

Tissue culture and genetic analysis of somaclonal variations of *Solanum melongena* L. cv. Nirrala

Research Article

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Abstract: The present study was designed to analyze genetically somaclonal variants using biochemical and molecular markers. Efficient tissue culture protocol for *Solanum melongena* L. cv. Nirrala was developed. Maximum callus induction (100%) was observed for Murashige and Skoog (MS) media supplemented with 2.0 mg L⁻¹ naphthalene acetic acid + 0.5 mg L⁻¹ 6-benzylaminopurine; and nodal explants gave best callusing response (88.8%) as compared to internodes (88.3%) and leaves (87.7%). The best shooting was induced on nodal and internodal callus in the presence of 2.0 mg L⁻¹ 6-benzylaminopurine. Total soluble protein content of callus and regenerated variant plants was estimated for biochemical analysis, and largest amount of soluble protein was found in callus (6.54 mg g⁻¹ fresh tissue) followed by variant plant grown on 2.0 mg L⁻¹ 6-benzylaminopurine (5.96 mg g⁻¹ fresh tissue). Random amplification of polymorphic DNA technique was done with five decamer primers (OPC1-OPC5) and maximum polymorphism was detected by OPC 2 (26.99%) among all samples, whereas nodal callus on media containing 1.0 mg L⁻¹ naphthalene acetic acid + 1.0 mg L⁻¹ 6-benzylaminopurine showed highest polymorphism producing 22 bands, out of which 8 bands were polymorphic. The study shows that this marker system can provide better evaluation of genetic variation induced by tissue culture.

Keywords: Brinjal • Genetic Variation • Micropropagation • Protein

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Abbreviations

BAP – 6-Benzylaminopurine;
CTAB – Cetyltrimethylammonium bromide;
2,4-D – 2,4- dichlorophenoxyacetic acid;
KN – kinetin;
MS – Murashige and Skoog;
NAA – Naphthalene acetic acid;
TAE – tris acetate EDTA.

1. Introduction

Solanum melongena L. of family Solanaceae is an economically important and one of the popular vegetable

crops of tropical, subtropical and temperate regions of the world [1]. More than 4,000,000 acres (1,600,000 ha) are devoted to the cultivation of eggplant in the world [2]; it represents an economically and nutritionally important crop in Asia and southern Europe [3]. It is mainly cultivated on small scale, but is a potential source of income for farmers in Pakistan [4]. Important commercially grown varieties of *Solanum melongena* in Pakistan are Dilnasheen, Qaiser, Purple Queen, Cluster king, Bemissal and Nirrala [5]. It has limited nutritional value, but the presence of considerable amount of good fiber, proteins, carbohydrates, fats, and various vitamins and minerals, makes its total nutritional value comparable with that of tomato [6].

Due to the high morphogenetic potential of eggplant, the application of various *in vitro* methods

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have been a great success and several protocols of *in vitro* regeneration have been developed using various explants such as [7-11], hypocotyl [8,11,12], leaf [8,10,13], root [6,8,14], shoot tip [7,12], midrib [12], epicotyl and stem nodes [13]. Similarly, various agronomic traits of valuable importance in eggplant have been selected on the basis of *in vitro* regeneration systems, such as resistance to nematodes and atrazine was obtained from protoplast fusion [15,16] and *Fusarium* resistance was achieved from anther cultures [17]. Genetic variation occurs in plants during *in vitro* regeneration of plants from callus cultures. The term 'somaclonal variation' has been coined to describe these changes [18]. Due to the high stability and heritability, somaclonal variation has proved to be a problem for *in vitro* propagation and genetic transformation of plants by producing undesirable changes in plant cultures [19]. Despite the limitations associated with somaclonal variation, it is considered to be an additional tool for crop improvement [20]. The frequency of somaclonal variation is influenced by many factors such as genotype, explants origin, hormone composition, cultivation period, numbers of subcultures and culture conditions [21]. The somaclonal variation from *in vitro* derived plants can be evaluated by phenotypic or cytogenetic analysis, study of proteins, DNA of plant or isoenzyme markers [22], but many of them have limitations.

Biochemical markers are also a good tool to analyze somaclonal variation as these are based on the variability of proteins and isozymes. These have proved to be effective in the assessment of somaclonal variations in *Stevia rebaudiana* (Bertoni) Bertoni, [23], *Mandevilla velutina* (Mart. Ex Stadelm) and many other plants. However, due to the less sensitivity of secondary metabolites and isozymes, biochemical markers have less preference to analyze the variations in plant cultures [24]. The molecular markers like Random Amplified Polymorphic DNA (RAPD) [25] generally assess even small variations of the genome, and have been frequently used in recent studies to detect the somaclonal variation in a number of plant species including potato [26], costmary [27], date palm [28], and olive [29]. However, very limited number of reports is available regarding the use of RAPD markers for the assessment of somaclonal variations in eggplant. Rotino *et al.* [30] reported that the main cause of somaclonal variation in eggplant was hormone concentration in culture media. In a study, Zayova *et al.* [31] assessed the somaclonal variation by the analysis of phenotypic variation of regenerated plants of *Solanum melongena*. Whereas, Ferdausi *et al.* [12] has used the biochemical markers to select the eggplant genotypes tolerant to brinjal shoot and root borer insects.

The main objective of the present study was to establish and characterize an efficient callus culturing and regeneration protocol for *Solanum melongena* cv. Nirrala and to determine the somaclonal variations on the basis of different hormonal concentrations used for *in vitro* propagation of callus, using biochemical and molecular markers.

