New Forests (2007) 34:73–81
DOI 10.1007/s11056-007-9038-7

Regeneration of *Melia volkensii* Gürke (Meliaceae) through direct somatic embryogenesis

S. A. Indieka · D. W. Odee · G. M. Muluvi · K. N. Rao · J. Machuka

Received: 13 March 2006 / Accepted: 11 December 2006 / Published online: 24 January 2007

Abstract Experiments were conducted to study plant regeneration through direct somatic embryogenesis using mature zygotic embryo and cotyledonary explants from seeds of *Melia volkensii* stored for <3 and >12 months. Explants were cultured on Murashige and Skoog (MS) medium supplemented with BAP, NAA and 2,4-D (0.5, 1.0 and 2.0 mg l^{-1}) alone, and BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) in combination with 2,4-D or NAA (0.2 and 0.5 mg l^{-1}). After 4 weeks in culture, up to 60% of cotyledonary explants from the seeds stored for <3 months produced direct somatic embryos on BAP (0.5–4.0 mg l^{-1}) in combination with 2,4-D (0.2 mg l^{-1}). The number of somatic embryos ranged from 5 to 14 per explant in BAP (0.5 mg l^{-1}) and 2,4-D (0.2 mg l^{-1}) combination. Only 20% of cotyledonary explants from seeds stored for >12 months produced somatic embryos Mature zygotic embryos failed to produce any somatic embryos. Subcultures of somatic embryos from cotyledonary explants of seeds stored for <3 months formed clusters of shootlets on semi solid MS and 1/2 MS media. After 6 weeks of subculture on multiplication MS media augmented with BAP (0.5 mg l^{-1}) and IAA (0.2 mg $^{-1}$), 70% of the shoot tips formed 4–7 shoots per explant. Up to 33% of the multiplied shoots were rooted in MS

S. A. Indieka · D. W. Odee (✉)
Biotechnology Laboratory, Kenya Forestry Research Institute, P.O. Box 20412, 00200 Nairobi, Kenya
e-mail: dodee@africaonline.co.ke

G. M. Muluvi · J. Machuka
Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844, 00100 Nairobi, Kenya

K. N. Rao
International Plant Genetic Resources Institute-Regional Office for Sub Saharan Africa, C/o ICRAF, P.O. Box 30677, 00100 Nairobi, Kenya

Present Address:
K. N. Rao
International Centre for Biosaline Agriculture, P.O. Box 14660, Dubai, United Arab Emirates

medium supplemented with 2.0 mg l^{-1} IBA. Plantlets developed normally into seedlings in the greenhouse.

Keywords Cotyledon · Mass-propagation · Seed storage · Somatic embryos · Zygotic embryos

Introduction

Melia volkensii Gürke (Meliaceae) is among the most popular indigenous multipurpose tree species endemic to the arid and semi arid lands (ASALs) of East Africa. It is fast growing, tolerant to dry conditions and used for dryland agroforestry in Kenya (Stewart and Blomley 1994; Broadhead et al. 2003). The species has problems of propagation by seed due to difficulties in extraction, poor germination and high post-germination mortality. However, seed germination rate of up to 60% under laboratory conditions has been reported. Rooting of stem cuttings has also been reported to be difficult and erratic (Stewart and Blomley 1994).

Plant regeneration through somatic embryogenesis has been recognized as a powerful tool for multiplication and improvement of forest tree species (Komamine et al. 1992). This method has great potential especially for woody species having propagation constraints similar to those described for *M. volkensii*. Somatic embryogenesis has been achieved in tree species using a wide range of explant sources such as cotyledons, zygotic embryos, leaves and internodes (Vila et al. 2003; Ipekci and Gozukirmizi 2004; Amoo and Ayisire 2005). For example, Sawara cypress (*Chamaecyparis pisifera* Sieb. Et Zucc., Cupressaceae) has been multiplied via adventitious shoot bud production from somatic embryo cultures (Murayama et al. 2003). Additionally, somatic embryos can also provide opportunities for in vitro conservation of plant germplasm (Toribio et al. 2004) and target tissue during genetic transformation (Escobar et al. 2000).

