Tree and Forestry Science and Biotechnology ©2011 Global Science Books

Direct Somatic Embryogenesis from Mature Zygotic Embryo and Conversion to Plants in Medicinal Tree *Terminalia chebula* Retz.

C. Anjaneyulu • Charu C. Giri*

Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad- 500007, A.P, India

Corresponding author: * giriccin@yahoo.co.in

ABSTRACT

Terminalia chebula Retz. mature zygotic embryo (MZE) explants were evaluated for induction of direct somatic embryogenesis on Murashige and Skoog medium (MS) supplemented with 3% (w/v) sucrose, various combinations and concentrations of 2,4 dichlorophenoxy acetic acid (2,4-D) and kinetin (Kn). Seeds of *T. chebula* collected from different geographic and climatic zones such as Srisailam Tiger Forest Reserve, Srisailam, Andhra Pradesh, forest area in eastern part of Mayurbhanj district of Orissa and Ananthagiri Hill forest area, Vikarabad, Andhra Pradesh, India. Seeds of geographic zone in Mayurbhanj district of Orissa showed maximum frequency of direct somatic embryo induction (62.00 ± 1.15 fully developed somatic embryos per explants) and was obtained on MS medium containing 1.0 mg/l 2,4-D and 0.01 mg/l Kn after 6 weeks of culture. The influence of seed age (duration after collection) as MZE source on direct somatic embryogenesis revealed that one-month old MZEs promoted maximum frequency; however, this declined with an increase in age. Somatic embryo maturation was obtained on MS media supplemented with 50 g/l sucrose. Direct and indirect (via intervening callus phase) somatic embryogenesis was a parallel process. A maximum of 26.33 ± 1.20 explants showed direct somatic embryogenesis compared to 80.0 ± 2.33 indirect somatic embryogenesis. Direct somatic embryogenesis can be advantageous in terms of curtailing more than four weeks of culture passage *in vitro* compared to indirect somatic embryogenesis. Conversion of somatic embryos to plants was possible on ½-strength MS medium fortified with 0.5 mg/l BA (6-benzyladenine) and 30 g/l (w/v) sucrose. Direct somatic embryogenesis and the conversion of somatic embryos to plants is reported for the first time in *T. chebula*.

Keywords: geographic zones, *in vitro* culture, seed age, somatic embryo induction, zygotic embryo explants
Abbreviations: **AC**, activated charcoal; **ABA**, abscisic acid; **BA**, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; **GA_3**, gibberellic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MZE**, mature zygotic embryo; **NAA**, α-naphthalene acetic acid

INTRODUCTION

Somatic embryogenesis offers number of applications as an alternative pathway of mass multiplication of elite plants within short time, for easy delivery system, and germplasm conservation (Giri *et al.* 2004; Wu *et al.* 2007; Vengadesan and Pujit 2009; Konan *et al.* 2010; Zhang *et al.* 2010). Somatic embryogenesis pathway provides immense potential to speed up the propagation of forest tree in particular. Further, somatic embryogenesis finds importance in tree biotechnology for its capability to produce unlimited numbers of somatic embryos in the form of propagules (Laxmisita and Raghavaswamy 1998; Sharry *et al.* 2006; Valladares *et al.* 2006; Martinez *et al*. 2008; Barbon *et al*. 2008; Krajnakova *et al*. 2009; San-Jose *et al.* 2010). In addition, it can be utilized for production of synthetic seeds or synseeds (Chen and Chang 2006; Valladares *et al.* 2006). Further, it can also be exploited as somatic embryogenesis system for genetic transformation studies (Giri *et al.* 2004; Chen and Chang 2006; Moromoto *et al.* 2006; Li *et al.* 2007; Shyamkumar *et al.* 2007; Silva *et al*. 2009). Two of the basic strategies used for micropropagation of forest tree species are direct regeneration and indirect regeneration via intermediate callus phase. Indirect regeneration often results in somaclonal variation making the strategy less desirable for large-scale clonal multiplication. Micropropagation without intervening callus phase is advantageous over conventional vegetative propagation in terms of quality, quantity and economics (Altman and Loberant 1998).

In some cases somatic embryo formation occurred directly on the explants in callus induction medium whereas in other findings different media combinations and hormones were needed for callus induction, somatic embryo formation, embryo maturation and plant conversion in a stepwise process (Paiva *et al.* 2003; Giri *et al.* 2004; Lan *et al*. 2009; Shekhawat *et al*. 2009). Hence, direct regeneration without callus phase is a reliable method for propagation to achieve true to type clones. Direct somatic embryogenesis pathway and efficient conversion of somatic embryos to plants may supplement and accelerate the process of tree propagation.

