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RESEARCH ARTICLE

SOMATIC EMBRYOGENESIS AND PLANTLET REGENERATION FROM NODAL CULTURES OF *CELASTRUS PANICULATUS* WILLD. – A RARE ENDANGERED MEDICINAL PLANT

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ABSTRACT

An efficient protocol has been developed for the induction, maturation and germination of somatic embryos from nodal segments of *Celastrus paniculatus* Willd. Somatic embryos were induced on Murashige and Skoog (1962) (MS) basal medium containing 1.0 mg l^{-1} benzyladenine (BA) + 1.0 mg l^{-1} kinetin (Kn). The explants with primary embryogenic callus was subsequently transferred onto MS + B_5 vitamins Gamborg's *et al.* (1968) liquid and semi-solid medium supplemented with 0.5 mg l^{-1} α- naphthaleneacetic acid (NAA) + 1.5 mg l^{-1} BA resulted in highest number of somatic embryos. The development of somatic embryos took place in the same medium. The well-formed embryos germinated on B_5 medium supplemented with 0.5 mg l^{-1} abscisic acid (ABA) + 1.0 mg l^{-1} BA. Further development into healthy plantlets was obtained on basal B_5 medium. Hardened plantlets produced normal plants upon transfer to soil. A distinct feature of this study is the somatic embryogenesis in nodal cultures seems to be first report on this plant.

INTRODUCTION

Celastrus paniculatus Willd (Celastraceae) is a medicinal woody climber distributed at 1200 m altitudes in hilly tracts of India and other countries of South-East Asia. Seeds of this plant are source of Ayurvedic drug 'Jyothishmati' used in treating rheumatism, gout and depressive illness (Baranwal and Singh, 2000). A black empyreumatic oil oleum nigrum, distilled from the seeds is a tranquilizer and widely used as antifatigue, antistress, antipyretic and analgesic (Patel *et al.*, 1995). The seed extract exhibits antibacterial, antifertility and antispermatogenic effects in male rats (Wangoo, 1988). Leaf sap is emmenagogue and an antidote for opium poisoning (Warrier *et al.*, 1994). Flowers possess analgesic and anti-inflammatory activity (Ahmad *et al.*, 1994). Bark is reported to be abortifacient, depurative and a brain tonic and also taken internally for snake bite (Govil, 1993). Due to over exploitation and destructive harvesting hampered natural vegetation through seeds while, seeds exhibit poor viability and germination.

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Conventional propagation through seeds is slow and cumbersome, raising concern about the possible extinction of species and has been declared threatened (Kirtikar and Basu, 1987; FRLHT, 1997; Seetharam *et al.*, 1998). The multiplication of the species through tissue culture technique is urgently needed for enhancing population size to counteract genetic stochasticity. Somatic embryogenesis has been successfully exploited for clonal propagation, artificial seeds production, cryopreservation for the storage of elite clones and most importantly *in vitro* manipulation through genetic transformation (Machado *et al.*, 1995). *In vitro* multiplication through shoot and callus has been reported in C. *paniculatus* (Lakshmi and Seeni, 2001; Sharada *et al.*, 2003). We report here a simple protocol for induction, maturation and germination and plantlet formation from nodal cultures of *C. paniculatus.*

MATERIALS AND METHODS

Plant material

C. paniculatus was collected from botanic garden, Gulbarga University, Karnataka, India. Nodal explants (1-2 cm long), excised from 6-7 years old healthy plant were washed in running tap water and rinsed in 5% (v/v) Teepol detergent for

10 min. Surface sterilization was done by immersion in 0.1% (w/v) mercury chloride ($HgCl_2$) for 3-5 min and followed by 4-5 washes in sterile distilled water. Nodal explants were inoculated one per borosil culture tube (15 cm x 2.5 cm) with their cut ends implanted vertically on the medium.

