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Rapid and efficient plant regeneration of eggplant (*Solanum melongena* L.) from cotyledonary leaf explants

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The aim of this study was to develop an efficient protocol for establishment of plant regeneration through cotyledonary leaf explants of Eggplant (*Solanum melongena* L.). Two varieties of brinjal [Pusa purple long (PPL) and Black beauty (BB)] were used. High frequency and rapid regeneration protocol was developed from cotyledonary leaf explants on MS medium supplemented with either 6-Benzyl amino purine (BAP), Kinetin (Kn), Thidiazuron (TDZ) or Zeatin (Ze). The highest number of shoots (23.3±0.10) was obtained on MS medium containg 2.0 mg/BAP+0.5 mg/L Kn. The *in vitro* regenerated small shoots were further elongated on MS medium supplemented with gibberellic acid (GA_3) at 1.5 mg/L. Elongated shoots were then excised from shoot clumps and transferred to rooting medium containg indole butyric acid (IBA) at 3.0 mg/L. The rooted plantlets were hardened on MS basal liquid medium and subsequently transferred to polycups containg vermiculate:soil:sand (1:2:2). Plantlets, thus developed were successfully established and finally transferred to a greenhouse. The plantlets showed high survival rate (80%) in the soil.

Keywords: Brinjal, cotyledonary leaf, eggplant, plant regeneration, IBA, *Solanum melongena*

Introduction

Eggplant (*Solanum melongena* L.) belonging to the family Solanaceae is grown in the sub-tropics and tropics. It is one of the most popular vegetable crops in many parts of the world including India. The crop is cultivated on small family farms and considered to be important source of nutrition and cash income for many resource poor farmers[1]. Eggplant can be cultivated and grown round the year but the productivity and quality of this crop suffers due to its susceptibility to a number of diseases and insect-pests[2]. In India, it is also used for the treatment of diabetes, bronchitis, asthma, dysuria, dysentery, etc[3]. The progress towards the improvement of this crop for insect-pest and disease resistance and introduction of its new varieties has been hampered mainly due to the wide prevalence of sterility in the progeny and occurrence of genetic incompatibility following intergeneric and interspecific crosses, respectively[4,5]. To overcome such problems of conventional breeding, advanced biotechnological methods such as somatic hybridization and genetic transformation can be applied as an alternative approach for the development of disease-and pest-resistance in this crop[6]. An efficient and reproducible *in vitro* regeneration system is considered as an integral part of successful transformation.

There are some reports available regarding the *in vitro* regeneration of brinjal from different explants via organogenesis[7-10] and somatic embryogenesis[11,12]. But these methods are confronted with problems of low rate of regeneration. In this investigation, we have succeeded in overcoming these problems and report an easy and reproducible protocol using cotyledonary leaf explants of two Indian popular varieties of eggplant.

Materials and Methods

Plant Material

Seeds of eggplant (*Solanum melongena* L.) cultivar Pusa purple long (PPL) and Black beauty (BB) were obtained from the Agriculture Research Station, Gulbarga. They were germinated in pots containg sterilized soil. Cotyledonary leaf from 9-10-d-old seedlings were used as explants for regeneration studies. The cotyledonary leaf explants were surface sterilized with 1% Bavisten for 3-4 min, which was followed by successive washing with distilled water

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four times to make explants free from Bavisten. Then these cotyledonary leaf explants were surface sterilized with 0.1% (w/v) mercuric chloride for 1-2 min and rinsed with sterile distilled water 4-5 times to remove the traces of mercuric chloride. Cotyledonary leaf explants were inoculated on MS medium[13] supplemented with different cytokinins viz., BAP, TDZ, Zeatin and Kn alone or in combination with Kn for induction of multiple shoots. The medium contained 3% sucrose (w/v). The *p*H of the medium was adjusted to 5.8 and solidified with 0.8% agar before autoclaving at a pressure of 1.06 kg cm^2 for 20 min. The cultures were maintained at a temperature of 25±2°C under 16/8 h (light/dark) under 3000 lux intensity provided by white cool florescent light.

