African Journal of Biotechnology Vol. 10(3), pp. 442-448, 17 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1968
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro propagation and whole plant regeneration from callus in Datura (*Datura stramonium*. L)

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Accepted 27 May, 2010

Leaf explants and mature embryos of Datura were separately cultured in MS basal medium supplemented with 30 g/l sucrose, 8 g/l agar and different combination of 2,4,D (at three levels, 0, 1 and 2 mg/l) and kinetin (0, 0.25, 0.5 and 1 mg/l). The formed calli were transferred to regeneration media supplemented with BAP alone (at three levels, 1, 2 and 3 mg/l) or in combination with NAA (at four levels, 0, 0.02, 0.2 and 1 mg/l). The regenerated shoots were rooted in media containing IBA at three levels (0.5, 1 and 1.5 mg/l). The media containing 2 mg/l 2,4-D + 0.5 mg/l kinetin and 2 mg/l of 2,4-D alone were found to be the best treatments for callus induction from leaf and embryo explants, respectively. Moreover, the media containing 3 mg/l BAP + 1 mg/l NAA and 2 mg/l BAP + 1 mg/l NAA were found to be the best hormonal treatments to shoot regeneration from calli of leaf and embryo explants, respectively. Also 0.5 mg/l IBA was found to be the best treatment for rooting of regenerated shoots.

Key words: Datura, leaf explants, embryo explants, calli.

INTRODUCTION

Datura (*Datura stramonium* L.) is a pharmaceutical plant that belongs to Solanaceae family which produces some secondary metabolites such as tropane alkaloids (Sato et al., 2001). It was found that *in vitro* culture can enable plants to produce secondary metabolites under controlled culture conditions. Furthermore, the establishment of cell culture of medical plant may be considerable potential in the future as an alternative for the production of new secondary metabolites (Gumuscu et al., 2008). Besides, tissue culture techniques are a prerequisite to regeneration of transformed tissues. There are only few reports about tissue culture of Datura. Therefore, by considering the pharmaceutical importance of this plant, it is necessary to provide efficient tissue culture protocols for it. Leaf explants have been used extensively for callus production in many plants, for example in *Tylophora indica* (Jayanthi and Mandal, 2001), *Cynedon dactylon* (Ramgareeb et al., 2001). On the other hand, it was found that immature embryos are suitable explants to different tissue culture approaches, for instance to somatic embryogenesis in *Quercus acutissima* (Kim et al., 1997), *Mella azedarach* (Vlla et al., 2003) and Acacia (Xie and Hong, 2001), to callus induction or direct shoot regeneration in soybean (Barwal et al., 1996), etc. Culture of proembryos of *D. stramonium* at the first time has been done in 1942 in media enriched with vitamins and organic substance such as succinic acid and adenine, but these explants failed to grow or grew feebly. It has been found that nutritional requirements of proembryos is more exact than immature or mature embryos (Raghavan, 2003) and so, culture of mature embryos can eliminate this problem. Mature embryos were found to be suitable explants for callus production and other approaches in some plants, such as *Allium cepa* (Zheng et al., 1998). In this study, we decided to compare these two explants sources (leaf and embryo explants) for callus induction and plant regeneration from callus and also determination of best hormonal treatments for each type of explant.

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Abbreviations: 2,4-D, 2,4-Dichloro-phenoxyacetic acid; **MS,** murashige and skoog medium; **BAP,** 6-benzylaminopurine; **IBA,** indole-3-butyric acid; **NAA,** α- naphthalene acetic acid.

MATERIALS AND METHODS

Preparation and sterilization of leaf explants

Immature plants of Datura were gathered up from their natural habitat around of Sari Agricultural Sciences and Natural Resources University. The leaves were excised and washed with many drops of dish washing liquid under tap water for 30 min and subsequently immersed in 70% ethanol for 45 s. After this stage, under sterile condition, the leaves were transferred into a sealed bottle containing sodium hypochlorite solution (1% NaOCl) in company with four drops of Tween 20 and gently agitated. After 20 min, the solution was decanted and the leaves were rinsed three times with sterile distilled water and then were cut as explants to culture (with the dimensions of 1.5 × 1.5 cm).

Preparation of embryo explants

After the seed setting stage, the seeds were gathered up from mature Datura plants, washed with many drops of dish washing liquid under tap water for 15 min and then surface-sterilized by immersing in 70% ethanol for 1 min, subsequently in a 3% sodium hypochlorite solution. After 10 min, the solution was emptied, the seeds were rinsed three times and soaked overnight in sterile distilled water to soften the seeds in order for easy isolation of embryos.

