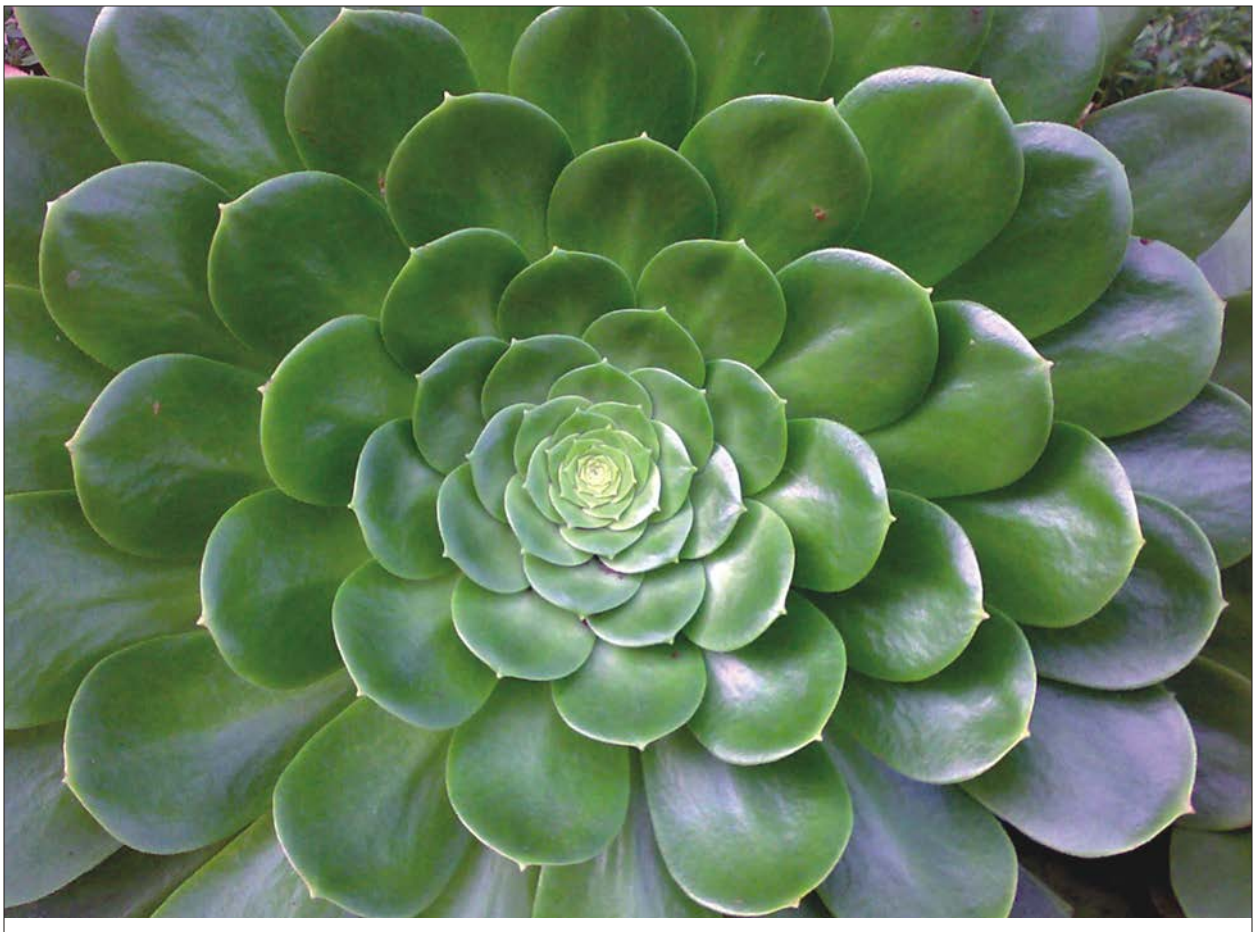

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Regeneration via Direct Organogenesis from Leaf Segments of Eggplant (*Solanum melongena* L.)