The development of a robust and reliable somatic embryogenesis protocol is therefore crucial for multiplication, genetic transformation and in vitro conservation of woody plants. However, there are no protocols currently available for *M. volkensii*. This paper reports for the first time results on regeneration of *M. volkensii* through direct somatic embryogenesis using mature zygotic embryo and cotyledonary explants.

Materials and methods

Mature seeds of *M. volkensii* from Nuu provenance (Kitui district, Kenya), which had been in cold (4°C) storage for <3 and >12 months with laboratory germination rates of 65 and 43%, respectively, were used in this study. The outer and inner seed coats were removed, followed by separation of zygotic embryos and cotyledons. The explants were surface sterilized for 10 min with a solution of 6% sodium hypochlorite containing two drops of Tween® 20 detergent and then rinsed in three changes of sterile distilled water. The cotyledons were split longitudinally into two and rinsed again in three changes of sterile distilled water. The explants were placed with abaxial surface in contact with the embryo induction medium containing MS (Murashige and Skoog 1962) vitamins, micro- and macro-elements, 30 g l^{-1} sucrose, and supplemented with

plant growth regulators (PGRs) either alone or in combination. The pH of the formulations was adjusted to 5.8 using 1 M NaOH or HCl, prior to the addition of 0.8% agar, and sterilized at 121°C and 1.06 kg cm^{-2} for 15 min. Approximately 25 ml of sterile embryo induction medium was dispensed per sterile plastic Petri dish (Ø 9 cm).

Two experiments were conducted to evaluate the response of mature cotyledonary and zygotic embryo explants on induction of direct somatic embryos. In the first experiment the effects of MS medium supplemented with PGRs using explants from seeds stored for <3 months were evaluated as follows: BAP, NAA and 2,4-D (0.5, 1.0 and 2.0 mg l^{-1}) alone, BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with NAA (0.5 and 0.2 mg l^{-1}), and BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.5 and 0.2 mg l^{-1}). In the second experiment, BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.5 and 0.2 mg l^{-1}) were evaluated on explants from seeds stored for >12 months. All control treatments were devoid of PGRs. Two explants were placed in each dish as a replicate. After 4 weeks of culture, sections of cotyledonary explants containing direct somatic embryos were excised and subcultured on semi solid MS and 1/2 MS (micro- and macro-elements) without PGRs. Shoot tips harvested from the clusters of shootlets formed on MS and 1/2 MS media were used for shoot multiplication on semi solid MS media supplemented with BAP (0.5 mg l^{-1}) and IAA (0.2 mg l^{-1}) for 6 weeks. Shoots of ~3–5 cm long were subcultured on MS media supplemented with IBA (0.5, 1.0, 2.0 and 5.0 mg l^{-1}) for rooting.

For histological studies, pieces of cotyledonary tissues containing somatic embryos at different developmental stages were fixed in formaldehyde/ glacial acetic acid/ ethanol (FAA, 5/5/90, v/v/v) for 24 h. The tissues were dehydrated in ethanol series then followed by xylene series each for 3 h, infiltrated and embedded in paraffin wax. Tissues were then sectioned (transverse) to 6 μM with a rotary microtome, mounted on glass slides, stained with 1% (v/v) safranin and excess stain washed with water. Excess wax was removed with xylene prior to making permanent slides. Photographs were then taken with a binocular Nikon compound microscope with a Nikon camera (Model FX–35DX, Japan)

All cultures in this study were incubated in a growth chamber (NK SYSTEM BIOTRON LH-200, Nippon Chemical Instrument Co., Ltd, Osaka, Japan) maintained at 28 ± 2°C under a 16:8 h photoperiod with coo white fluorescent lamps providing light intensity of 70 μmol m^{-2} s^{-1}. The somatic embryo induction experiments consisted of 10 replicates (20 explants) per treatment, while the rooting experiment consisted of three replicates each consisting of five shoots. Each experiment was laid out in the growth chamber in a completely randomized design. Data on percentage (%) somatic embryogenesis and rooting, number of embryos per explants and callus formation were assessed after 4 weeks in culture. The data were analyzed by ANOVA and means compared using the Newman–Keuls Test ($P < 0.05$). The number of embryos per explant, percentage somatic embryogenesis and rooting were transformed [$X' = \sqrt{(X + 0.5)}$] prior to the statistical analysis (Zar 1974).

