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Callus induction and plantlet regeneration in *Abelmoschus esculentus* (L.) Moench.

A.H. Kabir, K.K. Sarker, S.A. Sharmin, M.S. Islam, and M.F. Alam*

Biotechnology and Microbiology Laboratory, Department of Botany, Rajshahi University, Rajshahi 6205, Bangladesh

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The present study was undertaken to evaluate the most suitable concentration of growth regulators for callus induction and subsequent organogenesis in Okra (*Abelmoschus esculents* (L.) Moench). The best callus induction (95.0%) was found in MS medium supplemented with 0.5 mg/l BAP and 2.0 mg/l NAA from hypocotyl explant. The study of two explants, hypocotyl was found good for producing high amount of organogenic callus. Combination of 2.0 mg/l BAP + 0.1 mg/l IAA and 2.0 mg/l BAP + 0.5 mg/l NAA gave the most effective for plant regeneration from callus. Hypocotyl derived calli showed better performance than leaf disc derived callus. Callus derived plants rooted most effectively in MS medium containing 2.0 mg/l IBA. In this case spontaneous rooting was achieved. The success of plant tissue culture for *in vitro* culture of okra was encouraged by acclimatization of the plantlets in the field conditions.

Key words: callus, organogenesis, regeneration, acclimatization

Introduction

Okra (*Abelmoschus esculentus* (L.) Moench.) is one of the most widely grown vegetables in tropics and subtropics including Bangladesh. It is an important vegetable crop because it is being grown successfully throughout the year (Singh, 1990). It is known as powerhouse of valuable nutrients. The fruit is highly proteinaceous. It is a good source of vitamin A and vitamin C. It is low in calories and is fat-free. It has also considerable medicinal and industrial value (Kirtiker and Basu, 1984). A mucilagenous preparation from the pod can be used as a plasma replacement (Purseglove, 1968; Chopra *et al.*, 1986). The okra growth and yield is satisfactory in Bangladesh but is still low in average yield as compared to other countries of the world viz. USA and India (Choudhury, 1979).

*Corresponding author: M.F. Alam; e-mail: mfalambot@yahoo.com

The importance of vegetables in the over all agriculture economy significantly increased in terms of area, economic value and employment during the past two decades. Now the vegetable is a key component of agriculture and can not be ignored in agriculture policy planning. Okra is frequently infected by a number of systemic diseases caused by fungi, viruses, bacteria, mycoplasma and nematodes. Conventional breeding technique has a boundary of limitation. In the cases of inbreeder, the progress in the improvement is hampered because of lacking of genetic variability. Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques to widen the possibilities for the development of transgenic lines or somaclonal variants of different cultivars. A few previous works have been done in vitro culture of okra (Ganesan *et al*., 2007; Haider *et al*., 1993). Many reports are available on callus initiation in vegetable crop plant species (Roy and De, 1990; Nishio *et al*., 1987; Sexena *et al*., 1987; Asao *et al*., 1989). The present study describes an optimized regeneration system in *Abelmoschus esculentus* from callus derived from different seedling explants cultured on a variety of medium composition.

Materials and methods

Plant materials

Seeds of Okra (*Abelmoschus esculentus*) cv. SL-44, were collected from Bangladesh Agriculture Research Institute. The seeds were rinsed in 70% ethanol followed by 3-5 times washing with sterilize distilled water. After washing, they were treated in 0.1% $HgCl_2$ solution with gently shaking for 4 min and followed by 3-5 times washing with sterilize distilled water.

Callus induction

To induce callus, hypocotyl and leaf segments were taken and were cultured in MS (Murashige and Skoog, 1962) medium supplemented with different callus inducing substances and incubated in the light of $25\pm2^{o}C$ for 3-6 weeks. MS medium supplemented with different concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid), NAA (Napthalene acetic acid), BAP (Benzyl amino purin), IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), and KIN (6-furfural amino purine) were used as sources of carbon (30 gm/l) for massive callus induction (Fig. 1). After 21 days of incubation the callus induction frequency was estimated. After callus induction from the explants, the calli were transferred into the fresh medium after every 18-24 days for

further proliferation and maintenance. Brown, watery and dead calli were removed during each subculture. Friable, having green roundish callus was considered as putative embryogenic calli. These were selected for plant regeneration.