The *in vitro* initiated individual shoots (4-5 cm long) were separated and transferred to MS basal medium containg different concentrations of GA_3 for elongation of shoots. These elongated shoots bearing at least 4-5 internodes excised from the mass of proliferated shoots were transferred to rooting media supplemented with different concentrations of NAA and IBA. The rooted plants were taken out from the culture tubes, washed to free agar gel with distilled water and transferred to MS liquid medium for two wks for hardening and subsequently transferred to plastic pots with sterile vermiculate, sand and soil (1:2:2). The plantlets were kept in a polychamber at 80% relative humidity, 32±2°C under a 16 h photoperiod for acclimatization. The plants were given fertilizer with 1/8th MS macronutrients bi-weekly. Established plants were transplanted in earthen pots under natural condition and the survival rate was recorded.

Statistical Analysis

The experimental design was random and factorial with cytokinins as independent variables. The data pertaining to the number of shoots, shoot length and roots were subjected to analysis of variance (ANOVA) test and mean were determined by Duncan's New Multiple Range Test (DNMRT). Twenty five cultures were raised for each treatment and all the experiments were repeated thrice.

Results and Discussion

For induction of multiple shoots, different explants viz., cotyledonary leaf, leaf and hypocotyl explants of eggplant were inoculated on MS medium supplemented with different cytokinins. Among these explants, the cotyledonary leaf explants showed maximum response for shoot initiation (Fig. 1a) and frequency of shoot induction in both varieties of eggplant (Table 1). Several other protocols for plant regeneration via direct and indirect organogenesis have been developed from different eggplant tissues.

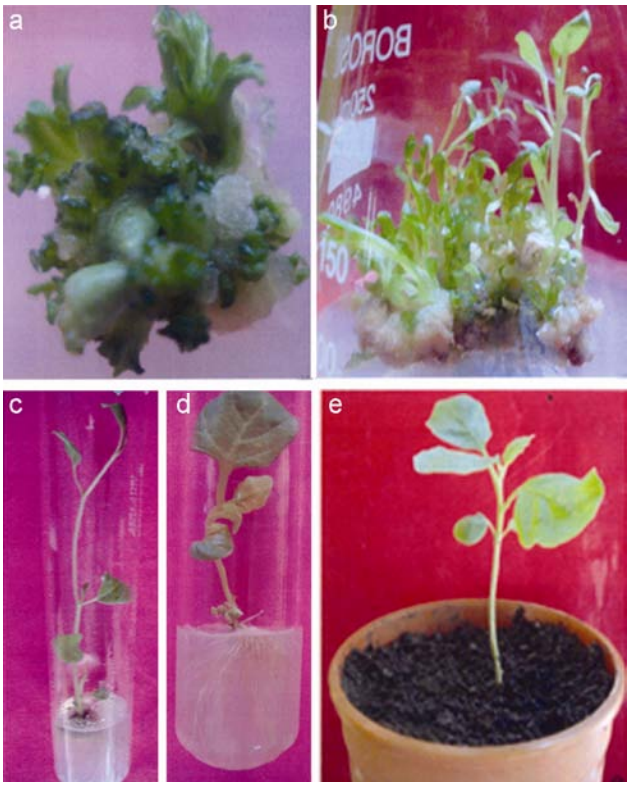

Fig. 1—*In vitro* propagation of eggplant: a. Initiation of multiple shoots from cotyledonary leaf explants on MS medium containing 2.0 mg/L BAP+0.5 mg/L Kn after 12 d of culture; b. Proliferation of shoots from cotyledonary leaf explants on MS medium containing 2.0 mg/L BAP+0.5 mg/L Kn after one month of culture; c. Elongation of *in-vitro* shoots on MS medium supplemented with 1.5 mg/L GA_3; d. Direct rooting from regenerated shoots on MS medium containing 3.0 mg/L IBA after 30 d of culture; & e. Hardened plants in polycups containing vermiculate, sand and soil (1:2:2).