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ABSTRACT

Direct organogenesis from leaf explant of Indian variety of *Solanum melongena* L. (PLR1) was successfully achieved. Eggplant leaves cultured for 10–12 days on MS medium supplemented with (2iP) 2.0 mg L^{-1} and Naphthalene acetic acid (NAA) 1.0 mg L^{-1} induced high frequency shoot organogenesis (79-81%) and favored shoot elongation. Shoots developed from leaf explant directly after two weeks of explant inoculation. Shoot proliferation reached maximum in the presence of 2.0 mg L^{-1} 2iP with induction of 4.5-5.0 average mean number of shoots per explant. High frequency of root induction (83-85%) was observed on medium supplemented with 1.0 mg L^{-1} Naphthalene acetic acid (NAA) with 6-7 mean number of roots whereas increase in the level of (IBA) Indole butyric acid (2.0 mg L^{-1}) resulted in induction of roots along with development of callus from the base of the shoots. The plantlets with developed roots are hardened with 2:1 ratio of soil and vermiculite shifted to controlled environmental growth chamber with 8 h photoperiod for acclimatization and after ten days the plantlets are transferred to field condition. This protocol can be efficiently used for mass multiplication and also for regeneration of genetically transformed *Solanum melongena* tissue.

Key words: Benzyl adenine, multiple shoots, MS medium, leaf explant and hardening

INTRODUCTION

Eggplant, *Solanum melongena* L., is a common and popular vegetable crop grown in the subtropics and tropics. Eggplant is a perennial but grown commercially as an annual crop. Eggplant is essentially a warm weather crop which is grown extensively in India, Bangladesh, Pakistan, China, Japan and the Philippines. According to the 2009 (FAO., 2009) Production Yearbook, Asia has the largest eggplant production which comprises more than 90% of the world production area and 87% of the world production. Eggplant is a bushy plant and grows to a height of 60-120 cm. The plant is erect, compact and well branched. The leaves are large, simple, lobed and alternate on the stems. It has a rather fibrous or lignified root system. Micropropagation is the only aspect of plant tissue culture which has been most convincingly documented with regard to its feasibility for commercial application and consequently it has been extensively used for rapid and large scale propagation of a number of plant species. The economic importance of some vegetable crops in the family *Solanaceae* necessitates the application of this technique for their clonal multiplication (Kumar *et al*., 1998) during the past few years.

The application of *in vitro* methodologies to eggplant has resulted in considerable success. Eggplant tissues present a high morphogenetic potential that is useful for developmental studies as well as for establishing biotechnological approaches to produce improved varieties, such as embryo rescue, *in vitro* selection, somatic hybridization and genetic transformation. Taken together, these characteristics also make eggplant a complete model for studies on different areas of plant science. Although various sources of explants have been reported for direct organogenesis (Kashyap *et al*., 2003), there has been no report on regeneration from leaf directly in *Solanum melongena* L. Leaf explant has higher regeneration potential and good susceptibility for *Agrobacterium* transformation (Franklin *et al*., 2004). The main objective of the present study is to develop multiple shoots directly from leaf explant.

MATERIALS AND METHODS

Plant material: Seeds of *Solanum melongena* L; (cv. PLR 1) were collected from TNAU Research Station, Palur, Tamil Nadu, India.

***In vitro* seed germination:** Seeds were washed with running tap water for 30 min followed by sterile distilled water for four times. Subsequently seeds were washed with 5% teepol solution (w/v) for 3 min and treated with 70% (v/v) ethyl alcohol for 45 sec, rinsed with sterile distilled water thoroughly followed by surface disinfection with 0.1% (w/v) $HgCl_2$ solution for one min and rinsed thoroughly with sterile distilled water under aseptic condition. The surface sterilized seeds were inoculated on half strength MS (Murashige and Skoog, 1962) medium containing 8.0 g L^{-1} Agar as gelling agent and 15 g L^{-1} sucrose as carbon source in MS medium without hormones for germination. This setup kept to 16/8 h photoperiod with low light intensity (1000 Lux). Seeds germinated after two weeks of inoculation and the germinated seedlings produced leaves after three weeks which served as explant source for shoot regeneration.

Culture media and conditions for shoot regeneration: The explants (leaves), was excised from 20 days old seedlings and then inoculated in culture bottles, each containing 50 mL of MS medium supplemented with appropriate plant growth regulators such as BAP, 2iP and Kin (1-2 mg L^{-1} each hormone) and tightly closed with screw cap. The cultures were kept in culture rooms maintained at 25±2°C for 16/8 h photoperiod of 35 mM^{-2} S^{-2} light intensity and 70% relative humidity.

Shoot elongation and rooting: Two weeks after inoculation the explants developed shoots directly from the margins of the leaf segments and the cultures were immediately transferred to shoot elongation medium supplemented with cytokinins like 2iP and BAP (2 and 1 mg L^{-1}) on half strength and full strength MS medium with agar and sucrose. After ten days of subculture, shoots were elongated on elongation medium with significant frequencies.