2. Experimental Procedure

2.1 Plant material and experimental design

Seeds of Nirrala variety of *Solanum melongena* were collected from the National Agriculture Research Centre (NARC), Islamabad, Pakistan. For germination, seeds were dipped in tap water for about 24 hours to break dormancy. Later the seeds were dipped in 70% ethanol for 1 minute followed by washing with distilled water. Then the seeds were surface sterilized with 15% commercial bleach for 10 minutes and finally washed three times with sterile distilled water to remove the bleach. Whole procedure of surface sterilization was kept inside a laminar flow hood. The surface sterilized seeds were then inoculated into glass bottles (10-12 seeds in each) containing 50 ml MS media [32] supplemented with 3% (wt/vol) sucrose for germination. The pH of media was adjusted to 5.8 with the use of 1 M L⁻¹ NaOH and 0.5 M L⁻¹ HCl before adding Gellan gum powder (Culture Gel, Phytotechnology laboratories, Shawnee Mission, KS) at a concentration of 2 g L⁻¹. The media was autoclaved at 120°C and 15 lbs pressure for 15 min. The glass bottles containing seeds were kept in a growth chamber (K & K, K-HB301S-3, Korea) at 16-hrs photoperiod, 55±5 relative humidity at 25±1°C and light intensity of 1000 lux.

2.2 Culture medium

For callus induction full strength MS medium was used supplemented with 17 different concentrations and combinations of BAP, 2, 4-D and NAA (Table 1). After about 30 days of seed germination, different explants like leaves, nodes and internodes were cut into rectangular shaped small pieces (10-15 mm) with the use of sterilized surgical blade. These were then inoculated onto callus induction media under aseptic conditions. The cultures were kept inside growth chamber for callus induction under the same conditions as described above. In order to increase the callus induction rate the explants were sub-cultured on the similar medium after 30-35 days. The quantitative measurement of callus growth was

Table 1. Explant response, color and texture of callus on callus induction media supplemented with different phytohormones.

Plant growth regulator	Concentration (mg/l)	Media type	Explant used										Mean % response		
			Leaves			Nodes			Internodes						
			% response	Size	Texture	Color	% response	Size	Texture	Color	% response	Size	Texture	Color	
-	-	MS	0	-	-	-	60	+++	Fragile	Creamy brown	70	+	Compact	Brown	43.3
BAP	0.5	C1	100	+++	Compact	Yellowish green	100	+++	Compact	Creamy brown	100	+++	Compact	Light green	100
	1.0	C2	100	+++	Compact	Dark green	80	+++	Compact	Dark green	100	+++	Compact	Dark green	93.3
	2.0	C3	100	++++	Compact	Dark green	100	++++	Compact	Dark green	100	++	Compact	Brownish green	100
	0.5	C4	100	++++	Fragile	Yellowish green	100	++++	Fragile	Yellowish green	80	++	Fragile	Pale yellow	93.3
NAA	1.0	C5	100	+++	Fragile	Yellowish green	80	+++	Fragile	Light brown	70	++	Fragile	Light brown	83.3
	2.0	C6	100	++++	Fragile	Pale yellow	100	++++	Fragile	Pale yellow	90	+	Fragile	Light green	96.6
	0.5	C7	100	++++	Fragile and watery	Pale yellow	100	++	Fragile and watery	Pale yellow	100	++	Fragile and watery	Light green	100
	1.0	C8	100	+++	Fragile and watery	Light green	100	+++	Fragile and watery	Pale yellow	100	+++	Fragile and watery	Pale yellow	100
2,4-D	2.0	C9	100	++	Fragile and watery	Pale yellow	100	+	Fragile and watery	Pale yellow	100	+	Fragile and watery	Pale yellow	100
	1.0+0.5	C10	100	+++	Compact	Light green	60	+++	Slightly compact	Light green	100	++	Slightly compact	Yellow	86.6
	1.0+1.0	C11	90	+++	Compact	Light green	100	+++	Slightly compact	Light green	70	++	Slightly compact	Light green	86.6
	2.0+0.5	C12	100	+++	Fragile	Light green	100	+++	Fragile	Light green	100	+++	Fragile	Light green	100
NAA+BAP	2.0+1.0	C13	80	++	Slightly compact	Yellowish green	100	++	Slightly compact	Yellowish green	70	++	Slightly compact	Yellowish green	83.3
	1.0+0.5	C14	70	++	Fragile	Light green	60	+++	Slightly compact	Yellow	90	+	Fragile	Yellow	73.3
	1.0+1.0	C15	70	+++	Slightly compact	Yellowish green	80	+++	Slightly compact	Pale yellow	100	+	Fragile	Pale yellow	83.3
	2.0+0.5	C16	70	+	Fragile	Light green	100	+++	Slightly compact	Yellowish green	50	+++	Slightly compact	Light brown	66.6
2,4-D+BAP	2.0+1.0	C17	100	+++	Slightly compact	Light brown	80	++	Slightly compact	Light brown	100	+++	Slightly compact	Pale yellow	93.3

estimated in terms of percentage of callus induction and degree of callus growth on a regular basis.

The healthy calli were transferred to shooting media supplemented with different combinations and concentration of BAP and KN (kinetin) for shoot initiation. The number of shoots produced by explants was counted and it was expressed as an average number of shoots per explants. The *in vitro* initiated shoots (5-6 cm long) were separated and transferred to rooting medium for root initiation. The rooting media was either full strength MS alone or supplemented with different concentration of NAA.

2.3 Molecular analysis

Total genomic DNA from young leaves and calli formed on different hormonal concentrations and combinations was extracted using CTAB (Cetyl trimethyl ammonium bromide) method [33], with few modifications. The quality and integrity of extracted DNA samples was assessed by running them in 1% agarose in 0.5 x TAE (tris acetate EDTA) buffer. The gel was visualized under ultraviolet (UV) light and photographed using gel documentation system (Dolphin Doc ^{plus}, Dolphin-IDV 2.6, Wealtec). Five primers of OPC series (OPC 1-OPC 5) were tested in RAPD analysis to analyze the genetic variation among the sample plants (Table 2). The genomic DNA of leaves and callus was amplified by selected primers in 25µl reaction mixture containing 25-50 µg µL⁻¹ DNA, 25 pmol primer (e-oligos, Gene link), 12.5 µl 2x PCR master mix (K0171, Fermentas) and 10.5 µl of PCR water (R0581, Fermentas) using thermal cycler (Labnet, multigene II, Labnet international, Inc, Woodbridge, N.J). PCR conditions were optimized by increasing the annealing temperature in 0.5°C increments of amplification, and the conditions employed for amplification were as follows: initial denaturation at 94°C for 30 sec, annealing at 36°C, 36.5°C, 37°C, 37.5°C, 38°C, 38.5°C, 39°C, 39.5°C and 40°C for 1 minute, and extension at 72°C for 2 minutes, final extension for 7 minutes at 72°C. Amplified products were run on 1.5% agarose gel in 0.5X TAE buffer. Gel was stained in ethidiumbromide (0.1 mg/10ml), and was visualized by gel documentation system. The size of

each band was estimated by using 100bp DNA ladder plus (SM0302, Gene ruler, Fermentas).