Terminalia chebula Retz. belongs to the Combretaceae family and is a tree of both pharmaceutical and industrial importance (Khare 2004). In the present communication, we report direct somatic embryogenesis from mature zygotic embryo (MZE) explants of *T. chebula* and its comparative advantage over indirect (via intervening callus phase) pathway. Further, the influence of different geographic/climatic zones and the age of seed (storage duration) for MZE explants source, on direct somatic embryogenesis were investigated.

MATERIALS AND METHODS

Mature seeds from trees growing in its natural forest habitat of Tiger Forest Reserve, Srisailam, Ananthagiri Hills forest, Vikarabad, Andhra Pradesh, India and Mayurbhanj district in the eastern part of Orissa, India were collected during the month of January to October of the year starting from 2002 to 2008. The seed material brought to laboratory conditions in CPMB, OU, Hyderabad, pro-

Received: 6 June, 2009. Accepted: 1 October, 2010.

Original Research Paper

cessed by shade or sun drying and used for their evaluation in different experimental treatments. Excised mature zygotic embryos (MZE) were surface sterilized to avoid contamination of bacteria, fungi and for the establishment of *in vitro* cultures. The seeds were processed, mature zygotic embryo excised and made available for inoculation following the protocol reported earlier (Anjaneyulu *et al.* 2004).

The excised embryos of 15-17 mm size were cultured in 2.5 cm diameter tubes containing semisolid 0.9% (w/v) agar MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose and different concentrations of plant growth regulators. Various concentrations of 2,4 dichlorophenoxy acetic acid (2,4-D) (0.0, 0.2-5.0 mg/l) either alone or in combination with Kn (0.0, 0.01-0.5 mg/l) or BA (0.0, 0.01-0.5 mg/l), purchased from Sigma-Aldrich, St. Louis, MI, USA, were evaluated for direct somatic embryo induction. Somatic embryo maturation was evaluated using MS medium with different concentrations (30, 50, 60 g/l) of sucrose. Various strengths of MS nutrients (full, ½, ¼) supplemented with various concentrations of BA (0.25 to 2.0 mg/l). Cultures were maintained at 25 ± 2°C, 80% relative humidity (RH) in dark or light conditions under a 16-h photoperiod with a light intensity of 3000 lux provided by cool white fluorescent tubes (Philips India Ltd.). Each experiment was repeated thrice for final scoring of data. The number of somatic embryos produced, were scored during individual treatments and observations taken after regular intervals as per the experimental treatments. The experimental design was Latin square and mean ± SE were calculated for all parameters. Matlab version 5.3 and SPSS version 10.0 Math Works Inc., USA, statistical packages was used for analysis of data.

RESULTS AND DISCUSSION

Subsequent to the collection of seeds of *T. chebula* from different geographic and climatic zones, mature zygotic embryo (MZE) inside the hard testa was obviously the first explant to be available for *in vitro* study (**Fig. 1A, Table 1**). Undamaged intact mature zygotic embryo explants were found suitable and evaluated for induction of direct somatic embryogenesis using different concentrations and combinations of 2,4-D and Kn in MS medium (**Fig. 1B**). Globular somatic embryos and even advanced stages of somatic embryos were observed directly on the surface of the explants in 1.0 mg/l 2,4-D and 0.01 mg/l Kn medium after 28 days (**Fig. 1C**).

Mature zygotic embryo explants induced direct somatic embryogenesis, other explants (leaf, stem, nodal segments, etc.) of *T. chebula* did not produce embryogenic callus after prolonged subculture passages in the present study. However, in earlier studies, different explants such as axillary buds, hypocotyls, leaves, zygotic embryos and ovary was found suitable for induction of direct somatic embryogenesis without callus phase. This was evident from the studies with *Tectona grandis*, *Eleutherococcus senticosus*, *Coffea canephora*, *Hardwickia binata* and *Paulownia elongata*, *Cocus nucifera*, *Eucalyptus camaldulensi*, respectively (Kushalkar and Sharon 1996; Choi *et al.* 1999; Fuentes *et al.* 2000; Chand and Singh 2001; Ipekci and Gozukirmizi 2003; Prasanthi *et al.* 2007; Lan *et al.* 2009; Yadav *et al.* 2009; Prakash and Gurumurthi 2010).

