a medium containing different concentrations of NAA and BAP. Moreover, the concentration of the elements Ca, Mn, Mg, Fe and K were measured and analysed between embryogenic and non-embryogenic calli. For shoot elongation, embryogenic calli were transferred to a new culture medium containing 3.5, 4 and 4.5 mg l⁻¹ BAP plus 2 mg l⁻¹ GA3. Finally, produced shoots were rooted in a culture medium containing 1, 1.5 and 2 mg l⁻¹ NAA. Results showed that the best treatment for the embryogenic callus induction was MS medium containing 0.5 mg l⁻¹ BAP plus 0.25 mg l⁻¹ NAA. Two elements, Fe and K, had the highest amount in non-embryogenic calli compare to the embryogenic one. For plant regeneration, MS medium containing 4.5 mg l⁻¹ BAP plus 2 mg l⁻¹ GA3 and 2 mg l⁻¹ NAA were the best treatments for shooting and rooting, respectively. In this study, the best treatments for plant regeneration produced 35 shoots from an explant with 92 % shooting. This regeneration protocol could be useful for gene transformation and micro-propagation studies.

Key words: eggplant; tissue culture; somatic embryogenic regeneration; BAP; NAA

Abbreviations:

NAA Naphthaleneacetic acid
BAP 6-Benzylaminopurine
GA3 Gibberellic acid
MS medium Murashige & Skoog Medium
IBA Indole-3-butyric acid
IAA Indole acetic acid

Somatska embriogeneza hipokotilnih kalusov izbrane sorte jajčevca

Izvleček: Optimizacija tkivnih kultur jajčevca in regeneracijskih razmer sta potrebni za doseganje različnih ciljev kot sta genska transformacija in razvoj somaklonalne variabilnosti. V tej raziskavi so bili uporabljeni hipokotilni izsečki za pridobitev kalusa v gojiščih, ki so vsebovala različne koncentracije NAA in BAP. Med embriogenimi in neembriogenimi kalusi so bile izmerjene koncentracije elementov kot so Ca, Mn, Mg, Fe in K. Za dolžinsko rast poganjkov so bili embriogeni kalusi premeščeni v drugo gojišče, ki je vsebovalo 3,5, 4 in 4,5 mg l⁻¹ BAP in 2 mg l⁻¹ GA3. Na koncu so bili poganjki ukoreninjeni v gojišču, ki je vsebovalo 1, 1,5 in 2 mg l⁻¹ NAA. Rezultati so pokazali, da je bilo za indukcijo embriogenih kalusov najboljše MS gojišče, ki je vsebovalo 0,5 mg l⁻¹ BAP in 0,25 mg l⁻¹ NAA. Dva elementa, Fe in K, sta imela največjo vsebnost v neembriogenih kalusih v primerjavi z embriogenimi. Za regeneracijo rastlin, za nastanek in vkoreninjenje poganjkov, je bilo MS gojišče, ki je vsebovalo 4,5 mg l⁻¹ BAP, 2 mg l⁻¹ GA3 in 2 mg l⁻¹ NAA najboljše. V raziskavi je najboljše regeneracijsko obravnavanje dalo 35 poganjkov iz enega izsečka z 92 % vkoreninjenjem. Ta regeneracijski protokol bi lahko bil koristen za gensko transformacijo in raziskave mikro propagacije.

Ključne besede: jajčevce; tkivna kultura; regeneracija s somatsko embriogenezo; BAP; NAA

Okrajšave:

NAA Naftalen očetna kislina
BAP 6-Benzil aminopurin
GA3 Giberelinska kislina
MS gojišče Murashige & Skoog gojišče
IBA Indol-3-maslena kislina
IAA Indol očetna kislina

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1 INTRODUCTION

Eggplant (*Solanum melongena* L., $2n = 2x = 24$) is one of the important plants of the Solanaceae family (Portis, et al., 2018), which grown for its often purple edible fruit. Different cultivars of eggplant have different sizes, shapes and colours ranging from oval or egg-shaped to long club-shaped; and from white, yellow, green through degrees of purple pigmentation to almost black (Aminifard, et al., 2010). Eggplant after potatoes and tomatoes represents the third most important solanaceous crop species, and it is believed that eggplant is native to India and China (Magioli & Mansour, 2005). Its bulk production is concentrated in China, India, Iran, Turkey, Egypt, Italy and Spain (Portis, et al., 2018).