Table 1—Frequency and days taken for initiation of shoots from different explants of eggplant on MS medium

Explants	Initiation of regeneration (d)		Frequency of regeneration (%)	
	PPL	BB	PPL	BB
Cotyledonary leaf	12-13^a	15-16^a	100^a	100^a
Hypocotyl	16-17^c	19-20^c	80^b	60^c
Leaf	14-15^b	17-18^b	100^a	80^b

Data represents average of three replicates; each replicate consists of 25 cultures.
Mean ± Standard error.
Mean followed by the same superscript in a columns is not significantly different at P=0.05 levels.

In these protocols, the regeneration efficiency has been reported to be affected by different factors, such as combination of growth regulators, explants type and genotype. Different sources of explants have been used for induction of organogenesis in eggplant, including hypocotyl, leaf and cotyledon[8,9,14], epicotyl and stem node[9], root[15] and anther[16]. The regeneration efficiency reported in these systems was relatively low and approximately 7 shoots/explant[8,11,17], except in the one described by Sharma and Rajam[8], who achieved the production of 20 shoots/explant in one of the four cultivars studied.

Shoot induction was not found on MS basal medium even after four wks of culture. The explants were found to be swollen and they initiated shoots within two wks after inoculation on MS medium containg different concentrations of BAP, TDZ and Kn and medium with Ze only. Both the varieties responded identically in the same media. Cytokinins especially BAP, were reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation[18]. Hence, different concentrations of BAP, TDZ, Kn and Ze were evaluated on shoot formation. The higher frequency and maximum number of shoots were found on MS medium supplemented with 2.0 mg/L BAP (18.4±0.54) followed by 1.0 mg/L BAP (14.8±0.67) in the PPL cultivar. Similar results were also obtained by others[19,20] in eggplant using different cultivars. Further increase in the concentration of BAP reduced the frequency and number of shoots formation as compared to lower concentration. TDZ responded better to shoot induction as compared to Kn and Zeatin supplemented medium. The lower concentration of TDZ (0.20 and 0.30 mg/L) induced (12.6±0.50 and 13.7±0.45) shoots/explant. Magioli *et al*[9] also reported induction of shoots by using TDZ at lower concentrations and suggested that higher concentration of TDZ inhibited the shoot formation. Compared to all these four growth regulators, BAP induced maximum number of shoots followed by TDZ on cotyledonary leaf explants on MS medium (Table 2). Ze and Kn were not much effective in the formation of shoots in eggplant. In Kn containing medium, the shoot length was more as compared to other growth regulators.

Interaction of Kinetin on Multiple Shoot Induction

It is well established that proper ratio of different cytokinins is necessary for morphogenesis, leading to

Table 2—Frequency, number of shoot and shoot length obtained from cotyledonary leaf explants of eggplant supplemented with different concentrations of cytokinins on MS medium

