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Somatic Embryogenesis in Papaya (*Carica papaya* L. cv. TNAU Papaya CO.8)

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Authors' contributions

This work was done in collaboration among all the six authors. Authors KS and DS designed the study. Author CKR performed the experiment, statistical analysis and wrote the first draft of the manuscript. Authors KS, DS, KKK, CK and GK supervised the study. Authors KS, KKK and DS corrected the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

An efficient indirect somatic embryogenesis protocol for *Carica papaya* var TNAU Papaya CO.8 was developed using immature zygotic embryos as an explant. Two growth regulators namely 2,4-D and picloram each at 1, 2, 3 mg/L were tested for callus induction and the highest callus induction frequency (83.33%) was observed in MS medium supplemented with 3 mg/L 2,4-D. However the rate of conversion into somatic embryos was highest (63.33%) on MS medium supplemented with 2 mg/L 2,4-D. Maturation of somatic embryos was studied by using MS medium with different concentrations of abscisic acid (ABA) and benzyl amino purine (BAP) along with glutamine (400

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mg/L). The maturation of globular embryos was observed to be higher in the combination of ABA (1.5 mg/L), BAP (0.4 mg/L) along with glutamine (400 mg/L). Even though regenerative was observed from cotyledonary stage embryos in presence of different growth regulators like BAP, α-naphthalene acetic acid (NAA), phloridzin dehydrate kinetin and gibberellic acid, further growth was not observed due to abnormal regenerative structures. Regeneration of cotyledonary stage somatic embryos were highest (77.4%) in half strength MS medium without growth regulators. The well-developed plantlets with shoots and roots were subsequently transferred for hardening.

Keywords: Somatic embryogenesis; papaya; TNAU Papaya CO.8; regeneration; immature zygotic embryos.

1. INTRODUCTION

Papaya (*Carica papaya* L.) belongs to the family Caricaceae and it is one of the most economically important fruit crop of the tropical regions of the world. It is a dicotyledonous, polygamous and diploid species with the geographical origin being Southern Mexico and Costa Rica [1]. It is a nutritionally rich fruit crop with high carotene and vitamin C content. India leads the world in papaya production with an area of 1,39,000 ha in cultivation and registering an annual production of 61,04,000 MT [2]. This crop is also an essential source of industrially important proteolytic enzyme 'Papain' which is used in meat processing and pharmaceutical industries.

Crop improvement efforts have resulted in the development of several promising varieties from Tamil Nadu Agricultural University (TNAU), Coimbatore, Indian Agricultural Research Institute (IARI), Pusa and Indian Institute of Horticultural Research (IIHR), Bangalore.

The variety TNAU Papaya CO.8 released by TNAU Coimbatore is a high yielding (200-220 t/ha), red pulped dioecious variety. Although papaya is conventionally propagated through seeds there is difficulty in predicting the actual sexform of the plants until flowering. When cultivating a dioecious variety the grower has to plant at least 4-6 seedlings per hill and resort to thinning of excess males during flowering, which increases the cost of cultivation. In conventional seed production, maintenance of genetic purity is laborious due to highly heterozygous nature of the crop besides requiring proper knowledge on the selection of male and female parents. This difficulty can be overcome if protocols are developed for large scale clonal multiplication of female or bisexual lines. Propagation through cuttings and grafting is partially successful but is not commercially practiced due to the limited multiplication rate and these methods could spread the serious sap transmissible papaya ring spot virus (PRSV) disease. Hence the use of alternative techniques for large scale clonal propagation is essential.

Micropropagation techniques can be used to produce a large number of true to type quality planting materials. Attempts to produce *in-vitro* plants through shoot cultures in papaya have proved to be disadvantageous due to high levels of endogenous bacterial contamination, difficulty in culturing mature tissue and loss of viability in shoot cultures when repeatedly sub-cultured [3,4].

Somatic embryogenesis is an attractive alternative approach to develop clonal plants, and the bipolar nature of somatic embryos enables both shoot and root apices which in turn reduce a step of aseptic manipulation. In papaya, somatic embryos from immature zygotic embryos, leaf and young hypocotyl tissues have been developed earlier [1,4,5,6,7,8]. The technique can also be a base for genetic manipulation of desirable traits such as PRSV resistance [9].