Histological study of callus

Histological study of organogenic calli were conducted for identifying the nature of shoot formation on sections that obtained with a microtome (Esau, 1965). Putative organogenic calli were fixed in FAA (5% formalin, 5% acetic acid, 45% alcohol) solution. Dehydration of the fixed materials was accomplished by placing them in a grade series of ethyl alcohol (50%, 60%, 70%, 80%, 90% and 100%) and absolute alcohol with chloroform (2:1 1:1 and 1:2). Then, the dehydrated materials were embedded in paraffin which blocks and sections were prepared serially at 12 µm thickness using a rotatory microtome (The Cambridge Rocker). After staining with safranin-orange-G and tannic acid, the sections were mounted on glass slides using Canada balsam. Microphotographs were taken at ×100 magnification.

Plant regeneration

Plantlets were regenerated by transferring the selected calli in MS semisolid medium supplemented with different concentration and combination of growth regulators (Table 1-4). The culture was incubated at $25+20^{C}$ under white light for 16-8 light dark condition. After 4-6 weeks differentiation, of shoots and roots were observed. The number of calli producing shoots and the total number of shoots were counted for each treatment. The shoot from the selected callus was excised and transferred on MS medium for further growth. The plantlets from each of individual callus was further multiplied by node culture.

Acclimatization

The plantlets having sufficient root and shoot systems were taken out from the culture vessels and were washed under running tap water to remove the agar attached roots. The plantlets were then transferred to small pots containing sterilized ground soil, sand and cow dung manure at the ratio of 1:2:1. After keeping 7-10 days in the growth culture room, the plantlets were moved and adapted to the room temperature for the growth of plantlets. There after, They were transferred to the field condition where eventually developed into mature plants.

Data recording

Callus induction frequency: Frequency of callus induction was calculated using the following formula:

$$\text{Frequency of callus induction (\%)} = \frac{\text{Number of explants induced callus}}{\text{Total number of explants inoculated}} \times 100$$

Qualitative nature of callus: Cultured explants that showed callus formation were counted after 20 to 40 days culture. The colour, nature and physical condition of callus were recorded.

Shoot Induction: The percentages of explants induced to develop adventitious shoots that calculated using following formula:

$$\text{Explants induced shoot (\%)} = \frac{\text{Number of culture induced shoot}}{\text{Total number of explants inoculated}} \times 100$$

Number of shoots per explant was computed after required days of culture. Mean number of adventitious shoots per explants was calculated using following formula:

$$\bar{X} = \frac{\sum Xi}{N}$$

$\bar{X}$ = Average number of shoots; Σ = Summation; Xi = Total number of shoots; N = Number of observation

All shoot regenerated from callus produced sufficient amount of roots, so no parameters for root formation was used.

Results and discussion

When seeds were treated in 0.1% $HgCl_2$ solution for 4 and 5 minutes, contamination was occurred. About 90-100% of seeds were not contamination that treated in 0.1% $HgCl_2$ for 7-10 minutes. When the seeds treated for 8-12 minutes, then no contamination but partial or complete tissue killing was observed. But 100% contamination free and no tissue killing were observed when seeds treated for 7 minutes. So, for surface sterilization seed treatment with 0.1% $HgCl_2$ solution for 7 minutes of resulted the most effective. So this concentration and duration of $HgCl_2$ solution were similar with the other workers (Munjury, 1998; Banu, 1998; Das *et al.*, 2001).

Callus development

In BAP and NAA combination, the highest percentage of callus induction (85.2%) was observed in 1.0 mg/l BAP and 2.0 mg/l NAA in leaf disc explant (Fig. 1). These calli were white in colour and friable in nature. In BAP and NAA combinations, various type of calli were occurred. These calli were both white and greenish in colour and compact and friable in nature. This result was more or less similar to the others (Aloufa, 2002; Nagmani and Venketes Waran, 1987). In BAP-IBA combinations, the highest percentage of callus induction (70%) was observed in 0.5 mg/l BAP and 2.0 mg/l IBA. All these calli were white in colour and compact in nature. The lowest percentage was observed (30.3%) in 0.5 BAP mg/l and 1.0 IBA mg/l. In BAP and 2,4-D combination, and the highest percentage of callus induction (60.0%) was observed in 0.5 mg/l BAP and 2.0 mg/l 2,4-D. The highest percentage of callus induction (50.0%) was observed in 0.5 mg/l BAP and 1.0 mg/l IAA. Role of auxin alone or in combination with cytoknin for callus proliferation is well documented (Hammerschlag *et al.*, 1985; Jain *et al.*, 1988; Niedz *et al.*, 1989; Verhagen and Wann, 1989; Roy and De, 1990). The highest callus formation (95.0%) was recorded in the medium containing 0.5 mg/l BAP and 2.0 mg/l NAA by using hypocotyls (Fig. 1). Calli were greenish in colour and loose in nature. Combination of BAP and IBA found to produce appreciable amount of callus. The highest frequency of callus induction (75.2%) was observed in 1.0 BAP mg/l and 2.0 IBA mg/l containing medium. These calli were white in colour and loose in texture. Percentage of callus induction was also high when hypocotyls was treated with the combination of BAP and IAA. The highest percentage of callus induction was observed in 0.5 mg/l BAP and 2.0 mg/l IAA (75.1%). Callus induction was found good (55.3%) when it was cultured on MS medium supplemented with 0.1 BAP mg/l and 2.0 2,4-D mg/l. Effect of NAA, IBA, IAA and 2,4-D on callus induction was poor.