For root induction, regenerated shoots of 4.5 cm length were transferred to MS medium supplemented with auxins such as NAA, IBA and IAA (1-2 mg L^{-1}). Rooting was evaluated after 20 days in culture. The frequency of rooting the average number and length of root per shoot were determined and recorded after 45 days. Rooted plantlets were removed from the culture bottles without damaging the roots and then washed in running tap water and transferred to plastic cups containing sterilized soil and vermiculate at 2:1 ratio. Polyethylene bags were inverted over the cups to maintain humidity and kept under16/8 h photoperiod (1500 Lux) at 25±2°C. The plants were irrigated periodically with half strength MS nutrient solution. At the end of 4 weeks, they were transferred to the field, after which survival was assessed.

Statistical analysis: For each experiment 50 explants was repeated three times. Every two weeks counts were made on multiple shoot induction on the margins of the leaf explants. The analysis of variance and mean separation were carried out using Duncan's Multiple Range Test (DMRT) and the significance was determined at 5% level.

RESULTS

Induction of shoots from leaves: *In vitro* plant regeneration of *Solanum melongena* L. by direct shoot organogenesis has been achieved from the cultured leaf explants (Fig. 1). The size and the direction of leaves potentially influenced the response of organogenesis from leaf margins. Young leaves of size from 2-4 cm responded effectively on shoot organogenesis but the older leaves above 4 cm in size has not shown interesting result. In the present investigation, five days after inoculation, shooting response of explants was observed besides the hormonal effects on callus induction. Shoot bud induction was observed from leaves cultured with the abaxial surface in contact with the medium.

Effect of cytokinins on shoot induction: Induction of multiple shoots has been achieved by the incorporation of hormones like BAP, 2iP and Kin with some auxins such as NAA and IAA. More than five combinations have been tried on full strength and half strength MS medium. Addition of hormones induced shoot formation and number shoots depended on the types and concentrations of hormones. For multiple shoot induction from leaf explant, the combination 2iP and NAA with 2 and 1 mg L^{-1} was the most successful combination. More than 4.5 average mean number of shoots per explant has produced in that combination with the maximum of 79-81% response (Table 1). But individual treatment of BAP and 2iP doesn't produce any significant results (results not shown). The combined effect of BAP and 2iP with NAA has produced excellent response.

Shoot elongation: The multiplied shoots from the leaf explant excised and subcultured for shoot elongation. Various concentration and combinations of hormones has been tried (Table 2) but very few concentrations and combinations so far successful. Each shoots were separated from explant culture and subcultured on fresh medium supplemented with hormones such as BAP and 2iP for shoot elongation. For shoot elongation the influence of hormones individually are more effective then the combinations. Five concentrations (0.5-2.5 mg L^{-1}) of BAP have been tested but, as concentration of BAP increases the response decreases.

Influence of auxin on root induction: Two weeks after subculture of elongated shoot on rooting medium were assessed; the elongated shoots showed good sign of rooting when cultured on media containing auxin (1-2 mg L^{-1}), however, a 85% rooting efficiency was achieved by a simple method of transferring shoots from shooting media to rooting media containing IBA and NAA separately. The concentration of 1 mg L^{-1} NAA produced 6.5 mean numbers of roots (Table 3) among the five concentrations tested. When comparing NAA with IBA treatment, the percentage of success for NAA is relatively higher than IBA treatment, because less number of roots formed on IBA supplemented medium. The increasing concentration of NAA leads to the development of basal callus, as concentration of NAA increased the rooting response decreased. After the development of roots, the lengths of roots were measured.

Hardening and establishment of plants: Rooted shoots were successfully hardened in autoclaved vermiculite and sterilized soil at the ratio of 1:2 and kept under high relative humidity