Though a number of reports indicating successful somatic embryogenesis in papaya, the frequency of somatic embryogenesis vary considerably from genotype to genotype and hence different cultural conditions may be required for each genotype. Therefore this study was attempted to develop an effective somatic embryogenesis protocol for the high yielding dioecious cultivar TNAU Papaya CO.8.

2. MATERIALS AND METHODS

2.1 Preparation of Explants

The selfed 90-100 day-old unripe fruits of TNAU Papaya CO.8 were collected from the Orchard, Horticultural College and Research Institute, TNAU, Coimbatore. The unripe fruits were thoroughly washed with distilled water two to

three times. The fruits were surface decontaminated using 70% (v/v) ethyl alcohol for two minutes. They were soaked in 1.25% sodium hypochlorite (w/v) for an hour followed by washing with distilled water thrice. The immature seeds were collected from unripe fruits under the laminar air-flow chamber (Fig. 1b).

These immature seeds were first washed with sterile distilled water, then washed with sterile distilled water containing Tween 20 for one minute and again washed with sterile distilled water to avoid excess surfactants. Then these were surface decontaminated using 70% (v/v) ethanol and 1.25% sodium hypochlorite (w/v) sterilization for three minutes and finally washed thrice with sterilized distilled water to remove traces of sterilants. Immature zygotic embryos were excised under stereo-microscope (Zeiss Stemi DV4) by removing the sarcotesta layer of the seed and giving the gentle cut on the embryo sac with a sterilized blade.