Induction of somatic embryogenesis

The nutrient medium contained salts and vitamins of MS (Murashige and Skoog's., 1962) supplemented with 3 % (w/v) sucrose and varied concentrations of BA (0.5-2.0 mg l^{-1}) alone or in combination with Kn (0.5-2.0 mg l^{-1}) or NAA (0.5-2.0 mg l^{-1}) to check their embryogenic callus induction efficiency. The medium was adjusted to pH 5.7 before adding 0.8% (w/v) agar (Hi Media laboratories Pvt., Ltd., Mumbai), dissolved and dispensed in culture tubes and then autoclaved at 121^{o}C and 1.1 Kg cm^{-2} pressure for 15 min. All cultures were incubated in a culture room maintained at 25 ± 2^{0}C and 12 h photoperiod. A photon flux density of 40 µ $Em^{-2}S^{-1}$ was provided by cool white fluorescent tubes (Philips India Ltd., Mumbai).

Maturation of somatic embryos

Embryogenic calli were transferred to liquid and solid (shaking as well as static) medium having same concentration for development and maturation. The suspension cultures were initiated by transferring 4 week old embryogenic callus (1 g) fresh weight onto MS +B5 vitamins liquid medium (50 ml) containing various concentrations of NAA (O.5- 2.0 mgl^{-1}) in combination with BA (O.5- 2.0 mgl^{-1}) in Erlenmeyer flask (250 ml) and kept on gyratory shaker at 100 rpm. Suspension cultures were sub cultured at 15 days interval in the medium having same composition. All the cultures were incubated at 25 ± 2^{0}C and 14 h photoperiod. Number of somatic embryos formed in liquid medium was compared to semisolid medium for 30 days. The number of well developed embryos obtained were counted and averaged for 20 replicates and experiment was repeated thrice.

Germination of mature embryos

Mature embryos were transferred to (i) MS basal (semisolid) medium containing (2%sucrose) (ii) with ABA (O.5 - 2.0 mgl^{-1}) + BA (O.5 - 2.0 mgl^{-1}) (iii) basal B_5 medium (2% sucrose) and (iv) B_5 medium with ABA (O.5- 2.0 mgl^{-1}) + BA (O.5 - 2.0 mgl^{-1}). Medium which promoted the initiation of both root and shoot primordia was identified as best germination medium.

Plantlet formation

Germinated embryos with well developed root and shoot systems were transferred for further growth onto (i) semisolid basal MS medium (ii) basal B_5 medium (iii) ½ strength B_5 medium. The sucrose content was kept 1% in all cases. The number of plantlet with root and shoot systems was counted. The ratio of plantlet formed to the total number of mature embryos was calculated as the conversion percentage.

Hardening of plantlets

Hardening process, which took about 4 weeks, was critical to successful transfer of plants to soil. Complete plantlets in the tubes were kept open for 5-6 days by loosening cotton plugs. After a day they were transferred to plastic cups with sterile inert supporting powder SOILRITE (Supplied by Karnataka explosive, India) and ½ strength liquid medium without sucrose. Each plantlet was covered with a glass beaker and cups were maintained in the growth chamber at 90% relative humidity and 14 h light photoperiod. The well developed plants were transferred to sterile soil, farmyard manure and sand mixed in 3:1:1 ratio. The humidity was gradually reduced to 60% over 20 days. Regenerated plants then transferred to the field successfully.

Histology

To ascertain the embryogenic nature of differentiating structures were subjected to histological study. Somatic embryos were fixed in acetic acid and alcohol (1:3) then dehydrated in ethanol xylol series embedded in paraffin wax, sectioned free handly and at 8-10 µ thickness and stained with hematoxylin and basic fuschin.

Statistical analysis

The number of somatic embryos was counted from the 20 replicates and was repeated thrice. The data were analyzed by ANOVA and Duncan's multiple range test (α = 0.05) was used to compare treatment means.

RESULTS AND DISCUSSION

Effect of cytokinins on somatic embryo induction Somatic embryogenesis has been reported in tissues cultured from more than 30 plant families (Narayanswamy, 1997). The auxins and cytokinins play a vital role in somatic embryo induction, maturation and germination. The nodal explants of C. paniculatus developed four different types of embryogenic callus viz., white friable, white nodular friable, green compact and brown friable on MS medium supplemented with BA (O.5 - 2.0 mgl^{-1}) in combination with Kn (O.5 - 2.0 mgl^{-1}) and green compact, white nodular friable and brown compact with roots on MS medium supplemented with NAA (O.5 - 2.0 mgl^{-1}) in combination with BA (O.5 - 2.0 mgl^{-1}).