Preparation of culture media and culture of explants

MS basal medium supplemented with 30 g/l sucrose and plant growth regulators was prepared in 500 ml conical flasks. 2, 4-D in three levels (0.00, 1.0 and 2.0 mg/l) and kinetin in four levels (0.00, 0.25, 0.5 and 1 mg/l) in all possible combination were considered as hormonal treatments (on the whole 16 hormonal treatments). pH was adjusted to 5.9 before autoclaving; 8 g/l agar was added and then autoclaved for 20 min at 121°C. Two different sources of explants viz. lives (with the size of about 1.5 × 1.5 cm) and embryos were separately cultured in petri dishes containing 20 ml of prepared MS medium. Both experiments were performed by factorial arrangement based on complete randomized design with four repetitions. Four explants were placed in each petri dish, sealed with para film and then incubated at 25 ± 1°C in darkness. Fresh weights of formed calli in each treatment for both kind of explants (leaves or embryos) were estimated and compared. For determination of callus fresh weight, they were transferred on sterile pre-weighted aluminum foil and weighed again. Obtained data for each kind of explants were analyzed with SAS soft ware and also medium fresh weights of calli were compared based on Duncan multi range test.

Hormonal treatments for shoot regeneration from callus

For shoot regeneration study from callus, the calli derived from each kind of explants (leaf or embryo) were separately transferred to conical flasks containing 100 ml B5 basal medium supplemented with 30 g/l sucrose, 8 g/l agar and different plant growth regulators which is mentioned below. Five calli with the dimensions of about 1 × 1 ×1cm were placed in each container. BAP at three levels (1, 2 and 3 mg/l) and NAA at four levels (0, 0.02, 0.2 and 1 mg/l) in all possible combinations were considered as hormonal treatments (on the whole 12 hormonal treatments) to shoot regeneration study. The containers were sealed with sterile aluminum foil and transferred into incubator with photoperiod of 16 h light/ 8 h dark at 25 ± 1°C and subculture every ten days. The number of regenerated shoots per callus was recorded five weeks after culture.

Root formation from regenerated shoots

After regenerating shoots from each kind of calli (leaf or embryo-derived calli), those (the shoots) with the length of about 3 - 5cm were transferred into culture vessels containing 10 ml MS basal medium supplemented with 30 g/l sucrose, 7 g/l agar and different levels of IBA (0.5, 1 and 1.5 mg /l), the vessels were incubated 25 ± 1°C in photoperiod of 16 h light/ 8 h dark.

RESULTS

Callus formation from leaf explants

Leaf explants in some treatments initiate callus formation about 15 days after culture. The calli at first formed at excised edges of explants and then expanded throughout the explants. Among all the treatments, those containing 0.5 mg/l kinetin in combination with 2 or 1 mg/l of 2, 4-D and also those containing 0.25 mg/l kinetin in combination with 2 or 1 mg/l 2,4-D, initiated callus formation earlier than others. Callus fresh weight in these treatments at the forth weeks after culture was also more than others. Formed calli in these treatments were both friable and bulky (Figure 1). Although these treatments initiated callus formation concurrently, their proliferation rate was wearied from one to another. Among these treatments, the maximum callus fresh weight belonged to the treatment containing 2 mg/l 2, 4-D in combination with 0.5 mg/l kinetin (by mean of 2.25 g per petri dish). Callus fresh weight in other treatments has been listed in the Table 1. It was found that the presence of 2, 4-d is necessary for callus induction from leaf explants in *Darura stramonium* since the treatments lacking in 2,4-D failed in callus formation or produced too little calls. It was also found that the low concentrations of kinetin (0.25 or 0.5 mg/l) in company of 2,4-D can expedite callus initiation and proliferation from leaf explants, but this was not so in the higher concentration (1 mg/l). For instance, callus fresh weight in the treatment supplemented with 2 mg/l 2,4-D alone was lower than those supplemented with 2 m/l 2,4-D and 0.5 or 0.25 mg/l, but was higher than that supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin. With neglect of kinetin concentration, the formed calli in 2 mg/l 2,4-D was generally more friable than those formed in 1 mg/l 2,4-D. The leaf explants in 0.25 mg/l alone failed in callus formation but they produced numerous hairy roots in which some of them subsequently converted to long roots. The results of leaf culture of Datura are as summarized in Table 1. The results of variance analysis for callus formation from leaf explants confirms the significant effect of 2,4-D. The reciprocal effect of 2,4-D and kinetin on callus formation was also significant, whereas, the effect of kinetin was not significant. It indicates that kinetin can encourage the effect of 2,4-D on callus formation from leaf explants, but it is not able to