MS medium supplemented with PGRs such as 2,4-D + Kn were evaluated for induction of direct somatic embryogenesis. Among the combinations used for induction of somatic embryogenesis, a combination of 1.0 mg/l 2,4-D and 0.01 mg/l Kn only produced maximum direct somatic embryos. MS medium was used in most tree species for direct somatic embryogenesis. Different PGRs such as 2,4-D, BA, TDZ (thiodiazuron), 2-Isopentenyl adenine (2iP) were also used for direct somatic embryogenesis in tree species (Giri *et al.* 2004). Direct somatic embryogenesis was obtained using only 2,4-D as the sole PGR without incorporation of cytokinin in *Eleutherococcus senticosus* and *Hardwickia binata* (Choi *et al.* 1999; Chand and Singh 2001). In *Coffea canephora* induction of direct somatic embryogenesis was obtained with only one cytokinin 2-isopentenyl adenine 2iP at 5 µM (Fuentes *et al.* 2000). TDZ supplemented at 10 and 3.0 mg/l was used as the sole PGR for direct somatic embryogenesis in *Paulownia elongata* and *Phalaenopsis amabilis*, respectively (Ipekci and Gozukirmizi

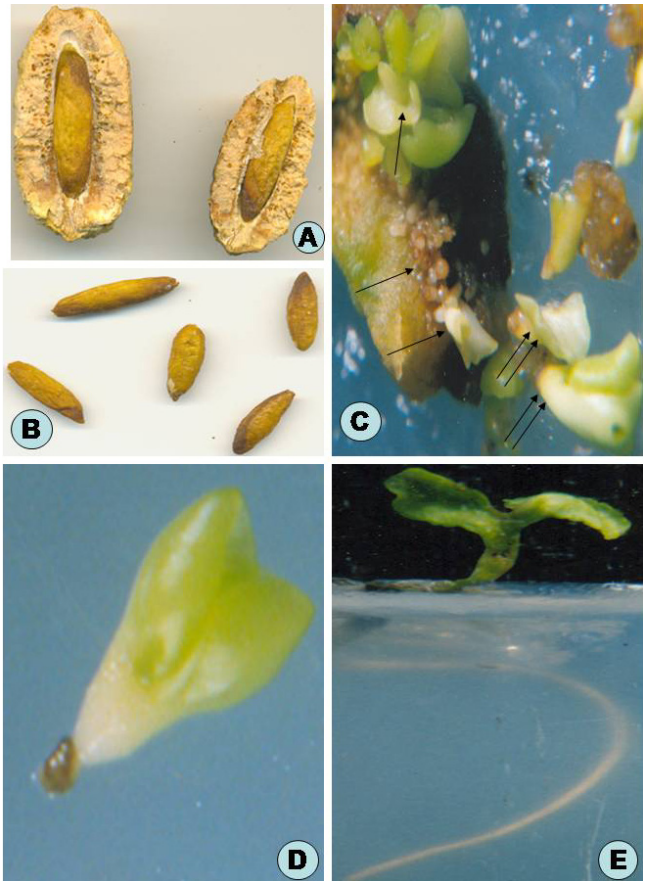

Fig. 1 Direct somatic embryogenesis in *T. chebula*. (**A**) Mechanically broken seeds showing MZE inside testa. (**B**) Excised individual intact undamaged MZE. (**C**) Direct somatic embryogenesis from MZE explants, isolated somatic embryos on the surface of the explant without intervening callus (single arrow) somatic embryo detached from the explant (double arrow). (**D**) Further maturation of single somatic embryo. (**E**) A fully germinated somatic embryo with well developed root and shoot.

Table 1 Evaluation of seeds collected from different geographic and climatic zones on direct somatic embryogenesis from excised MZE in *Terminalia chebula*.*

Geographical location	PGR (mg/l)		Direct somatic embryo induction	No of somatic embryos/explant
	2,4-D	KN	(% Mean ± SE)	(% Mean ± SE)
Srisailam Tiger Reserve Forest,	1.0	0.01	EC	-
Srisailam A.P	1.0	0.10	EC	-
Forest area of Mayurbhanj	1.0	0.01	19.66 ± 2.33	62.00 ± 1.15
district in eastern part of Orissa	1.0	0.10	9.62 ± 0.63c	12.62 ± 0.63
Ananthagiri Hill forest,	1.0	0.01	EC	-
Vikarabad A.P	1.0	0.01	EC	-

Mean ± SE: standard error of three repeated experiments; 60 excised explants per treatment for direct somatic embryo induction; MS medium with 50 g/l was used for the study; *Observations were recorded after 4 weeks; freshly collected (1 month old seeds) embryos were used EC: embryogenic callus

Table 2 Comparative assessment of direct and indirect somatic embryogenesis from excised mature zygotic embryo cultures of *Terminalia chebula*.

