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Callus induction and somatic embryogenesis of *Moringa oleifera Lam* an anti-radiation plant

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An efficient reproducible somatic embryogenesis was developed from leaf and zygotic embryo explants of *Moringa olifera*. An optimal fast growing white organogenic callus established from zygotic embryo explants on MS medium supplemented with 10.75 μM NAA with 95.8% efficiency. The induced calli maintained on MS medium with 4.52 μM 2,4-D and 11.09μM BAP for somatic embryo induction. The highest induction frequencies of somatic embryos were obtained on Murashige and Skoog (MS) medium containing 13.31 μM BAP and 3% sucrose with an average of 17.3 embryos per gram of callus. Mature embryos get germinated on the same medium with 77.2% efficiency. The shoots raised from induced somatic embryos were well rooted in 10.75 μM NAA with 78.4 % efficient. The plantlets with well developed roots and shoots were transplanted to biodegradable plastic pots containing garden soil: sand: vermicompost in 1:1:2 ratio, for hardening with 85% survives.

Key Words: Indirect somatic embryogenesis; Callus production; *Moringa olifera;* Somatic embryogenesis.

Introduction

Moringa (*Moringa oleifera* Lam) belongs to the Moringaceae family, and is considered to have its origin in the North-West region of India (Makkar *et al.*, 1997). *Moringa oleifera* is one of thirteen species most widely cultivated. The leaves contain more vitamin A compared to carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and that the protein quality of *Moringa* leaves rivals that of milk and eggs (Fuglie, 2000).

The leaves are known to be great source of vitamins and minerals being served raw, cooked or dried. 8 g serving of dried leaf powder will satisfy a

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child within ages 1-3 years with 14% of the protein, 40% of the calcium, 23% of the iron, and nearly all vitamin A that the child needs in a day (Fuglie, 1999).

The extracts of *Moringa* leaves shows antimicrobial and antifungal activity in addition to cancer preventive effect (Siddhuraju, 2003 and Devendra *et al.,* 2011). Some of the compounds isolated from *Moringa* preparations which are reported to have hypotensive, anticancer and antibacterial activity include 4-(4'-*O*-acetyl-a-L-rhamnopyranosyloxy), Benzyl isothiocyanate, 4-(a-L-rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate, and 4-(a-L-rhamnopyranosyloxy) benzyl glucosinolate (Bennett *et al.,* 2003).

According to ethanobotanical studies its roots are bitter, acrid, thermogenic, digestive, carminative, anthelmintic, constipating, anti inflammatory, emmenagogue, diuretic, ophthalmic, expectorant and stimulant. They are useful in dyspepsia, anorexia, verminosis, diarrhea, colic, flatulence, paralysis, inflammations, amenorrhea, dysmenorrheal fever, strangury, vesicle and renal calculi. It is used in cough, asthma, bronchitis, pectoral diseases, splenomegaly, epilepsy and cardiopathy (Nepolean *et al.,* 2009).

Due to these medicinal values, this plant have to be conserved and multiplied to reach commercial requirement for the preparation of drug against anti-radiation by the industries (Litz, 1988). The propagation of *Moringa* is also found to be less due to its low seed germination, viability and lack of vegetative propagation methods. The present investigation carried out to establish reproducible and extensive propagation of *Moringa oleifera* through somatic embryogenesis from zygotic explants. The results provided a practical means of mass clonal propagation in order to meet the requirement of pharmaceutical industries for the production of plant based medicines.

Materials and methods

Plant material

Leaf and Zygotic embryos of *Moringa oleifera* explants were used as explants for callus induction and its plant regeneration. These were collected from in the month of October near Sagar nagar, Visakhapatnam, India.

Medium composition and cultural conditions

MS medium consisting of MS mineral salts and vitamins supplemented with 2,4 – D (2.26 - 20.35 μM) and NAA(0.53 - 16.12 μM) alone containing 3% sucrose. The pH was adjusted to 5.8 ± 0.02 with1N NaOH or 1N HCl

before autoclaving at 121^0C for 20 min. The growth regulators (2,4-D, NAA, BAP and K*n*) were added to the culture media before autoclaving. Cultures were maintained in a growth chamber set at 25 ± 2 ^{0}C, 70% relative humidity and a 16/8- h (light/dark) photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 50-60 µmol $m^{-2}s^{-1}$.

