Phytomorphology 73(1&2) 2023, 29-35 DOI: 10.56024/ispm.73.1ampersand2.2023/29-35

Somatic Embryogenesis and Plantlet Formation in Tasar Food Plant

Lakshman Ankati[1], Praveen Mamidala[1,2], Rajinikanth Marka[1] and Rama Swamy Nanna
[1]*Plant Biotechnology Research Laboratory, Department of Biotechnology, Kakatiya University, Hanamkonda-506009, Telangana State, India*
[2]*Department of Biotechnology, Telangana University, Nizamabad-503322, Telangana State, India*

Manuscript received on 31 March, 2023; accepted on 5 June, 2023

ABSTRACT

A protocol for plant regeneration via somatic embryogenesis was developed in tasar food plant *Terminalia alata* B. Heyneex. Roth. Somatic embryoids were induced from leaf explants on Murashige & Skoog (MS) medium containing 30g/l sucrose fortified with different concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid) in combination with Kn (kinetin)/BAP(N^6-benzyl aminopurine) and 1.0 mg/L of BAP or Kn alone. Leaf explants were also cultured on MS medium with different levels of sucrose (3-10%) fortified with 2.0 mg/L of 2,4-D + 1.0 mg/L of Kn. The combination of PGRs (2.0 mg/L 2, 4-D + 1.0 mg/L Kn) at 5.0% sucrose level was better for somatic embryo induction and conversion into bipolar embryos. The well-formed, cotyledonary-shaped embryos germinated into plantlets with 45.7% frequency on MS medium supplemented with 0.5 mg/L of Indole butyric acid (IBA) + 1.0 mg/L of Kn. These plantlets were transferred to earthenware pots containing garden soil and compost (1:1) and grown to maturity with survival percentage of 40. This protocol was developed to induce somatic embryos with a high frequency of germination and their subsequent conversion into whole plantlets. Thus, the present investigation offers potential applications for use in gene transfer and development of transgenics in this important tasar food plant and also for the production of synthetic seeds.

Key words: *Terminalia alata,* somatic embryogenesis, sucrose, plantlet formation

***Author for correspondence:** Rama Swamy Nanna; e-mail: swamynr.dr@gmail.com; ORCID: 0000-0003-3617-5059

Introduction

Somatic embryogenesis is a potential area of research in tree biotechnology. Since trees are cross-pollinated, it is difficult to get true-to-type germplasm through natural breeding. Hence, there is a need to develop a protocol for induction of somatic embryogenesis in forest tree species in order to propagate and conserve elite germplasm.

Micropropagation through somatic embryogenesis has several advantages over organogenesis (Merkle, 1995). The success of regeneration by adventitious shoots depends on *in vitro* rooting which may be a constraint in many tree species. The multiplication rate is generally faster with somatic embryogenesis and this method has proved valuable in micropropagation of forest trees (Gupta et al., 1993).

Somatic embryogenesis offers the best multiplication system of high value clones for reforestation and provides a means of gene transfer and production of new plants from transformed cells for mass production of transgenic trees (Ravishankar Rai and McComb, 2002). Following the initial reports of Reinert (1958) and Steward et al. (1958), somatic embryogenesis has not only been achieved in crop species, but also in tree species (Hussain et al., 2000; Cardoza and D'Souza, 2000; Chand and Singh, 2001; Ravishankar Rai and Mc Comb, 2002). Somatic embryogenesis also has been reported in *Terminalia arjuna* by Kumari et al. (1998).