Although the eggplant has a low calorie content, it is a rich source of magnesium, calcium, potassium and iron (Michalajc & Buczkowska, 2008). In addition, in traditional medicine, eggplant is used to treat diabetes, arthritis, asthma and bronchitis (Magioli & Mansour, 2005). Also, nasunin, as the major component of anthocyanin of eggplant, prevents lipid peroxidation (Igarashi, et al., 1993).

The tissue culture technique allows researchers to grow and manipulate plants under in-vitro sterile conditions and can be very useful to plant breeders (Bridgen et al., 2018). In fact, this technique can provide the diversities needed for selection the desired traits by inducing somaclonal variation. On the other hand, by optimizing the complete regeneration of callus, it is possible to transfer the desired genes to the desired plants (Chakravarthi, et al., 2010). Due to the reasons mentioned above, studies of the full plant regeneration from explants are of special importance in plants.

The fruit of the eggplant is found in the diet of many people in the world. For this reason, several studies have been carried out on regeneration of this plant through somatic embryogenesis using different explants such as leaf and cotyledon (Foo, et al., 2018), root (Franklin, et al., 2004) and hypocotyl (Statish, et al., 2015). However, it has been shown that plant regeneration can be affected by the cultivar. For example, Gandonou et al. (2005) showed that there is a significant difference between 9 genotypes of sugar beet in terms of callus induction capacity, embryogenic response and plant regeneration ability (Gandonou, et al., 2005), which indicates that the overall regeneration rate of the plant is genotype dependent. Hoque and Mansfield (2004), Schween and Schwenkel (2003) and Zale et al. (2004), working respectively on *Oryza sativa* L., *Primula* ssp. and wheat came to the same conclusions (Hoque & Mansfield, 2004; Schween & Schwenkel, 2003; Zale, et al., 2004). For this reason, in this study, regeneration of a native cultivar of Iran, Ghal-

ami Varamin, which is commonly cultivated, was studied using somatic embryogenesis using hypocotyl explants.

2 MATERIALS AND METHODS

2.1 SEED SURFACE STERILIZATION AND IN-VITRO GERMINATION

This research was carried out on an Iranian eggplant variety called 'Ghalami Varamin'. Seeds of this variety were purchased from Pakan Bazr Esfahan Company (<http://www.pakanbazr.com/en/>). The seeds were surface-sterilized in 70 % ethanol for 30 seconds and then 2 % sodium hypochlorite for 10 minutes and rinsed 3 times with sterile distilled water. The MS medium was used for germination and seedlings growth of eggplant (Park & Facchini, 2000). The MS medium (Duchefa Company) was prepared as 4.43 g l⁻¹. After adjusting to pH 5.7 and adding agar (7.5 g l⁻¹) and sucrose (3 %), the culture medium was autoclaved. The MS medium was poured into autoclaved glass containers under sterile laminar hood. Seeds were cultured on the medium for germination, and placed in a growth chamber at 25 ± 2 °C with a 16/8 h light/dark period, light intensity of 32.38 μmol s⁻¹ m⁻².

2.2 CALLUS INDUCTION CULTURE MEDIUM

After 15 to 20 days, hypocotyl explants were placed in MS culture medium containing different concentrations of auxin (NAA) and cytokinin (BAP) to produce callus (Table 1). All treatments were carried out in 3 replicates. Then, the percentage of callus induction and embryogenic calluses, and fresh mass and dry mass of calluses were measured. All data were analysed by ANOVA as a factorial experiment based on a completely randomized design. BAP as first factor and NAA as second factor had seven and three levels, respectively. The mean comparisons were done based on the Duncan's Multiple Range Tests (Duncan, 1955).