Results and discussions

Zygotic embryo explants

Germination of zygotic embryo explants was observed after 3 weeks of culture in MS media without PGRs (control) as well as those supplemented with BAP, NAA

and 2,4-D alone at 0.5 mg l^{-1}. Similar results were also observed in BAP (0.5 mg l^{-1}) either combined with 2,4-D (0.2 mg l^{-1}) or NAA (0.2 and 0.5 mg l^{-1}). After 4 weeks in culture, embryo explants in all the concentrations of NAA and 2,4-D tested alone had formed loose friable callus. However, callus formation was higher in 2,4-D than NAA treatments (data not shown). The embryo explants cultured in the medium consisting of BAP alone were furrowed and slightly callused. On the other hand, the zygotic explants in 2.0 and 4.0 mg l^{-1} BAP combined with either 2,4-D or NAA (0.2 and 0.5 mg l^{-1}) had more than 60% of the explants callused with no visible signs of direct somatic embryos after 4 weeks in culture. Callus score on the explants was not significantly ($P < 0.05$) influenced by the combination of BAP with NAA or 2,4-D. The embryo explants cultured in the control treatments germinated into small plantlets after 4 weeks in experiments using explants from seeds stored for <3 months. The zygotic explants from seeds stored for >12 months responded poorly to induction medium containing PGRs after 4 weeks in culture. They were slightly callused and brown in color. Failure to induce direct somatic embryogenesis using mature zygotic embryos of *M. volkensii* is in contrast with the results reported on immature zygotic embryos of *Melia azedarach* Linnaeus (Meliaceae) using 0.1–3.0 mg l^{-1} thidiazuron (Vila et al. 2003). In addition, Chand and Kumar (2001) obtained direct somatic embryos on *Hardwickia binata* Roxb. (Leguminoseae. Caesalpiniaceae) from semi-mature zygotic explants on MS supplemented with 2,4-D (0.1–3.0 mg l^{-1}) alone.

Cotyledonary explants

The cotyledonary explants cultured in treatments consisting of 2,4-D and NAA alone were callused after 3 weeks, and had no visible signs of somatic embryos by the 4th week. However, callus induced in NAA was less friable than 2,4-D. In addition, more than 30% of NAA cultures formed roots primarily at the proximal end of the explants. In most BAP concentrations tested alone, the explants were swollen and slightly callused on the cut surfaces after 3 weeks in culture. On the other hand, more than 50% of the cotyledonary explants were swollen in all combinations of BAP and 2,4-D treatments. Direct somatic embryos at globular stage either singly or fused (Fig. 1a), heart shaped (Fig. 1b) and cotyledonary (Fig. 1c) were visible on the surface of swollen cotyledonary explants in all the BAP concentrations when combined with 0.2 mg l^{-1} 2,4-D. After 4 weeks, 30–60% of the cotyledonary explants in BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.2 mg l^{-1}) had formed direct somatic embryos ranging from 3 to 14 per explant (Table 1). Cotyledonary explants inoculated on control treatments only formed green meristematic nodules on the cut surface after 4 weeks in culture. In this study, BAP alone did not induce somatic embryogenesis, but only in combination with 2,4-D. Other workers have induced direct somatic embryogenesis on *Azadirachta indica* A. Juss. (Meliaceae) mature cotyledonary explants in MS media supplemented with 0.3–10.0 mg l^{-1} thidiazuron (Murthy and Saxena 1998). Moreover, induction of direct somatic embryos using cytokinins alone has also been reported in non-woody plant species. For example, Chung et al. (2005) initiated direct somatic embryos on leaf explants of *Dendrobium chiengmai* (Orchidaceae), using BAP, thidiazuron, kinetin and zeatin alone.