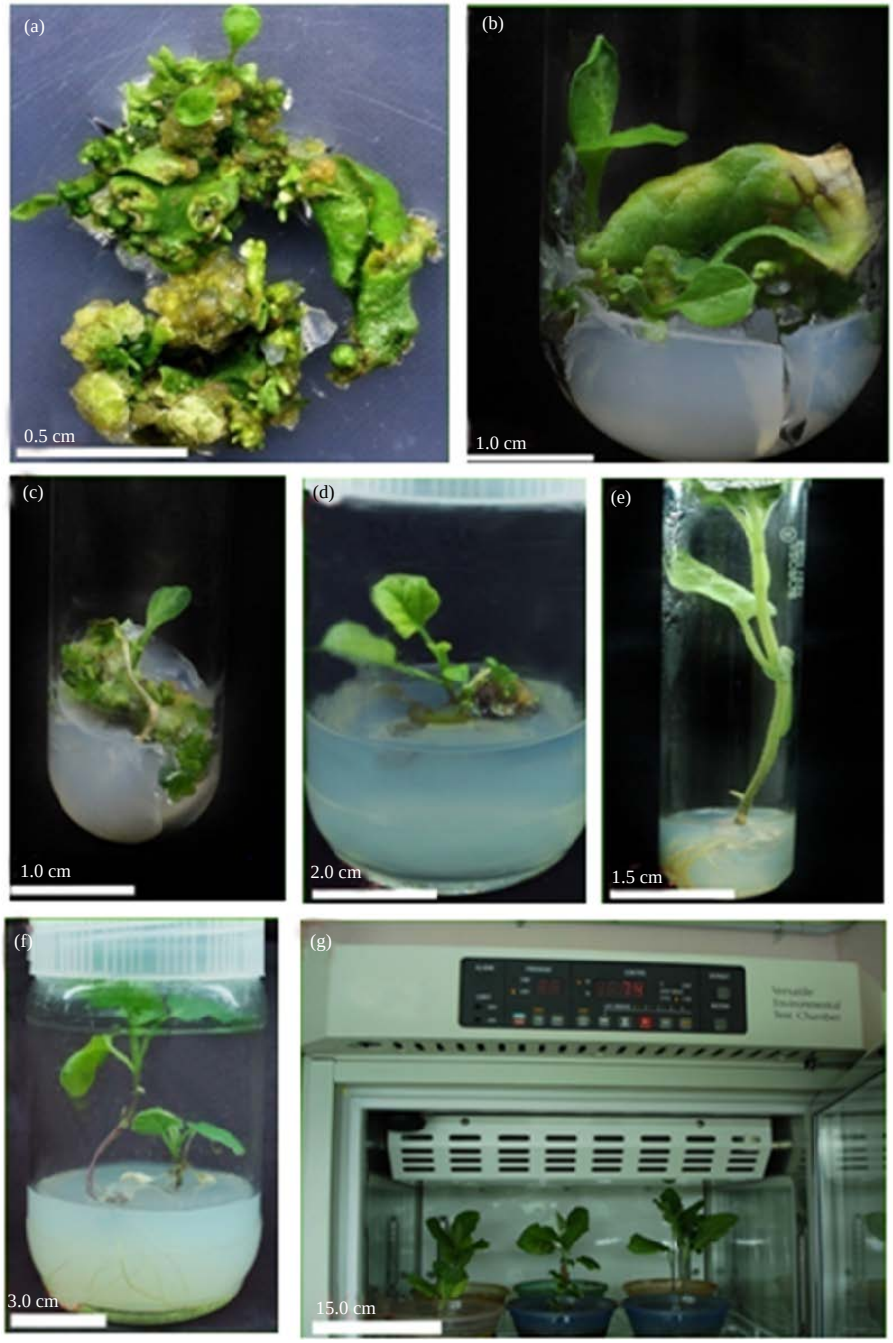

Fig. 1(a-g): Complete Plant regeneration of *Solanum melongena* L. using leaf explant through Direct organogenesis, (a and b) Multiple shoot bud induced directly from leaf on MS medium with 2iP, (c and d) Shoot elongation on MS medium with GA_3, (e) Induced roots on half strength MS medium with NAA, (f) Root elongated plant on half strength medium and (g) Hardened plant acclimatized in environmental growth chamber

(60-70%), gradual decreasing air humidity and temperature maintained at 26±2°C in controlled environmental growth chamber with the light intensity of 35 mM S^{-1} for four weeks. After about

Table 1: Effect of various cytokinin on shoot induction of *Solanum melongena* using leaf segments as explant