No of experiments*	Explants showing direct somatic embryogenesis		Explants showing indirect somatic embryogenesis	
	% of explants induced direct somatic embryos (% Mean ± SE)	No of somatic embryos/ explant (% Mean ± SE)	% of explants induced callus (% Mean ± SE)	No of somatic embryos/ explant (% Mean ± SE)
1	24.33 ± 1.20	51.33 ± 0.89	75.66 ± 1.20	41.66 ± 1.20
2	19.66 ± 2.33	62.00 ± 1.15	80.33 ± 2.33	37.66 ± 0.90
3	20.00 ± 2.08	48.33 ± 1.77	80.00 ± 2.08	40.16 ± 0.80
4	26.33 ± 1.20	54.66 ± 1.76	73.66 ± 1.20	39.66 ± 1.66
5	23.33 ± 1.33	55.33 ± 1.76	76.66 ± 1.33	43.33 ± 0.88

Mean ± SE-standard error of three repeated experiments; MS medium containing 2,4-D 1.0 mg/l and kinetin 0.01 mg/l was used for the study with 3% sucrose; early developmental stage of somatic embryos were counted on the explants for scoring number of somatic embryos; freshly collected (1 month old seeds) embryos were used
* Each experiment mentioned above is the outcome of three replicates of individual experiment performed. In case of direct somatic embryogenesis observations were taken after 4 (28 days) weeks of culture whereas in the indirect somatic embryogenesis the data was collected after 58 days of culture.

2003; Chen and Chang 2006). A combination of two cytokinins (BA, 2iP) was used for direct somatic embryogenesis in *Tectona grandis* (Kushalkar and Sharon 1996). In the woody shrub *Camellia sinensis* direct somatic embryogenesis was obtained using both auxin, cytokinin with 1.0 mg/l indole-3-butyric acid (IBA), 0.5 mg/l 6-benzyladenine (BA) and 0.0 mg/l gibberellic acid (GA_3)(Akula and Dodd 1998). These findings indicate that the indigenous level of PGRs in the mother explants of plant species is important besides the exogenous supply of growth regulators.

In the present study, during the process of induction of somatic embryogenesis, using MZE explants simultaneous occurrence of direct and indirect somatic embryogenesis was observed amongst the explants cultured. A detailed comparative study on direct and indirect somatic embryogenesis was also undertaken. A maximum percentage of 26.33 ± 1.20 and a minimum of 19.66 ± 2.33 MZE explants showed direct somatic embryo formation on the surface of the explants after 4 weeks of culture initiation (**Table 2**). In case of indirect somatic embryogenesis the percentage of cultures ranged from 73.66 ± 1.20 to 80.33 ± 2.33 for the induction of embryogenic callus after 6 weeks of culture and subsequent induction of somatic embryos after two weeks. A maximum of 62.00 ± 1.15 and a minimum of 48.33 ± 0.88 direct somatic embryos per explants were obtained after 4 to 6 weeks of culture. On the other hand, in the indirect somatic embryogenesis the number ranged from 37.66 ± 0.90 to 43.33 ± 0.88 somatic embryos per explant. A similar event of direct and indirect somatic embryogenesis was also obtained in *Tectona grandis* and *Quassia* amara (Kushalkar and Sharon 1996; Martin *et al.* 2005). Fully developed mature somatic embryos were obtained within 42 days in direct somatic embryogenesis pathway compared to 58 days following indirect pathway via intervening callus phase curtailing an *in vitro* passage of 16 days (**Fig. 2**).

Seeds collected from selected plants growing in different geographic and climatic zones such as Srisailam Tiger Forest Reserve, A.P, Srisailam, Forest area of Mayurbhanj district in eastern part of Orissa and Ananthagiri Hill forest, Vikarabad A.P were evaluated as a source for excised mature embryo explants on formation of direct somatic embryos. Amongst the seeds collected from selected plants growing in different geographic and climatic zones, maximum direct somatic embryogenesis was obtained from the Orissa seeds only and the direct somatic embryo frequency per explant was 62.00 ± 1.15. Direct somatic embryogenesis was not obtained with seeds collected from the Tiger Forest Reserve, Srisailam and Ananthagiri Hill forest area Vikarabad (**Table 1**). Keeping in view the production of direct somatic embryos from Orissa seeds, all further experiments were conducted using seeds collected from eastern part forest area in Orissa. Evaluation of seeds collected from different geographic and climatic zones (Srisailam Tiger Reserve Forest, Srisailam, A.P, Forest area of Mayurbhanj district in eastern part of Orissa, Ananthagiri Hill forest, Vikarabad, A.P) as a source for excised mature embryos on somatic embryogenesis showed different responses. The differences in competence for somatic embryogenesis in seeds of different climatic and geographic locations revealed a possible genotypic effect as suggested in other woody species (Carron *et al.* 1995; Merkle *et al.* 1997; Martin *et al.* 2005).