2.4 Biochemical analysis

Total soluble proteins of variants including callus sample and leaves of regenerated plants were analyzed both quantitatively and qualitatively. From the 4 week old regenerated and control plants, 0.5 g fresh leaves were taken. Similarly, 0.5 g of 4 week old callus sample used for shoot induction was also taken for protein extraction. These plant materials were crushed in liquid nitrogen in a mortar by pestle containing 1.0 ml of 0.1 M L⁻¹ TrisHCl buffer. The obtained slurry was centrifuged at 12,000 rpm for 10 minutes at 4°C in a refrigerated eppendorf centrifuge (Bench top centrifuge, K3 series, Centurion scientific Ltd, UK). The supernatant was stored at -20°C and was used for further estimation. Biuret method of Roenson and Johnson [34] was adopted for the estimation of total soluble proteins in plant samples. For the estimation of proteins, samples were prepared as follows:

Sample tubes	Protein extract	Biuret reagent	Distilled water
Experimental	0.1 ml	1.0 ml	-
Control	-	1.0 ml	0.1 ml

Both experimental and control tubes were vigorously shaken and allowed to stand for 30 minutes at room temperature. The optical density of both samples was measured at 545nm on a spectrophotometer. The amount of proteins was calculated from standard curve for proteins prepared from BSA (bovine serum albumin). Qualitative analysis of proteins was carried out on SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) with 7.5% polyacrylamide gel following the procedure of Laemmle [35] in an electrophoresis apparatus (Claever, Scientific Ltd).

3. Results and Discussion

Germination of seeds was observed within 10-15 days of culture and no sign of contamination was observed. The seeds developed into well-established plants within six to eight weeks. Each plant had a well developed root system, attained the height of up to 15-20 cm, having 5-6 nodes. Eighty percent of seeds responded and developed shoots together with roots.

Table 2. Primer series for RAPD analysis.

Sr. No.	Primer	Base sequence(5'-3')
1.	OPC1	TTCGAGCCAG
2.	OPC2	GTGAGGCGTC
3.	OPC3	GGGGGTCTTT
4.	OPC4	CCGCATCTAC
5.	OPC5	GATGACCGCC

3.1 Callogenesis

Callus formation is a process which depends not only on cultivation conditions, but also on the genotype of the investigated plant material [36]. In our study callus was induced on all the explants (leaf, nodes and internodes) and on all the media used with different induction percentage (Table 1). Among all the media used, MS media without any growth regulator induced callus (43.3%) only on nodal and internodal explants. Earlier Ray *et al.* [37] also tried MS medium without any phytohormone for callus induction of stem, root and leaf explants of eggplant, but explants cultured on this medium did not produce any callus. In the other report Kaur *et al.* [38] has studied the effect of media composition and explant type on the regeneration potential of three brinjal genotypes and found that hypocotyl induced highest percent callusing, but cotyledon showed best results for somatic embryogenesis on all media compositions.

3.2 Callus initiation period

The time of callus induction is different in different varieties of *Solanum melongena*; it ranges from 10-20 days in case of Loda variety [39]; 1 week for Kalpatru, and Rohinivarities [40]. In case of Nirrala variety, the callus induction occurred within the period of 5 to 18 days, and a mass of callus was formed after 4 to 5 weeks. It was observed that callus induction was slow in media having low concentration of BAP (8-18 days), but by increasing concentration of BAP callus induction occurred after 7-14 days. However, quicker callus induction was observed in media supplemented with NAA and 2,4-D (5-10 days) whereas the callus induction time varied from 5-15 days in media having combinations of BAP, NAA and 2,4-D.

3.3 Color and texture of callus

The effect of different hormones on the color and texture of callus was observed on regular basis during the course of study; and in the presence of a particular hormone color and texture of callus was observed (Table 1). It was found that media having BAP alone produced hard and compact callus that varied from dark green to brownish green in color; whereas media having NAA alone produced fragile and soft callus that varied from pale yellow to light green in color. Highly fragile, watery and pale yellow to light green callus was produced on media having different concentrations of 2,4-D (Table 1). Earlier, Anwar *et al.* [41] obtained the greenish callus in the presence of NAA, and fast growing green

callus in the presence of 2,4-D, from the leaf explants of eggplant. However, the effect of these hormones on the color and texture of callus is different for different varieties. Rahman *et al.* [39] obtained spongy white or hard white callus from the cotyledon explants of Loda variety in the presence of NAA, 2,4-D, and 2,4-D+BAP; whereas in case of combination of BAP and NAA, fragile green callus was obtained. In the present experiment, the media having combinations of auxins and cytokinins gave a mixed response; such as media having different combinations of NAA and BAP produced slightly compact and light green to yellowish green callus, whereas media having different combinations of 2,4-D and BAP produced fragile to slightly compact and light brown to yellowish green callus (Table 1).