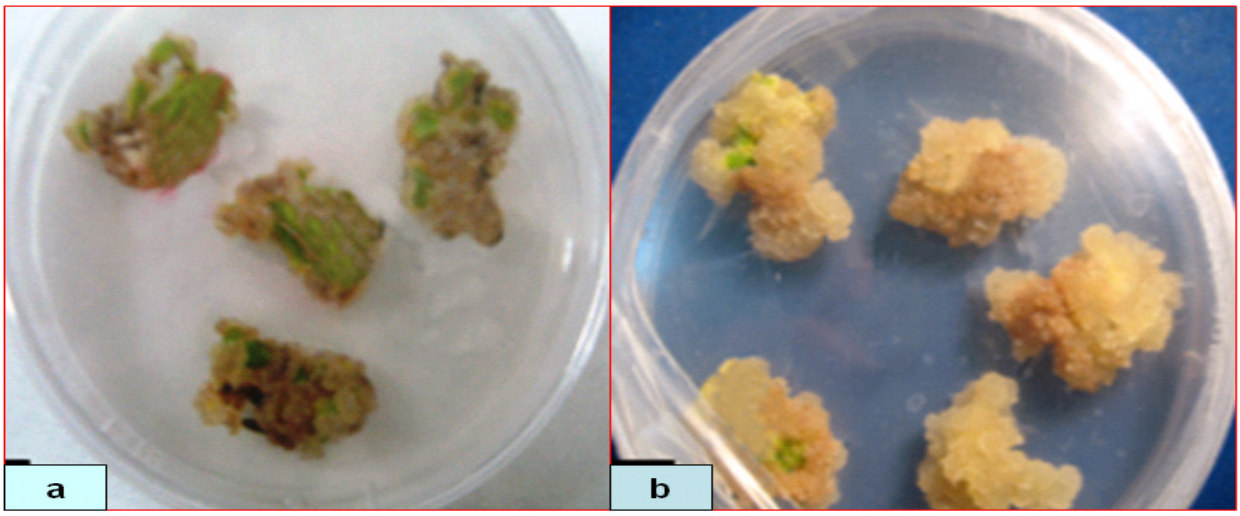

Figure 1. The left panel (a) shows the callus initiation from leaf explants of *D. stramonium* in the treatment with 2 mg/l 2,4-D in combination with 0.5 mg/l kinetin at the third week after culture and the right panel (b) shows the formed calli in the same treatment at the forth week after culture.

form callus solely (Table 2).

Callus formation from embryo explants

The embryo explants in 2 mg/l 2,4-D alone initiate callus formation earlier than other treatments. The formed calli in this treatment were creamy in appearance with some greenish parts which became thoroughly cream within subsequent days (Figure 2). Besides these treatments, those containing 2 mg/l 2,4-D in combination with 0.25 or 0.5 mg/l kinetin were found to be suitable treatments for callus formation from embryo explants. The formed calli in all the treatments were friable and white. The results of variance analysis revealed that against kinetin, 2,4-D has significant effect on callus formation from embryo explants. Addition of kinetin to some extent, reduced the effect of 2,4-D on callus formation, so that this negative effect can be increased from 0.5 to 1 mg/l kinetin. Mean comparison of callus fresh weight based on Duncan multi range test has been shown in Table 3.

Shoot regeneration from callus

About two weeks after transformation to regeneration media, the embryo-derived calli initiate shoot regeneration in most of the treatments. At first, green spots appeared on the surface of callus and these spots were then converted to shoot primordia and subsequently converted to shoots (Figure 3). The number of regenerated shoots per callus was different from one treatment to another. The maximum shoot regeneration percentage and also the maximum number of regenerated shoots belonged to the treatment containing 2 mg/l BAP plus 1 mg/l NAA. Besides, the treatment with 3 mg/l BAP plus 1 mg/l NAA or that containing 2 mg/l BAP plus 0.2 mg/l NAA was found to be suitable treatments for shoot regeneration from embryo-derived callus, respectively. It seems that the presence of NAA is necessary for suitable shoot regeneration since the treatments which lack NAA did not show suitable shoot regeneration. The response of leaf-derived callus to shoot regeneration was nearly different from those derived from embryo explants. All the treatments that contained 3 mg/l BAP plus 1 mg/l NAA were found to be the best hormonal treatment for shoot regeneration from leaf-derived calli. Shoot formation in treatment containing 3 mg/l BAP plus 0.2 mg/l NAA or in that containing 2 mg/l BAP plus 1 mg/l NAA were more suitable than other treatments. The results of callus culture are summarily listed in the Table 4.