Sterilization and inoculation of an explants

The leaf and zygotic embryo explants were washed thoroughly with distilled water for 10 min and then washed with a 2% (*v/v*) Tween-20 solution for 10 min, followed by three rinses with double-distilled water. The explants were further surface sterilized using 70% ethanol for 60 sec, followed by 0.1% mercuric chloride for 5 min. The explants were surface dried on sterile filter paper and cultured on MS basal medium supplemented with different levels of plant growth regulators for callus induction and somatic embryogenesis after three rinses with sterile double distilled water.

Induction of embryogenic calluses

The leaf and zygotic embryo explants were placed on MS medium supplemented with 2 to 21 μM 2,4-D or 0.53 to 16.12 μM NAA for the induction of embryogenic callus formation was examined. The data was recorded for the mean frequency of explants producing friable calli after two weeks of culture. Calli that formed on embryo explants were transferred to MS basal medium supplemented with 5.37 μM NAA and 9.29 μM kinetin for the induction of somatic embryos. Each treatment consists of 10 explants per dish with three replicates. The data was recorded for the mean frequency of calli explants producing somatic embryos after two weeks of culture.

Somatic embryo development

Somatic embryos were induced within 3 weeks on MS media supplemented with 13.31μM BAP and 9.29 μM kinetin (Table 2). The greenish-yellow, globular pro- embryoids were observed and these were sub-cultured on the same medium for maturation. Matured somatic embryos (heart- and torpedo shaped) were transferred to somatic embryo regeneration medium containing MS salts; 100 mg/l myo- inositol, 0.02-0.2 mg/l media thiamine, 30 g/l glucose together with 0.20-8.88 μM BAP were supplemented with alone and in combination. Maturation and germination of the somatic embryos were tested on the media with sub-culturing every 2 weeks. The number of globular, mature and germinated embryos formed on the surface of the callus explants were

counted and recorded at the end of each subculture. Frequencies were the means of three experiments using 30 explants in each treatment.

Plant regeneration

The plantlets developed from somatic embryos were cultured on MS basal medium supplemented with 5.37 μM NAA for elongation. These *in vitro* raised plantlets were subjected to acclimatization, transplanted to biodegradable plastic pots containing garden soil: sand: vermicompost in 1:1:2 ratio, for hardening.

Statistical analysis

The data shown represent the mean ± standard error of three independent experiments for somatic embryogenesis and its plant regeneration. ANOVA was performed on the results of each experiment, and the data were analyzed using DMRT ($P<0.05$).

Results

Induction of callus

A white friable callus was formed from cut ends of the leaf and embryo explants of *Moringa oleifera*, cultured on MS medium supplemented with 2,4 – D (2.26 - 20.35 μM) and NAA(0.53 - 16.12 μM) alone formed on the surfaces after three weeks of culture (Figure 1). Embryonic explants formed callus at a frequency of 95.0% on medium containing 10.75 μM NAA as the sole growth regulator (Figure 2). The frequency of callus formation increased from 0.53-5.37 μM NAA. Leaf explants formed few calli at a frequency of 91.8% on medium containing 11.31 μM 2,4-D.

Induction of somatic embryogenesis

The callus formed from the embryo and leaf explants of *Moringa oleifera* were initially cultured on MS medium supplemented with 2.21—17.75 μM BAP or 2.32-13.93 μM kinetin alone or along with 0.53 – 16.12 μM NAA. An optimal response for somatic embryo induction from callus explants were obtained after 7 days on MS medium supplemented with 0.53 μM NAA and 18.58 μM kinetin. When smeared and viewed under microscope after 30 days, numerous small white, clumps of somatic embryos appeared on outer periphery of the friable calli within 4 weeks. The induced calli showed greenish- yellow,

globular pro- embryoids as well as small plantlets. Globular somatic embryos developed from calli were maintained on MS medium supplemented with 18.58 μM kinetin. The induced embryoids were cultured on same medium for further growth.

