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Difference in somatic embryogenetic ability of cultured leaf explants of four genotypes of *Solanum melongena* L

PV Lakshmana Rao *

Defense Research Laboratory, Post Bag No 2, Tezpur 784001 Assam, India

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Summary — High frequency of somatic embryogenesis was obtained from leaf explants of 4 genotypes of *Solanum melongena* L. Varietal response was noticed with reference to optimum concentration of NAA required for somatic embryogenesis. Percent explants forming somatic embryos varied from 15.2–90.3, mean number of plants regenerated/explant from 1.5–29.5. In terms of their embryogenic competence and plant regeneration the genotypes could be rated in decreasing order as F₁ hybrid Suphal followed by PPL, DRLS and Kalalong. Initial inoculum size had a distinct correlation with number of plants regenerated. Decline in embryogenic competence was observed in all 4 genotypes under long-term culture. Plantlets regenerated from somatic embryos were established on soil and grown to maturity.

***Solanum melongena* = egg plant / leaf explant / somatic embryogenesis / genotypic response**

Résumé — Embryogenèse somatique d'explants de feuilles de quatre génotypes de *Solanum melongena*. Des explants foliaires de 4 génotypes de *Solanum melongena* L donnèrent lieu, avec une fréquence importante, à de l'embryogenèse somatique. Les réponses variétales furent notées, en corrélation avec la concentration d'acide α naphthylacétique (ANA) nécessaire pour l'embryogenèse somatique. La proportion d'embryons somatiques formés varia de 15,2 à 90,3 pour 100 embryons et le nombre moyen de plantes régénérées par embryon de 1,5 à 29,5. On peut classer les génotypes en fonction de leur compétence pour l'embryogenèse et la régénération de plantes de la façon suivante : hybride F₁ Sulphal, PPL, DRLS, Kalalong, par ordre décroissant. Le nombre de plantes régénérées était en rapport direct avec la taille initiale de l'inoculum. Un déclin de la compétence à l'embryogenèse fut observé par les 4 génotypes sur les cultures âgées. Les plantules régénérées à partir d'embryons somatiques furent cultivées sur sol, jusqu'à maturité.

***Solanum melongena* = aubergine / explant foliaire / embryogenèse somatique / réponse génotypique**

INTRODUCTION

Regeneration of plantlets from cultured cells is an essential part of plant biotechnology. Somatic embryogenesis is one of the preferred pathways of induced plant regeneration. It can be either indirect from callus, cell suspension, or protoplast culture or direct from organ tissues such as leaf, stem, hypocotyl etc (Williams and Maheswaran, 1986). Successful induction and realization of somatic embryogenesis in a number of species have opened the possibility of us-

ing somatic embryos as a potential propagation system for seed sown crops (Stuart *et al*, 1987).

Plantlet regeneration in *S. melongena* L through organogenesis and embryogenesis is possible from sexual embryos (Yamada *et al*, 1967) hypocotyl (Kamat and Rao, 1978; Matsuoaka and Hinata, 1979) leaf explants (Gledie *et al*, 1983; Rao and Singh, 1991). The present study reports the difference in somatic embryogenesis ability of 4 varieties of *S. melongena* L viz DRLS (DRL Selection), Kalalong, F₁ hybrid Suphal and PPL (Pusa Purple Long). The effect

* Correspondence and reprints; present address: Toxicology and Pharmacology Division, Defence Research and Development Establishment, Gwalior, 474002, India

of inoculum size on plant regeneration and the embryogenic competence in long term culture were also studied.

MATERIAL AND METHODS

Plant material

Seeds of different genotypes tested in the present study and their sources were as follows: DRLS (Defence Research Laboratory, Tezpur) PPL (National Seeds Corporation, New Delhi), F₁ hybrid Suphal (Indo-American Hybrid Seeds, Bangalore), Kalalong (locally purchased). These 4 origins were selected because of their high yields and suitability for cultivation in mid and high altitude regions of north-eastern India.