3.4 Effect of explants on callogenesis

It has been shown by the previous reports on *Solanum melongena* that callus induction and regeneration is significantly affected by the type of explants used and the developmental stage of the seedlings [40,42]. In the present study it was found that the callus induction potential varied in different parts of used plant. Efficient callus induction occurred at stem nodes (88.8%) as compared to internodes (88.3%) and leaves (87.7%) (Figure 1). Variation in the callus induction potential of these explants may be due to the difference of their cellular totipotency [43]. Furthermore, the use of younger tissues significantly increases the frequency of callus induction thus indicating that the process is age-dependent [42]. Similarly in the present study it was observed that the young and green explants were more efficient and frequent in callus formation as compared to old, diseased and spotted explants.

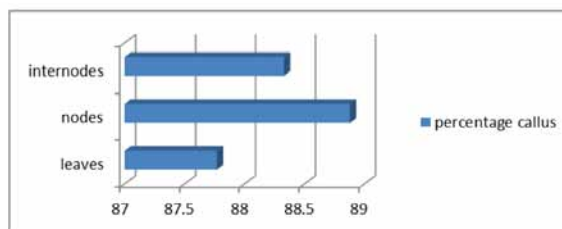


Figure 1. Overall percent callus response of different explants. Leaf, 87.7%; Nodes, 88.8%; and Internodes 88.3%.

3.5 Callus induction percentage

Among all the media types, the lowest callus induction of 66.6% was found on medium C16 (2 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BAP); whereas 100% callus induction was observed on media C1 (BAP 0.5 mg L⁻¹), C3 (BAP 2.0

mg L⁻¹), C7 (0.5 mg L⁻¹ 2,4-D), C8 (1.0 mg L⁻¹ 2,4-D)s, C9 (2.0 mg L⁻¹ 2,4-D), and C12 (2.0 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP) respectively (Figure 2, Table 3). However, due to the slow growth rate and relatively small size of callus in media C1 and highly fragile and watery nature of callus on media C7, C8, and C9 these media were found to be not effective for the callus induction. Similarly, direct regeneration was observed during the course of callus development on media C3 (Figure 3A). Chkravarthi *et al.* [44] also found that the concentrations of BAP ranging from 0.5-3.5 mg L⁻¹ induced direct regeneration instead of callus induction. Whereas the medium C12 supplemented with 2.0 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP was found to be quite effective for the proliferation of callus as compared to other media types (Figure 3B-C). Present results are in accordance with Zayova *et al.* [42] They also found that MS medium supplemented with NAA in concentration of 2.0 mg L⁻¹ and BAP 0.5 mg L⁻¹ was very effective in callus induction in two eggplant cultivars; LargaNegra and Black Beauty.

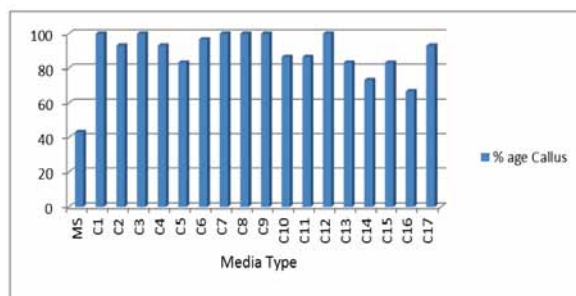


Figure 2. The callus induction percentage on different media. MS without any phytohormone, 43.3%; C1, 100%; C2, 93.3%; C3, 100%; C4, 93.3%; C5, 83.3%; C6, 96.6%; C7, 100%; C8, 100%; C9, 100%; C10, 86.6%; C11, 86.6%; C12, 100%; C13, 83.3%; C14, 73.3%; C15, 83.3%; C16, 66.6%; and C17, 93.3%.

3.6 Organogenesis

After about 30-35 days, the best calli from each explant *i.e.*, leaf, node and internode from callus induction media were transferred into shooting medium supplemented with different concentration of BAP and KN (Table 3). Shoot formation occurred after 35-50 days of incubation on nodal and internodal calli, whereas no shooting was observed on leaf derived calli. Previous studies on the organogenesis of eggplant show that the most responsive explants for indirect regeneration are leaves and hypocotyls [45, 46]. Present results are contradictory to their results as no regeneration was observed for leaf explants. Earlier it has been shown that the increase in the concentration of KN and BAP can increase the number of shoots [44]. Similar behavior was observed in the present study when BAP was used. It was noted that an increase in concentration of BAP increased the rate of development and number of shoots. Moreover, it was also observed that media supplemented with BAP were found to be quite effective for the proliferation of shoots and highest number of shoots (5.5 per explant) was induced on medium supplemented with 2.0 mg L⁻¹ BAP from nodal callus, and differentiated shoots reached up to 9.3 cm in length. The response of internodal callus on same regeneration medium was also quite good with 3.5 shoots per explant reaching up to the length of 1.6 cm. Media supplemented with 0.5 mg L⁻¹ and 1.0 mg L⁻¹ BAP also responded well for shooting giving the total induction percentage of 90% for both nodal and internodal calli (Figure 3D-E). Similar results were also found by Zayova *et al.* [31] on similar media for nodal explants. Recently Bhat *et al.* [47] achieved *in vitro* plant regeneration in a brinjal genotype using different explants such as cotyledonary leave, nodal segment and shoot tip. Among all these explants the maximum regeneration efficiency was observed for cotyledonary leaf pair (100%) followed by shoot tip (96.6%) and

Table 3. Shoot induction percentages on different shoot induction media. Any two means sharing a letter in common are not significantly different according to LSD test at the level of P>0.05.