Maturation and germination of somatic embryos and plantlet regeneration

The induced globular, heart and torpedo shaped somatic embryos were matured on MS medium supplemented with 13.31 μM BAP at an average of 17.3 embryoids per explants after 4 weeks of inoculation (Figure 3). The induced single shoots from embryos were well rooted on MS medium supplemented with 7.38 μM IBA with an efficiency of 78% and regenerated into plantlets after 1 month (Table 3).

Acclimatization

In vitro regenerated plantlets through somatic embryogenesis with well developed roots and shoots were transplanted to biodegradable plastic pots containing garden soil: sand: vermicompost in 1:1:2 ratio at 23 ± 2^0C temperature and 16 h photoperiod, with 30 µmol m^{-2} s^{-1} at light intensity under sterile condition with a survival rate of 85% (Figure 4). The plantlets with well elongated roots and shoots were transferred to green house for hardening.

Table 1. Effect of Auxin (2, 4-D and NAA) on organogenic callus from leaf and Zygotic embryo explants of *Moringaoleifera*

Plant growth regulators(µM)		**% of explants exhibiting callus induction**	
2,4-D	NAA	Leaf	Zygotic embryo
0	0	-	-
2.26		10.2 ± 0.41 ^{l}	36.4 ± 0.36 ^{j}
4.52		19.1 ± 0.95 ^{i}	69.6 ± 1.06 ^{g}
6.78		29.4 ± 0.73 ^{f}	77.2 ± 0.76 ^{e}
9.04		36.3 ± 0.36 ^{e}	86.5 ± 0.43 ^{cd}
11.31		48.3 ± 0.57 ^{c}	91.8 ± 0.92 ^{a}
13.57		59.2 ± 0.32 ^{b}	82.1 ± 0.70 ^{d}
15.83		68.7 ± 0.20 ^{a}	56.7 ± 0.15 ^{h}
18.09		25.4 ± 0.61 ^{g}	42.3 ± 0.29 ^{i}
20.35		17.0 ± 0.89 ^{j}	35.2 ± 0.48 ^{j}
	0.53	15.9 ± 0.23 ^{k}	31.9 ± 0.24 ^{k}
	2.68	28.4 ± 0.18 ^{fg}	54.3 ± 0.76 ^{h}
	5.37	40.1 ± 1.00 ^{d}	74.9 ± 0.21 ^{f}
	8.06	48.3 ± 0.99 ^{c}	89.9 ± 0.35 ^{c}
	10.75	22.0 ± 1.10 ^{h}	95.0 ± 0.16 ^{b}
	16.12	19.1 ± 0.95 ^{i}	72.6 ± 0.99 ^{f}

Data were recorded after 3 wk of culture.

n.d not determined. Values represent means ± SE from 5 replicates.

Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test (P<0.05).

Table 2. Effect of cytokinins (BAPand Kn) on shoot regeneration from calli induced from leaf and Zygotic embryo explants of *Moringaoleifera*.

Cytokinin (µM)		**% of explants showing response for somatic embryo production**	**Mean number of somatic embryos induced**
BAP	Kn		
0	0	-	-
2.21	--	28.1 ± 0.15 ^{i}	2.6 ± 0.19 ^{h}
4.43	--	50.7 ± 0.28 ^{f}	7.5 ± 1.10 ^{f}
6.65	--	65.4 ± 0.51 ^{d}	8.2 ± 0.24 ^{e}
8.87	--	72.1 ± 0.70 ^{c}	10.0 ± 0.69 ^{cd}
11.09	--	81.3 ± 0.70 ^{b}	13.1 ± 0.69^{b}
13.31	--	90.8 ± 0.68 ^{a}	17.3 ± 0.45 ^{a}
15.53	--	66.0 ± 0.91 ^{d}	11.7 ± 0.24 ^{c}
17.75	--	48.1 ± 1.04 ^{f}	5.2 ± 0.63 ^{h}
--	2.32	--	--
--	4.64	10.0 ± 0.31^{k}	1.1 ± 0.15^{i}
--	6.96	29.4 ± 0.25 ^{h}	2.4 ± 0.77 ^{h}
--	9.29	37.3 ± 0.43 ^{g}	6.6 ± 0.91 ^{g}
--	11.61	59.8 ± 0.49 ^{e}	7.0 ± 0.24 ^{ef}
--	13.93	82.0 ± 0.27 ^{b}	10.6 ± 1.30 ^{d}
13.31	2.32	61.3 ± 0.15 ^{de}	7.8 ± 1.21 ^{f}
13.31	4.64	30.2 ± 1.06 ^{h}	10.4 ± 0.59 ^{d}
13.31	6.96	24.6 ± 0.59 ^{j}	2.6 ± 0.45 ^{h}

Data were recorded after 3 wk of culture.
n.d not determined. Values represent mean ± SE from 5 replicates.
Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test (P<0.05).