Seedlings were established from seeds in the nursery during the months of December – February. Leaf explants were taken from 20–25 d-old seedlings. Preparation of explants and other culture conditions were the same as described earlier (Rao and Singh, 1991). Briefly, mature dark green leaves from 3–4 week old seedlings were surface sterilized in 0.1% (w/v) mercuric chloride for 2 min followed by a 30-s rinse in 70% alcohol and repeated washings in sterilized distilled water. The leaves were transversely dissected into 3 pieces and the apical ends discarded. The middle portion was again transversely cut into 3–5 mm sections and placed abaxial side down on the medium.

Culture medium

The basal medium used in the present study was that of Murashige and Skoog (1962) supplemented with 3% (w/v) sucrose, 100 mg/l myo-inositol, and 0.8% (w/v) agar and hereafter referred to as MS basal medium. NAA was incorporated at 0.5–12.0 mg/l in 7 concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 10.0 and 12.0 mg/l) in combination with 0.1 mg/l kinetin as it was found beneficial in promoting somatic embryogenesis (Rao and Singh, 1991). The pH of the medium was adjusted to 5.6–5.8 before autoclaving. For callus initiation, the explants were cultured on 15 ml of medium in 25 x 150-mm tubes. Suspension cultures were raised in 250-ml conical flasks containing 50 ml of medium. The flasks were maintained on gyratory shaker at 100 rpm. Cultures were incubated at 25 ± 1 °C at 16 h photoperiod and 3 000 lux intensity.

Preparation of samples for scanning electron microscopy

Embryos at various stages of development were fixed for 2 h at room temperature in 2% glutaraldehyde buf-

ferred to pH 7.2 with cacodylate and post-fixed in 1% osmium tetroxide overnight. After dehydration through a graded series of ethanol to absolute ethanol, embryos were critical point dried and sputter coated with gold. Observations and photographs were made on Jeol scanning electron microscope at 20 kV.

Data collection and analysis

All the experiments described below were repeated 4 times. Each replicate consisted of 40–50 explants unless otherwise mentioned. All the treatments were arranged in a completely randomized design. Relating to each genotype data on explants forming embryos and mean number of embryos/embryo forming explants (EFEX) were recorded after 35 d of culture. For plant regeneration all the somatic embryos formed on single leaf explant were transferred to basal MS medium without hormones. Number of plants regenerated/EFEX was recorded after 30 d of culture. For the experiment on embryogenic competence in long-term culture, embryogenic stock callus was maintained by subculture on basal MS medium with 0.1 mg/l NAA and without NAA at 30-d interval. Mean separation was performed by LSD after Fisher's *F* test. Callusing from the cut end of the leaf explants was noticed after 8–10 d of culture.

RESULTS

Somatic embryogeny

Somatic embryos were visible on the leaf explants after 3 weeks of culture. They appeared as round globular masses, loosely attached without vascular connections to parent tissue. Some of the leaf explants directly formed globular embryos without intervening callus. The developmental stages of somatic embryos included globular, heart and cotyledonal stages (figs 1, 2).

In all the 4 genotypes with increase in NAA concentration significant increase in explants forming embryos was observed up to 8.0 mg/l (table I). Optimum NAA concentration for highest embryogenic response was different for different genotypes; 8.0 mg/l was optimum for highest response in Kalalong (67.2%), and Suphal (94.3%) whereas it was 10.0 mg/l for DRLS (69.8%) and PPL (81.5%). At 12.0 mg/l NAA concentration there was a significant decrease in embryo formation in all 4 varieties. Embryogenesis was highest in Suphal, followed by PPL, DRLS and Kalalong in decreasing order.

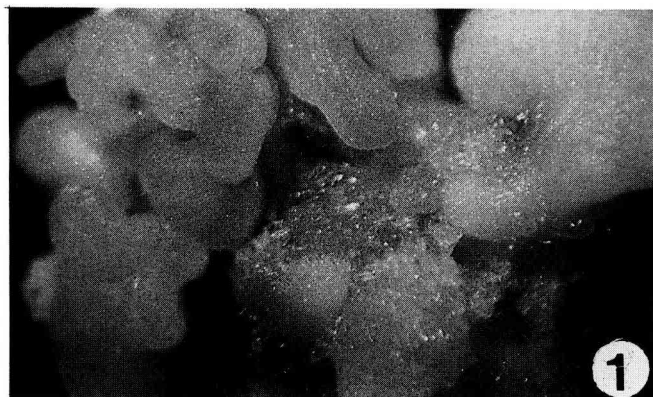


Fig 1. A mass of embryoids from leaf callus (x 8).