Plant growth regulator	Hormone concentration mg/l	Media type	Nodal callus			Internodal callus		
			No. of shoots	Shoot length (cm)	Percent shooting	No. of shoots	Shoot length (cm)	Shooting %
BAP	0.5	S1	2.5a	3.5b	100a	1a	2.5a	80a
BAP	1.0	S2	4a	5.5b	90b	4.4a	1.5a	90b
BAP	2.0	S3	5.5a	9.3a	100a	3.5a	1.6a	100c
BAP+KN	2.0+1.0	S4	1a	0.75b	25c	-	-	-
LSD			6.25	8.52	5.41	6.03	2.36	1.59

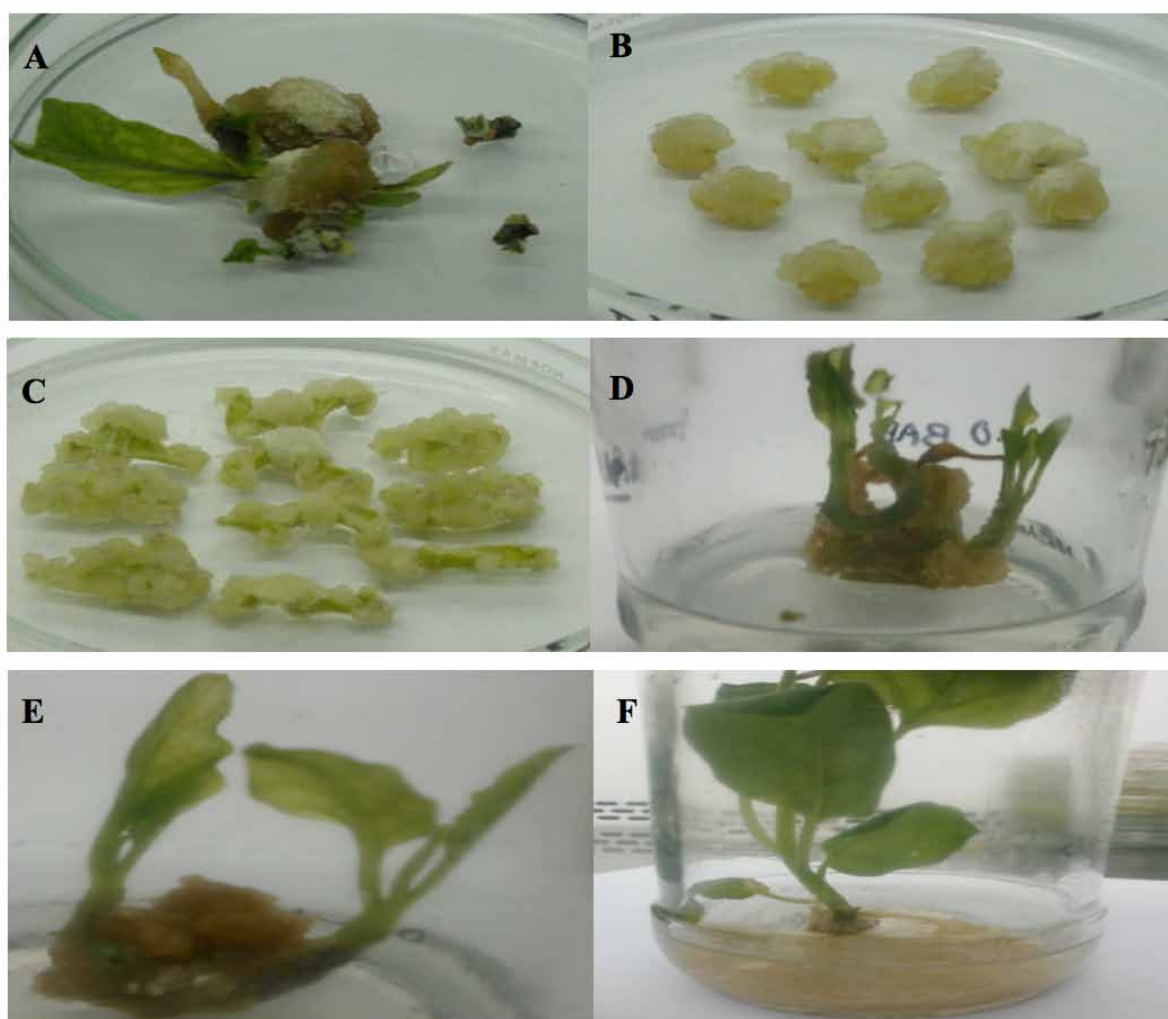


Figure 3. *In vitro* propagation of *Solanum melongena*. A: Direct regeneration from nodal explants on C3 medium containing 2.0 mg/l BAP; B: Light green and fragile callus from internodes on C12 medium containing 2.0 NAA and 0.5 mg/l BAP; C: Light green and fragile callus from the leaf explants on C12 medium; D: Proliferation of shoots on S3 medium containing 2.0 mg/l BAP after 45 days of culture; E: Multiple shoots from internodes on S3 medium containing 1.0 mg/l BAP after 45 days of culture; F: Fully developed roots on MS medium after one month of inoculation.

nodal segment (93.33%) when cultured on MS medium supplemented with 2 mg L⁻¹ BAP and 1 mg L⁻¹ kinetin.

It was reported by Sarker *et al.* [7] that the addition of KN in regeneration medium increases the number of multiple shoots, but our results are in contradictory with their study. It was observed that KN alone did not promote any shooting, even in combination with BAP its shooting efficiency was very low (25%) inducing just one shoot of 0.75 cm length from node derived callus after 45-50 days. It was also noted that hormone concentration, size, color and origin of callus affected the regeneration potential, as reported by Taha and Tijan [48]. The shoot induction percentage was maximum (100%) in medium having high concentration of BAP (2.0 mg L⁻¹), it decreased (up to 80%) with decrease of BAP concentration (0.5 mg L⁻¹). Furthermore, the callus which was young, green in color and large in

size showed rapid rate of regeneration as compared to small sized and old calli. It has been reported earlier that young and green calli have greater organogenic potential in comparison to old calli [31].

After shoot induction, another important step of micropropagation is the successful rooting of regenerated shoots. The differentiated shoots (5-6 cm long) were separated and transferred to rooting medium for root initiation. The rooting media was either MS alone or supplemented with different concentration of NAA (Table 4). Very little or no rooting was observed on media supplemented with NAA. However, best rooting was observed on MS medium without any phytohormone (Figure 3F). Earlier studies on the micropropagation of eggplant also showed that the MS basal medium devoid of any growth regulator promotes the germination of somatic embryos into plantlets with roots [49,50]. Similar

Table 4. Rooting media with different hormonal concentration.