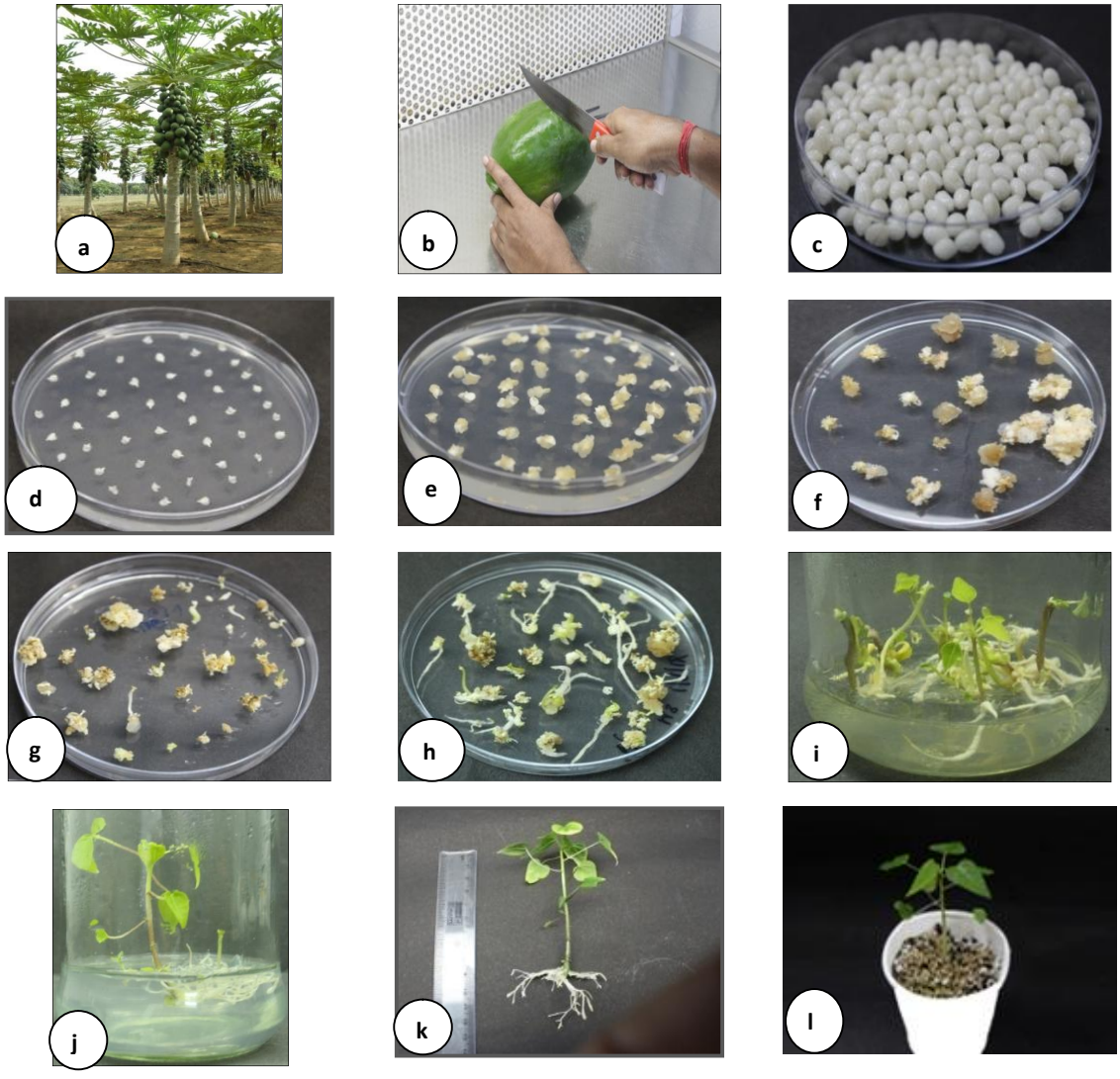

Fig. 1. Schematic representation of steps involved in somatic embryogenesis of papaya cultivar TNAU Papaya CO.8

a. Papaya field; b.90-100 days old selfed immature fruit; c. Seeds from immature fruits; d. Excised immature zygotic embryos; e. Callus formation after 13 days of culturing in CIM containing 2,4-D; f. Embryogenic calli after 60 days of culturing in CIM containing 2,4-D; g. Somatic embryos at the maturation stage in maturation medium; h. Somatic embryos at the regeneration stage in regeneration medium; i. Plantlets with well- developed leaves on regeneration medium; j: Well developed plantlet; k. Plantlet with roots: l. Hardened plantlets

2.2 Embryogenic Callus Initiation

The excised immature zygotic embryos were inoculated on the petri plates containing Callus Induction Media (CIM) supplemented with full strength MS [10] basal salts, MS vitamins, proline (500 mg/L), glutamine (400mg/L), casein hydrolysate (300 mg/L), 3% sucrose, gelrite (4 g/L) with varied concentrations *viz*., 1.0, 2.0, 3.0 mg/L of 2,4-D and picloram (Fig. 1d). The pH was normalized to 5.8 with 0.1 N NaOH before adding the gelrite and autoclaved at 121ºC for 15 min. Twenty immature zygotic embryos were inoculated per petri plate and plates were kept at 25 ± 2ºC in darkness for 45 days for callus induction and somatic embryo formation. The callus culture of papaya var. TNAU Papaya CO.8 was examined for different somatic embryo developmental stages by morphological observations. The calli were sub-cultured on the initiation medium for another 15 days for embryo development. The percentage of callus showing somatic embryogenesis and callusing percentage were recorded based on the visual observations.

2.3 Maturation of Somatic Embryo

To induce embryo development, the embryogenic calli were placed on maturation media. The MS basal medium supplemented with 4 g/L of gelrite, 1.5 mg/L of ABA, 3% sucrose, 400 mg/l of glutamine and 30 g/L sucrose with varying concentrations *viz*., 0.4 mg/L and 0.6 mg/L of BAP was adopted. The cultures were maintained for two weeks in dark condition and then incubated under the light with a 16/8 hour photoperiod for four weeks (sub-cultured on fresh medium at two weeks interval). Somatic embryo development was examined under the microscope on a regular basis.

2.4 Regeneration of Somatic Embryos

After six weeks, fully-grown cotyledonary embryos were placed on the regeneration medium *i.e*., half strength MS basal medium, MS vitamin, 0.4% gelrite, 3% sucrose supplemented with varying combinations of growth regulators and subjected to light with 16/8 hour photoperiod for two weeks. For shoot formation, 2-3 subcultures were done at every two-week interval.

2.5 Hardening of Plants

After 4-6 weeks, plantlets of 2-3 cm height with 2-3 trilobed leaves were excised and root dip treatment was given for two to five seconds with 500 ppm of indole-3 butyric acid (IBA), for initiating rooting in the plantlets. The rooted young plantlets were transferred to coir pith and incubated in a culture room under 16 hours photoperiod for hardening by covering it with plastic cover with few holes in them. Then the plantlets were transferred to 1:1:1 ratio of coir pith: zeolite: soilrite and were maintained at high humidity before transferring to the greenhouse.