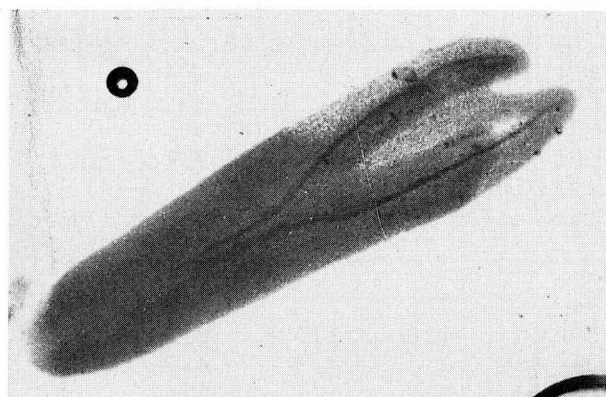


Fig 2. Mature somatic embryo with distinct cotyledons (x 70).

For the experiment on mean number of embryos/EFEX, only those embryos with distinct shoot and root meristems were included for data analysis. The mean number of embryos/EFEX also showed a similar trend of increasing response with increase in NAA concentration. At 0.5 and 4.0 mg/l NAA concentration, mean number of embryos formed were not significantly different in all 4 varieties. Lowest and highest mean number of embryos/EFEX varied from 3.5 in

DRLS to 35.0 in Suphal respectively. Stages of embryoid development which were evident in scanning electron micrographs (figs 3, 4) included globular, heart-shaped somatic embryos with distinct shoot development. Occasionally secondary somatic embryogenesis was also observed in which adventive embryos were formed on somatic embryos. Continued growth of these on parent somatic embryos resulted in abnormal embryo formation.

Table I. Effect of NAA concentration on somatic embryogenesis in four high yielding genotypes of *Solanum melongena*.

| Genotypes | NAA concentrations (mg/l) | | | | | | |
|--|---------------------------|------|------|--------------------|------|------|------|
| | 0.5 | 1.0 | 2.0 | 4.0 | 8.0 | 10.0 | 12.0 |
| <i>Explant forming embryos (%)</i> | | | | | | | |
| DRLS | 15.2 | 18.5 | 21.0 | 54.0 | 66.3 | 69.8 | 52.5 |
| Kalalong | 22.3 | 25.0 | 33.5 | 54.0 | 67.2 | 61.7 | 54.0 |
| Suphal | 28.8 | 35.5 | 55.5 | 73.3 | 94.3 | 85.2 | 68.8 |
| PPL | 16.0 | 30.8 | 45.5 | 58.7 | 74.0 | 81.5 | 66.7 |
| LSD (5%) | 4.82 | 6.19 | 6.57 | 8.12 | 7.44 | 7.28 | 8.49 |
| <i>Mean number of embryos / EFEX</i> | | | | | | | |
| DRLS | 3.5 ^{ns} | 5.3 | 6.3 | 14.5 ^{ns} | 14.0 | 18.5 | 18.5 |
| Kalalong | 4.3 ^{ns} | 5.8 | 9.0 | 17.0 ^{ns} | 15.5 | 13.5 | 12.0 |
| Suphal | 7.2 ^{ns} | 8.7 | 12.3 | 17.7 ^{ns} | 35.0 | 23.0 | 18.3 |
| PPL | 4.5 ^{ns} | 8.5 | 10.3 | 16.0 ^{ns} | 21.0 | 21.7 | 15.8 |
| LSD (5%) | 2.93 | 2.44 | 1.77 | 3.97 | 5.23 | 3.28 | 3.83 |
| <i>Number of plants regenerated / EFEX</i> | | | | | | | |
| DRLS | 1.5 | 3.0 | 4.0 | 11.5 ^{ns} | 11.0 | 15.0 | 14.3 |
| Kalalong | 2.3 | 3.3 | 6.0 | 14.0 ^{ns} | 12.8 | 10.0 | 7.5 |
| Suphal | 4.8 | 6.5 | 9.3 | 14.0 ^{ns} | 29.5 | 17.8 | 13.3 |
| PPL | 2.0 | 5.8 | 7.3 | 12.8 ^{ns} | 17.8 | 17.3 | 11.7 |
| LSD (5%) | 2.14 | 2.57 | 1.86 | 3.43 | 4.41 | 3.47 | 3.57 |

All media were supplemented with 0.1 mg/l kinetin. Values are means of 4 replicates. Mean separation was performed separately for each group. ns: non-significant.