Table 3. Effect of Auxin (IBA & NAA) concentration on Root Induction from *In Vitro* raised shoots of *Moringaoleifera*

Plant growth regulators (µM)		**% of rooting**	**Mean no. of roots per shoot**
IBA	NAA		
Control	--	--	--
2.46	--	22.1 ± 0.11^{g}	1.2 ± 0.6 ^{g}
4.92	--	42.0 ± 0.24^{d}	2.8 ± 0.5 ^{c}
7.38	--	76.5 ± 0.26 ^{b}	1.2 ± 0.4 ^{g}
9.84	--	65.5 ± 0.74 ^{c}	4.1± 1.0 ^{a}
19.68		32.2 ± 1.01 ^{f}	2.5 ± 0.8 ^{d}
--	2.68	31.1 ± 0.87 ^{f}	1.3 ± 1.3 ^{f}
--	5.37	20.3 ± 0.53 ^{h}	2.8 ± 0.6 ^{c}
--	8.06	42.4 ± 1.30 ^{d}	2.3 ± 0.6 ^{e}
	10.75	78.4 ± 0.40 ^{a}	3.3 ± 0.7 ^{b}
	21.50	39.3 ± 0.13 ^{e}	1.3 ±1.0 ^{f}

Data were recorded after 4 wk of culture.
n.d not determined. Values represent mean ± SE from 5 replicates.
Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test (P<0.05).

Fig. 1. Friable callus was formed on MS medium with 11.31 μM 2,4 - D after three weeks incubation.

Fig. 2. Embryonic explants producing callus on MS medium with 10.75 μM NAA.

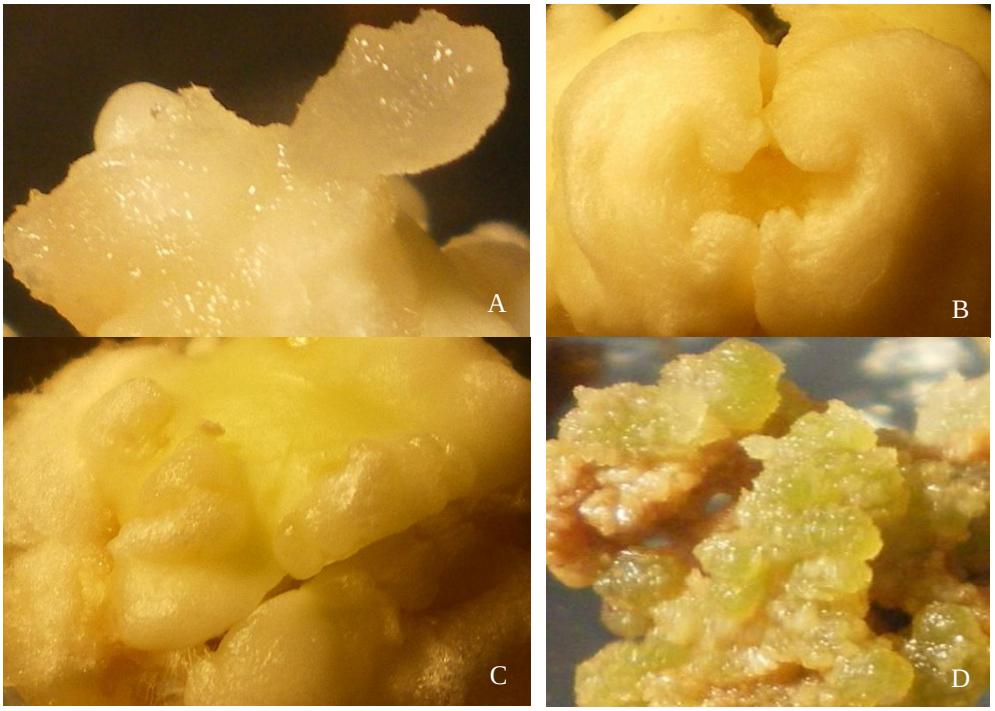

Fig. 3. A) Torpedo, B) Heart and C)Globular D) Cotyledonary staged somatic embryos induced on MS medium containing 13.31 μM BAP with 17.3 embryoids per explants after 4 weeks.