Table 3 Effect of the age of the seed as a source of excised MZE on direct somatic embryo induction *Terminalia chebula*.*

Age of MZE in months	PGR (mg/l)		No of somatic embryos per explant (% Mean ± SE)
	2,4-D	KN	
1	1.0	0.01	62.00 ± 1.15
	1.0	0.10	46.62 ± 0.63
3	1.0	0.01	28.88 ± 4.77
	1.0	0.10	16.62 ± 1.63
6	1.0	0.01	12.69 ± 1.39
	1.0	0.01	7.62 ± 0.63
9	1.0	0.01	6.62 ± 0.63
	1.0	0.01	4.52 ± 0.63
12	1.0	0.01	2.53 ± 1.18
	1.0	0.01	C

Mean ± SE: standard error of three repeated experiments; 60 explants per treatment was used; MS medium was used for the study; *Observations were recorded after 4 weeks; Age of the MZE refers to the time duration from the date of collection of seeds from the mature tree C: Callus

In the present research work, the effect of the age of the seed (storage duration) on direct somatic embryogenesis was evaluated using excised zygotic embryo explants. Maximum somatic embryogenesis frequency was obtained when one month old excised zygotic embryos cultured on MS medium supplemented with 1.0 mg/l 2,4-D + 0.01 mg/l Kn and the highest percentage of (62.00 ± 1.15) direct somatic embryos was observed. Three month old excised embryos when cultured on MS supplemented with 1.0 mg/l 2,4-D and 0.01 mg/l Kn produced 28.88 ± 4.77 somatic embryos per explants. Six-, 9- and 12-month old excised embryos, when cultured on MS supplemented with 1.0 mg/l 2,4-D and 0.01mg/l Kn, showed a decline in somatic embryo formation and resulted only in callus induction (**Table 3**). Maximum direct somatic embryogenesis frequency was obtained with one-month old excised zygotic embryos when compared to 3 months old seeds. In the present study, one month old excised zygotic embryos were suitable for efficient direct somatic embryo induction. Therefore, it is advisable to use one-month old seed as a source of MZE to achieve optimum culture response in terms of direct somatic embryogenesis. Maturation of somatic embryos was obtained on MS medium supplemented with 50 g/l sucrose (**Fig. 1C, 2**).

In earlier studies, it has been reported that somatic embryos can differentiate either directly from the explant without an intervening callus phase or indirectly with callus phase (Williams and Maheswaran 1986; Cuenca *et al.* 1999). The distinction between direct and indirect somatic embryogenesis is unclear. According to older hypothesis, direct somatic embryos should originate from embryogenically predetermined cells (EPDC) and indirect somatic embryos should originate from undetermined cells. Pathway leading to the development of somatic embryos in the present study from MZE explants follows two roots namely direct and indirect somatic embryogenesis. Indirect way of somatic embryogenesis was more predominant in terms of frequency of occurrence than the direct route of somatic embryo induc-