Terminalia alata, a medium-sized large deciduous tree which grows up to 35 meters tall has huge medicinal value and their leaves are considered to be an important meal for Tasar Silkworm. The wood of *T. alata* is routinely used in preparation of tools and in constructions as well. Further, it is often used as a fuel and reported to possess tritrerpenoid glycosides in their roots which have proved good for their antifungal activities (Srivastava et al., 2001). Due to these properties, *T. alata* is considered as a multipurpose tree and is exploited in urban and rural areas. Despite its importance, not much work has been done on

conservation of this multi-propose tree either using *ex-situ* or *in-situ* conservation methods. Therefore, we attempted to develop a reproducible *in vitro* regeneration protocol for the most neglected multipurpose plant, *T. alata*, which is medicinally important, which widely beneficial to the tasar silkworm industry and also to ayurvedic practitioners who use its roots as an active ingredient in medicinal preparations. In the current study, we report on induction of somatic embryogenesis and plantlet formation for the first time in *T. alata.*

Materials and Methods

Plant Material

Leaf explants were used from healthy trees of *T. alata* growing in the research field, Department of Botany, Kakatiya University. Leaves were washed thoroughly under running tap water and treated with Tween-20 for 20-30 minutes followed by 3 rinses in sterile distilled water. These were surface sterilized with 1.0% (w/v) sodium hypochlorite for 3-5 minutes followed by 3 rinses with sterile distilled water. Subsequently, these were treated with 1.0% (w/v) Bavistin for 10-15 minutes, 1% (w/v) of Streptomycin for 30 minutes and (6%) H_2O_2 for 10 minutes and washed with several changes of sterile distilled water. The sterilized explants were dried on sterile tissue paper and inoculated.

Culture Media and Culture Conditions

The sterilized leaf explants (1 cm^2) were inoculated on MSO, MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose along with 0.5 mg/L 2,4-D, various concentrations of Kn (1.0-4.0 mg/L), Kn/BAP or BAP/Kn alone. To find out the efficacy of sucrose on induction of somatic embryogenesis, the leaf explants were also cultured on MS basal salts containing 3-10% (w/v) sucrose levels and augmented with 2.0 mg/L 2,4-D + 1.0 mg/L Kn. In order to avoid the phenolics, the medium was also amended with 250 mg/L PVP, 250 mg/L ascorbic acid 250 mg/L citric acid. The pH of the medium was adjusted to 5.8 either with 0.1 N NaOH or 0.1 N HCl prior to addition of 0.8 % (w/v) Difco-Bacto agar and autoclaved at 121 °C under 15 psi for 15-20 minutes.

All the cultures were incubated at 25±1 °C under 16 hrs of light and 8 hrs of dark as photoperiod cycle. A light intensity of 40-50 µmol $m^{-2}s^{-1}$ was provided by white-cool fluorescent tubes. For sub-culturing, the cultures were transferred onto fresh medium every 3 weeks of incubation.

For further proliferation and maturation of somatic embryos, the calli consisting of globular embryos were transferred onto MS medium with 50 g/L sucrose augmented with 2.0 mg/L 2,4-D +1.0 mg/L Kn. Bipolar embryos were cultured on MS medium fortified with 0.5 mg/L IBA+1.0 mg/L Kn for germination and plantlet formation.

Plantlet Establishment

The plantlets developed through somatic embryogenesis were washed to remove remains of agar medium and transferred to plastic cups containing sterile soil: vermiculite (1:1). These plants were covered with polythene bags to maintain relative humidity (70-80%) and kept in the culture room for three weeks. Later the plantlets were shifted to earthenware pots containing garden soil: compost (1:1) and maintained in the green house.

Data on somatic embryogenesis were recorded for every 6 weeks of culture. Each experiment was repeated at least thrice consisting of 20 replicates.

Results

The leaf explants were cultured on MS medium without growth regulators (MSO) and MS medium containing 30 g/L sucrose augmented with various concentrations of 2, 4-D in combination with 1.0 mg/L Kn/BAP or on Kn/BAP alone. The leaf explants were also cultured on MS medium supplemented with 0.5 mg/L 2,4-D in combination with 1.0-4.0 mg/L Kn. The leaf explants cultured on MSO medium did not respond, whereas somatic embryogenesis was induced in all the combinations and concentrations of growth regulators tested but with varied results. Callus was formed from the cut ends of the explants after 2 weeks of culture in all the concentrations and combinations of auxins and cytokinins used. The gradual initiation of somatic embryogenesis was noticed after 3 weeks of culture. The calli pieces sub-cultured on the same fresh medium showed the somatic embryogenesis with globular to different forms depending upon the growth regulators present in the medium (Table 1).