Root formation

Green-compact calli were formed at the base of explants in 1.5 mg/l IBA and no root formation took place. About six days after culture, the mini shoots in 0.5 mg/l IBA initiated root formation form their bases dipped in culture medium. The formed roots in this treatment were nearly both long and delicate with many hairy roots, but those cultured in 1 mg/l IBA were shorter in length and had less hairy roots. The other results have been shown in the Table 5.

DISCUSSION

We achieved the best callus formation from leaf explants of Datura in media containing both 2,4-D and kinetin. It

Table 1. The results of leaf culture of *D. stramonium* on different combination of 2,4-D and kinetin.

Treatment	Days taken for callus initiation	Medium callus fresh weight (gr/per Petri dish) (28 days after culture)	Response
2 mg/l 2,4 –D + 1 mg/l kinetin	≥15	1.76	Friable- cream callus
2 mg/l 2,4 –D + 0.5 mg/l kinetin	13 – 15	2.25	Friable-cream callus. The most suitable callus formation.
2 mg/l 2,4 -D + 0.25mg/l kinetin	13 – 15	2.13	Friable- white callus. Suitable callus formation
2 mg/l 2,4 –D) 0 mg/l kinetin	≥ 15	1.75	Friable- cream callus
1 mg/l 2,4 –D + 1 mg/l kinetin	≥15	1.62	non- friable white callus
1 mg/l 2,4 –D + 0.5 mg/l kinetin	13 – 15	2.08	Nearly friable- cream callus Suitable callus formation.
1 mg/l 2,4 –D + 0.25mg/l kinetin	15 – 17	2.06	Nearly friable callus. Suitable callus formation.
1 mg/l 2,4 –D + 0 mg/ l kinetin	16 – 18	1.51	non- friable white callus
0 mg/l 2,4 -D + 1 mg/l kinetin	≥21	0.04	Condonable Callus formation
0 mg/l 2,4 –D + 0.5 mg/l kinetin	≥21	0.06	Condonable Callus formation (too little callus formation)
0 mg/l 2,4 –D+ 0.25 mg/l kinetin	-	0.00	No callus formation. Many long roots were formed
0mg/l 2,4 –D + 0 mg/l kinetin	-	0.00	No callus formation

Table 2. Variance analysis of the effect of kinetin and 2,4-D on fresh weight of leaf-derived callus.

S.O.	df	MS	F-value
Kinetin	3	0.00074929	0.64^{ns}
2,4-D	2	0.03834256	32.84^{**}
Kinetin × 2,4-D	6	0.02348203	20.11^{**}
Error	36	0.00116746	

ns, Not significant; **significant at level 0.01 of statistical probability.

was revealed that, the presence of kinetin can encourage the effect of 2,4-D on callus formation from this type of explants. Whereas the best callus formation from embryo explants was observed in 2 mg/l 2, 4-D alone, adding of kinetin reduced the callus formation from embryo explants. As it was mentioned before, tissue culture information about Datura is somewhat deficient, therefore we compared the results of our study with related species. There are some reports about the other plants in this family (Solanaceae). For instance, Brasileiro et al. (1999) studied the callus formation and plant regeneration in tomato (*Lycopersicon esculentum*). They reported that the presence of cytokinins encourages the effect of auxins on callus formation in this plant. But against our results, they showed that auxin in the form of 2,4-D had no callus formation in tomato. These researchers achieved the shoot regeneration from callus in the medium containing 0.01 mg/l BAP plus 0.001 mg/l NAA, whereas for Datura, shoot regeneration from each kind of callus (calli of leaf or embryo) was not suitable in low concentrations of these hormones (Table 4). In another study, Alagumanian et al. (2004) cultured two explant sources (leaf and steam segments) of *Solanum trilobatum* in media containing coconut milk supplemented with different concentrations of kinetin (2.3, 4.6, 9.3, 13.9, 18.6 and 23.2 µM) or NAA (0, 2.7, 5.4 and 10.7 µM). They found that coconut milk is necessary for callus maintenance in *S. trilobatum* since without coconut milk, the formed calli turned brown and died after a few days,