All the embryogenic callus developed were induced from 10-28 days after inoculation of which white nodular friable callus has shown early induction and was found to posses maximum embryogenic potential with 90% in 1.0 mgl^{-1}BA + 1.0 mgl^{-1} Kn and 50% in 1.0 mgl^{-1}NAA + 1.5 mgl^{-1} BA. In the present study (BA and Kn) were used for embryo induction; presumably, endogenous auxin in the explant stimulated cell proliferation and initiated the somatic embryos (Zimmerman, 1993). MS + 1.0 mgl^{-1}BA + 1.0 mgl^{-1} Kn was successful in producing maximum number of somatic embryos (Table 1) and shows early developmental stages of embryogenesis distinctly (Figure 1A). Regular subculturing of calli (4 weeks intervals) and addition of activated charcoal (0.5%) prevented cessation of growth due to heavy leaching of phenolics resulting in blackening of callus.

Effect of auxin and cytokinin on maturation

In a significant development, the embryogenic callus (1 g fresh weight of 4 weeks old) when transferred to MS + B_5 vitamins liquid and semisolid medium containing 0.5 mgl^{-1} NAA + 1.5 mgl^{-1}BA, development and maturation was noticed.

Table 1. Somatic embryo induction in MS solid medium from nodal segments of *C. paniculatus* Willd

Growth regulators (mgl^{-1})		Induction period of callus (days)	Nature of callus	Embryogenic calli (%)
*BA	Kn			
0.5	1.0	15	White friable	60^{ab}
1.0	1.0	10	White nodular friable	90^{a}
1.5	1.0	25	Green compact	70^{ab}
2.0	1.0	28	Brown friable	50^{c}
**NAA	BA			
0.5	1.0	20	Green compact	40^{b}
1.0	1.5	18	White nodular friable	50^{a}
1.5	1.5	23	White friable with roots	30^{c}
2.0	1.5	28	Brown compact with roots	20^{d}

* BA + Kn ANOVA F = 2.806; P<0.05
** NA + BA ANOVA F = 1.348; P<0.05
Means followed by same letters are not significantly different according to DMRT at α - 0.05