Fig. 1 Direct somatic embryogenesis and plant regeneration of *Melia volkensii*: (**a**) globular somatic embryos initiated on slightly callused region of cotyledonary explant (bar = 0.5 mm); (**b**) heart shaped (HSE) and globular shaped (GSE) somatic embryos appearing simultaneously at the proximal end of cotyledonary explant (bar = 0.5 mm); (**c**) cotyledonary somatic embryos on swollen explant after 4 weeks of culture on MS supplemented with 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} 2,4-D (bar = 1 mm); (**d**) shootlets produced when sections of cotyledonary explants containing clusters of somatic embryos were subcultured on MS and 1/2 MS after 4 weeks of culture (bar = 10 mm); (**e**) shoot multiplication using shoot tip explants from the shootlets on MS supplemented with 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} IAA after 6 weeks of culture (bar = 12 mm); (**f**) rooted shoots on MS supplemented with 2 mg l^{-1} IBA after 4 weeks of culture (bar = 12 mm)

The production of somatic embryos on cotyledonary explants was significantly ($P < 0.0001$) influenced by the concentration and combination of BAP and 2,4-D. For instance, increasing the concentrations of BAP and 2,4-D reduced the number of somatic embryos initiated per explant (Table 1). This is an indication that relatively higher concentration of the PGRs was detrimental to direct somatic embryogenesis, a pattern also observed by Wachira and Ogada (1995) on *Camellia sinensis* (L.) O. Kuntze (Theaceae). Clusters of somatic embryos at different developmental stages were more frequently observed at the proximal than distal ends of the cotyledonary explant. Somatic embryos were also formed on slightly callused regions of the cotyledonary explants, especially on the cut surfaces. These results suggest that occurrence of somatic embryogenesis in *M. volkensii* cotyledonary explant was direct and may have been influenced by orientation. For example, Rancillac et al. (1996) reported the influence of leaf explants orientation of the red Oak tree (*Quercus rubra* L., Fagaceae) on occurrence of direct somatic embryos. Similarly Vila et al. (2003) reported that somatic embryos were frequently initiated on hypocotyls and cotyledonary axils of immature zygotic embryo explants of *M. azedarach*. Differences in embryogenic response with respect to explant orientation has been linked to the differences in cell structure and physiology (Choi et al. 1997).

Cotyledonary explants cultured in BAP and NAA combinations at 0.5 mg l^{-1} formed callus on the cut surface. The callus tissue consisted of whitish nodular-like protrusions, but without somatic embryos. Further subculturing using the same media formulations did not induce somatic embryos or shootlets. Failure to induce somatic embryos with BAP combined with NAA in this study, also contrasts the results obtained with mature cotyledonary explants of *Arachis hypogaea* L. (Leguminosae) on MS supplemented with BAP (0.5–10.0 mg l^{-1}) and 0.5 mg l^{-1} NAA (Venkatachalam et al. 1999). However, nodular protrusions similar to those found in this study have previously been reported on *Cola nitida* Malvales (Sterculiaceae) on MS supplemented with 0.8 mg l^{-1} kinetin combined with 2,4-D at 0.2 and 0.6 mg l^{-1} (Ombembe et al. 1999). Most of the cotyledonary explants from seeds stored for >12 months did not respond to BAP and 2,4-D combinations, but turned yellow instead. Direct somatic embryogenesis was observed in only 20% of the cotyledonary explants on the medium consisting of 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} 2, 4-D

Table 1 The effect of BAP in combination with 2,4-D on direct induction of somatic embryos after 4 weeks in culture of mature cotyledonary explants from *Melia volkensii* seeds stored for <3 months