Plant regeneration

After sufficient callus induction, the explants were initiated subsequent organogenesis by the effect of different growth regulators (Fig. 2). The necessity of cytokinin for shoot initiation is well established (Beck and Coponetti, 1983; Evans *et al.*, 1984). When hypocotyl derived callus was cultured in MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA, the highest 60.82% shoot differentiation was observed (Table 2). The highest mean number of shoots per callus was (6.5±0.04) also recorded in the same

medium. Many workers have been described application of BAP with NAA for regeneration (Kowalozyuk *et al*., 1983; Otani *et al*., 1996). In combinations of

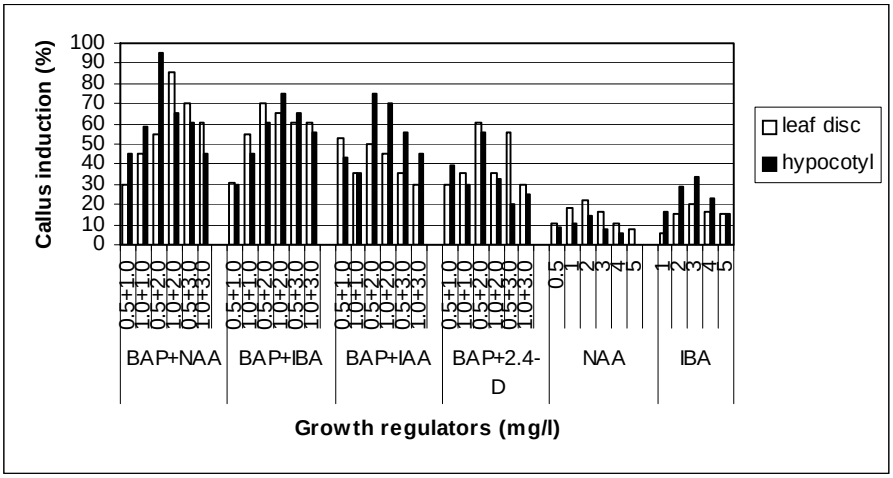

Fig. 1. Effect of growth regulators on callus induction.

KIN and IBA combination showed maximum of 50.90% callus regenerated into shoot when they cultured on 1.0 mg/l KIN and 0.5 mg/l IBA. In this case, mean number of shoot per callus was 5.2±0.21. In BAP-IBA combinations, shoot differentiation rate was very low. Among all combinations, the highest (60.82%) callus regenerated into shoots when they were cultured in medium supplemented with 2.0 mg/l BAP and 0.1 mg/l IAA. Similar results were reported by Kamat and Rao (1978). The best regeneration (52.36%) was found in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA from leaf disc derived callus (Table 1). The maximum callus was regenerated as 44.65% into shoots when cultured on 2.0 mg/l BAP and 0.1 mg/l IAA. The hypocotyl-derived calli showed better shoot differentiation with a good degree of growth.

Introduction of roots on calli-regenerated shoots are essential for successful establishment of the plantlet on the soil. In hypocotyl derived callus, highest frequency (80%) of rooting and mean number of roots/shoot (10.00±0.9) was recorded in media containing 2.0 mg/l IBA (Table 4; Fig. 2). Efficient effect of IBA on root induction was also reported by other workers (Zang *et al*., 1997; Venkatachalam *et al*., 2000). The highest mean length of root was 9.23±0.42 cm in the same media composition. Effect of NAA on root induction was also good. The highest frequency of rooting (70%) was recorded in MS medium containing 1.0 mg/l NAA. The highest mean number of roots/shoot was observed 8.11±0.34. The highest frequency of rooting (65%)

was recorded in media containing 3.0 mg/l IAA. Effect of IBA in combination with GA3 on root induction was also noticeable from leaf disc derived callus. For root induction from leaf disc derived callus, the highest of rooting (70%) was recorded in MS medium containing 2.0 mg/l IBA (Table 3). The highest mean number of roots/shoot was 9.00±0.43 and mean length of root was 8.24±0.37 cm that observed in the same media. Root induction of 60% found to be suitable in MS medium supplemented with 2.0 mg/l NAA. The highest mean number of roots/shoot was 7.25±0.26 in this media. Comparatively root induction was found better in hypocotyl-derived callus.

