Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 2 (4): 407-415, 2007
ISSN 1816-4951

Establishment of Cell Suspension Culture and Plantlet Regeneration of Brinjal (*Solanum melongena* L.)

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Abstract: The aim of this study to show, an efficient protocol for establishment of cell suspension culture and plantlet regeneration through cell culture from the cotyledonary explants of Brinjal (*Solanum melongena* L.). In this investigation, three varieties of Brinjal cv. Loda, China and Jhotika were used. In first step, the somatic embryogenic calli formation was done using MS medium supplemented with different concentrations of auxin and cytokinin singly or in combination. Cells of the three varieties were isolated from the rapidly growing embryogenic and friable calli using orbital shaker. For callus induction the isolated cells were transferred to MS liquid medium containing different hormonal concentrations and after 37-63 days of incubation the micro-calli were appeared. The Loda and China varieties showed the best result (8.0 and 8.2%, respectively) in 2 mg L^{-1} NAA+0.05 mg L^{-1} BAP and 2 mg L^{-1} 2,4-D+0.05 mg L^{-1} BAP. For embryo formation, micro-calli were subcultured on MS solid medium and the Loda variety showed the best result (21%) in the medium containing 1.0 mg L^{-1} BAP+0.05 mg L^{-1} GA_3. The bipolar embryos were selected and cultured in MS medium with different combinations and concentrations of auxin (NAA) and cytokinin (BAP and IBA) for shoot and root formation. Optimum shoot and root formations were recorded in MS medium supplemented with 0.75 mg L^{-1} NAA+1.5 mg L^{-1} BAP and 2.0 mg L^{-1} NAA+0.5 mg L^{-1} IBA, respectively. The plantlets appeared in the embryo mass were cultured and acclimatized.

Key words: Brinjal, cotyledon, embryogenic calli, cell suspension, somatic embryogenesis and regeneration

INTRODUCTION

Brinjal is an economically important vegetable comprising an imperative supply of dietary protein, carbohydrate, vitamin and mineral particularly for the vegetarian population of developing countries. Brinjal can be cultivated round the year but the productivity and quality of this crop suffer due to its susceptibility to a number of diseases and insect pests (Sadilova *et al*., 2006). In South and South-East Asia eggplant is extensively damaged by the infestation of a Lepidopteron insect, *Leucinodes orbonalis* commonly known as shoot and fruit borer. During its cultivation the total loss caused by this insect pest is 5-20% in shoot and 10-70% in fruit (Das *et al*., 2000). The principal methods used for the improvement of these crops are selection from inbred lines and intervarietal crosses (Anisuzzaman *et al*., 1993). The progress towards the improvement of this crop for insect pest resistance and introduce new varieties is hampered mainly due to the wide prevalence of sterility in the progeny and occurrence of genetic incompatibility following intergeneric and interspecific crosses, respectively (Rao, 1979; Daunay *et al*., 1991). To overcome such problems of conventional breeding,

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advanced biotechnological methods such as micropropagation and genetic transformation can be applied as an alternative approach for the development of disease and pest resistance of this crop (Franklin and Lakshmi Sifa, 2006). An efficient and reproducible *in vitro* regeneration system is considered as an integral part of successful transformation. There are a number of reports available regarding the *in vitro* regeneration of brinjal from different explants *via* organogenesis (Kamat and Rao, 1978; Fassuliotis *et al*., 1981; Sarma and Rajam, 1995; Fari *et al*., 1995; Magioli *et al*., 1998) and somatic embryogenesis (Matsuoka and Hinata, 1979; Gleddie *et al*., 1983; Yadav and Rajam, 1998). But these methods bear some probems. In this investigation, we tried to remove these problems and became able to describe an easy procedure for cell culture of Brinjal and plantlet regeneration through cell suspension culture of Brinjal.

Investigation, the plantlet regeneration from the culture cotyledonary explants of Brinjal is involved the following phases such as embryogenic calli formation, cell isolation, cell expansion, calli initiation, formation of early stage embryos, maturation of embryos and shoot and root formation.