BAP: 2,4-D (mg l^{-1})	Percentage of explants with somatic embryos	Range in number of embryos explant^{-1}	Mean number of embryos explant^{-1}
0:0	0	0	0a*
0.5:0.2	60	5–14	6.2c
1.0:0.2	50	4–12	5.0bc
2.0:0.2	30	5–8	2.2ab
4.0:0.2	30	3–7	1.9ab
0.5:0.5	0	0	0a
1.0:0.5	0	0	0a
2.0:0.5	0	0	0a
4.0:0.5	0	0	0a

* Data were transformed [$X' = \sqrt{(X + 0.5)}$] prior to ANOVA (n = 20). Values in the column followed by the same letter are not significantly different according to the Newman–Keuls test ($P < 0.05$)

(data not shown). These results may indicate that cotyledonary explants from seeds stored for >12 months required higher levels of BAP. However, further studies are required to evaluate the effect of storage duration and conditions of *M. volkensii* seeds on induction of direct somatic embryos.

Plant regeneration

The clusters of somatic embryos transferred onto semi solid MS and 1/2 MS did not germinate into plantlets. Instead, they formed clusters of shootlets which we further used as sources of explants for multiplication of *M. volkensii* (Fig. 1d). Nevertheless during shoot multiplication, 70% of the shoot tip cultures formed 4–7 shoots per explant on MS media augmented with BAP (0.5 mg l^{-1}) and IAA (0.2 mg l^{-1}) (Fig. 1e). Root formation ranged from 5 to 33% in all the IBA concentrations tested, with 2 mg l^{-1} producing the highest score. It was also observed that root initiation and formation was preceded by callus formation at the base of the shoots (Fig. 1f). Initiation of direct somatic embryos and failure to develop into plantlets on MS media devoid of PGRs in this study is in contrast with the results obtained on *M. azedarach* (Vila et al. 2003). Interestingly, our results are similar to those reported by Puchooa (2004) for *Litchi chinensis* Sonn. (Sapindaceae). However success in the development and germination of direct somatic embryos into plantlets has been linked to various factors including explant type, time of withdrawing the PGRs, culture conditions and medium composition (Eudes et al. 2003; Ipekci and Gozukirmizi 2003). For instance, Venkatachalam et al. (1999) reported failed somatic embryo germination in *A. hypogaea*, which was attributed to delayed subculturing. On the other hand, Zhang et al. (2006) obtained germination of direct somatic embryos of *Syngonium podophyllum* Schott (Araceae) on MS supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1}NAA. Consequently there is need to investigate the effects PGRs and embryo developmental stage on maturation and germination of *M. volkensii* direct somatic embryos.

In this study, both the cotyledonary and zygotic embryo explants produced callus, irrespective of the type of auxin used. This indicates that mature cotyledon and zygotic embryo explants of *M. volkensii* may not be auxin specific in terms of callus induction. However our results are in contrast with those obtained with cotyledonary explants *Parkia biglobosa* (Jacq.) Benth. (Leguminoseae) where only 2,4-D, but not NAA induced callus formation (Amoo and Ayisire 2005). Histological examination of explant sections containing somatic embryos confirmed that they were directly initiated from the epidermal and sub-epidermal cells. Relatively small and compactly arranged meristematic cells led to the development of nodular structures (Fig. 2a). Somatic embryos at cotyledonary stage with and without secondary somatic embryos were also observed (Fig. 2b, c). These results further confirm that interaction of PGRs (mainly cytokinins and auxins) may be essential for induction of somatic embryos in woody species as outlined by Komamine et al. (1992).

Conclusions

Our success in direct somatic embryogenesis using mature cotyledonary explants demonstrates the usefulness of mature tissues in somatic embryogenesis studies.