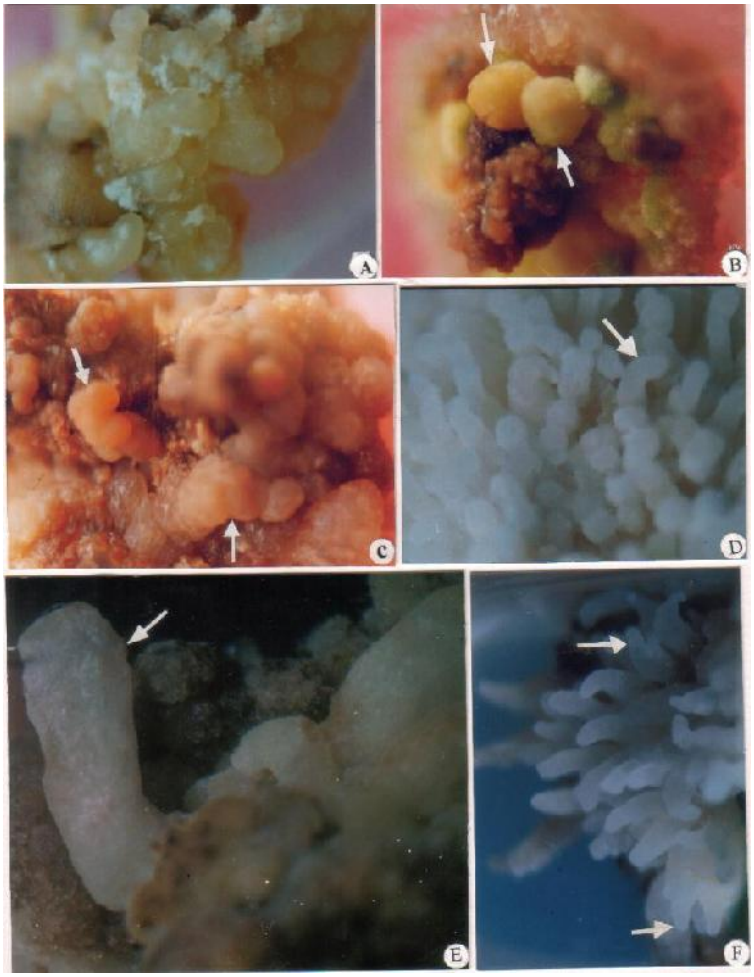

Figure 1. Stages in somatic embryo development in *Celastrus paniculatus* Willd

A) Nodular embryogenic callus
B) Early (arrow) and late globular embryo
C) Heart shaped embryos
D) Torpedo shaped embryo
E) Magnified view of torpedo shaped embryo under stereomicroscope
F) Matured embryo

Earlier the effect of NAA and BA on development and maturation of somatic embryos was reported in *Astragalus adsurgens* (Luo and Jia, 1998). The white clumps of somatic embryos sporadically appeared on outer periphery of the friable calli within four weeks. Microscopic examination revealed numerous aggregates of somatic embryos (Table 2) with distinct early and late globular (Figure 1B), heart shape (Figure 1C), Torpedo (Figure 1D and1E) and cotyledonary stages (Figure 1F). It is evident from the observation that more number of somatic embryos was formed in liquid medium when compared to semisolid medium after 30 days culture (1.2 times on per gram bases and 1.3 times on absolute basis).

Table 2. Somatic embryo development, maturation and conversion from embryogenic callus cultures of *C. paniculatus* Willd

Growth regulators		MS + B_5 Vitamins liquid media Development and maturation		Growth regulators		B_5 semi solid media Germination and conversion	
NAA	BA	Mean number of heart shaped embryos ± S.E.	Mean number of mature embryos ± S.E.	ABA	BA	Regenerated plantlets	Conversion percentage (%)
0.5	1.5	114.5 ± 1.08^{a}	67.9 ± 1.26^{a}	0.5	1.0	30.0 ± 0.63^{a}	44.18
1.0	1.5	58.3 ± 1.08^{b}	36.0 ± 1.41^{b}	1.0	1.0	11.4 ± 0.32^{b}	31.66
1.5	1.5	55.0 ± 1.0^{c}	18.8 ± 1.02^{c}	1.5	1.0	6.8 ± 0.32^{c}	36.17
2.0	1.5	37.4 ± 1.6^{d}	14.0 ± 0.71^{d}	2.0	1.0	3.2 ± 0.32^{d}	22.85

* Means followed by same letters are not significantly different according to DMRT at α - 0.05

Auxin is implicated as playing a major role in embryogenesis, providing positional information for the coordination of correct cellular patterning from the globular stage onwards, and auxin transport holds a key role within the plant and is a candidate for the influx carrier (Benett *et al.,* 1996). About 80% of the total cultures developed well formed embryos and were loosely attached to the calli, which could be easily separated. In few cases, the somatic embryos arose from the base of the primary somatic embryos to form clusters indicating secondary embryogenesis. The somatic embryos varied in shape and size having one or two unequal cotyledons sometimes fused to look like a cup (data not shown).

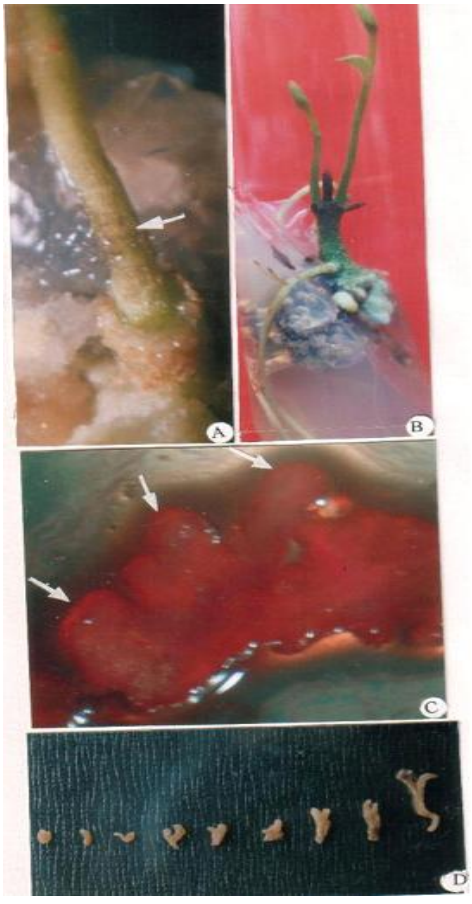

Figure 2. Germination and conversion of somatic embryos in *Celastrus paniculatus*Willd