Concentrations of growth regulators (mg/L)	PPL			BB		
	Frequency (%)	No. of shoots/culture	Shoot length/culture (cm)	Frequency (%)	No. of shoots/ culture	Shoot length/culture (cm)
BAP						
1.0	100	14.8±0.67^{b}	6.2±0.10^{b}	100	8.0±0. 10^{b}	4.3±0. 75^{b}
2.0	100	18.4 ± 0.54^{a}	4.7 ± 0. 97^{d}	100	9.4 ± 0. 00^{a}	4.1 ± 0. 56^{b}
3.0	100	11.3 ± 0.75^{c}	3.2 ± 0. 25^{e}	70	7.0 ± 0. 00^{c}	3.6 ± 0. 00^{c}
4.0	50	6.8 ± 0.50^{e}	2.7 ± 0. 54^{f}	40	4.0 ± 0. 00^{d}	3.0 ± 0. 00^{c}
Kn						
1.0	90	5.4 ± 0.25^{f}	6.9 ± 0.25^{b}	100	5.2 ± 0.50^{d}	5.7 ± 0.44^{a}
2.0	90	6.8 ± 0.45^{e}	8.3 ± 0.33^{a}	80	6.6 ± 0.22^{c}	5.9 ± 0.40^{a}
3.0	60	3.6 ± 0.00^{g}	7.1 ± 0.75^{b}	80	2.3 ± 0.44^{e}	5.2 ± 0.00^{a}
TDZ						
0.20	100	12.6 ± 0.50^{c}	3.2 ± 0.75^{e}	90	8.2 ± 0.35^{b}	3.3 ± 0.15^{c}
0.30	100	13.7± 0.45^{b}	4.2 ± 0.00^{d}	80	9.2 ± 0.97^{a}	4.8 ± 0.20^{b}
0.40	80	10.8± 0.25^{c}	4.3 ± 0.45^{d}	80	6.7 ± 0.43^{c}	3.9 ± 0.58^{c}
0.50	80	8.3± 0.78^{d}	5.6 ± 0.33^{c}	80	4.1 ± 0.70^{d}	3.1 ± 0.44^{c}
Zeatin						
0.20	100	5.2 ± 0.15^{f}	5.2 ± 0.33^{c}	90	4.8 ± 0.10^{d}	4.4 ± 0.70^{b}
0.40	100	7.9 ± 0.20^{d}	6.7 ± 0.87^{b}	70	5.3 ± 0.23^{d}	5.1 ± 0.00^{a}
0.60	100	4.3 ± 0.58^{g}	4.3 ± 0.45^{d}	70	3.6 ± 0.34^{e}	3.8 ± 0.50^{c}
0.80	100	3.8 ± 0.44^{g}	3.3 ± 0.50^{e}	80	2.4 ± 0.66^{e}	3.1 ± 0.35^{c}

Data represents average of three replicates; each replicate consists of 25 cultures.
Mean ± Standard error.
Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

the formation of complete plantlets[21]. The combined effect of BAP and TDZ with different concentrations of Kn on MS medium was studied to determine their effect on regenerating multiple shoots directly from cotyledonary leaf explants. Results of this experiment have been presented in Table 3. In the case of PPL, maximum multiple shoot regeneration was obtained when cotyledonary leaf explants were cultured on MS medium containg 2.0 mg/L BAP +0.5 mg/L Kn with innervate the callus at the base of the plants (Fig. 1b). The addition of Kn increased the frequency and number of multiple shoot induction on MS medium as compared to cytokinin alone tested. The similar results were also reported by Sarker *et al*[19] in other cultivars of eggplant. Among the two cytokinins, combination of BAP and Kn showed better response in terms of number of shoots per explant in both the varieties of eggplant. The plants were very healthy, thick and dark green in colour. The addition of TDZ in Kn medium also increased the number of shoots as compared to TDZ alone medium.

Elongation of Shoots

Separated individual shoots from multiple shoots were transferred on MS medium supplemented with different concentrations of GA_3 (0.5-1.5 mg/L) for elongation of shoots. The highest shoot length (13.3±0.50) with 100% frequency was recorded on medium containing 1.5 mg/L GA_3 (Fig. 1c) followed by 1.0 mg/L GA_3 (Table 4). These results are in agreement with other results[10,22] of shoot bud elongation using GA_3 but our results differed with the results of Frankalin *et al*[15], who reported that hormonal free medium was sufficient for shoot elongation.

Rhizogenesis

Roots were not induced during shoot multiplication in the cytokinins regime. Individual shoots when implanted in half or full strength MS medium free from growth regulators, poor and little numbers of roots were elicited with low frequency. Addition of auxins viz., NAA and IBA on MS medium enhanced the rate of frequency as well as number of roots. Among these two auxins tested, 3.0 mg/L IBA induced maximum number (89.3±0.75) of roots per shoot (Fig. 1d) as compared to other concentrations of IBA and NAA tested (Table 5). Similar results were also reported[10,22,23] but Hossain *et al*[10] reported that there was no root formation on MS medium containg any concentrations of IBA. This result is contrary to our results. The roots formed in IBA were thick, long and dark coloured as compared to NAA.