Table 1. Effect of different concentrations and combination of cytokinin and auxin in medium on shoot regeneration from leaf disc derived callus.

Growth regulators		Morphogenic response	
		No. of calli Regenerated shoot (%)	Mean no. of shoots per callus ($\overline{X} \pm S.E.$)
BAP + NAA	0.5+0.1	24.60	3.3±0.49
	1.0+0.5	29.91	4.0±0.35
	2.0+0.5	52.36	5.9±0.59
	2.0+1.0	45.76	4.4±0.45
	3.0+0.5	25.28	4.1±0.42
	3.0+1.0	22.16	3.8±0.37
BAP + IAA	1.0+0.1	29.54	3.8±0.19
	1.0+0.5	35.91	4.2±0.19
	2.0+0.1	44.65	5.1±0.31
	2.0+1.0	36.95	4.3±0.23
	3.0+0.5	25.80	3.7±0.26
	3.0+1.0	22.80	3.2±0.25
KIN + IBA	1.0+0.1	26.30	3.6±0.34
	1.0+0.5	33.55	4.7±0.4
	2.0+0.1	28.45	3.8±0.37
	2.0+0.5	24.91	3.4±0.21
	3.0+0.1	18.32	3.3±0.35
	3.0+0.5	13.45	3.2±0.00
BAP + IBA	1.0+0.1	10.91	3.1±0.21
	1.0+1.0	19.33	3.4±0.9
	2.0+0.1	32.91	4.0±0.25
	2.0+1.0	26.30	3.6±0.34
	3.0+0.1	15.60	3.2±0.39

Table 2. Effect of different concentrations and combination of cytokinin and auxin in MS medium on shoot regeneration from hypocotyl derived callus.

Growth regulators		Morphogenic response	
		No. of calli Regenerated shoot (%)	Mean no. of shoots per callus ($\overline{X} \pm$ S.E.)
BAP + NAA	0.5+0.1	14.15	3.0±0.05
	1.0+1.0	22.50	3.1±0.05
	2.0+0.1	35.20	4.5±0.18
	2.0+1.0	28.18	4.0±0.52
	3.0+1.0	22.50	3.1±0.13
BAP + IAA	0.5+1.0	26.80	3.2±0.54
	1.0+0.5	35.28	4.3±0.25
	2.0+0.1	60.82	6.5±0.04
	2.0+0.5	50.52	6.0±0.17
	3.0+0.1	45.19	5.4±0.35
KIN + IBA	0.5+0.1	25.50	3.5±0.17
	1.0+0.5	50.90	5.2±0.21
	2.0+0.1	34.00	5.0±0.57
	2.0+0.5	23.50	3.3±0.49
	3.0+0.5	18.50	2.9±0.29
BAP + IBA	0.5+0.1	13.52	2.3±0.17
	1.0+0.5	16.58	2.6±0.28
	2.0+0.5	24.12	3.6±0.16
	2.0+1.0	19.12	3.0±0.31
	3.0+0.5	15.00	2.5±0.15

Table 3. Induction of roots from leaf disc derived callus in MS medium with various concentration of growth regulators.

Treatment		Mean length of root in cm ($\overline{X} \pm$ SE)	Roots/shoot ($\overline{X} \pm$ SE)	Rooting frequency (%)
IBA	0.5	6.00±0.85	7.20±0.45	50
	1.0	7.33±0.42	8.45±0.22	60
	2.0	8.24±0.37	9.00±0.43	70
	3.0	6.90±0.22	7.50±0.75	55
	4.0	5.95±0.12	6.92±0.55	45
NAA	0.5	4.85±0.13	5.40±0.77	45
	1.0	4.70±0.21	6.55±0.10	55
	2.0	6.20±0.10	7.25±0.25	60
	3.0	5.40±0.44	6.09±0.55	45
	4.0	4.50±0.13	5.09±0.78	40
IAA	1.0	4.80±0.91	5.12±0.12	35
	2.0	6.00±0.39	6.35±0.60	55
	3.0	5.30±0.16	5.81±0.71	45
	4.0	5.00±0.22	5.25±0.45	40
	5.0	5.50±0.76	5.00±0.55	30
IBA+GA3	0.5+1.0	4.42±0.28	5.10±0.45	30
	1.0+1.0	4.68±0.26	5.65±0.81	45
	1.0+2.0	5.80±0.43	6.00±0.73	50
	1.0+3.0	4.50±0.54	5.30±0.33	35
	1.0+4.0	4.30±0.11	4.72±0.58	25

Table 4. Induction of roots from hypocotyl derived callus in MS medium with various concentration of growth regulators.