MATERIALS AND METHODS

The cotyledonary explants of three varieties of Brinjal cv. Loda, China and Jhotika were used as experimental materials in this investigation. Seeds of these varieties were collected from Bangladesh Agricultural Development Corporation (BADC), Rajshahi, Bangladesh. Seeds were washed thoroughly under running tap water and after then treated with 1% savlon supplied by ACI and 2-3 drops of Tween-80 for about 10 min. This was followed by successive three washing with distilled water to make free the seeds from savlon and Tween-80, surface sterilization was carried out with 0.1% $HgCl_2$ for 6-7 min followed by gentle shaking. After this treatment, the seeds were rinsed 4-5 times in sterile distilled water to make free the seeds from $HgCl_2$. Sterilized seeds were aseptically germinated in glass bottle containing 50 mL of autoclaved (121°C temperature and 15 psi for 15 min) half strength MS (Murashige and Skoog, 1962) medium and double autoclaved soil separately. Germinating seeds were maintained at 25±2°C temperature and 60° RH in darkness. After germination, seedlings were maintained under 16/8 h light and dark region. The experiment was conducted in the Institute of Biological Sciences, Rajshahi University, Bangladesh in 2006.

Embryogenic Calli Induction

Cotyledons from 8 days old aseptically grown seedlings were used as explants. Explants (20 pieces) were cultured in 9 cm petridish and placed horizontally in the callus induction medium. The MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and different concentrations of 2,4-D (2, 4-Dichlorophenoxy acetic acid) NAA (α-Naphthalene acetic acid), IAA (Indole-3-acetic acid) and BAP (6-Benzyl aminopurin) singly or in combination were compared for the induction of embryogenic calli. The medium was adjusted to pH 5.8 and autoclaved (as described earlier). All the cultures were maintained as described earlier for aseptic seed germination. The data for callus initiation were scored after 28-30 days of culture. Induction frequencies for all types of callus were calculated as the percentage of cultured pieces of cotyledons.

Cell Isolation and Callus Induction

Embryogenic and rapidly proliferating friable calli subcultured for 18 days, were transferred to MS liquid medium supplemented with 1.0 mg L^{-1} NAA+0.05 mg L^{-1} BAP. The culture bottles, wrapped with brown paper, were placed on a rotary shaker (100 rpm). After 6-7 days, the liquid medium containing cells and micro calli were filtered through a 500 μm sieve. The cells collected in liquid medium were kept in a stationary position for 20-25 min for sedimentation. The supernatant was discarded keeping the cells settled at the bottom. The cells were distributed to petridishes (4 cm)

containing the fresh liquid medium to observe the growth efficiency of cells and to obtain calli. To observe the growth efficiency of cells, some petridishes were kept on a orbitary shaker and the weight of cells in 5 mL liquid medium was taken in every other two days. On the other hand, to obtain callus some of the cultured petridishes were kept at stationary position at 25°C in dark and after 37-63 days of incubation, micro calli were appeared in the plates initiating induction of callus.

Embryo Formation and Regeneration

For embryo formation the calli derived from isolated cells, were subcultured on the agarifide MS medium supplemented with different concentration of NAA+BAP, BAP+GA_3 and KIN+GA_3. After 20-21 days the embryos were appeared in the solid MS medium. The embryos showed various polarities like unipolar and bipolar. The number of embryos per callus was calculated under microscope from the beginning of embryogenesis and their average number were calculated. For shoot and root formation, the bipolar embryos were transferred in MS medium containing different concentrations of NAA with BAP, NAA with IBA and IBA singly.

RESULTS AND DISCUSSION

Induction and Maturation of Embryogenic Calli

For callus induction, cotyledons of three varieties were cultured on MS media supplemented with auxin and cytokinin. Different varieties of brinjal took different time for callus induction. Data on percentage of explants responded, weight and nature of callus were recorded and the results are presented in Table 1. When the cotyledons of Loda, China and Jhotika varieties were cultured on MS medium containing 2,4-D, the lowest percentages were 19, 20 and 21%, respectively. These rates gradually increased with the increasing of hormonal concentrations and the highest rates were obtained 91, 93 and 92% for Loda, China and Jhotika, respectively. But when the cotyledons were cultured on MS medium containing NAA, the highest callusing rates were 100, 100 and 92%, respectively. All the calli, mentioned above, were not embryogenic. These results are similar to Nasir (2002) and Huda and Sikdar (2003). They obtained 100% callus induction rate of Brinjal using MS medium containing mg L^{-1} 2,4-D. Nasir used leaf and Huda used cotyledons as explants.