A high percentage of somatic embryogenesis (62%) was observed on 0.5 mg/L 2,4-D+2.0 mg/L Kn with 38% of globular embryos formation (Fig.1A-B). As the concentration of Kn increased along with 0.5 mg/L 2,4-D, the somatic embryos induction percentage decreased. The maturation of globular embryos into bipolar embryos was absent at 3.0 and 4.0 mg/L Kn.

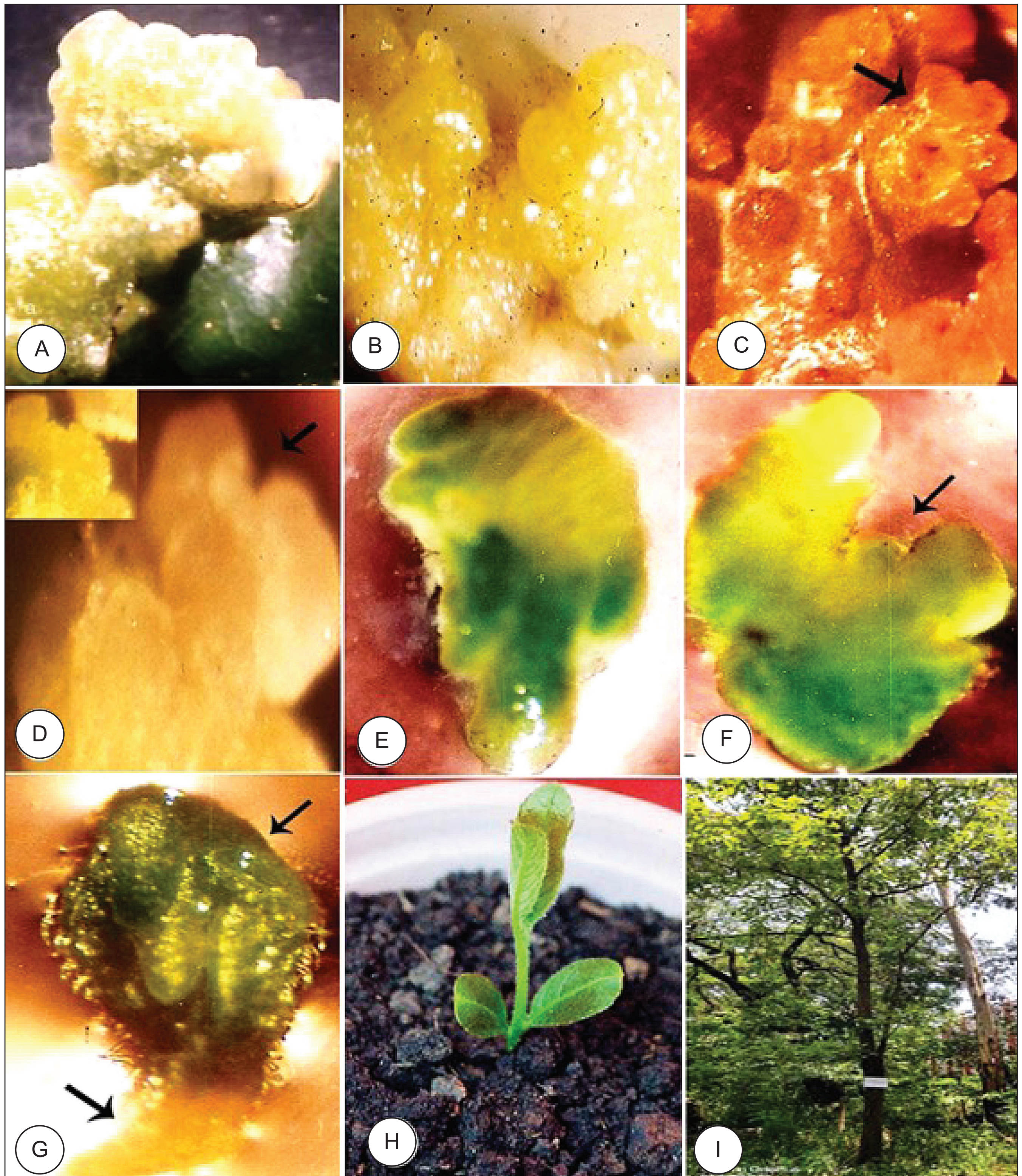

Figure 1. Somatic embryogenesis from leaf explants of *T. alata.* (A, B) Induction of proglobular and globular embryoids on MS (30g/l sucrose)+0.5 mg/L 2,4-D+ 2.0 mg/L Kn respectively; (C) High frequency of globular embryoids on MS (50 g/L sucrose) + 2.0 mg/L 2,4-D+1.0 mg/L Kn (Note the formation of cluster of embryoids); (D, E) Formation of heart-shaped and also torpedo-shaped (bipolar) embryos on the same medium respectively; (F, G) Germination of somatic embryoids on MS (30 g/L sucrose)+0.5 mg/L IBA+1.0 mg/L Kn; (F) cotyledonary stage embryo with primary axis; (G) Root formation; (H) Transplanted plantlet developed through somatic embryogenesis pathway; (I) Plant growing in the research field (10 years old).

Table 1. Induction of somatic embryogenesis from leaf explants of *T. alata* on MS with different concentrations of 2,4-D+Kn/ BAP and BAP/Kn

Growth regulator Conc. (mg/L)	% of cultures with somatic embryogenesis	% of cultures with globular embryos	% of cultures with bipolar embryos
MSO	---	---	---
2,4-D + Kn			
0.5 + 1.0	51	21	5
0.5 + 2.0	62	38	6
0.5 + 3.0	54	13	--
0.5 + 4.0	48	09	--
2,4-D + Kn			
1.0 + 1.0	67	39	12