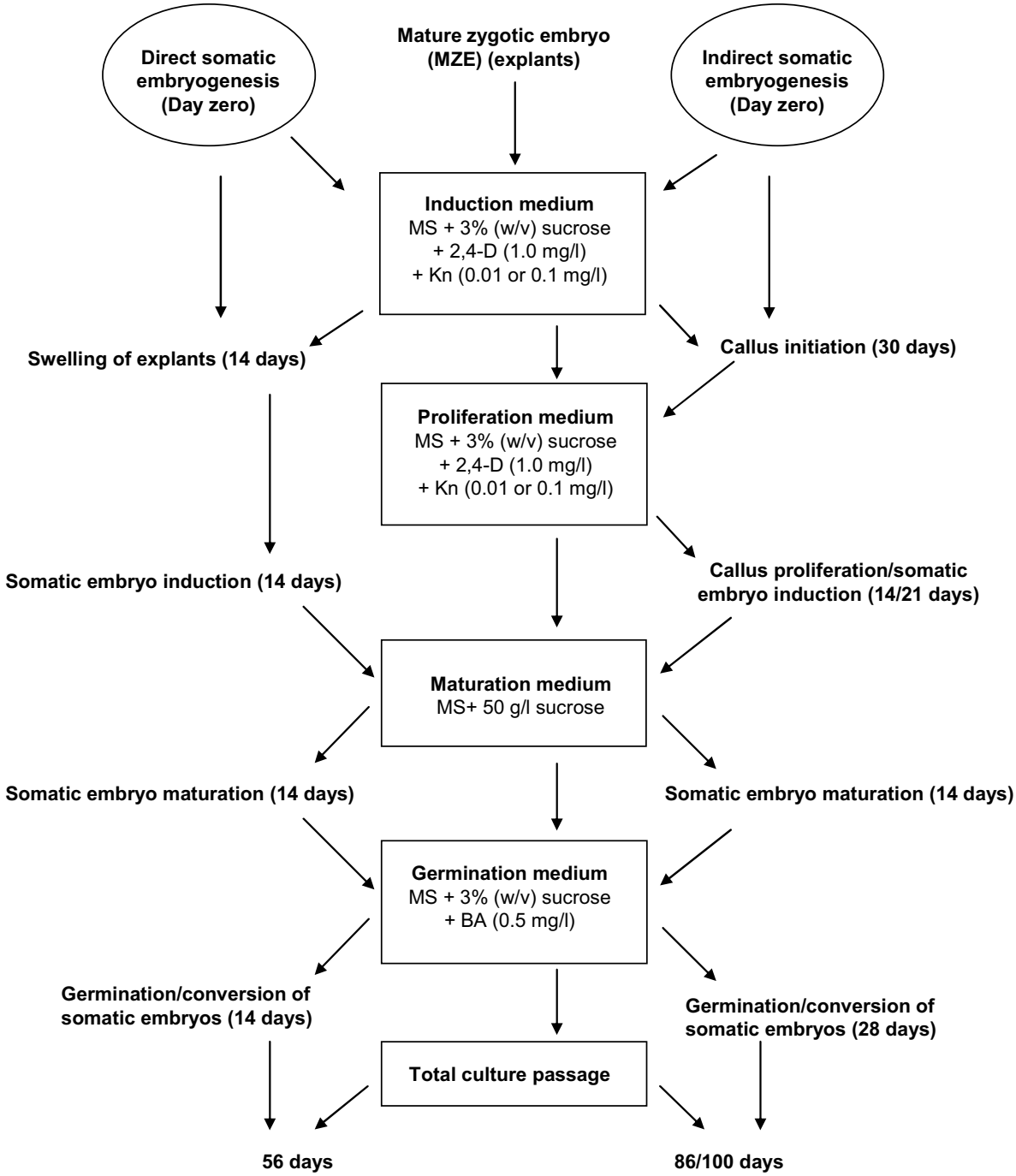

Fig. 2 Schematic representation of the comparative account of direct and indirect somatic embryogenesis using MZE explants in *T. chebula*.

tion from MZE explants in the present study. The direct somatic embryogenesis obtained in the present study may be attributed to the presence of EPDCs as found in some of the MZE explants (Arnold *et al.* 2002).

Direct somatic embryogenesis avoids the passage through callus and thus avoids the genetic instability often associated with somatic embryos obtained indirectly from callus (Ipekci and Gozukirmizi 2003). Explants of embryonic tissue origin such as immature embryo or unfertilized ovary have been found to be a suitable source for the induction of somatic embryogenesis as has been found in the present study (Mach *et al.* 1993; Douglas *et al.* 2007). Amongst the different strengths of MS nutrients and BA combinations were tested for somatic embryo germination, ½ MS medium supplemented with 0.5 mg/l BA was found suitable for germination (**Fig. 1E**).

In over assessment of the direct and indirect embryogenesis certain advantages were evident in the duration of *in vitro* passage. Initiation of direct somatic embryogenesis, and even advanced developmental stages of the somatic embryos wereobserved within 28 days following onset of culture (**Fig. 2**). On the other hand, in the indirect pathway, somatic embryos were observed on the callus surface after 44 days and some of the cultures after 51 days after culture. Somatic embryo maturation was complete within 42 days in direct compared to 58 to 65 days in indirect pathway of somatic embryogenesis. Subsequent to maturation even the germination of somatic embryo was comparatively quicker, in direct pathway i.e. 14 days whereas 28 days was required in indirect method via intervening callus phase. Total duration of somatic embryogenesis process took 56 days in case of direct somatic embryogenesis compared to 86 or 100 days following indirect embryogenesis path. There was a distinct advantage of curtailing the *in vitro* passage up to 30 to 44 days in the direct pathway of somatic embryogenesis (**Fig. 2**).