Media code	PGRs	Concentration mg/l
R1	NAA	1.0
R2	NAA	2.0
R3	-	-

results of rooting were obtained by Ferdausi *et al.* [12] as well with an average number of 12-15 roots per plant.

3.7 Biochemical analysis of somaclonal variants of *Solanum melongena* cv. Nirrala

Since the sixties of last century, the biochemical analysis of total soluble proteins and enzymes served as a powerful tool for the analysis of genetic variability of *Solanum* species [51]. In the present study, qualitative analysis of protein content was carried on SDS gel electrophoresis, and proteins were quantified using biuret assay for protein quantification. In our study, increase in protein content was found in callus sample and all variants in comparison with seed derived control plant (Table 5). Highest amount of soluble protein (6.54 mg g⁻¹ fresh tissue) was found in callus sample that was used for regeneration of plantlets. Our findings are in accordance with those of Ali *et al.* [52], they estimated total protein and peroxidase contents in callus cultures of *Stevia rebaudiana*, an important sweetening herb. Their study proved that callus of any age has more total soluble protein contents than any other part of plant. Similar findings were observed by Singh *et al.* [23] in callus cultures of the same herb. They found out that during the process of root and shoot differentiation the metabolites like starch, total soluble sugars and total

phenols decrease while total soluble proteins increase in callus cultures.

During the indirect organogenesis of plants the growth regulators in the culture media induce variability in regenerated plants. Earlier Rotino *et al.* [30] reported that hormonal concentrations in culture media can induce somaclonal variations in eggplant, and the rate of variation increased with overall increase in the concentration of growth regulators [53]. Similar observations were found in the present study where an increase in the concentration of BAP in regeneration media increased the protein content in regenerated plants. The maximum increase of 66.01% was observed in plants regenerated on media supplemented with BAP 2.0 mg L⁻¹, followed by plant regenerated on media supplemented with BAP 1.0 mg L⁻¹ (23.12%) and BAP 0.5 mg L⁻¹ (16.15%) respectively.

The present study is a preliminary step to determine somaclonal variations in eggplant on the basis of protein analysis. This type of work has been done on various other plants but eggplant remained neglected. For example, Taighian and Fahmy [54] evaluated somaclonal variation in sugar cane based on variation in protein content. In another study, Al-Naggar *et al.* [55] *in vitro* selected the salt tolerant canola plants on the basis of molecular and biochemical analysis and it was found that the salt tolerant plants had high content of total proteins.

3.8 Molecular analysis of somaclonal variants of *Solanum melongena* cv. Nirrala

In the present study RAPD was used to assess somaclonal genetic variability among 54 samples of *Solanum melongena*, because RAPD is the far most sensitive and simplest technique for the assessment of genetic fidelity of *in vitro* propagated plants [56]. Out

Table 5. Change in soluble protein content and its percentage increase in somaclonal variants of *Solanum melongena* cv. Nirrala. Any two means sharing a letter in common are not significantly different according to LSD test at the level of P>0.05.

Plant Samples/ Variants	Growth regulators (mg/l)	Soluble Protein (mg/g fresh tissue)	Percentage Increase (%)
Control	-	3.59c	0
V1	BAP 0.5	4.17bc	*+16.15
V2	BAP 1.0	4.42abc	+23.12
V3	BAP 2.0	5.96ab	+66.01
Callus	NAA 2.0+BAP 0.5	6.54a	+82.17
LSD (0.05)		2.45	

*+ = increase in protein content

of five RAPD primers, four primers (OPC 1, OPC 2, OPC 4 and OPC 5) showed clear polymorphic amplification, whereas no amplification profile was generated by OPC 3 in any sample. There was difference in the intensity and resolution of banding pattern at different annealing temperatures for different primers. Among all the five primers, OPC 1 and 2 produced sharp DNA bands at annealing temperature of 40°C, and OPC 4 and 5 produced sharp bands at annealing temperature of 37°C.

Total number of loci traced by these primers was 25, out of which 11 were polymorphic. Overall polymorphism among these samples was 23.97% (Table 6), and size of amplified fragments ranged from 100-700 bp. Previously, Xing *et al.* [57] also used RAPD markers to study the genetic variation in directly regenerated four eggplant genotypes (Meizi, Xianfeng I, Heijuren and Jiuye), and found no variation. This may be due to the fact that genetic variations occur more frequently in plants regenerated from intermediate callus stage, than those regenerated directly without passing through callus stage [58,59].

The ability to detect somaclonal variations among samples varied among different primers, and highest polymorphism was detected by OPC 2 (26.99%), while OPC 1 has shown lowest polymorphism of just 18.32%. Earlier, Mahmood *et al.* [60] also used RAPD markers to detect somaclonal genetic variations between *in vitro* and *in vivo* grown tissues of *Silybum marianum* L. Gaertn. by using ten primers from OPC series (OPC1- OPC10). In their study OPC 10 generated highest polymorphism on the basis of producing ten unique bands in each sample ranging in size from 200-100 bp. Similarly somaclonal variation among callus samples of desiree cultivar of potato was analyzed by Munir *et al.* [61] using RAPD primers from OPC series and found highest polymorphism (57%) with primer OPC 7. Recently Khan *et al.* [62] used three RAPD primers (OPB-07, OPL-04 and OPC-05) to assess the degree of polymorphism

among five brinjal cultivars and reported higher level of genetic polymorphism (50%) with OPB-07 primer, whereas the other two primers (OPL-04 and OPC-05) gave 25% and 30% polymorphism respectively.