2.0 + 1.0	90	54	26
3.0 + 1.0	75	48	22
4.0 + 1.0	52	41	16
2,4-D + BAP			
1.0 + 1.0	46	16	--
2.0 + 1.0	51	24	--
3.0 + 1.0	42	13	--
4.0 + 1.0	40	11	--
BAP			
1.0	20	07	--
Kn			
1.0	25	10	--

The leaf explants when cultured on MS medium containing 30g/L sucrose supplemented with various concentrations of 2,4-D in combination with 1.0 mg/L Kn showed maximum percentage of somatic embryogenesis (90%) at 2.0 mg/L 2,4-D+1.0 mg/L Kn (Fig. 1C). At high concentration of 2, 4-D, the percentage of somatic embryo induction was found to be low (Table 1). The conversion of globular embryos to bipolar embryos was higher at 2.0 mg/L 2, 4-D + 1.0 mg/L Kn followed by 3.0 mg/L 2, 4-D +1.0 mg/L Kn. All the concentrations of 2, 4-D along with 1.0 mg/L Kn showed the conversion of globular embryoids to bipolar embryos (Fig. 1D-E).

Somatic embryogenesis was also observed on MS medium supplemented with different concentrations of 2, 4-D+1.0 mg/L BAP. High percentage of somatic embryogenesis was recorded at 2.0 mg/L 2, 4-D+1.0 mg/L BAP including the percentage of globular embryos formation (Table 1). Bipolar embryos formation was not found in all the concentrations of 2, 4-D+1.0 mg/L BAP combination.

To induce the somatic embryogenesis, leaf explants were also cultured on 1.0 mg/L BAP/Kn alone. Somatic embryo induction percentage was less on both the cytokinins used. It is also interesting to find that there was no conversion of globular embryos into bipolar even after two subcultures on the same medium containing 1.0 mg/L BAP/Kn.