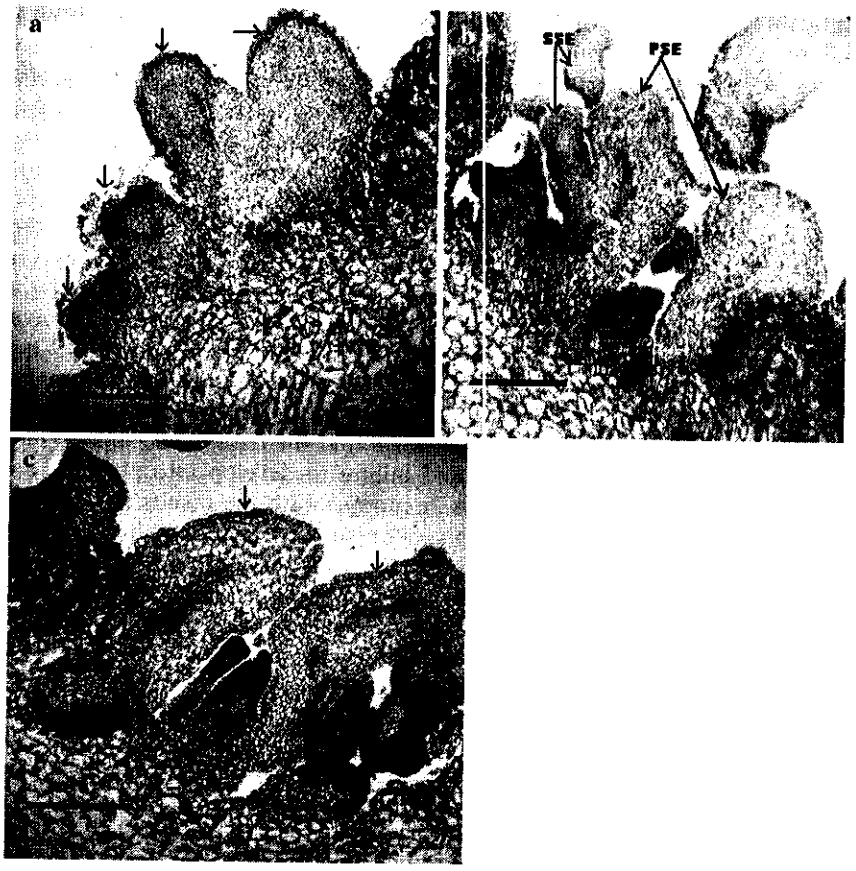

Fig. 2 Histological examination of direct somatic embryogenesis (bars = 0.5 mm): (**a**) development of globular somatic embryos from the epidermal and sub-epidermal cells of cotyledonary explants; (**b**) primary and secondary somatic embryos (PSE and SSE); (**c**) longitudinal section of somatic embryos without SSE (shown with *arrows*)

Further work on the development of robust direct somatic embryogenesis protocol for *M. volkensii* could open up opportunities for breeding, conservation and genetic transformation of this species. However, there is also need to test immature cotyledonary and zygotic embryo explants of *M. volkensii* since it has been shown to be successful with *M. azedarach*, a closely related tree species (Vila et al. 2003). Similarly, trials with thidiazuron may also avoid the use of combinations of PGRs on direct somatic embryogenesis with *M. volkensii* explants, as has also been demonstrated with other related species (Murthy and Saxena 1998).

Finally, the positive response of shoot multiplication and rooting from shoot tip explants obtained through somatic embryogenesis has demonstrated the potential of this technique for applicability to mass propagation of *M. volkensii*.

Acknowledgments This research was supported by the Drylands Research Programme of KEFRI, ISAAA *Afri*Centre and IPGRI-SSA Abdou-Salam Ouédraogo Fellowship to S.A.I.