It has been reported that somaclonal variations in callus samples arise due to the growth regulators present in callus induction medium [21]. The impact of different growth regulators on somaclonal variations of eggplant were analyzed by Hitomi and Amagaki [63]. They obtained high frequency of morphological variants in the presence of NAA and 2, 4-D. Likewise, Zayova *et al.* [31] also found more somaclonal variations in eggplant in terms of morphological parameters, such as plant height, leaf size, leaf number, number of roots etc., in the presence of NAA even in low concentrations. Similarly, in our study samples grown on NAA alone or in combination with BAP produced more polymorphism in terms of band number; and highest level of polymorphism (8 polymorphic bands) was found in node derived callus sample grown on media supplemented with 1.0 mg L⁻¹ NAA + 1.0 mg L⁻¹ BAP (sample # 35), followed by leaf derived callus on media supplemented with 1.0 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP (samples # 31), node derived callus on media supplemented with 1.0 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP (sample # 32) and internodal callus on media supplemented with 1.0 mg L⁻¹ NAA (sample # 19) each producing 7 polymorphic bands. So our results confirm that NAA can cause more variations at DNA level than any other plant growth regulator checked presently.

Amplification profile of RAPD analysis with OPC 2 is shown as representative gel (Figure 4). OPC2 generated a band of size 425 bp in samples 31 (leaf derived callus on media C10), 32 (node derived callus on media C10), 34 (leaf callus on media C11), 35 (node callus on media C11) and 36 (internodal callus on media C11) that was not observed in any other sample. Whereas OPC 1 generated maximum variability in sample 54 (internodal callus on media C17), which produced three polymorphic bands, and a unique band of 450 bp

Table 6. Number of bands generated and polymorphism percentage as revealed by RAPD among 54 samples of *Solanum melongena* cv. Nirrala.

Sr.No.	Primers	Total Bands	Monomorphic Bands	Polymorphic Bands	Rare Bands	Percentage Polymorphism
1	OPC1	251	205	46	3	18.32%
2	OPC2	363	265	98	8	26.99%
3	OPC4	140	109	31	9	22.14%
4	OPC5	201	147	54	3	26.86%
5	Total	955	726	229	23	23.97%

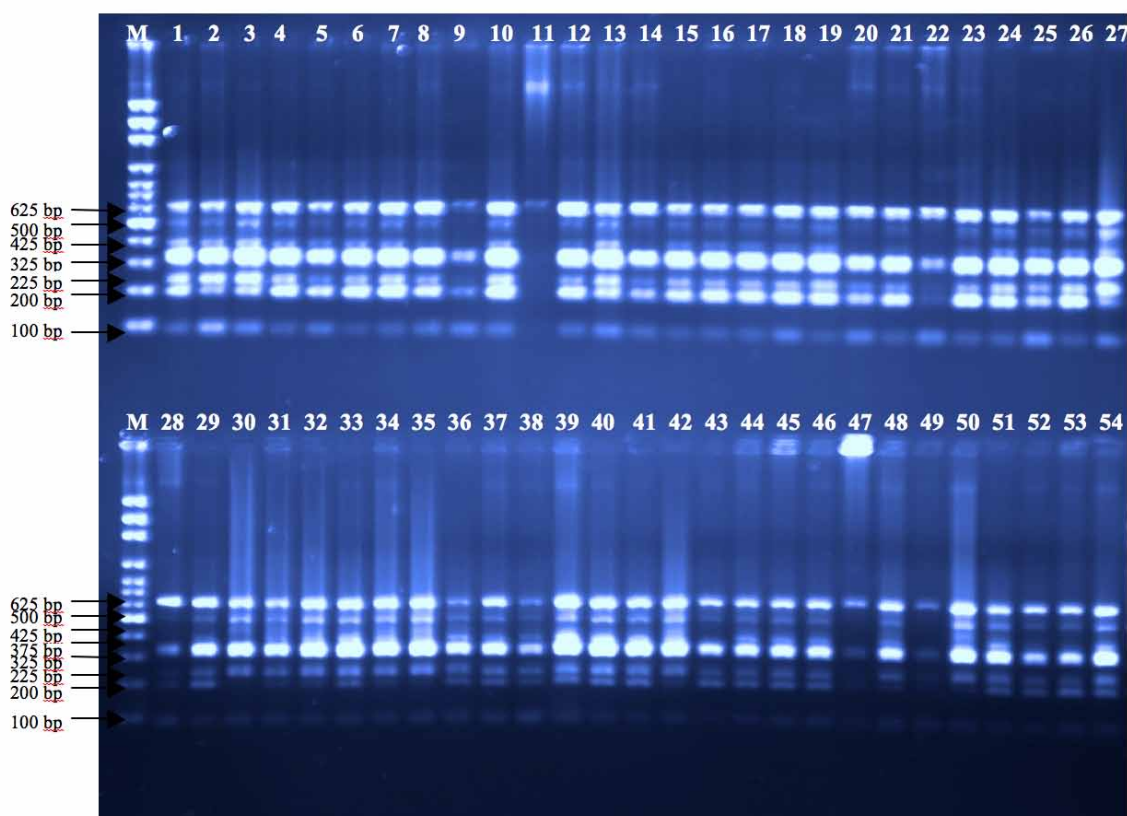


Figure 4. RAPD based banding patterns among 54 samples of *Solanum melongena* cv. Nirrala by using OPC 2. M: DNA ladder marker (100 bp plus Fermentas), 1: Seed derived leaf DNA, 2-54: Callus DNA samples grown on different phytohormones.

was found in samples 20 and 21 (node and internode derived callus on media C6); and sample 54 (internodal callus on C17) that was not present in any other sample. Similarly, OPC 5 produced an unique band of size 200 bp in sample 1 and a fragment of size 400 bp in sample 6 (internodal callus on media C1) and 7 (leaf callus on media C2) that was uniquely present in these samples. Whereas, OPC 4 produced maximum three polymorphic bands in samples 29 (node callus on media C9), 30 (internodal callus on media C9), 31 (leaf derived callus on media C10), 35 (node callus on media C11), 37 (leaf derived callus on media C12), 38 (internodal callus on media C12), 40 (leaf derived callus on media C13), 41 (node callus on media C13), 42 (internodal callus on media C13), 44 (leaf derived callus on media C14) and 45 (internodal callus on media C14) respectively.