A) Germination of somatic embryos (arrow) observed under stereomicroscope
B) Transplantable plantlet with well developed vegetative leaves and roots
C) A free hand section of a synchronized development of somatic embryos showing late (arrow) globular, heart and torpedo shaped embryo with suspensor
D) Sequential developmental stages of somatic embryos

Effect of ABA on germination

Maturity and white appearance of embryo seems to be an important precondition for germination. Translucency of the embryos may reflect their lack of starch and or protein storage (Tulecke and Mc Granahan, 1985). This may explain why translucent embryos are unable germinate. Biochemical studies were needed to verify this hypothesis.

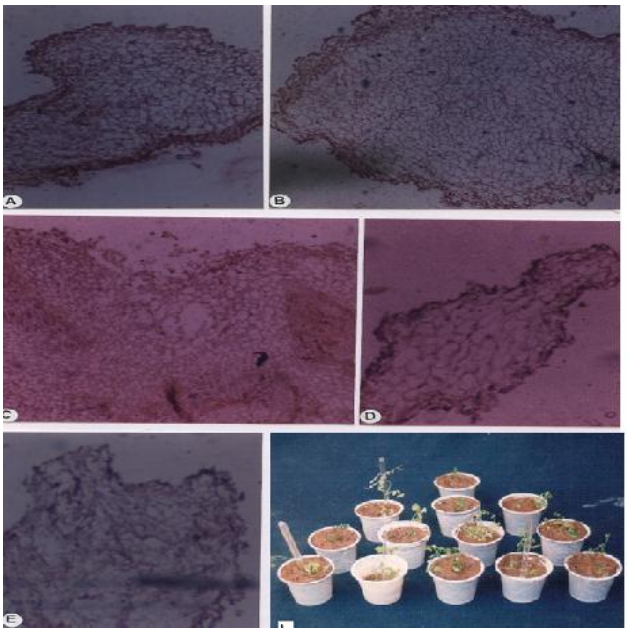

Figure 3. Histological sections of somatic embryos

A) Preglobular
B) Globular
C) Heart shaped
D) Torpedo shaped
E) Cotyledonary
F) Pot transfer

The germination and conversion of mature white somatic embryo was accomplished when transferred to B_5 semisolid medium supplemented with different concentrations of ABA + BA (Table 2). Fuji *et al.,* (1990) reported the influence of ABA on many developmental stages in zygotic embryos and conversion of somatic embryos into plantlets. Among the various concentrations tested, 0.5 mgl^{-1} ABA + 1.0 mgl^{-1} BA favored the germination and conversion of mature embryos into distinct root and shoot system after 30 days culture. Addition of even low concentration of auxin lowered the germination rate and embryos starting recallusing at the base. Germination of somatic embryos is characterized by cotyledonary expansion and chlorophyll formation, followed by radical hypocotyl elongation (Figure 2A) with subsequent

vegetative leaf formation (Figure 2B). The lack of synchrony is one of the major problems encountered with somatic embryo production for commercial realization (Figure 2C). The sequential developmental stages of somatic embryos has been observed (Figure 2D). However, some progress has been made in inducing synchronization of somatic embryogenesis (Ammirato, 1987). The germinated embryos developed into complete plantlets on basal B_5 medium and transfer to the field after acclimatization (Figure 3F).

The malformed embryos reduced on medium containing higher concentration did not germinate and showed poor conversion into plantlets (Table 2). Light microscopic observations of the histological sections of different stages of somatic embryo development revealed preglobular (Figure 3A) globular embryo with suspensor (Figure 3B) and subsequently embryos differentiated into heart shape (Figure 3C) torpedo shape (Figure 3D) and cotyledonary shaped (Figure 3E) structures, thus embryos resembled zygotic embryos. The regenerated plants were morphologically similar to the parents starting from part of nodal explant it is possible to raise about 40 plants within 16 weeks and this protocol is easy to implement in the commercial production (Figure 3F). It is apt to mention that so far there are no reports on somatic embryogenesis from nodal cultures in *C. paniculatus.* In conclusion good quality embryo production is important for increased rate of germination and conversion, which are limiting steps for a practical use of somatic embryogenesis. Presently efforts are aimed at improving the frequency of germination and conversion of somatic embryos in *C. paniculatus*.