Treatment		Mean length of root in cm ($\overline{X} \pm SE$)	Roots/shoot ($\overline{X} \pm SE$)	Rooting frequency (%)
IBA	0.5	7.00±0.14	8.25±0.40	55
	1.0	8.34±0.27	9.50±0.32	65
	2.0	9.23±0.42	10.00±0.93	80
	3.0	7.20±0.12	8.00±0.19	60
	4.0	6.90±0.15	7.00±0.00	50
IAA	1.0	5.45±0.34	5.80±0.23	45
	2.0	5.61±0.34	6.15±0.49	55
	3.0	6.50±0.39	7.19±0.00	65
	4.0	5.50±0.22	6.00±0.23	50
	5.0	4.96±0.29	5.92±0.42	40
NAA	0.5	5.95±0.79	6.00±0.21	50
	0.1	6.27±0.42	7.20±0.73	60
	1.0	7.29±0.32	8.11±0.34	70
	2.0	6.00±0.77	7.09±0.73	55
	3.0	5.40±0.33	5.70±0.77	45
IBA + GA3	0.5+1.0	5.40±0.88	5.85±0.55	40
	1.0+1.0	5.90±0.33	6.17±0.42	45
	1.0+2.0	6.12±0.20	7.20±0.39	60
	1.0+3.0	5.20±0.45	6.00±0.32	50
	1.0+4.0	5.00±0.77	5.50±0.88	35

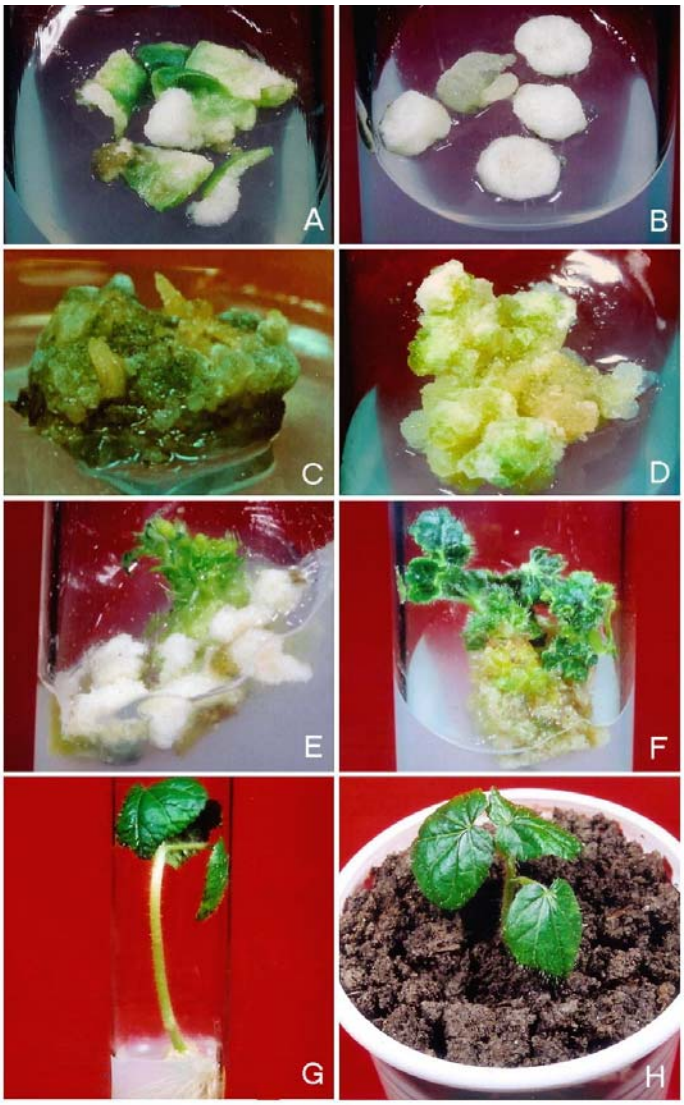

Fig. 2. Callus induction and plantlet regeneration in *Abelmoschus esculentus* L.

A - Initiation of callus from leaf disc explants.
B - Initiation of callus from hypocotyl explants.
C - Proliferation of callus (after four weeks of culture).
D - Putative organogenic calli showing green pots and primary shoot initiation.
E & F - Plant regeneration from callus.
G - Regenerated plantlets with well developed roots.
H - Acclimatization of plantlet.

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