2.6 Observations and Statistical Analysis

The experiments were repeated thrice. A completely randomized design (CRD) was used for statistical analysis. Observations on callus induction, maturation of somatic embryos, and regeneration of shoots were registered periodically in each treatment. The data were analyzed computing ANOVA and critical difference at 5% probability.

3. RESULTS AND DISCUSSION

3.1 Induction of Embryogenic Callus

The number of days for callus initiation was counted from the day of inoculation to day of first response from the excised embryos. The minimum callus induction period was registered with an increased concentration of 2,4-D *i.e.,* the embryos inoculated in MS media fortified with 2,4 D @ 3 mg/L recorded the lowest number of days (12.35 days) for callus initiation (Fig. 1e), followed by MS media supplemented with 2,4-D @ 2 mg/L (13.28 days). No callus formation was observed in MS basal media alone (Table 1). Similarly, callus formation using zygotic embryos was observed after 15 days of culturing in the callus initiation medium [11].

After 30 to 45 days, callus formation was observed with all the treatments used in the experiment. Higher callus induction was recorded in MS media fortified with 2,4-D @ 3 mg/L (83.33%), and in the treatment MS fortified with 2,4-D @ 2 mg/L (81.66%) (Table 1). Similarly Roy et al. [12] reported that 85 per cent of explants produced embryogenic calli when culture media was supplemented with 1.5 mg/L of 2,4-D. Anandan et al. [4] reported that highest callus induction frequency (87.16%) in callus induction medium in presence of 2,4-D @ 2 mg/L was the optimum for embryogenic callus formation. in cultivar CO.7. In general 2,4-D

promoted friable calli formation while picloram resulted in formation of watery nature of calli with poor growth.

Table 1. Effect of auxins concentrations on callus induction and somatic embryogenesis in TNAU Papaya CO.8

Sl. no.	Treatments	Days for callus Initiation	Callus induction (%)	Percent of embryogenesis
1	Control(MS alone)	----	Nil (0.62)	Nil (0.62)
2	2,4-D @ 1mg/L	14.56[b]	75.00 (60.07)[b]	38.33 (38.24)[c]
3	2,4-D @ 2mg/L	13.28[d]	81.66 (64.80)[a]	63.33 (52.74)[a]
4	2,4-D @ 3mg/L	12.35[e]	83.33 (66.25)[a]	55.00 (47.87)[b]
5	Picloram @ 1mg/L	15.55[a]	45.00 (42.12)[d]	31.67 (34.23)[d]
6	Picloram @ 2 mg/L	14.81[b]	53.33 (46.92)[c]	41.67 (40.19)[c]
7	Picloram @ 3 mg/L	14.00[c]	58.33 (49.79)[c]	36.67 (37.25)[c]
	SEd	0.17	2.84	1.48
	CD @ 5 %	0.35	6.09	3.18

Figures in parenthesis are arcsin transformed values; values followed by similar alphabets within a column are not significantly different at 0.05 probability

Table 2. Effect of various concentrations of ABA and BAP on the maturation of somatic embryos in papaya cv TNAU Papaya CO.8

Sl. no.	Treatment details	Percent maturation of globular embryos
1	MS alone	0.00[d] (0.62)
2	MS + ABA (1.5 mg/L)	21.67[c] (27.74)
3	MS + ABA (1.5 mg/L) + BAP (0.4 mg/L)	53.33[a] (46.91)
4	MS + ABA (1.5mg/L) + BAP (0.6 mg/L)	36.67[b] (37.27)
5	MS + ABA (2.0 mg/L)	5.00[d] (12.92)
6	MS + ABA (2.0 mg/L)+ BAP (0.4 mg/L)	0.00[d] (0.62)
7	MS + ABA (2.0 mg/L)+ BAP (0.6 mg/L)	0.00[d] (0.62)
	SEd	**2.67**
	CD @ 5 %	**5.73**

Figures in parenthesis are arcsin transformed values; values followed by similar alphabets within a column are not significantly different at 0.05 probability