To find out the efficacy of sucrose in induction of somatic embryogenesis in *T. alata,* leaf explants were cultured on MS medium containing different concentrations of sucrose (3-10%) supplemented with 2.0 mg/L 2,4-D+1.0 mg/L Kn (Table 2). Maximum percentage of somatic embryo induction was recorded at 5.0% of sucrose (Fig. 1C). The percentage of conversion of globular embryos to heart-shaped embryos was also found to be higher at 50 g/L sucrose compared to all other sucrose concentrations used (Fig. 2). It was also interesting to note that the cluster of globular embryoids (6-10) formation was found at 50 g/L sucrose (Fig. 1C). At higher concentrations the conversion of somatic embryos decreased. The development of bipolar embryos from heart-shaped was higher at 4 and 5% (g/L) sucrose in *T. alata* (Fig. 1D). As the concentration of sucrose increased beyond 5.0%, there was a gradual decrease in the percentage of somatic embryogenesis. The explants consisting of somatic embryoids when sub-cultured on MS medium containing 50 g/L sucrose with 2.0 mg/L 2,4-D+1.0 mg/L Kn enhanced the formation of bipolar embryos (Fig. 1E). However they did not show further maturation of these bipolar embryos. Later

these cultures with bipolar embryos were cultured on MS medium containing 30 g/L sucrose fortified with 0.5 mg/L IBA+1.0 mg/L Kn (Fig. 2). All the bipolar embryos were converted into plantlets within 2 weeks on the same medium (Fig. 1 F-I).

Plantlet Establishment

The plantlets developed through somatic embryogenesis were hardened in the culture room for 3 weeks. Later these were shifted to pots containing garden soil: compost (1:1) and maintained in the green house. Survival of plantlets was found to be 40%. The plants developed through this technology were similar to parental plants (Fig. 1H).

Discussion

A major factor for somatic embryogenesis is the nature of growth regulators present in the induction medium. The type of auxin or auxin in combination with cytokinin in the medium greatly influences the somatic embryos induction frequency and plantlet formation.

High frequency of somatic embryoids was found at 2.0 mg/L 2,4-D+1.0 mg/L Kn in *T. alata* in comparison to all other combinations and concentrations of growth regulators used. The combination of both auxin and cytokinin elicited the maximum number of somatic embryos induction. Whereas the globular embryoids were not converted into bipolar in all the concentrations and combinations of growth regulators except at the concentrations of 0.5 mg/L 2,4-D+1.0/2.0 mg/L Kn and 2,4-D (1.0-4.0 mg/L)+Kn(1.0 mg/L) used in the present investigations. The medium containing 30g/L sucrose supplemented with 2.0 mg/L 2,4-D+1.0 mg/L Kn was found to be the best for induction of maximum percentage of somatic embryogenesis and also bipolar embryos formation (Table 2).

The requirement of cytokinin in addition to auxin was observed in *Sapindus trifoliatus* (Desai et al., 1986) and *Terminalia arjuna* (Kumari et al., 1998) as it was noticed in the present investigation. Kumari et al. (1998) have found the globular embryo formation from the leaf calli initiated on 5.0 mg/L 2,4-D+0.01 mg/L Kn in *T. arjuna.*

Somatic embryogenesis is generally believed to be triggered by an auxin and for many plants, 2,4-D has been widely regarded to be effective for somatic embryogenesis (Litz and Conover, 1983; Ammirato, 1983; Finner, 1988; Baker and Wetzstein, 1994). Similarly, 2,4-D induced somatic embryogenesis in *Mangifera indica* (Hussain et al., 2000). Despite the crucial role of 2,4-D in inducing somatic embryogenesis,