Table II. Effect of initial inoculum size on plant regeneration.

| Initial embryogenic callus (mg FW) | Plants regenerated / g FW of E callus | | | |
|--|---------------------------------------|----------|--------|------|
| | DRLS | Kalalong | Suphal | PPL |
| 75 | 34.6 | 27.0 | 50.0 | 40.0 |
| 150 | 46.0 | 30.0 | 60.0 | 56.0 |
| 300 | 33.3 | 42.6 | 41.7 | 36.0 |
| 600 | 20.8 | 19.1 | 23.3 | 21.6 |
| 900 | 16.7 | 15.5 | 17.7 | 16.1 |

Values are means of 20 flasks. FW: fresh weight, E: embryogenic callus.

The entire leaf sections with embryogenic callus and embryos were carefully removed and individually transferred to basal MS medium without hormones for plant regeneration (fig 5). Those embryoids formed directly on the edges of the leaves could be removed and plated singly on basal medium. Some of the transferred embryos developed only shoots or roots. Only those embryos which formed complete plants were included for data analysis. Somatic embryos which were initiated on media with low NAA concentration, *ie* 0.5–2.0 mg/l, showed faster plant regeneration and a better developed root system than those formed on high NAA concentration. The highest number of plants regenerated/EFEX in DRLS, Kalalong, Suphal and PPL were 15.0, 14.0, 29.5 and 17.8 respectively. Plants with a well developed root system (fig 6) were hardened under high humidity conditions before transfer to the field (fig 7) and were successfully grown to maturity (fig 8).

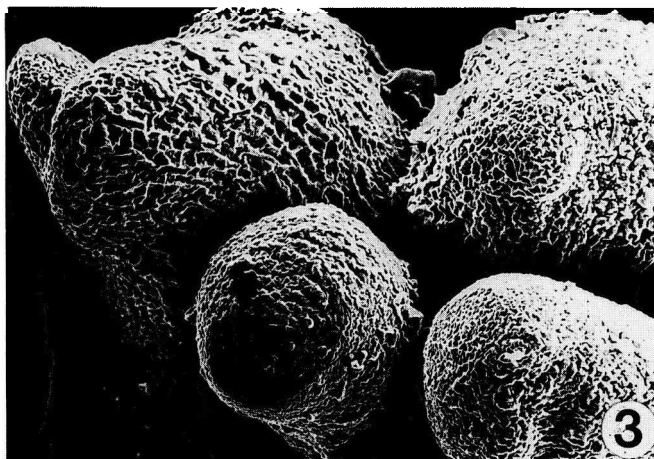


Fig 3. Scanning electron micrograph of globular embryos (x 110).

Effect of initial inoculum size on plant regeneration

Embryogenic calli of 75–900 mg fresh weight (fw) were inoculated on basal MS medium without hormones. Number of plants regenerated was recorded after 4 weeks of culture (table II). The maximum number of plants regenerated per g fw of embryogenic callus was produced when inoculum size was 150 mg fw in varieties DRLS, Suphal and PPL. However, it was 300 mg in the case of Kalalong. Any increase in inoculum size beyond the optimum fw in individual genotypes decreased the frequency of plant regeneration.

Effect of long-term culture on embryogenic competence

Random pieces of embryogenic calli (300–320 mg) from stock callus were transferred to basal medium with and without 0.1 mg/l NAA. On media supplemented with 0.1 mg/l NAA, the embryogenic competence in terms of percent callus explants forming somatic embryos and number of plants regenerated were significantly reduced by the 4th passage. Fifty and 75% decline (in percent explants forming somatic embryos) were noticed by 8th and 12th passage respectively. Number of plants regenerated/callus explant showed a 7-fold decrease from the 2nd to 12th passage in all 4 genotypes. On NAA free media 50% decline was observed in the 4th passage in percent callus explants forming somatic embryos. The number of plants regenerated/callus explant did not differ significantly on media with and without NAA in contrast to percent explants forming somatic embryos. Plants regenerated after prolonged culture were found to be phenotypically normal and were successfully established on soil.