The calli derived from 2,4-D 2 mg/L and 3 mg/L were found to be white, friable and compact in nature, which led to formation of higher percentage of embryogenic calli on MS media fortified with 2,4-D @ 2 mg/L (63.33%) after 45 to 50 days from callus initiation and followed by MS media fortified with 2,4 D @ 3 mg/L (55.00%) (Table 1). While in other media compositions only yellowish to brownish nonfriable calli was observed. This may be due to the hypersensitive reaction of phenol metabolizing enzymes like peroxidases, phenolases which leads to the browning of callus [13]. Earlier reports also show that medium supplemented with 2,4-D is the most efficient for embryogenic callus induction and somatic embryos formation in papaya [14,15,12,7,1,16,4], as 2,4-D stimulates an increased endogenous level of natural auxin [17].

3.2 Maturation of Somatic Embryos

The budding of globular stage somatic embryos (Fig. 1f) were observed under stereomicroscope after 60-70 days of culturing of embryogenic calli on embryo induction medium with 2,4-D 2 mg/L. For the effective maturation of somatic embryos to the cotyledonary stage, embryogenic cultures were transferred to MS medium fortified with different concentrations of ABA or in combination with different concentrations of BAP (Fig. 1g). Among the treatments, MS media fortified with ABA (1.5 mg/L) in combination with BAP (0.4 mg/L) recorded significantly highest per cent cotyledonary embryos (52.50%) (Table 2). Similar results were obtained by including ABA in the maturation medium which increased embryo size and improved somatic embryo development [18,19,20,7,5,1]. This is in contrast with the report given by Litz and Conver et al. [3], wherein the somatic embryos of papaya matured in the absence of exogenous growth regulators.

Earlier studies have shown that ABA is an essential plant growth regulator for embryo formation and maturation [21,22,23]. Several authors have also suggested the positive effects of ABA on somatic embryo development and maturation in several other crop species [24,25,26]. Among the different concentrations of ABA tested, only 1.5 mg/L was favourable for embryo maturation while 2 mg/L resulted in drying of callus and while lower concentrations did not significantly promote embryo maturation. Exogenously applied ABA might not allow tissue proliferation and promote embryo growth through alteration of nucleotide biosynthesis [27]. In this study also arresting of callus growth and embryo formation was observed when ABA was included in the maturation medium. It was also observed in few species, ABA prevents the secondary embryogenesis process, inhibiting early germination [28,29] and there by stimulating deposition of reserve products [30].

3.3 Regeneration of Plantlets

The matured cotyledonary embryos (Fig. 2d) were transferred onto regeneration media consisting of half MS fortified with casein hydrolysate (100 mg/L) in combination with different growth regulators *Viz.,* BAP, GA_3, NAA and phloridzin. Among the different treatment combinations used, the half strength MS without any growth regulators performed better, recording 77.4% germination of embryos within 13 days and registering the first leaf at 16th day (Fig. 1h). The emerging shoots with roots gave