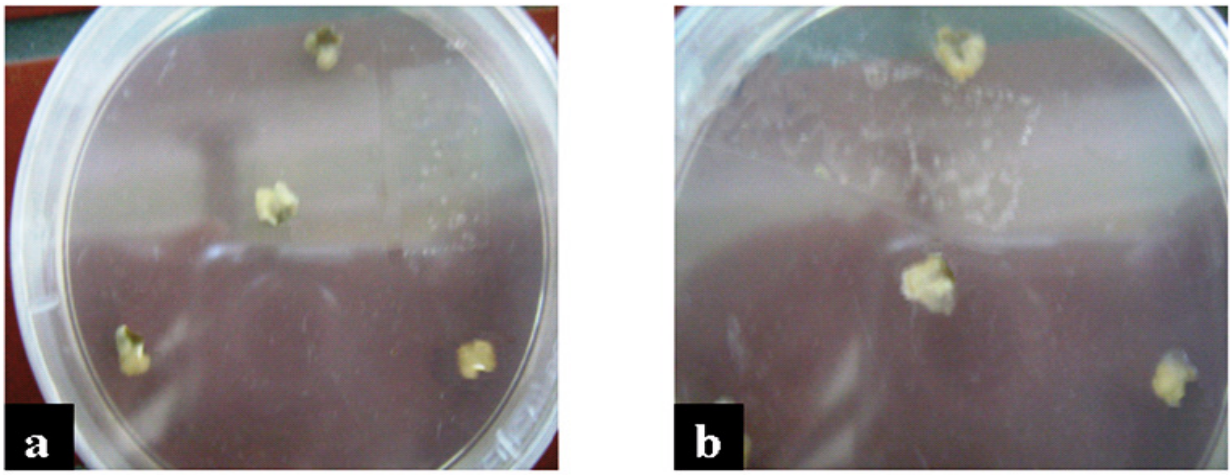

Figure 2. The left panel (a) shows the embryo-derived calli in the treatment containing 2 mg/l 2,4-D 15 days after culture. Some greenish parts are seen in the two upper calli. The right panel (b) shows the same petri dish 2 days later. As it is seen, all greenish parts turned thoroughly cream in appearance.

Table 3. Mean comparison of embryo-callus fresh weight in different combinations of 2,4-D and kinetin multi range test.

Kinetin (mg/l)	2, 4-D (mg/l)		
	0	1	2
0	0^{f}	0.447^{bcd}	0.570^{a}
0.25	0^{f}	0.475^{bc}	0.528^{ab}
0.5	0.05^{f}	0.475^{bc}	0.522^{ab}
1	0.03^{f}	0.395^{cde}	0.440^{bcd}

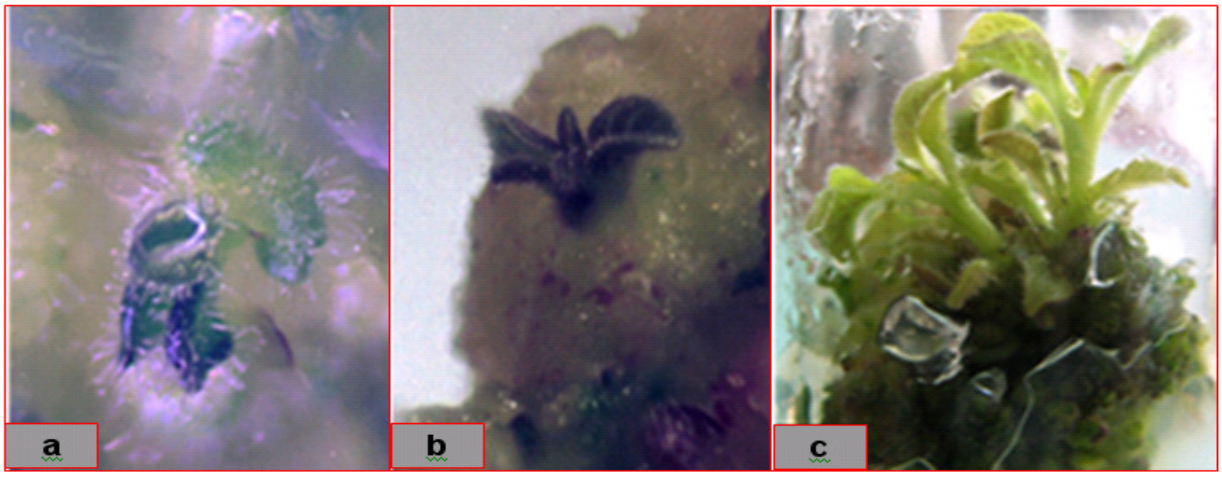

Figure 3. Shoot regeneration from embryo-derived calli in MS basal medium supplemented with 2 mg/l plus 1 mg/l NAA. Green regions appeared about two weeks after callus culture (a). Shoot regeneration in the third week after culture (b). Elongated shoots in the fourth week after culture (c)