Embryogenic calli were induced when cotyledons were cultured on MS medium containing auxin with cytokinin (BAP). When NAA was used with BAP, the three varieties Loda, China and Jhotika showed the highest (100%) embryogenic callus induction rate in MS medium supplemented with 2.0 mg L^{-1} NAA+0.05 mg L^{-1} BAP. But when the cotyledons of these three varieties were cultured on MS medium with 2,4-D and IAA with BAP, the callusing rates were comparatively lower. The nonembryogenic calli were large, white and friable while the embryogenic calli were green and compact. Production of embryogenic calli was increased considerably with carefully selection of embryogenic regions in the growing callus and their subsequent collection in the maintenance medium.

Isolation of Cells, Formation of Embryogenic Calli and Regeneration

Better performance of the isolated cells depends on the condition of embryogenic calli from which they were derived and the duration of their subculture. Greenish, friable, rapidly growing and embryogenic calli subcultured for 18 days were suitable for cell isolation. These calli produced the maximum yield of cells. This result was much better than those were obtained from shorter or longer duration of subculturing. Isolated cells from embryogenic calli were relatively uniform in size after purification (Fig. 1G and H). They were able to undergo cell divisions. The cell (Fig. 1I) is going to produce three daughter cells. Similarly, the cell (Fig. 1J) is going to produce two daughter cells. The cultured petriplates were kept on orbital shaker to observe the growth efficiency of cells and a growth curve was plotted in Fig. 2. The results presented in the growth curve, indicate that the three varieties,

Table 1: Effect of different concentrations of auxin (2, 4-D, NAA and IAA) and cytokinin (BAP) singly or in combination employed in MS medium on callus induction (Each treatment consisted of 20 explants)

		Using varieties								
		Loda			China			Jhotika		
Growth regulators	Concentration of growth regulators (mg L^{-1})	Percentage of explants responded	Weight/ callus (g)	Nature of callus	Percentage of explants responded	Weight/ callus (g)	Nature of callus	Percentage of explants responded	Weight/ callus (g)	Nature of callus
2,4-D	0.01	19	0.20	SW	20	0.26	SW	21	0.28	SW
	0.05	22	0.33	SW	24	0.32	SW	24	0.34	SW
	0.10	32	0.60	SW	31	0.61	SW	35	0.63	SW
	0.50	36	0.68	SW	38	0.72	SW	39	0.71	SW
	1.00	78	0.96	SW	80	0.98	SW	74	0.88	SW
	2.00	91	1.20	SW	93	1.26	SW	92	1.18	SW
	3.00	72	1.06	SW	76	1.20	SW	68	1.01	SW
	5.00	70	1.12	SW	70	1.00	SW	70	1.10	SW
NAA	0.01	31	0.65	SG	32	0.62	SG	28	0.52	SG
	0.05	36	0.68	SG	35	0.68	SG	38	0.64	SG
	0.10	43	0.75	SG	44	0.70	SG	45	0.71	SG
	0.50	64	0.90	SG	62	0.91	SG	63	0.86	SG
	1.00	76	1.10	SG	78	1.05	SG	75	0.99	SG
	2.00	100	1.50	SG	100	1.45	SG	92	1.40	SG
	3.00	83	1.90	SG	81	1.10	SG	88	1.21	SG
	5.00	81	1.00	SG	76	1.00	SG	84	1.18	SG
IAA	0.01	20	0.19	HW	21	0.20	HW	23	0.21	HW
	0.05	34	0.31	HW	35	0.32	HW	38	0.42	HW
	0.10	48	0.41	HW	40	0.36	HW	43	0.48	HW
	0.50	52	0.49	HW	56	0.48	HW	54	0.52	HW
	1.00	63	0.61	HW	64	0.51	HW	67	0.62	HW
	2.00	72	0.72	HW	78	0.76	HW	76	1.00	HW
	3.00	71	0.81	HW	71	0.73	HW	72	0.78	HW
	5.00	71	0.82	HW	68	0.71	HW	71	0.78	HW
2,4-D+BAP	1.0+0.05	66	0.72	SW	68	0.88	SW	67	0.86	SW
	2.0+0.05	78	0.93	SW	76	0.90	SW	74	0.90	SW
	3.0+0.05	75	0.89	SW	57	0.85	SW	55	0.85	SW
	1.0+0.10	55	0.99	SW	51	1.02	SW	55	0.99	SW
	2.0+0.10	60	1.01	SW	66	1.01	SW	66	1.05	SW
	3.0+0.10	52	1.50	SW	48	0.81	SW	44	1.02	SW
	2.0+0.5	44	0.70	SW	50	0.78	SW	50	0.87	SW
NAA+PAB	1.0+0.05	67	0.98	SG	72	1.00	SG	73	1.01	SG
	2.0+0.05	100	1.16	SG	100	1.20	SG	100	1.18	SG
	3.0+0.05	81	0.91	SG	84	0.96	SG	82	1.00	SG
	1.0+0.10	69	0.82	SG	82	0.90	SG	82	0.95	SG
	2.0+0.10	76	0.82	SG	83	1.00	SG	75	0.92	SG
	3.0+0.10	65	1.00	SG	76	0.98	SG	81	0.89	SG
	2.0+0.5	75	0.87	SG	75	0.92	SG	78	0.91	SG
IAA	1.0+0.05	63	0.37	HG	65	0.30	HG	60	0.71	HW
	2.0+0.05	76	0.43	HG	72	0.41	HG	71	0.55	HW
	3.0+0.05	72	0.55	HG	60	0.55	HG	70	0.45	HW