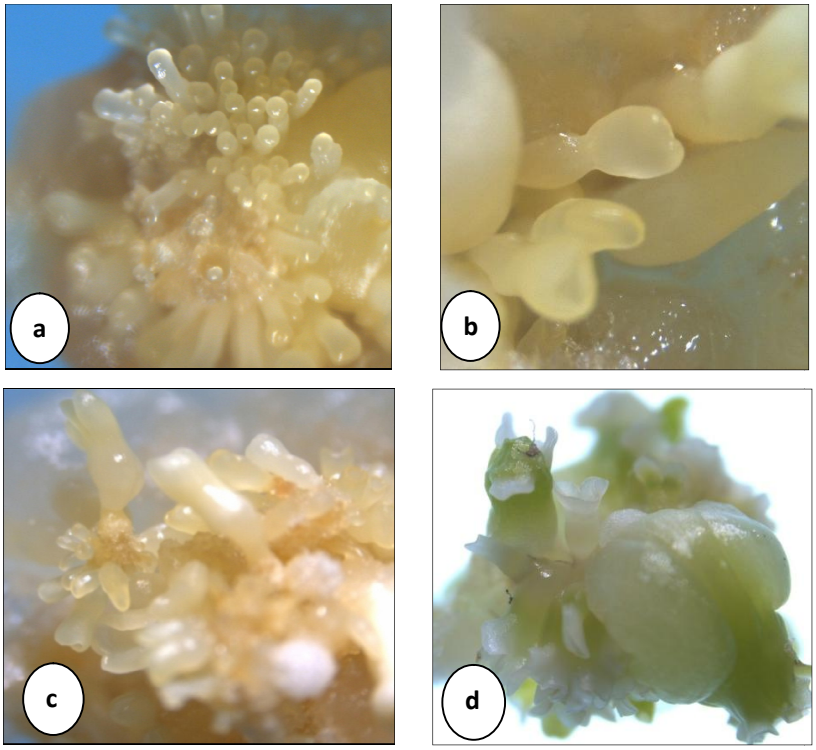

Fig. 2. Different stages of somatic embryo development in TNAU Papaya CO.8 cultivar at the 90th day

a. Globular stage; b. Heart stage; c. Torpedo stage and d. Cotyledonary stage

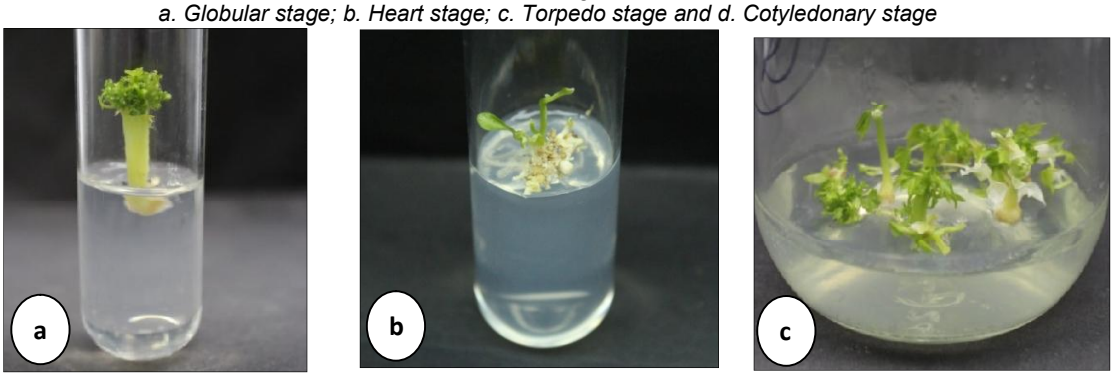

Fig. 3. Abnormal growth of plantlets in regeneration media supplemented with different growth regulators

a. BAP 0.4 mg/L + NAA 0.1 mg/L., b. BAP 0.4 mg/L + NAA 0.1 mg/L + Phloridzin 3 mg/L and c. BAP 0.2 mg/L + NAA 0.1 mg/L

rise to complete plantlets within 45- 60 days of culturing in half strength MS medium (Fig. 1j-k). The regenerated plantlets were hardened under greenhouse conditions (Fig. 1l). The results are found to be in accordance with the reports of Malabadi et al. [1] and Koehler et al. [19] where the efficient germination of somatic embryos and elongation of papaya shoots was achieved on half strength MS basal medium without any growth regulators, whereas the results obtained in the present study are in contrast with Roy et al. [12] in which it was reported that MS supplemented with 1.0 mg/L GA_3 and coconut water 10% resulted in highest (68%) percentage of somatic embryogenesis and proper growth of plantlets. In the present study plantlets obtained from the media consisting of combinations of growth regulators like BAP, NAA, phloridzin were found to be abnormal in leaf shape, branching in the main stem of the plantlets, fasciation like structures and there was callusing at the base of the shoots instead of roots (Fig. 3). Hence for regeneration of plantlets, half MS media without any supplementation of growth regulators proved to be sufficient for the cultivar TNAU Papaya CO.8.

4. CONCLUSION

In the present study the somatic embryogenesis in papaya cultivar TNAU Papaya CO.8 was attempted and demonstrated. Embryogenic calli in higher frequency were induced from immature zygotic embryos cultured on MS medium supplemented with 2,4-D @ 2 mg/L. The maturation of globular stage embryogenic calli was found to be satisfactory calli matured successfully in the MS medium in combination of ABA (1.5 mg/L), BAP (0.4 mg/L) along with glutamine 400 mg/L. Further regeneration of matured embryos occurred in half strength MS medium free from growth regulators and subsequently hardened. This protocol can be effectively utilized as an alternative for large scale clonal propagation and genetic transformation studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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