The rooted shoots were kept for 4-6 wks in the rooting medium and then transferred to MS liquid

Table 4—Effect of GA_3 on *in vitro* shoots elongation in eggplant on MS medium

Concentration of growth regulator (mg/L)	PPL		BB	
	Frequency (%)	Shoot length/ culture (cm)	Frequency (%)	Shoot length/ culture (cm)
GA_3				
0.5	100	8.5 ± 0.33^{c}	100	6.5 ± 0.50^{c}
1.0	100	10.0 ± 0.33^{b}	100	8.3 ± 0.10^{b}
1.5	100	13.3 ± 0.50^{a}	100	10.8 ± 0.15^{a}

Data represents average of three replicates; each replicates consist of 25 cultures.
Mean ± Standard error.
Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table 3—Frequency, number of shoot and shoot length obtained from cotyledonary leaf explants of eggplant supplemented with different concentrations cytokinins along with Kn on MS medium

Concentrations of growth regulators (mg/L)	PPL			BB		
	Frequency (%)	No. of shoots/ culture	Shoot length/ culture(cm)	Frequency (%)	No. of shoots/ culture	Shoot length/culture (cm)
BAP+Kn						
2.0+0.5	100	23.3 ± 0.10^{a}	9.0 ± 0.30^{b}	100	14.0 ± 0.10^{a}	5.3 ± 0.45^{d}
2.0+1.0	100	21.4 ± 0.23^{bc}	9.3 ± 0.67^{b}	100	11.8 ± 0.00^{b}	7.1 ± 0.33^{c}
2.0+1.5	100	17.3 ± 0.65^{c}	11.2 ± 0.25^{a}	100	9.0 ± 0.00^{c}	8.6 ± 0.50^{b}
TDZ+Kn						
0.5+0.5	100	15.1 ± 0.00^{de}	7.1 ± 0.25^{d}	100	11.0 ± 0.20^{b}	5.5 ± 0.75^{d}
0.5+1.0	100	13.6 ± 0.75^{e}	8.2 ± 0.35^{c}	100	9.7 ± 0.75^{c}	8.2 ± 0.10^{b}
0.5+1.5	100	11.3 ± 0.50^{f}	8.9 ± 0.85^{b}	100	9.5 ± 0.33^{c}	9.3 ± 0.86^{a}

Data represents average of three replicates; each replicate consists of 25 cultures.
Mean ± Standard error.
Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table 5—Frequency, number of root and roots response of *in vitro* obtained in eggplant supplemented with different concentration of NAA and IBA on MS medium

Concentrations of growth regulators (mg/L)	PPL		BB	
	Frequency (%)	No. roots/ culture	Frequency (%)	No. roots/ culture
NAA				
1.0	100	22.4 ± 0.45^{f}	100	34.3 ± 0.45^{d}
2.0	100	33.8 ± 0.60^{e}	100	41.3 ± 0.32^{c}
3.0	100	53.6 ± 0.76^{c}	100	48.7 ± 0.54^{c}
IBA				
1.0	100	43.9 ± 0.34^{cd}	100	32.3 ± 0.65^{d}
2.0	100	74.8 ± 0.56^{b}	100	56.8 ± 0.75^{b}
3.0	100	89.3 ± 0.75^{a}	100	62.3 ± 0.86^{a}

Data represents average of three replicates; each replicate consists of 25 cultures.
Mean ± Standard error.
Mean followed by the same superscript in a column is not significantly different at P=0.05 levels

medium for 2 wks. None of the plantlets survived when directly transformed from rooting medium to the pots under natural condition. About 75-80% of the transplanted eggplants survived, if the plants in the culture tubes were kept under normal room temperature for 7-8 d before transplantation in pots and reared for three wks (Fig. 1e). The plants were reared under semi-controlled temperature (32±2°C) and light (2000 lux) in a chamber with 80% humidity. During this period of acclimation the shoots elongated, leaves expanded and turned deep green looking good and healthier.

After 3 wks, plants were transferred to an open place and gradually acclimated to outdoor condition, where 80% of plants survived and they produced flower and fruit. The technique described here appears to be readily adaptable for large-scale clonal propagation and the genetic transformation studies.

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