SW: Spongy white ; HW: Hard white; SG: Spongy green and HG: Hard green

Loda, China and Jhotika showed the similar trend of cell growth in the treated medium composition and their highest growths were found within 4-6 days after incubation. The growth of cells of Loda and China varieties were stopped after 14 days while the growth of cells of Jhotika variety was stopped after 16 days. But when the cultured petriplates were kept at stationary position at 25°C in dark to obtain calli, the cells of the three varieties, Loda, China and Jhotika kept growing gradually on MS liquid media containing different concentrations of 2,4-D, NAA and IAA with different concentrations of BAP. Loda variety showed the highest (8%) callusing rate on MS medium supplemented with 2.0 mg L^{-1} NAA+0.05 mg L^{-1} BAP while China and Jhotika varieties showed the highest callusing rate

Fig. 1: A-V show the pathway of complete plant production through cell culture; A, B and C are 8 day old calli; D, E, F are embryogenic calli of Loda China and Jhotica varieties respectively; G and H are isolated cells; I, J and K show the micro calli of Loda, China and Jhotica respectively derived from isolated cell; L, M and N indicate the calli with embryos derived from micro calli; O and P are embryos; Q and R show shoot formation in shooting medium; S and T show root formation in rooting medium; U is a plant in natural condition and V is a complete plant with fruits; W and X are *in vitro*

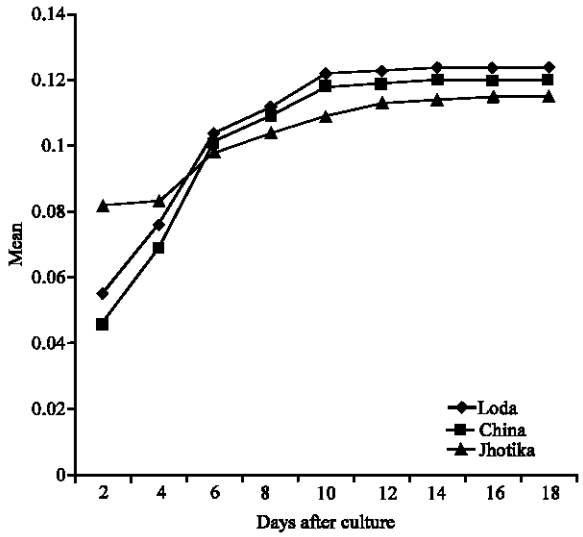

Fig. 2: Graph showing the cell growth in liquid MS medium

Table 2: Effect of auxin in combination with cytokinin (BAP) employed in MS liquid medium on callus induction from isolated cells