Fig. 4. A & B: The Shoot induction of the aroused callus on MS medium with13.31 *µM* BAP

Discussion

The present investigation was concentrated on regeneration protocol from the leaf and zygotic embryo explants of *Moringa oleifera* through somatic embryogenesis from induced callus. Callus formation was demonstrated on explants taken from naturally grown plants. The conditions and medium provided were optimized for encouraging callus proliferation. Friable organogenic callus developed directly from embryo explants were periodically sub-cultured on MS medium supplemented with 11.31 *µM* 2, 4-D with 2.21 *µM* BAP. Callus induction through an auxin-cytokinin combination has been reported for several systems including *Diffen bachia*; *Chlorophytum arundinacem* and *Brassica napus* (Latto *et al.,* 2006; Shan *et al.,* 2007 and Burbulis *et al.,* 2008).

The globular, heart shaped embryos developed on the surface of the induced calli on MS medium supplemented with 13.31 *µM* BAP. A highest response was reported on MS media containing IBA and BAP for hypocotyl explants of *Jatropha integerrima* (Sujatha *et al.,* 2008). The somatic embryos induced from embryo explants of *Moriinga oleifera* were successfully regenerated, but in some plants somatic embryos do not germinate into complete plantlets (Mohanraj *et al.,* 2009).

The globular structures induced from callus was passed through heart, torpedo and cotyledonary developmental stages with an efficacy of 25%

globular structures converted into cotyledonary embryos, similar results were reported for *Pennisetum americanum* with 1 mg/l 2,4 – D at early stages of embryo development (Devendra *et al.,* 2011), but there is no response obtained with 2,4 – D in combination with TDZ for somatic embryogenesis of *Paspalum scrobiculatum* (Vikrant, 2002).

The successfully induced somatic embryos separated during late maturation stage and sub-cultured for further maturation to plantlets. The plant regeneration through callus derived somatic embryogenesis was reported for *Elaeagnus angustifolia* (Hassan *et al.,* 2008; Zeng *et al.*, 2009 and Hassan *et al.,* 2010). In *Eclipta alba,* rooting of shoots were well on MS media up on supplementation of IBA (16.12 µM) was found to be effective (Faisal *et al.,* 2005).

The *in vitro* regenerated plantlets were then transplanted in the pots containing 1:1:1 ratio of garden soil: sand: vermicompost. Each pot covered with polythene bags with small holes to maintain high humidity and kept them in the culture room to get acclimatized. The plantlets were initially irrigated with MS medium on alternate days. The plantlets with well elongated roots and shoots were transferred to green house for hardening.

Conclusion

The present investigation was concentrated for the conservation and multiplication for the plant regeneration through somatic embryogenesis for *Moringa oleifera*. Micropropagation of this plant was beneficial for the preparation of medicine against radiation by the industry. The protocol was standardized for clonal propagation of selected medicinal plant. It is possible to achieve a tenfold increase in the products per unit area of cultivation. Clonally propagated plants would also have identical phytochemical profiles by reducing the somaclonal variations. Likewise it could be possible to propagate important medicinal plants for cultivation and sustainable use and consequently to conserve them from their extinction.

References

Bennett, R.N., Mellon, F.A., Foidl, N., Pratt, J.H., DuPont, M.S., Perkins, L. and Kroon, P.A. (2003).Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish tree) and *Moringastenopetala*L. Journal of Agricultural and Food Chemistry 51: 3546-3553.

Burbulis, N., Kupriene, R. and Blinstrubiene, A. (2008). Callus induction and plant regeneration from somatic tissue in spring rapeseed (*Brassica napus*L.). Biologia Plantarum 54:258-263.