2.3 NUTRIENTS MEASUREMENT

Embryogenic and non-embryogenic calli were washed in distilled water, oven dried at 70 °C for 48 h and grounded after being weighed. Different ions concentration was taken from the chloride acid (2 N) extract of the samples that were measured using atomic absorption spectrometer (Varian Spectra aa220; made in Italy). All data were analysed by ANOVA based on a completely

Table 1: Various hormonal treatments used to induce callus

Hormonal combination code	BAP(mg l ⁻¹)	NAA(mg l ⁻¹)
A	0	0
B	0	0.25
C	0	0.5
D	0.25	0
E	0.25	0.25
F	0.25	0.5
G	0.5	0
H	0.5	0.25
I	0.5	0.5
J	3.5	0
K	3.5	0.25
L	3.5	0.5
M	4	0
N	4	0.25
O	4	0.5
P	4.5	0
Q	4.5	0.25
R	4.5	0.5
S	5	0
T	5	0.25
U	5	0.5

randomized design with three replications. The mean comparisons were done based on the Duncan's Multiple Range Tests.

2.4 SHOOT PROLIFERATION MEDIUM

The embryogenic calli isolated from the whole callus masses and transferred to the MS medium culture with different concentrations of BAP (3.5, 4 and 4.5 mg l⁻¹) in order to shooting. Then, the percentage of shoot induction, shoot length, number of shoots and leaves were measured. In facts, the only treatments in this section were different concentrations of BAP that were analysed by ANOVA based on a completely randomized design with three replications. The mean comparisons were done based on the Duncan's Multiple Range Tests.

2.5 ROOT PROLIFERATION MEDIUM

For further prolongation and development of stem and leaf system, shoots were transferred to MS medium

supplemented with 2 mg l⁻¹ gibberellic acid (GA3). For root induction, shoot lengths of 3 to 4 cm were transferred to MS culture media containing different concentrations of NAA (1, 1.5 and 2 mg l⁻¹). The studied traits included root induction percentage and root length. The only treatments in this section were different concentrations of NAA that were analysed by ANOVA based on a completely randomized design with three replications. The mean comparisons were done based on the Duncan's Multiple Range Tests.

3 RESULTS AND DISCUSSIONS

3.1 CALLUS INDUCTION

In this study, callus induction from hypocotyl explant was successfully performed. Callus initiation and appearance of embryogenic calli were occurred after 7 and 16 days, respectively (Figure 1 and 5). Embryogenic calli were seen in white, frangible and sponge-like forms so that after a while, the formations of shoot primordia in these calli were observed (Figure 1). But, non-embryogenic types appeared as compact, non-frangible, and sometimes greenish callus masses (Figure 1), similar to what was reported by other researchers (Corral-Martinez & Segui-Simarro, 2012; Rivas-Sendra, et al., 2015). Eventually, these types of calli were necrosed and died on the medium.

The measured traits were the percentage of callus induction, percentage of embryogenic callus induction and fresh and dry mass of calluses. Variance analysis of data showed that there is a significant difference between different concentrations of BAP, NAA and their interactions at 1 % probability level (Table 2).

The mean comparison of interaction effects of NAA*BAP for induction of callus showed that the highest percentage of callus induction (85 %) observed in the MS media supplemented with the combination of 0.5 mg l⁻¹ NAA + 4 mg l⁻¹ BAP, and the lowest percentage of callus induction (zero) observed in combinations of 0 mg l⁻¹ BAP + 0 mg l⁻¹ NAA, 4.5 mg l⁻¹ BAP + 0 mg l⁻¹ NAA and 5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA (Figure 2). Zayova et al., (2008) reported the highest induction of callus using hypocotyls (63.3%) in the treatment MS media supplemented with 0.5 mg l⁻¹ BAP + 2.0 mg l⁻¹ NAA (Zayova, et al., 2008). In another study, callus induction using cotyledons as explant, the highest callus induction (100%) occurred in some concentrations of Kinetin, BAP and combinations of both (Foo, et al., 2018).