		Used varieties								
		Loda			China			Jhotika		
Growth regulators	Concentration of growth regulators (mg L^{-1})	Percentage of cell responded	Weight/ callus (g)	Nature of callus	Percentage of cell responded	Weight/ callus (g)	Nature of callus	Percentage of cell responded	Weight/ callus (g)	Nature of callus
2,4- D+BAP	1.0+0.05	4.7	0.63	SW	5.2	0.63	SW	6.3	0.69	SW
	2.0+0.05	7.0	0.67	SW	8.2	0.65	SW	7.0	0.71	SW
	3.0+0.05	5.7	0.52	SW	5.0	0.60	SW	5.4	0.63	SW
	1.0+0.10	6.0	0.52	SW	6.1	0.56	SW	5.3	0.60	SW
	2.0+0.10	6.4	0.50	SW	5.6	0.57	SW	5.4	0.55	SW
	3.0+0.10	6.2	0.43	SW	5.4	0.53	SW	5.1	0.51	SW
	2.0+0.5	4.5	0.47	SW	6.2	0.51	SW	5.0	0.55	SW
NAA+BAP	1.0+0.05	7.8	0.62	SG	6.8	0.76	SG	6.7	0.71	SG
	2.0+0.05	8.0	0.77	SG	5.8	0.81	SG	7.7	0.77	SG
	3.0+0.05	6.1	0.70	SG	5.7	0.63	SG	6.2	0.68	SG
	1.0+0.10	5.7	0.58	SG	5.7	0.45	SG	6.2	0.63	SG
	2.0+0.10	5.6	0.63	SG	4.8	0.40	SG	5.7	0.60	SG
	3.0+0.10	5.8	0.45	SG	4.8	0.34	SG	5.3	0.50	SG
	2.0+0.5	4.4	0.40	SG	4.7	0.30	SG	5.3	0.47	SG
IAA+BAP	1.0+0.05	5.4	0.34	HG	4.9	0.57	HG	4.8	0.20	HW
	2.0+0.05	6.0	0.38	HG	5.2	0.51	HG	5.0	0.42	HW
	3.0+0.05	4.9	0.30	HG	4.0	0.57	HG	4.8	0.48	HW
	1.0+0.10	4.1	0.29	HG	4.0	0.54	HG	4.1	0.31	HW
	2.0+0.10	4.0	0.31	HG	5.0	0.39	HG	4.3	0.39	HW
	3.0+0.10	3.4	0.28	HG	3.2	0.47	HG	5.0	0.32	HW
	2.0+0.5	3.7	0.28	HG	3.2	0.35	HG	4.8	0.30	HW

SW: Spongy white; HW: Hard white; SG: Spongy green and HG: Hard green

were 8.2% and 7.7 on the MS medium supplemented with 2.0 mg L^{-1} 2,4-D+0.05 mg L^{-1} BAP and 2.0 mg L^{-1} NAA+0.05 mg L^{-1} BAP, respectively. Among these three combinations (2,4-D+BAP, NAA+BAP and IAA+BAP) of growth regulators, IAA+BAP showed the lower callusing rate than 2,4-D+BAP and NAA+BAP (Table 2) but all produced calli were found embryogenic.

Table 3: Effect of different concentrations of auxin (2,4-D, NAA and IAA) and cytokinin (BAP) on somatic embryo formation from calli derived from isolated cells

Combination of growth regulators used in mg L^{-1}		Loda		China		Jhotika	
Pre-culture (63 days)	Sub-culture (26 days)	Days of embryo initiation	No. of embryos/ callus	Days of embryo initiation	No. of embryo callus	Days of embryo initiation	No. of embryos/ callus
2.0 NAA+0.05 BAP	1.0 NAA+0.05 BAP	23-27	15	21-23	13	18-21	11
	2.0 NAA+0.05 BAP	20-22	17	18-19	16	22-25	9
	2.0 NAA+1.0 BAP	-	-	-	-	-	-
	1.0 BAP+0.05 GA_3	20-21	21	13-14	12	23-26	13
	1.0kin+0.05 GA_3	-	-	-	-	-	-
2,4-D+0.05 BAP	1.0NAA+0.05 BAP	18-20	12	25-16	10	22-24	11
	2.0NAA+0.05 BAP	15-16	15	21-22	13	20-25	14
	2.0NAA+1.0 BAP	-	-	-	-	-	-
	1.0BAP+0.05 GA_3	12-13	18	19-20	11	24-25	13
	1.0kin+0.05 GA_3	-	-	-	-	-	-