Hormone con. (mg L^{-1})	Response (%)	Mean number of shoots
BAP+NAA		
1.0+1.0	74	4.0 ± 0.2^{cd}
1.5+1.0	74	3.9 ± 0.3^{d}
2.0+1.0	73	3.8 ± 0.2^{de}
1.0+1.5	79	3.9 ± 0.2^{d}
1.5+1.5	76	3.7 ± 0.3^{e}
2.0+1.5	75	3.5 ± 0.4^{f}
1.0+2.0	77	3.6 ± 0.2^{ef}
1.5+2.0	75	3.5 ± 0.1^{f}
2.0+2.0	73	
2iP+NAA		
1.0+1.0	75	3.4 ± 0.1^{fg}
1.5+1.0	79	4.2 ± 0.2^{bc}
2.0+1.0	81	4.3 ± 0.2^{b}
1.0+1.5	74	4.5 ± 0.1^{a}
1.5+1.5	75	4.1 ± 0.3^{c}
2.0+1.5	74	4.0 ± 0.3^{cd}
1.0+2.0	74	4.0 ± 0.1^{cd}
1.5+2.0	73	3.9 ± 0.2^{d}
2.0+2.0	71	3.7 ± 0.2^{e}

Mean±SE of two separate experiments with 50 replicates each. Means within a column followed by same letter are not significantly different according to Duncan's multiple range test (DMRT) ($p\leq0.05$)

Table 2: Effect of hormones on shoot elongation using leaf segments as explant

Hormone conc.	Response (%)
NAA	
0.5	57±0.5
1.0	59±0.5
1.5	61±1.0
2.0	56±0.5
2.5	53±1.0
GA_3	
0.5	60±0.5
1.0	65±1.0
1.5	67±1.0
2.0	59±0.5
2.5	55±1.0
NAA+GA_3	
0.5	55±0.5
1.0	58±0.5
1.5	61±1.0
2.0	54±0.5
2.5	51±0.5

Values are Means ±SE of three repeated experiments. Each treatment consisted of four replicates. Means within a column followed by the same letters are not significant at $p\leq0.05$ according to DMRT

Table 3: Effect of auxin on root induction of *Solanum melongena* using leaf segments as explant

Hormone con. (mg L^{-1})	Response (%)	Mean number of shoots
NAA		
1.0	85	6.5 ± 0.5^{a}
1.5	80	6.0 ± 0.4^{bc}
2.0	81	6.1 ± 0.4^{b}
IBA		
1.0	80	5.9 ± 0.5^{cd}
1.5	79	5.8 ± 0.4^{de}
2.0	75	5.6 ± 0.5^{ef}

Mean±SE of two separate experiments with 50 replicates each. Means within a column followed by same letter are not significantly different according to Duncan's multiple range test (DMRT) ($p\leq0.05$)

one month, the hardened plants were transferred to earthen pots (15 cm) containing soil and vermiculite (2:1). Then it was transferred in the field condition in green house. The percentage of survival was calculated after five to six weeks in the greenhouse.

DISCUSSIONS

Direct regeneration from leaf is another alternative step for clonal propagation. In the present investigation we studied the effect of 2iP with NAA for shoot induction and multiplication using leaf explant of *Solanum melongena* L. Reports on *in vitro* morphogenesis of explant usually describe hypocotyl (Kamat and Rao, 1978; Matsuoka and Hinata, 1979) and leaf explants (Gleddie *et al*., 1983; Mukherjee *et al*., 1991) as the most responsive type. High shoot regeneration potential of proximal end explants as to leaf explants has been emphasized in *Beta vulgaris* (Zhang *et al*., 2001) and *Euphorbia nivulia* (Martin *et al*., 2005, 2003). Midrib portion of expanded leaf in Indian spinach (*Beta palonga*) and base petiolar transition zone has been used for direct organogenesis (Mitra and Mukherjee, 2001). We made an attempt on direct organogenesis from leaf explant of *S. melongena* L. Misra (2002) has already reported leaf explant as an excellent source for regeneration via direct organogenesis in pigeon pea. The previous reports on the responsiveness of different explants were undertaken only by Alicchio *et al*. (1982) and Sarma and Rajam (1995) in *S. melongena* L. The regeneration of adventitious shoots from leaf explants was dependent on the presence of both auxin and cytokinin in the medium. The continuous presence of BAP in the medium did not affect further development of axillary buds, thus indicating that the effect of BAP is induced at the beginning of the application. Multiple shoots were successfully achieved within six weeks from immature leaf bits on media supplemented with 2iP. We found 2iP to be a more effective enhancer for shoot induction and subsequent formation of normal shoots when compared with other cytokinins enriched medium. Increased number of shoots reported by several investigators using BAP (Gleddie *et al.,* 1983; Sarma and Rajam, 1995), Zeatin (Gleddie *et al*., 1983; Mukherjee *et al*., 1991) and TDZ (Magioli *et al*., 1998). The exposure to high concentration of cytokinins for extended periods resulted in new shoots became shorter, stunted and leaf size decreased, this type of problems were experienced by Dunstan *et al*. (1985) in apple. We used proximal end to get connected with the medium and shoots are induced at the proximal end of leaf because high frequency shoot induction at the proximal region may also be due to the higher level of IAA and abscisic acid (Rajasekharan *et al*., 1987). In other report (Welander, 1988), the high potential of the proximal end to the distal may be due to the difference in the maturity between proximal and distal end of the leaf and which is supported by the fact that leaves reach maturity first at distal (tip) and subsequently in a basipetal progression. Combined effects of BAP and Kin on leaf explants of *Stevia rebaudiana* responding towards *de novo* shoot regeneration after 7 weeks (Sreedhar *et al*., 2008). In our case we got shoot regeneration with in four weeks. The effect of BAP with NAA on shoot induction was already reported by Franklin *et al*. (2004) in *Solanum melongena* L. but we tried both BAP with NAA and 2iP with NAA and latter produced superior results. The effect of 2iP showed a better result for multiplication of shoots. This was probably due to variation in endogenous growth regulator contents and physiological status of the plant species. Like our results, 2iP triggered high frequency of shoot induction has been observed in *Cichorium intybus*.