The cluster analysis was carried out on the basis of RAPD amplification data using Simqual subprogram of NTSYS-pc software. It revealed two major clusters C1 and C2 in the cladogram (Figure 5) showing total genetic divergence of 37%. The cladogram showed grouping among the samples on the basis of polymorphism.

Among all these groups maximum polymorphism was found among the members of group G2 on the basis of producing seven polymorphic bands. However, within the group G2, sample 35 showed 4% genetic divergence that produced eight polymorphic bands. Other groups included samples showing polymorphism in a range of five (Groups G4, G6) to six (Groups G1, G3, G5) bands. However, groups G7, G8 and G9 included three samples each. One thing common among all these groups was the presence of four polymorphic bands. In general we can conclude that that biochemical and molecular markers are quiet effective for screening variant lines. Among the biochemical markers, changes in total soluble protein content in regenerated plants are good indicator of biochemical variation, but these are influenced by physiological conditions of plants and growth conditions. It was observed that RAPD technique provides more efficient analysis of somaclonal variants as it is more effective, simple and cost effective method, and in our study it gave genetic basis of observed phenotypic changes.

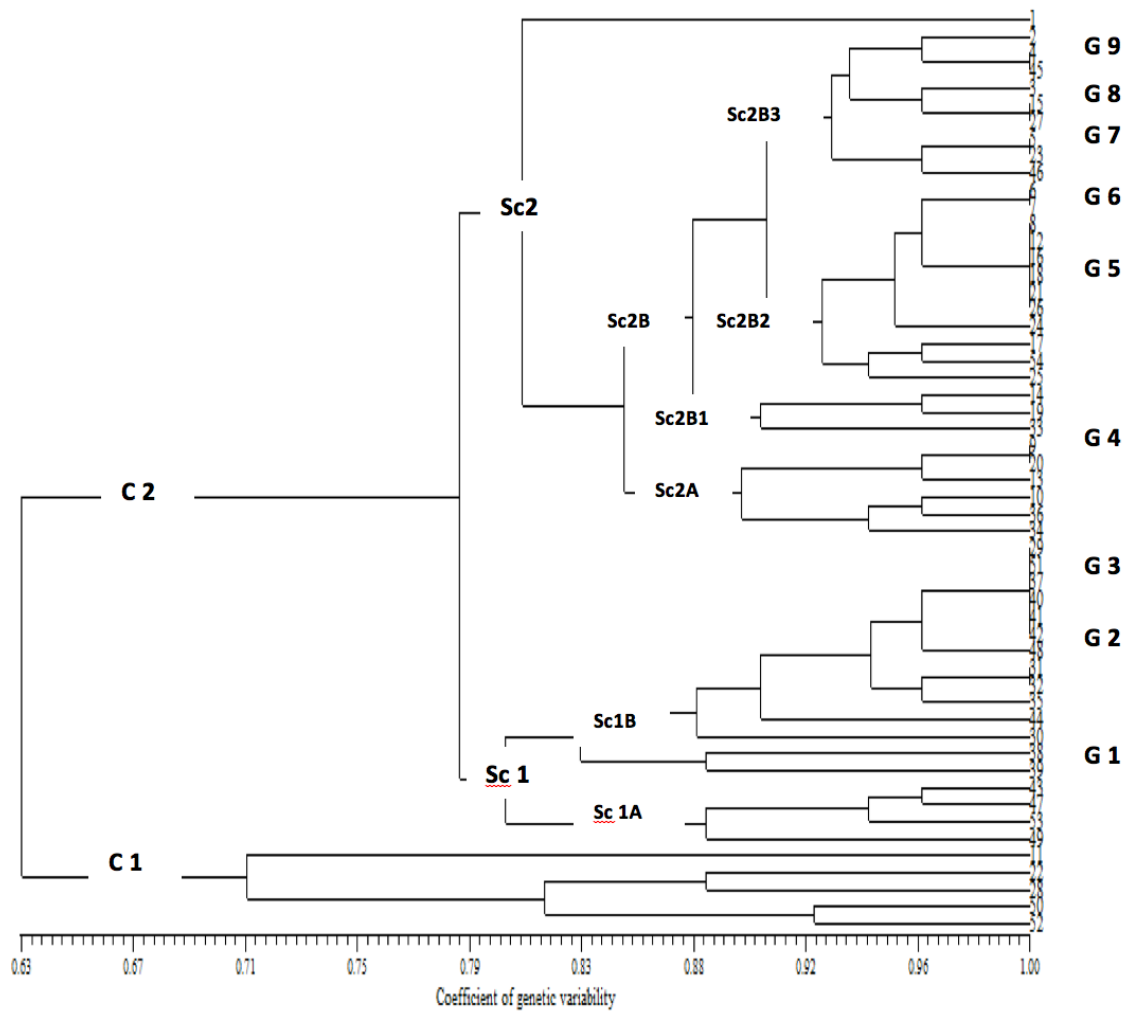


Figure 5. Dendrogram representing the genetic relationship among 54 samples of *Solanum melongena* using NTSYS clustral analysis generated from RAPD primers OPC 1, OPC 2, OPC 4 and OPC 5.
C1: Cluster 1, C2: Cluster 2, Sc 1: Sub cluster 1, Sc 2: Sub cluster 2, Sc 1A: Sub cluster 1A, Sc 1B: Sub cluster 1B, Sc 2A: Sub cluster 2A, Sc 2B: Sub cluster 2B, Sc 2B1: Sub cluster 2B1, Sc 2B2: Sub cluster 2B2, Sc 2B3: Sub cluster 2B3, G 1: Group 1, G 2: Group 2, G 3: Group 3, G 4: Group 4, G 5: Group 5, G 6: Group 6, G 7: Group 7, G 8: Group 8, G 9: Group 9.

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