In this study, the highest percentage of embryogenic calli (52 %) was related to 0.5 mg l⁻¹ BAP + 0.25 mg l⁻¹ NAA. In the treatment with BAP alone, induction of cal-

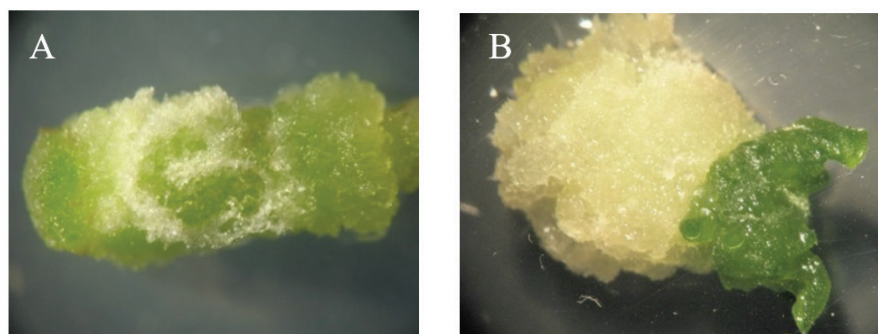


Figure 1: A: non-embryogenic callus. B: embryogenic callus

Table 2: Variance analysis of traits related to the callus induction section

Sources of variation	Degrees of freedom	Mean Square			
		Callusing percentage	Percentage of embryogenic calluses	Fresh mass (g)	Dry mass (g)
a (BAP)	6	0.35**	0.11**	0.027**	0.01**
b (NAA)	2	0.17**	0.067**	0.029**	0.017**
a,b	12	0.19**	0.06**	0.018**	0.009**
error	42	0.02	0.01	0.002	0.002

** Significant differences between the treatments at the level of 1 %

lus in Ghalami Varamin cultivar occurred in all BAP concentrations except for the 4.5 mg l⁻¹ BAP, and 0.5 mg l⁻¹ was the best concentration (Figure 2). However, embryogenic calli occurred in 0.25, 0.50, 3.5 and 4 mg l⁻¹ treatments, and no embryogenic calli were recorded in the other two concentrations (4.5 and 5 mg l⁻¹). In the study of Foo et al., (2018), the highest shoot formation from the cotyledon explants was induced on Kinetin (2 mg l⁻¹) alone (Foo, et al., 2018). In our study, in addition to the best hormone composition selected, BAP alone induced embryogenic calli too while Foo et al. (2018), showed that kinetin alone is sufficient to induce shoots from the cotyledon explants (Foo, et al., 2018). It is likely, different concentrations of auxin and cytokinin hormones in the two tissues caused different results. Therefore, it seems that in the eggplant, depending on the type of explant, certain concentrations of auxin or cytokinin alone have the potential to induce embryogenic calli.

In our study, the NAA hormone (as a type of auxin) alone induced calli, but at a much lower rate than the BAP alone. In addition, the NAA hormone did not induce the embryogenic calli (Figure 2). Foo et al. (2018)

revealed that all concentrations of another type of auxin, Kinetin, (0.5, 1, 1.5 and 2 mg l⁻¹) induced calli (Foo, et al., 2018). However, only two concentrations (1.5 and 2 mg l⁻¹) induced embryogenic calli (Foo et al., 2018). Therefore, depending on the type of auxin, explant and the concentration used, this hormone alone can induce calli, especially embryogenic calli.