whereas, the calli of *D. stramonium* grew normally without coconut milk. It was also reported that kinetin stimulates callus induction from both types of explant. Similarly, we found that this hormone has no significant effect on callus formation from both types of explants in Datura, although it had reciprocal effect with 2,4-D on callus formation from

Table 4. The results of callus culture of *D. stramonium* in different combination of NAA and BAP.

Treatment	Days taken to shoot regeneration		The number of shoot per callus		Length of auxiliary shoot(cm) (the fourth week after culture	
	Embryo-derived calli	Leaf-derived calli	Embryo-derived calli	Leaf derived calli	Embryo-derived calli	Leaf derived calli
3 mg/l BAP + 1 mg/l NAA	13 - 15	19 – 22	2 - 7	3 - 6	5.5-8.5	4.5 - 6
3 mg/l BAP + 0.2 mg/l NAA	15 - 18	20 – 25	2 - 5	3 - 5	4-7	3.5 - 7
3 mg/l BAP + 0.02 mg/l NAA	17 - 22	≥25	2 - 4	1 - 2	3.5 -5.5	2.5 - 4.5
3 mg/l BAP alone	≥ 21	≥25	1 - 3	1 - 2	3-5.5	3 ≤
2 mg/l BAP + 1 mg/l NAA	13 - 15	21 – 25	3 - 7	3 - 5	6.5 -9	4 - 6
2 mg/l BAP + 0.2 mg/l NAA	14 - 17	24 – 28	2 - 6	2 - 4	5-8.5	3.5 - 5
2 mg/l BAP + 0.02 mg/l NAA	15 - 18	-	2 - 4	No shoot formation	2.5-4	-
2 mg/l BAP alone	≥ 21	-	1 - 2	No shoot formation	2.5 ≤	-
1 mg/l BAP + 1 mg/l NAA	15 - 20	21 – 27	2 - 5	2 - 4	5- 7.5	2.5 - 5
1 mg/l BAP + 0.2 mg/l NAA	17 - 20	24 – 30	2 - 4	No shoot formation	3.5-5	-
1 mg/l BAP + 0.02 mg/l NAA	≥ 21	≥28	1 - 2	No shoot formation	3 ≤	-
1 mg/l BAP alone	≥ 21	-	1 - 2	No shoot formation	2 ≤	-

Table 5. The effect of different concentrations of IBA on *in vitro* rooting of regenerated shoots of Datura.

Concentration of IBA (µM)	No. of shoots cultured	days taken to root formation	No. of rooted Shoots (three weeks after culture)	No. of main roots	Length of roots(three weeks after culture)
0.5	12	6 - 9	10	3 - 6	6 - 9.5 cm
1	12	8 - 12	7	2 - 4	7 ≤
1.5	12	-	0	0	0

leaf explants. Moreover, these researchers achieved suitable shoot regeneration from callus of *S. trilobatum* by using BAP alone (from 2.2 to 22.2 µM), but we found that BAP alone is not able to have shoot regeneration from leaf-derived calli of Datura. Although shoot regeneration from embryo-derived calli of Datura occurred in the treatments without NAA, it was enhanced significantly by addition of this hormone (Table 4).

Conclusion

There is tangible difference between embryo and leaf explants of *D. Stramonium* in response to *in vitro* culture approaches. Embryo explants are generally better than leaf explants for both callus induction and plant regeneration from callus. 2,4-D is a suitable auxin source for callus induction from both leaves and embryo explants. Adding of kinetin can encourage the effect of callus formation from leaf explants of Datura, whereas, it reduces the effect of 2, 4-D on callus formation from embryo explants. Both BAP and NAA are

necessary for the desirable shoot regeneration from callus *D. stramonium*. Finally, IBA at level 0.5 mg/l is suitable for rooting of regenerated shoots.

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