Devendra, B.N., Srinivas, N., Prasad, V.S.S.L.T. and Swarnalatha, P. (2011). Antimicrobial activity of *Moringa oleifera* lam., leaf extract, against selected bacterial and fungal strains. International Journal of Pharma and Bio Sciences 2(3):13-18.

Devendra, B.N., Srinivas, N. and Naik, G.R. (2011).Direct somatic embryogenesis and synthetic seed production from*Tylophora indica* (Burm.f.) Merrill, an endangered, medicinally important plant. International Journal of Botany 7(3):219-222.

Faisal, M., Singh, S. and Anis, M. (2005). *In vitro* regeneration and plant establishment of *Tylophora indica* (Burm. F.) Merril: Petiol callus culture. In Vitro Cell Dev Biol Plant 41:511-515.

Fuglie, L.J. (1999). The Miracle Tree: *Moringa oleifera*: Natural Nutrition for the Tropics. Church World Service, Dakar, pp. 68.

Fuglie, L.J. (2000). The Miracle Tree: *Moringa oleifera*: Natural Nutrition for the Tropics. *Church World Service*, Dakar; revised in 2001 and published as The Miracle Tree: The Multiple Attributes of Moringa, pp. 172.

Gambino, G., Bondaz, J. and Gribaudo, I. (2006). Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. Eur J Plant Pathol 114: 397-404.

Hassan, S.A.K.M., Farhana, A., Laila, S.B., John, L.M., Miskat, A.A.J. and Rahima, K. (2008).Micropropagation of *Eclipta alba* (Linn.) Hassk- a Valuable Medicinal Herb.Bangladesh J Sci Ind Res. 43:215-222.

Hassan, S.A.K.M., Rebeka, S., Miskat, A.A.J. and Rahima, K. (2010). *In Vitro* Mass Propagation of *Mimosa pudica* L., Using Shoot Tip and Nodal Explants. Bangladesh J Sci Ind Res 45:95-100.

Latto, S.K., Bamotra, S., Sapuradhar, R., Khan, S. and Dhar, A.K. (2006). Rapid plant regeneration and analysis of genetic fidelity of in vitro derived plants of *Chlorophytum arundinaceum* Baker–an endangered medicnal herb. Plant Cell Rep. 25:499-506.

Litz, R.E., (1988). Somatic embryogenesis from cultured leaf explants of the tropical tree *Euphoria longan* Stend. J Plant Physiol 132:190–193.

Makkar, H.P.S. and Becker, K. (1997). Nutrients and antiquality factors in different morphological parts of the *Moringa oleifera* tree. Journal of Agricultural Science 128(3): 311-322.

Mohanraj, R., Ananthan, R. and Bai, V.N. (2009). Production of synthetic seeds in *Coelogynus breviscapa* Lindl. Asian J Biotech 1(3):124-128.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15:473-493.

Nepolean, P., Anitha, J. and. Emilin, R.R. (2009). Isolation, analysis and identification of phytochemicals of antimicrobial activity of *Moringa oleifera* Lam. Current Biotica (3)1:33-39.

Shan, X., Chen, J. and Kane, M.E. (2007). Indirect shoot organogenesis from leaves of *Diffeen bachia* cv. Camouflage. Plant Cell Tissue Organ Cult 89:83-90.

Siddhuraju, P. and Becker, K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. Journal of Agricultural and Food Chemistry 51:2144-2155.

Steward, F.C., Mapes, M.O. and Hears, K. (1958). Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. Am J Bot. 45:705-708.

Sujatha, M., Reddy, T.P. and Mahasi, M.J. (2008). Role of biotechnological interventions in the improvement of castor (*Ricinus communis* L.) and *Jatropha curcas* L. Biotech Adv 26:424–435.

Vikranth, A. (2002). Induction of multiple shoots by thidiazuron from caryopsis cultures of minor millet (*Paspalum scrobiculatum* L.) and its effect on the regeneration of embryogenesic callus cultures. Plant Cell Rep. 21:9-13.

Zeng, F.S., Wang, W.W., Zhan, Y.G. and Ying, X. (2009). Establishment of the callus and cell suspension culture of *Elaeagnus angustifolia* for the production of condensed tannins. African J Biotech. 8:5005-5010.

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