References

Amoo SO, Ayisire BE (2005) Induction of callus and somatic embryogenesis from cotyledon explants of *Parkia biglobosa* (Jacq.) Benth. Afri J Biotechnol 4:68–71

Broadhead JS, Black CK, Ong CK (2003) Tree leafing phenology and crop productivity in semi arid agroforestry systems in Kenya. Agroforest Syst 58:137–148

Chand S, Kumar AS (2001) Direct somatic embryogenesis from zygotic embryos of timber-yielding leguminous tree, *Hardwickia binata* Roxb. Curr Sci 80:882–887

Choi YE, Kim HS, Soh WY, Wang DC (1997) Developmental and structural aspects of somatic embryos formed on medium containing 2, 3, 5-triiodobenzoic acid. Plant Cell Rep 16:738–744

Chung HH, Chen JT, Chang WW (2005) Cytokinins induce direct somatic embryogenesis of *Dendrobium Chiengmai* Pink and subsequent plant regeneration. In Vitro Cell Dev Biol Plant 41:765–769

Escobar MA, Park J, Polito VS, Leslie CA, Uratsu SL, McGranahan GH, Dandekar AM (2000) Using GFP as a scorable marker in walnut somatic embryo transformation. Ann Bot 85:831–835

Eudes F, Acharya S, Laroche A, Selinger LB, Cheng K-J (2003) A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. Plant Cell Tiss Org Cult 73:147–157

Ipekci Z, Gozukirmizi N (2003) Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. Plant Cell Rep 22:16–24

Ipekci Z, Gozukirmizi N (2004). Indirect somatic embryogenesis and plant regeneration from leaf and internode explants of *Paulownia elongata*. Plant Cell Tiss Org Cult 79:341–345

Komamine A, Kawara R, Matsumoto M, Sunabori S, Toya T, Fujimura T (1992) Mechanism of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. In Vitro Cell Dev Biol 28:11–14

Murashige T, Skoog F (1962) A revised medium of rapid growth and assays with tobacco tissue culture. Physiol Plant 15:473–497

Murayama E, Hosoi Y, Ishii K (2003) Somatic embryo culture for propagation, artificial seed production and conservation of Sawara cypress (*Chamaecyparis pisifera* Sieb. Et Zucc.). J For Res Jpn 8:1–8

Murthy BNS, Sexena PK (1998) Somatic embryogeneresis and plant regeneration of neem (*Azadirachta indica* A. Juss.). Plant Cell Rep 17:469–475

Ombembe OO, Adebona AC, Esan EB (1999) Effect of plant growth regulators on callus growth of *Cola nitida* Malvales (Sterculiaceae). Biosci Res Comm 11:53–57

Puchooa D (2004) *In vitro* regeneration of Lychee (*Litchi chinensis* Sonn.). Afri J Biotechnol 3:576–584

Rancillac M, Klinguer A, Klinguer S, Millet B (1996) Preliminary investigation on somatic embryogenesis from leaf disks of red oak (*Quercus-rubra* L.). Plant Growth Reg 20:67–73

Stewart M, Blomley T (1994) Use of *Melia volkensii* in semi-arid agroforestry system in Kenya. Commonw For Rev 73:128–131

Toribio M, Fernandez C, Celestino C, Martinez MT, San José MC, Vieitez M (2004) Somatic embryogenesis in mature *Quercus robur* tree. Plant Cell Tiss Org Cult 76:283–287

Venkatachalam P, Geetha N, Khandelwal A, Shaila MS, Sita LG (1999) Induction of direct somatic embryogenesis and plant regeneration from mature cotyledon explants of *Arachis hypogaea* L. Curr Sci 77:269–273

Vila S, Gonzalez A, Rey H, Mroginski L (2003) Somatic embroygenesis and plant regeneration from immature zygotic embryos of *Melia azedarach* (Meliaceae). In Vitro Cell Dev Biol Plant 39:283–287

Wachira F, Ogada J (1995) *In vitro* regeneration of *Camellia sinensis* (L.) O. Kuntze by somatic embryogenesis. Plant Cell Rep 14:463–466

Zar JH (1974) Biostatistical analysis. Prentice-Hall Inc., Englewood Cliffs, 620 p

Zhang Q, Chen J, Henny RJ (2006) Regeneration of *Syngonium podophyllum* 'Variegatum' through direct somatic embryogenesis. Plant Cell Tiss Org Cult 84:181–188