CONCLUSIONS

In the present study, the direct somatic embryogenesis was

achieved for the first time in *T. chebula*. There is a distinct advantage with the direct somatic embryogenesis, which curtails the *in vitro* passage. The somatic embryogenesis culture system developed in the present study will be useful for rapid multiplication of this important medicinal tree as an alternative pathway of propagation. Further, it may be exploited for genetic transformation studies and germplasm conservation.

ACKNOWLEDGEMENTS

The authors would like to thank the AP Netherlands Biotechnology Programme, A.P. of The Netherlands, Ministry for Development Co-operation (presently Agribiotech Foundation) for the financial support. C. Anjaneyulu would like to thank CSIR, New Delhi for the award of a Senior Research Fellowship.

REFERENCES

Akula A, Dodd WA (1998) Direct somatic embryogenesais in a selected tea clone TRI-2025 (*Camellia sinensis* (L.) O. Kuntze) from nodal explants. *Plant Cell Reports* **17**, 804-809

Altman A, Loberant B (1998) Micropropagation: clonal plant propagation *in vitro*. In: Altman A (Ed) *Agricultural Biotechnology*, Marker Dekker, New York, pp 19-42

Anjaneyulu C, Shyamkumar B, Giri CC (2004) Somatic embryogenesis from callus cultures of *Terminalia chebula* Retz.: an important medicinal tree. *Trees – Structure and Function* **18**, 547-552

Arnold SV, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **69**, 233-249

Attree SM, Pomeroy MK, Fowke LC (1994) Production of vigorous desiccation tolerant white spruce [*Picea glauca* (Moench) Voss.] synthetic seeds in a bioreactor. *Plant Cell Reports* **13**, 601-606

Barbón R, Jiménez E, Preil W (2008) Influence of *in vitro* environment on somatic embryogenesis of *Coffea arabica* L. cv. Caturra rojo: The effects of carbon dioxide on embryogenic cell suspensions. *Plant Cell, Tissue and Organ Culture* **95**, 197-208

Carron MP, Etienne H, Lardet L, Campagna C, Perrin Y, Leconte A, Chaine C (1995) Somatic embryogenesis in rubber (*Hevea brasiliensis* Mull. Arg.). In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants (Vol 2), Angiosperms*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 117-136

Chand S, Singh AK (2001) Direct somatic embryogenesis from zygotic embryos of a timber-yielding leguminous tree, *Hardwickia binata* Roxb. *Current Science* **80**, 882-887

Chen JT, Chang WC (2006) Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. *Biologia Plantarum* **50**, 169-173

Choi YE, Yang DC, Yoon ES (1999) Rapid propagation of *Eleutherococcus senticosus* via direct somatic embryogenesis from explants of seedlings. *Plant Cell, Tissue and Organ Culture* **58**, 93-97

Cuenca B, San-Jose MC, Martinez MT, Ballester A, Vieitez A (1999) Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. *Plant Cell Reports* **18**, 538-543

Douglas AS, Charles RC, Miguel PG (2007) Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. *Plant Cell, Tissue and Organ Culture* **89**, 15-22

Konan EK, Durand-Gasselin T, Kouadio YJ, Flori A, Rival A, Duval Y, Pannetier C (2010) *In vitro* conservation of oil palm somatic embryos for 20 years on a hormone-free culture medium: characteristics of the embryogenic cultures, derived plantlets and adult palms. *Plant Cell Reports* **29**, 1-13

Fuentes SRL, Calheiros MBP, Manetti-Filho J, Vieira LGE (2000) The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell, Tissue and Organ Culture* **60**, 5-13

Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees – Structure and Function* **18**, 115-135

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

Khare CP (2004) *Terminalia chebula* Retz. In: Khare CP (Ed) *Encyclopedia of Indian Medicinal Plants*, Springer-Verlag, Berlin, pp 451-453

Krajňáková J, Häggman H, Gömöry D (2009) Effect of sucrose concentration, polyethylene glycol and activated charcoal on maturation and regeneration of *Abies cephalonica* somatic embryos. *Plant Cell, Tissue and Organ Culture* **96**, 251-262

Kushalkar R, Sharon M (1996) Direct and indirect somatic embryogenesis in teak (*Tectona grandis* L.). *Current Science* **71**, 712-715

Lan TH, Hong PI, Huang CC, Chang WC, Lin CS (2009) High-frequency direct somatic embryogenesis from leaf tissues of *Drimiopsis kirkii* Baker (giant squill). *In Vitro Cellular and Developmental Biology – Plant* **45**, 44-47

Laxmisita G, Raghavaswamy BV (1998) Application of biotechnology in forest trees - clonal multiplication of sandal wood, rose wood, teak, eucalypts and bamboos by tissue culture in India. In: Puri S (Ed) *Tree Improvement*, Oxford and Publishing Co. Pvt. Ltd, New Delhi, pp 233-248