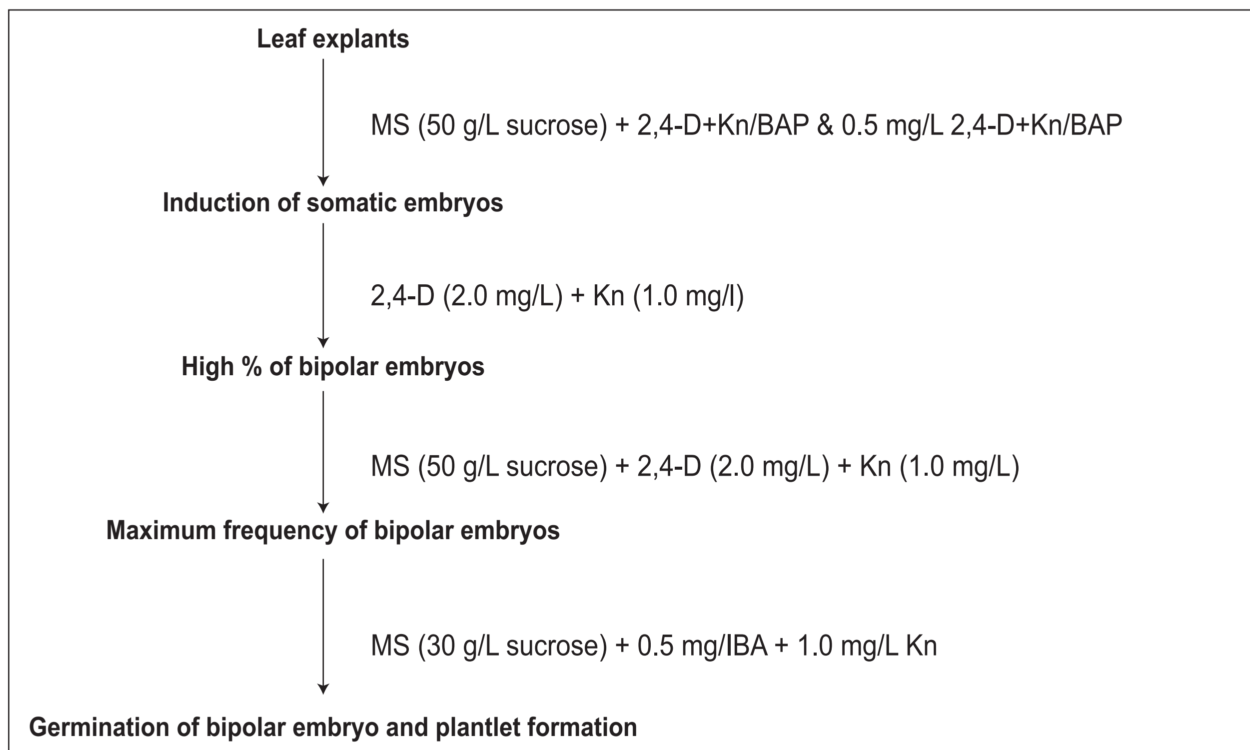

Figure 2: Flow chart showing the somatic embryogenesis in *T. alata*

Table 2. Induction of somatic embryogenesis from leaf explants of *T. alata* cultured on MS + 2,4-D (2.0 mg/L) + Kn (1.0 mg/L) and various levels of sucrose

% of Sucrose	% of somatic embryogenesis	% of cultures with globular embryos	% of cultures with heart-shaped embryos	% of cultures with bipolar embryos
3.0	88	63	35	26
4.0	93	78	43	38
5.0	95	83	52	47
6.0	63	49	29	22
10.0	52	31	16	09

continuous presence of auxin inhibited the embryo development into cotyledonary stage in both Amarapali and Chausa cultivars of mango (Hussain et al., 2000). The same phenomenon was also found in *T. alata* by not converting into cotyledonary stage in the present investigation.

According to Zimmerman (1993) new gene products are needed for the progression from the globular to heart-stage and these new products are synthesized only when an exogenous auxin is removed. But, we found the requirement of auxin and cytokinin combination for morphogenesis of somatic embryos into plantlet conversion in *T. alata.* At higher concentration of auxin, probably the population of embryogenic cells drop due to their disruption and elongation and embryogenic potential of the culture is lost (Bhojwani and Razdan, 1996). Similarly, in *T. alata* the embryogenic response was reduced at high concentrations of 2, 4-D+Kn or vice-versa and also with BAP/Kn.