In the case of fresh and dry weight of callus, the concentrations of 5 mg l⁻¹ BAP + 0.25 mg l⁻¹ NAA and 4.5 mg l⁻¹ BAP + 0.25 mg l⁻¹ NAA showed the highest results respectively (Figure 3).

3.2 COMPARISON OF IONS IN EMBRYOGENIC AND NON-EMBRYOGENIC CALLUS

Concentration of five elements, calcium, magnesium, iron, potassium and manganese were measured in embryogenic and non-embryogenic calli. The results of variance analysis showed that there is no significant difference between the two embryogenic and non-embryogenic calli for calcium, magnesium, and manganese. But

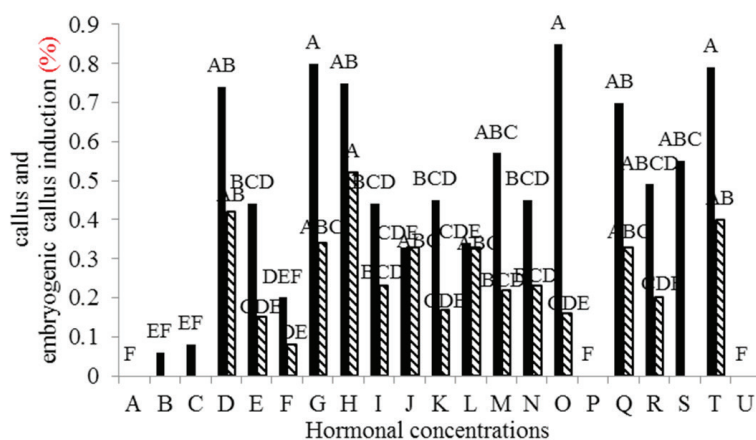


Figure 2: Mean comparison of the interaction effects of NAA*BAP for the percentage of callus induction and embryogenic callus induction. Black columns are percentage of callus induction and others are related to the percentage of embryogenic callus induction

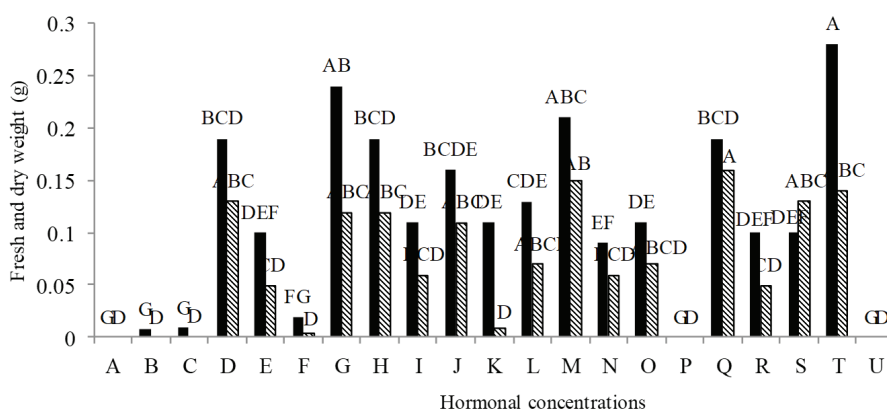


Figure 3: Mean comparison of the interaction effects of NAA*BAP for fresh and dry mass of calli. Black columns are fresh mass of calli trait and others are related to the dry mass of calli

iron and potassium were dissimilar between embryogenic and non-embryogenic calli (Table 3). Our results revealed that the amount of both iron and potassium were more in non-embryogenic calli than the embryogenic ones (Fig. 4). Unfortunately, there is no report so far on the difference between the embryogenic and non-embryogenic calli in terms of the amount of these elements.

3.3 PLANT REGENERATION

3.3.1 Shooting

Shooting was initiated by transferring embryogenic calli to MS medium containing 3.5, 4 and 4.5 mg l⁻¹ BAP. For shooting regeneration, some traits including shooting percentage, number of shoots, Shoots length and

number of leaves were measured. Variance analysis for these traits showed that there are significant differences between them (Table 4).