Li ZN, Fang F, Liu GF, Bao MZ (2007) Stable *Agrobacterium*-mediated genetic transformation of London plane tree (*Platanus acerifolia* Willed.). *Plant Cell Reports* **26**, 641-650

March GD, Grenier E, Miannay N, Sulmont G, David H, David A (1993) Potential of somatic embryogenesis in *Prunus avium* immature zygotic embryos. *Plant Cell, Tissue and Organ Culture* **34**, 209-215

Martin KP, and Joseph M (2005) Direct and indirect somatic embryogenesis on cotyledon explants of *Quassia amara* L., an antileukaemic drug plant. *In Vitro Cellular and Developmental Biology – Plant* **41**, 54-57

Martínez T, Corredoira E, Valladares S, Jorquera L, Vieitez AM (2008) Germination and conversion of somatic embryos derived from mature *Quercus robur* trees: The effects of cold storage and thidiazuron. *Plant Cell, Tissue and Organ Culture* **95**, 341-351

Moromoto M, Nakamura K, Sano H (2006) Regeneration and genetic engineering of a tropical tree *Azadirachta excelsa*. *Plant Biotechnology* **23**, 123-127

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Paiva NVB, Botelho MN, Aguiar R, Silva EAM, Otoni WC (2003) Somatic embryogenesis from immature zygotic embryos of annatto (*Bixa orellana* L.). *In Vitro Cellular and Developmental Biology – Plant* **39**, 629-634

Prakash MG, Gurumurthi K (2010) Effects of type of explant and age, plant growth regulators and medium strength on somatic embryogenesis and plant regeneration in *Eucalyptus camaldulensis*. *Plant Cell, Tissue and Organ Culture* **1**, 13-20

Prasanthi I, Perera P, Hoscher V, Verdeil JL, Doulbeau S, Deepthi M, Yakandawala D, Weerakoon LK (2007) Unfertilized ovary: A novel explant for coconut (*Cocus nucifera* L.) somatic embryogenesis. *Plant Cell Reports* **26**, 21-28

San-Jose MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón R, Vieitez AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. *Plant Cell Reports* **29**, 661-671

Sharry S, Ponce JLC, Estrella L.H, Cano RMR, Lede S, Abedini W (2006) An alternative path way for plant *in vitro* regeneration of chinaberry-tree *Melia-azedarach* L. derived from the induction of somatic embryogenesis. *Electronic Journal of Biotechnology* **9**, 188-194

Silva TER, Cidade LC, Alvim FC, Cascardo JCM, Costa MGC (2009) Studies on genetic transformation of *Theobroma cacao* L.: Evaluation of different polyamines and antibiotics on somatic embryogenesis and the efficiency of *uidA* gene transfer by *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture* **99**, 287-298

Shyamkumar B, Anjaneyulu C, Giri CC (2007) Genetic transformation of *Terminalia chebula* Ritz. and detection of tannin in transformed tissue. *Current Science* **92**, 361-367

Valladares S, Sánchez C, Martínez MT, Ballester A, Viéitez AM (2006) Plant regeneration through somatic embryogenesis from tissues of mature oak trees: True-to-type conformity of plantlets by RAPD analysis. *Plant Cell Reports* **25**, 879-886

Vengadesan G, Pijut PM (2009) Somatic embryogenesis and plant regeneration of northern red oak (*Quercus rubra* L.). *Plant Cell, Tissue and Organ Culture* **97**, 141-149

Williams EG, Maheswaran G (1986) Somatic embryogenesis: Factors influencing coordinated behaviour of cells as an embryogenic group. *Annals of Botany* **57**, 443-462

Wu Y-J, Huang X-L, Chen Q-Z, Li X-J, Engelmann F (2007) Induction and cryopreservation of embryogenic cultures from nucelli and immature cotyledon cuts of mango (*Mangifera indica* L. var Zihua). *Plant Cell Reports* **26**, 161-168

Yadav CB, Jha P, Mahalakshmi C, Anjaiah V, Bhat V (2009) Somatic embryogenesis and regeneration of *Cenchrus ciliaris* genotypes from immature inflorescence explants. *Biologia Plantarum* **53**, 603-609

Zhang N, Fang W, Shi Yan, Liu Q, Yang H, Gui R, Lin X (2010) Somatic embryogenesis and organogenesis in *Dendrocalamus hamiltonii*. *Plant Cell, Tissue and Organ Culture* **1**, 13-20