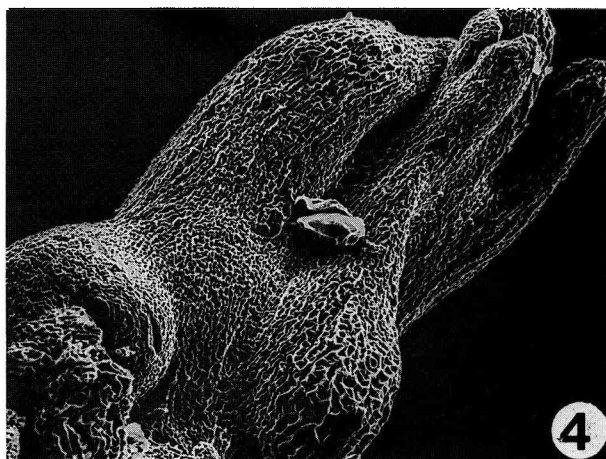


Fig 4. Scanning electron micrograph of shoot development from somatic embryo (x 110).

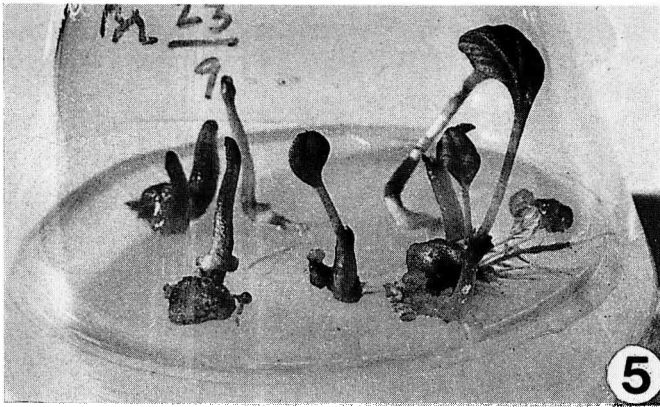


Fig 5. Early stages of plant regeneration from somatic embryos on basal medium.

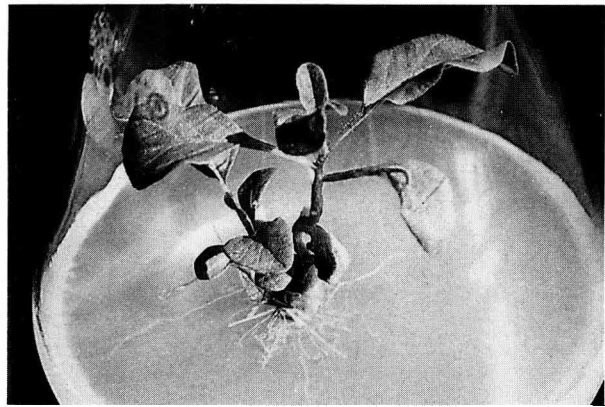


Fig 6. Complete plant with well developed root system.



Fig 7. Field-transferred *in vitro* plants.



Fig 8. Plants in bearing stage.

DISCUSSION

The present study has shown the induction of a high frequency of somatic embryogenesis from leaf explants of 4 high yielding varieties of *S. melongena* L. The frequency of somatic embryogenesis achieved was higher than that reported earlier for this species (Kamat and Rao, 1978; Matsuoka and Hinata, 1979). Gleddie *et al* (1983) obtained somatic embryogenesis in this species on media containing NAA only. But in the present study, the frequency of somatic embryo formation and plant regeneration were higher when the medium was supplemented with 0.1 mg/l kinetin in addition to NAA. Distinct genotypic response was noticed in respect of optimum NAA concentration, intensity of somatic embryo formation, and plant regeneration. Similar genotypic differences were noticed regarding embryogenic competence in long-term culture and initial inoculum size in plant regeneration.