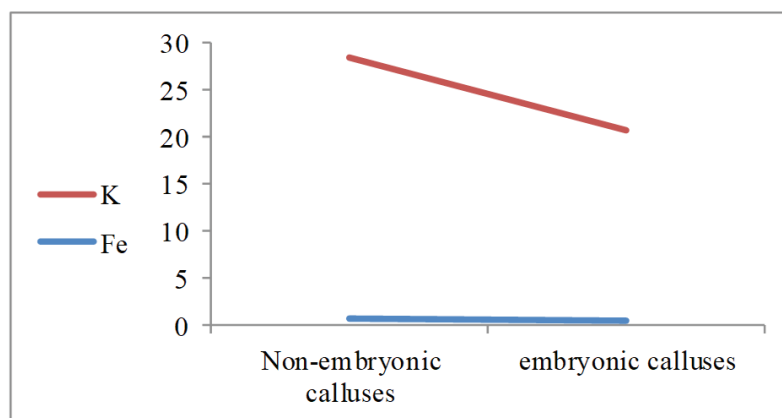
The mean comparisons showed that the highest percentage of shooting (94%), number of shoots (35 shoots), shoot length (25 cm) and leaf number (14 leaves) were observed with a MS medium containing 4.5 mg l⁻¹ BAP + 2 mg l⁻¹ GA3 (Table 5).

Different explants have been used for eggplant regeneration including hypocotyl (Mallaya & Ravishankar, 2013), leaf (Ray et al., 2011) and cotyledon (Rahman et al., 2006; Shivaraj & Rao, 2011), epicotyl and stem node, root (Ray et al., 2011). The highest percentages of shooting in various studies were different based on types of explant. Kaur et al. (2013) showed that cotyledon induced significantly the highest somatic embryogenesis on MS media fortified with 1.5 mg l⁻¹ IBA + 1.0 mg l⁻¹ BAP (89.62 %). In

Table 3: Variance analysis of iron and potassium amounts between embryogenic and non-embryogenic calli

Sources of variation	Degrees of freedom	Mean Square	
		K	Fe
treatment	1	81.99 **	0.133 **
error	1	1.47	0.002

** Significant differences at the level of 1 %

**Figure 4:** Mean comparison of iron and potassium amounts between embryogenic and non-embryogenic calli**Table 4:** Analysis of variance of shooting traits

Sources of variation	Degrees of freedom	Mean Square			
		shooting percentage	number of shoots	Shoots length (cm)	number of leaves
treatment	2	0.172**	540.7**	352.44**	121.33**
error	6	0.001	10.33	3.77	1.22

** Significant differences at the level of 1 %

Table 5: Mean comparison of different concentrations of BAP on traits related to shoot elongation

number of leaves	Shoots length (cm)	number of shoots	shooting percentage	GA ₃ (mg l ⁻¹)	BAP(mg l ⁻¹)
2 ^b	5 ^c	9 ^b	0.47 ^c	2	3.5
3 ^b	9 ^b	15 ^b	0.62 ^b	2	4
14 ^a	25 ^a	35 ^a	0.94 ^a	2	4.5

Table 6: Analysis of variance of rooting traits

Sources of variation	Degrees of freedom	Mean Square	
		rooting percentage	root length (cm)
Treatment	2	0.06**	30.33**
Error	6	0.001	1.22

** Significant differences at the level of 1 %

Table 7: Mean comparison of different concentrations of NAA on traits related to root induction