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REFERENCES

Ahmad, F., Khan, R.A. and Rasheed, S. 1994. Preliminary screening of methanolic extracts of *Celastrus paniculatus* and *Tocumella undulata* for analgesic and anti inflammatory activities. *J. Ethnopharmacol*, 42: 93-198.

Ammirato, P.V. 1987. Organisational events during somatic embryogenesis. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) Plant tissue and cell culture, AR Liss, New York pp 57-81.

Baranwal, S. and Singh, R.H. 2000. A clinical study on an Ayurvedic drug Jyothishmati in the treatment of depressive illness: Proceedings of international Congress on Ayurveda No. 1, 28-30 January, 2000 Chennai, T.N. India, pp117.

Benett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B. and Feldmann, K.A. 1996. Arabidopsis AUX1 gene- a permease- like regulator of root gravitropism. *Science*, 273: 948-950.

FRLHT 1997. New IUCN Red list criteria. Medicinal plants of India, Conservation Science Division, Ministry of Environment and Forests, Govt. of India, pp10.

Fuji J.S.D., Olsen, R., Ruzin, S.E., Redenbaugh, K. 1990. Alfalfa somatic embryo maturation and conversion of plant. *Plant Sci.*, 67: 43.

Govil J.N. 1993. Medicinal plants: New Vistas of research. Ed. Singh V.K. and Shamima H. Today and tomorrow's publishers, New Delhi, Part-2 pp393-395.

Kirtikar, K.R., Basu, B.D. 1987. Indian medicinal plants Vol I international book distributors, Dehradun, India. pp 574-577.

Lakshmi, G.N. and Seeni, S. 2001. Rapid *in vitro* multiplication and restoration of *Celastrus paniculatus* Willd. sub *sp. paniculatus* (Celastraceae), a medicinal woody climber. *Ind. J Exp Biol* 39:697-704

Luo, J.P., Jia, J.F. 1998. Plant regeneration from callus protoplasts of the forage legume. *Astragalus adsurgens Pall. Plant Cell. Rep*, 17: 313-317.

Machado, A.D.C., Puschmann, M., Puhringer, H., Kreman, P., Katinger, H. and Machado, M.L.D.C. 1995. Somatic embryogenesis of *Prunus subhirtella* autumno rosa and regeneration of transgenic plants after *Agrobacterium*-mediated transformation. *Plant Cell Rep.*, 335-340.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15: 473-497.

Narayanswamy, S. 1997. Regeneration of plants from tissue cultures. In: Applied and fundamental aspects of plant cell, tissue and organ culture, ed. J. Reinert and Y.P.S. Bajaj Springer-Verlag, Berlin, pp 179-206.

Patel, D.K., Amin, K.S. and Nanavathi, D.D. 1995. *Celastrus paniculatus*- a review. Ind Drugs 72: 556-573

Sharada, M., Ashok, A., Kaul, M.K. 2003. Regeneration of plantlets via callus cultures in *Celastrus paniculatus* Willd - A rare endangered medicinal plant. *J. Bioche and Biotech.*, vol 12: 65-69.

Seetharam, Y.N., Haleshi, C. and Vijay. 1998. Medicinal plants of north eastern Karnataka and their status. My Forest 34(2): 767-762.

Tulecke, G. and Mc Granhan, W. 1985. Somatic embryogenesis and plant regeneration from cotyledons of Walnut, *Juglans regia*. *Plant Sci.*, 40(1) 57-64.

Wangoo, D. 1988. Antispermatogenic effects of *Celastrus paniculatus* seed extract on the testis of albino rats. Fitoterapia. 59:377-387.

Warrier, P.K., Nambiar, V.P.K. and Ramankutty, C. 1994. Indian Medicinal Plants- a compendium of 500 species.Vol. II. Orient and Longman publishers, Madras, India, pp 47-51.

Zimmerman, J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5:1411-1423

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