The cellular origin of embryogenic callus or embryoids was not studied. However, in those cases where somatic embryos were formed from

callus, the origin may be from differentiated cells which have dedifferentiated and subsequently been redetermined for embryogenic development. This process has been reported in a number of species and this also holds true for androgenesis (Raghavan, 1976; Tisserat *et al*, 1979; Williams and Maheswaran, 1986). Results of the present study on the effect of initial inoculum size on callus growth and plant regeneration are in agreement with similar studies in several species (Caplin, 1963; Bhattacharya *et al*, 1981). In the present study, when inoculum was either smaller or larger than the optimal callus size, plant regeneration was reduced. Based on their study in Pokkali rice, Raghava Ram and Nabors (1985) have suggested that callus may release some factor into the medium which influences plant regeneration. This conditioning factor could not be produced in sufficient amounts when a smaller than optimal callus was used. A larger callus could deplete the medium of this factor or required nutrient.

In the present study though there was a decline in embryogenic competence with increase

Table III. Effect of long-term culture on embryogenic competence and plant regeneration in four genotypes of *Solanum melongena* L, on media containing 0.1 mg/l NAA (A) and without NAA (B). 40 calluses were transferred in each case.

| Genotypes and medium | | Number of passages | | | | | |
|----------------------|---|--------------------|------|------|------|------|------|
| | | 2 | 4 | 6 | 8 | 10 | 12 |
| DRLS | A | 65.0* | 58.0 | 40.0 | 30.2 | 20.0 | 15.0 |
| | | 14.0** | 11.0 | 7.3 | 4.0 | 3.1 | 1.8 |
| | B | 58.0 | 46.2 | 28.4 | 15.0 | 9.2 | 6.0 |
| | | 10.0 | 6.0 | 4.0 | 3.0 | 3.0 | 1.2 |
| Kalalong | A | 62.0 | 54.0 | 35.0 | 28.0 | 21.0 | 14.2 |
| | | 12.0 | 8.1 | 6.8 | 3.5 | 2.3 | 2.0 |
| | B | 51.0 | 45.2 | 25.0 | 13.6 | 9.0 | 5.0 |
| | | 9.0 | 6.2 | 4.5 | 3.0 | 2.0 | 2.0 |
| Suphal | A | 90.0 | 75.0 | 56.5 | 44.2 | 35.4 | 22.0 |
| | | 27.0 | 20.0 | 12.4 | 8.1 | 5.4 | 3.6 |
| | B | 80.0 | 63.0 | 39.2 | 23.0 | 16.8 | 10.0 |
| | | 22.4 | 14.0 | 8.2 | 5.0 | 4.0 | 3.0 |
| PPL | A | 78.0 | 62.0 | 47.3 | 32.4 | 22.5 | 16.0 |
| | | 15.7 | 12.6 | 7.8 | 5.2 | 3.6 | 2.2 |
| | B | 68.5 | 50.0 | 33.5 | 18.0 | 11.0 | 7.0 |
| | | 11.0 | 8.0 | 5.0 | 3.4 | 2.8 | 2.0 |

Values are means of 4 replicates. Each passage is of 30 d duration. * callus explants forming somatic embryos (%), ** = plants regenerated / callus explant.

in the number of subcultures, the embryogenic callus with potential for regeneration under prolonged culture was observed on both medium with 0.1 mg/l NAA and without NAA. NAA-free medium did not significantly alter the number of plants regenerated/callus explants in comparison to those explants on NAA supplemented media. Many cultures tissues exhibit high potential for embryogenesis/ morphogenesis when first initiated but gradually a decline sets in as the subculture proceeds, with eventual loss of response (Smith and Street, 1974). In exceptional instances, however, the morphogenic potential was retained even after prolonged subculture (Padmanabhan *et al*, 1974). Decline in embryogenic and morphogenic capacities in long-term culture may be due to change in the genetic system concerned, cytological disturbances (D'Amato, 1978) and partly due to the

changed metabolism in competent cells so that they are no longer responsive to external growth regulators.

In conclusion, the results of the present study and our earlier report on plant regeneration from encapsulated somatic embryos of this species (Rao and Singh, 1991) demonstrate the possibility for exploiting the embryogenic potential of leaf explants of *Solanum melongena* L for improvement and large-scale propagation.

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