NAA(mg l ⁻¹)	rooting percentage	root length (cm)
1	0.6 ^b	4.5 ^b
1.5	0.71 ^b	5 ^b
2	0.9 ^a	10 ^a

leaf explant, it was also the maximum on 1.5 mg l⁻¹ IBA + 1.0 mg l⁻¹ BAP (69.60 %). However, hypocotyl achieved the maximum of 38.41 % somatic embryogenesis on 0.5 mg l⁻¹ IBA + 1.0 mg l⁻¹ BAP (Kaur, et al., 2013). Mir et al., (2011) studied plant regeneration from different cultivars of eggplants with different explants. Their results showed that shoot induction percentage were different among the cultivars. The highest shoot induction percentage using hypocotyl (88.31 %), cotyledon (90 %) and root (60 %) were observed from PBSR-11 cultivar on MS medium containing 2.5 mg l⁻¹ IAA + 0.5 mg l⁻¹ BAP (Mir et al., 2011).

The number of shoots and shoot length in the most studies were low. For example, Ray et al. (2011) used stem, root and leaf explants for eggplant regeneration. They observed that the highest number of shoot regenerated through callus from stem containing 2.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA was 3.4 (Ray, et al., 2011). Shivaraj and Rao (2011) used cotyledonary leaf as explants for eggplant regeneration. Their results showed the highest number of shoots was 23.3 at 2 mg l⁻¹ BAP + 0.5 mg l⁻¹ Kinetin (Kn), and the highest shoot length was 11.2 cm at 2 mg l⁻¹ BAP

+ 1.5 mg l⁻¹ Kn from cultivar Pusa Purple long (Shivaraj & Rao, 2011).

Mallaya & Ravishankar (2013) showed that the maximum number of shoot buds elongated and shoot length using hypocotyl explant were 19 and 3.3 cm respectively on 0.5 mg l⁻¹ TDZ + 0.1 mg l⁻¹ GA3 (Mallaya & Ravishankar, 2013). By comparing the study of other researchers with this study, it can be concluded that the use of 4.5 mg l⁻¹ BAP and 2 mg l⁻¹ GA3 can provide a better result.

3.3.2 Rooting

Variance analysis of data showed that there was a significant difference between treatments for rooting percentage and root length traits (table 6).

In this study, rooting percentage and root length were 92 % and 10 cm respectively at 2 mg l⁻¹ NAA (table 7; Figure 5). For root regeneration, the results of Shivaraj and Rao (2011) showed that the highest frequency and number of roots were 100% and 89.3 respectively on 3 mg l⁻¹ IBA (Shivaraj & Rao, 2011). Mallaya, & Ravishankar (2013) reported that the highest number of root and root length were 4 and 5.6 cm respectively on 1 mg l⁻¹ IBA (Mallaya & Ravishankar, 2013).

5 CONCLUSION

In this study, the best treatments for plant regenera-

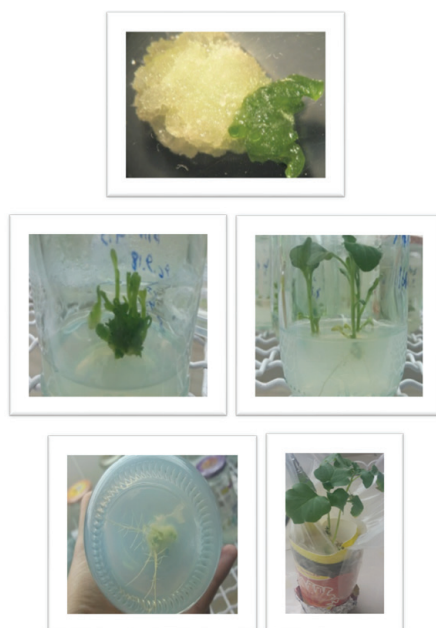


Figure 5: A: embryogenic callus. B: production of multiple shoots from embryogenic callus. C: production of roots in elongated shoots. D: production of roots. E: production of adult eggplant

tion resulted in 35 shoots and 90 % rooting from embryogenic calli. With our protocol, we could produce adult eggplant from embryogenic calli and then transferred to the pots (Figure 5). It seems that this protocol could be an efficient protocol for plant regeneration of eggplant specially Ghalami Varamin cultivar.

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