Table 4: Effect of different concentrations of NAA, BAP and IBA singly or in combination employed in MS medium on shoot and root formation from regenerated embryos

	Loda		China		Jhotika	
Growth regulators and concentration	Percentage of shoots	Percentage of roots	Percentage of shoots	Percentage of roots	Percentage of shoots	Percentage of roots
NAA+BAP	-	-	-	-	-	-
2.0+0.05	-	-	-	-	-	-
2.0+0.1	-	-	-	-	-	-
2.0+0.5	-	-	-	-	-	-
2.0+1.0	-	-	-	-	-	-
2.0+2.0	-	-	-	-	-	-
2.0+3.0	-	-	-	-	-	-
2.0+5.0	-	-	-	-	-	-
1.5+0.5	35	-	32	-	25	-
1.0+1.0	62	-	55	-	49	-
0.75+1.5	76	-	62	-	70	-
0.50+2.5	53	-	51	-	57	-
0.10+3.0	27	-	39	-	18	-
NAA+IBA						
2.0+0.5	-	85	-	82	-	76
2.0+1.0	-	77	-	71	-	72
2.0+1.5	-	65	-	60	-	65
2.0+2.0	-	53	-	55		52
2.0+3.0	-	-	-	-	-	-
IBA						
1.0	-	-	-			-
2.0	-	-	-	-	-	-
3.0	-	-	-	-	-	-

The embryogenic calli, derived from isolated cells were transferred to the MS solid medium containing different hormone of different concentrations for embryo formation and maturation. Among the three varieties, Loda variety showed the highest rate (21%) of embryo formation in MS medium containing 1.0 BAP+0.05 GA_3 while China and Jhotika varieties showed the highest rate (16 and 14, respectively) of embryo formation in 2.0 NAA+0.05 BAP (Table 3). But no variety produced any embryo in 2.0 NAA+1.0 BAP, 1.0 Kin+0.05GA_3, 2.0 NAA+1.0 BAP and 1.0 Kin+0.05 GA_3 All kinds of somatic embryos such as globular, heart-shaped, unipolar and bipolar embryos were observed.

The bipolar embryos were separated by gentle shaking with double autoclaved water and used for shoot and root formation. The embryos were transferred in medium containing different concentrations (0.10-2.0 mg L^{-1}) of NAA with different concentrations (0.05-3.0 mg L^{-1}) of BAP. Brinjal varieties failed to produce any shoot bud from their embryos under the increasing rate of BAP concentrations keeping NAA fixed (2.0 mg L^{-1}). But they started to form shoot buds and many of

them developed into shoots when NAA concentration was decreased. Sarker *et al*. (2006) also reported the similar result in brinjal using Zeatin. But Guri and Sink (1988) reported low regeneration frequencies in eggplant cv. Black Beauty for the production of transgenic shoot using 2.0 mg L^{-1} NAA. However, the percentage of shoot formation was different in different concentrations of auxin with cytokinin. The highest percentage of shoot formation of Loda, China and Jhotika were 76, 62 and 70, respectively in the media with 0.75 mg L^{-1} NAA and 1.5 mg L^{-1} BAP in combination (Table 4). But under the trend of decreasing NAA and increasing BAP from the best performer level (0.75+1.5), the percentage of shoot formation going decreased.

The regenerated shoots were transferred to rooting medium containing different concentrations of IBA singly and with NAA in combination for root induction. No shoots of any variety produced any root in the media containing IBA only. But when the different concentrations of IBA were used with NAA, the shoots of three varieties (Loda, China and Jhotika) produced roots and the highest rooting percentages were 85, 82 and 76, respectively (Table 4). Similar results of increased root formation was also reported by Sarker *et al*. (2000) in brinjal got 90% rooting using 2.0 mg L^{-1} BAP. Increased rate of BAP for enhancing rooting in Malaysian eggplant was reported by Taha and Tijan (2002). After the sufficient development of root the plantlets were taken and transplanted to small plastic pots containing sterilized soil. Plantlets were successfully acclimated with natural condition through gradual increase of duration of exposure to sunlight. The *in vitro* regeneration protocol described here is easily reproducible, requires minimum hormonal supplements. Present protocol can effectively be used for genetic manipulation of brinjal cells by culturing and transferring the genes in cells and protoplasts.

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