In the present study BAP replaced by 2iP and combined with NAA got nearly 5 shoots per explant. Increasing IBA concentration favored callus induction especially from the cut ends of explants. Although, most of the published protocols resulted in relatively low regeneration frequencies of shoot production resulted in the development of present protocol.

Shoot elongation was enhanced when the concentration of cytokinins in the culture medium was reduced. The size of the shoots determined the subsequent rate of buds and shoot production. Adequate shoot growth may be important for root induction.

In medium supplemented with NAA and GA_3, the elongation of shoots was observed. Besides promoting elongation, GA_3 suppressed shoot vitrification. So the combination of NAA with GA_3 was reduced and different concentration was tested.

In our study, the shoots could be easily rooted on the half strength MS medium with NAA and the number of roots formed on each shoot was also significantly higher than in other rooting media. All the excised shoots developed roots within four weeks of culture in the rooting medium. Root number increased when the medium supplemented with NAA and the number of roots reaches maximum at 1 mg L^{-1} of NAA. Half strength MS medium with NAA showed good number of root compared to full strength MS medium was found to be more effective than full strength MS medium. Magioli *et al*. (1998) advised half strength MS medium suitable for shoot induction and well supported by the results of Franklin *et al*. (2004). In cottonwood (*Populus deltoides*), IAA has been used to induce roots after shoot multiplication (Yadav *et al*., 2009). The production of basal callus at the base of explants was higher for those explants grown with BAP at full strength MS medium, an indication that tissue differentiation in the zone of contact with the medium is related to the IAA/cytokinins ratio.

The results indicated that NAA at higher concentration has obvious inhibitory impact on root production. The similar results were also achieved in *Robinia pseudoacacia* (Wang *et al*., 1999). However in the trials presented here, NAA was successfully used in the rooting process. In term of the adventitious rooting percentage NAA was clearly dominant to IBA. It is already reported by Durkovic (2008) in Carnusmas. The findings that an optimum balance of NAA and BAP is required to obtain a model organogenesis system without undesirable callusing or abnormal shoots is justified from the work described earlier in sugar beet (Detrez *et al*., 1988). The present investigation has been possible to establish a reliable and reproducible protocol for high frequency regeneration of eggplant through *in vitro* culture.

CONCLUSION

In conclusion, a simple, high frequency, reproducible and improved multiple shoot bud induction competence approach is able to produce and perpetuate large numbers of disease-free plants of elite clones of *Solanum melongena* L, cv. PLR and will aid in eggplant genetics research (an emerging field of high priority) by keeping a constant supply of competent and efficient plant material for future genetic transformation protocols. Besides this, are liable plasticity potential of tissue culture raised plants could reduce problems of selecting transformants due to direct shoot regeneration from co-cultured leaves. The regeneration system so developed will serve as platform for undertaking future genetic transformation studies.

ABBREVIATIONS

- BA 6-benzyladenine
- IBA Indole butyric acid
- 2iP 2-isopentanyladenine
- NAA a-naphthalene acetic acid
- IAA Indole acetic acid

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