Sinha et al. (2000) have found the efficiency of Kn in inducing somatic embryogenesis from leaf explants in *Sapindus mukorossi.* In contrast to their findings, Kn with 2,4-D was found to be superior in inducing high percentage of somatic embryogenesis in *T. alata*. Thus, auxin (2, 4-D) and cytokinin (Kn) combination showed the best results for somatic embryogenesis in *T. alata*. Maximum frequency number of somatic embryoids was found on MS medium containing at 50 g/L sucrose augmented with 2.0 mg/L 2,4-D+1.0 mg/L Kn. The present protocol can be used for large-scale production of true-to-type of tasar food plants and also for the production of transgenic plants using gene gun or *Agrobacterium* -mediated genetic transformation and also for production of synthetic seeds in *T. alata.*

Acknowledgements

AL is grateful to the Department of Biotechnology, Govt. of India, New Delhi for project assistantship.

References

Ammirato, P.V. 1983. The regulation of somatic embryos development in plant cell cultures, suspension cultures technique and hormone requirements. Bio/Technology, 1: 68-74

Baker, C.M. & Wetzstein, H.Y. 1994. Influence of auxin type of concentration on peanut somatic embryogenesis. Plant Cell Tissue and Organ Culture, 35: 151-156.

Bhojwani, S.S. & Razdan, M.K. 1996. Studies in plant science, pp.125-166. Plant Tissue Culture Theory and Practice. Elsevier, Amsterdam.

Cardoza, V. & D'Souza, L. 2000. Direct somatic embryogenesis from immature zygotic embryos in cashew (*Anacardium occidentale* L.). Phytomorphology, 50: 201-204.

Chand, S. & Singh, A.K. 2001. Direct somatic embryogenesis from zygotic embryos of a timber-yielding leguminous tree *Hardwickia binata* Roxb. Current Science, 80: 882-888.

Desai, V.H., Bhat, P.N. & Mehta, A.R. 1986. Plant regeneration of *Sapindus trifoliatus* L. (soapnut) through somatic embryogenesis. Plant Cell Reports, 3: 190-191.

Finner, J.J. 1988. Apical proliferation of embryogenic tissue of soyabean (*Glycine max* L. Merill). Plant Cell Reports, 7: 238-241.

Gupta, P.K., Pullman, G., Timmis, R., Kreitinger, M., Carison, W.C., Grob, J. & Welty, E. 1993. Forestry in the 21st century-The biotechnology of somatic embryogenesis. Bio/Technology, 11: 454-4590.

Hussain, A., Jaiswal, U., & Jaiswal, V.S. 2000. Somatic embryogenesis and plantlet regeneration in Amrapali and Chausa cultivars of Mango (*Mangifera indica* L.). Current Science, 78: 164-169.

Kumari, N., Jaiswal, U. & Jaiswal, V.S. 1998. Induction of somatic embryogenesis and plant regeneration from leaf callus of *Terminalia arjuna*. Current Science, 25: 1052-1055.

Litz, R.E., & Conover, R.A. 1983. High frequency somatic embryogenesis from *Carica* suspension culture. Annals of Botany, 51: 683-686.

Merkle, 1995. Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tissue Culture Biotechnology, 1: 112-121.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum, 159: 473-497.

Reinert, J. 1958. Morphogenese und ihre Kontrolle an Gewebekulturen aus Karotten. Naturwissenschaften, 45: 344-345.

Ravi Shankar Rai, V. & McComb, J. 2002. Direct somatic embryogenesis from mature embryos of sandalwood. Plant Cell Tissue and Organ Culture, 69: 63-70.

Sinha, R.K., Majumdar, K. & Sinha, S. 2000. Somatic embryogenesis and plantlet regeneration from leaf explants of *Sapindus mukorossi* Gaertn.: A soapnut tree. Current Science, 78: 620-623.

Srivastava, R.K., Fahey, L. & Christensen, H.K. 2001. The resource-based view and marketing: The role of market-based assets in gaining competitive advantage. Journal of Management, 27: 777-802.

Steward, F.C., Mapes, M.O. & Mears, K. 1958. Growth and organized development of cultured cells. II. Organization in Cultures Grown from freely suspended cells. American Journal of Botany, 45: 705-708.

Zimmerman, J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. Plant Cell, 5: 141-143.