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ORIGINAL ARTICLE

Factors influencing somatic embryo maturation, high frequency germination and plantlet formation in *Terminalia chebula* Retz.

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Abstract The factors influencing somatic embryo maturation, high frequency somatic embryo germination, and plantlet formation were studied in *Terminalia chebula* Retz. Maturation of somatic embryo were influenced by a number of factors such as in vitro culture passage, concentrations of sucrose, levels of abscisic acid (ABA), basal media and media additive combinations. Maximum frequency of somatic embryo maturation (57.22 ± 2.02), was obtained on MS medium supplemented with 50 g/l sucrose. Different factors such as strengths of MS nutrients, plant growth regulators, media additives and their combinations controlling somatic embryo germination and plantlet formation were studied. High frequency of germination and plantlet formation (58.80 ± 1.47) were achieved by subsequent subculture of mature somatic embryos on MS medium containing 30 g/l sucrose and 0.5 mg/l benzyladenine (BA). However, although duration of in vitro passage of the callus tissue was critical, contribution of the combinations of plant growth regulators and media additives showed nugatory effect on somatic embryo maturation and germination as evident from variable responses.

Keywords Somatic embryogenesis · Plant growth regulators · Media additives · Maturation · Germination · Plantlet formation

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Abbreviations

AC	Activated charcoal
ABA	Abscisic acid
BA	Benzyladenine
2,4-D	2,4 Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
IBA	Indole-3-butyric acid
KN	Kinetin
NAA	Napthalene acetic acid

Introduction

Terminalia chebula Retz. (Family: Combretaceae) is an economically important plant for medicine and raw material in the tanning industry (Chadha 1989). *T. chebula* shows antispasmodic, anti-HIV, antibacterial activity and antioxidant activities (Sandip 2003; Khare 2004; Bonjar 2004). In addition, *T. chebula* has also been shown to influence dermal wound healing, to have an inhibitory effect on human prostrate cancer cell line, a cardio-protective effect and can be used in therapies for managing rheumatoid arthritis (Suguna et al. 2002; Jaya et al. 2004; Yi et al. 2004; Suchalatha and Shyamala 2004; Lee et al. 2005). The distribution and natural regeneration of *T. chebula* from seeds is poor due to low germination capacity (Chadha 1989; Bhardwaj and Chakraborty 1994; Shankar 2001; Ramesh Singh et al. 2003).

Somatic embryogenesis offers a number of applications as an alternative pathway for mass multiplication of elite trees within a short time, germplasm conservation and genetic transformation (Jain and Ishii 2003; Giri et al. 2004; Moon et al. 2005; Valladares et al. 2006; Sharry et al. 2006; Li et al. 2007; Wu et al. 2007; Lelu-Walter

et al. 2008; Gonzalez-Arnao et al. 2008). The somatic embryogenesis process possesses immense potential for large-scale propagation with high commercial prospects. However, in addition to mere induction of somatic embryogenesis, the maturation and efficient somatic embryo germination and plantlet formation remains one of the serious constraints for practical exploitation. In the past, it has been found that under appropriate in vitro conditions the intrinsic and induced capability of plant cells and tissues to undergo different developmental stages of somatic embryogenesis is extremely critical (Krishnamurthy 1999; Feher et al. 2003; Silveira et al. 2004; Perán-Quesada et al. 2004; Zhang et al. 2007).

Few reports are available on in vitro propagation of *T. chebula*, a tree of medicinal and economic importance. We have developed, for the first time, protocols for the propagation of *T. chebula* through multiple-shoot induction and somatic embryogenesis (Shyamkumar et al. 2003; Anjaneyulu et al. 2004). However, much research input and further refinement considering different key factors for devising efficient protocol with particular reference to somatic embryogenesis pathway of plant propagation in *T. chebula* is still required.

The present communication reports on the influence of different factors and their possible role on somatic embryo maturation, germination and plantlet formation in *T. chebula*. Different factors such as in vitro culture passage, concentrations of basal media, media additives, and plant growth regulators were studied to achieve high frequency germination and plant formation from somatic embryos of *T. chebula*.

Materials and methods

Induction of callus, somatic embryogenesis and culture conditions

Callus and somatic embryogenesis were induced using mature zygotic embryo (MZE) explants as reported by Anjaneyulu et al. 2004. Cultures containing developing somatic embryos were maintained at 25 ± 2°C, 80% relative humidity in light (dark whenever necessary) conditions under 16-h photoperiod with a light intensity of 30,000 lux provided by cool white fluorescent tubes.

Maturation of somatic embryos

Various concentrations (30, 40, 50, 60 g/l) of sucrose and different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 mg/l) of abscisic acid were evaluated for maturation of somatic embryos. The effect of in vitro culture passage of initial cultures on somatic embryo maturation were also investigated. The number of fully developed somatic embryos produced was scored during individual treatments, and observations were taken after 4 weeks of culture.

Somatic embryo germination and plantlet formation

Fully developed cotyledonary stage somatic embryos derived from MZE-derived callus cultures were evaluated for somatic embryo germination and plantlet formation response. Different strengths of MS nutrients such as full, one-half and one-quarter were evaluated for somatic embryo germination. In addition, MS (Murashige and Skoog 1962) basal medium supplemented with media additives such as glutamine (0.05, 0.1, 0.2, 0.4 g/l), maltose (10, 20, 40 g/l), mannitol (10, 20, 40 g/l), sorbitol (10, 20, 40 g/l) and polyethylene glycol (10, 20, 40 g/l) were evaluated for somatic embryo conversion.

A combination of GA_3 (0.1, 0.2, 0.5, 1.0, 2.0, 4.0 mg/l), IBA (0.1, 0.2, 0.5, 1.0, 2.0 mg/l) and BA (0.1, 0.2, 0.5, 1.0, 2.0, 4.0 mg/l) were also evaluated for conversion of somatic embryos. A combination of activated charcoal (0.2, 0.5, 1.0, 2.0 g/l), IBA (1.0 mg/l) and activated charcoal (0.2, 0.5, 1.0, 2.0 g/l) + BA (0.5 mg/l) were also tested for conversion of somatic embryos.

Statistical analysis

Latin square experimental design was followed for the experiments and statistical analysis. In the experiments, each explant was referred to as one replicate. A minimum of 22–38 replicates for somatic embryo maturation and conversion (germination) were used in each experiment. Statistical analysis of data, such as mean, SE, one-way ANOVA, and multiple comparisons, was done using Matlab Version 5.3, and SPSS version 10.0 Math Works (USA) statistical packages.

Results and discussion

Maturation of somatic embryos

Somatic embryogenesis was achieved in *T. chebula* using mature zygotic embryo explants (Anjaneyulu et al. 2004). Mature zygotic embryo explants have proved to be good starting material for the induction of somatic embryogenesis (Giri et al. 2004; Steinmacher et al. 2007; Prasanthi et al. 2007). MS medium supplemented with various concentrations (30, 40, 50, 60 g/l) of sucrose evaluated for somatic embryo maturation revealed variable culture responses. The number of early cotyledonary or late heart stage somatic embryos inoculated and fully developed cotyledonary somatic embryos with clear cotyledons and

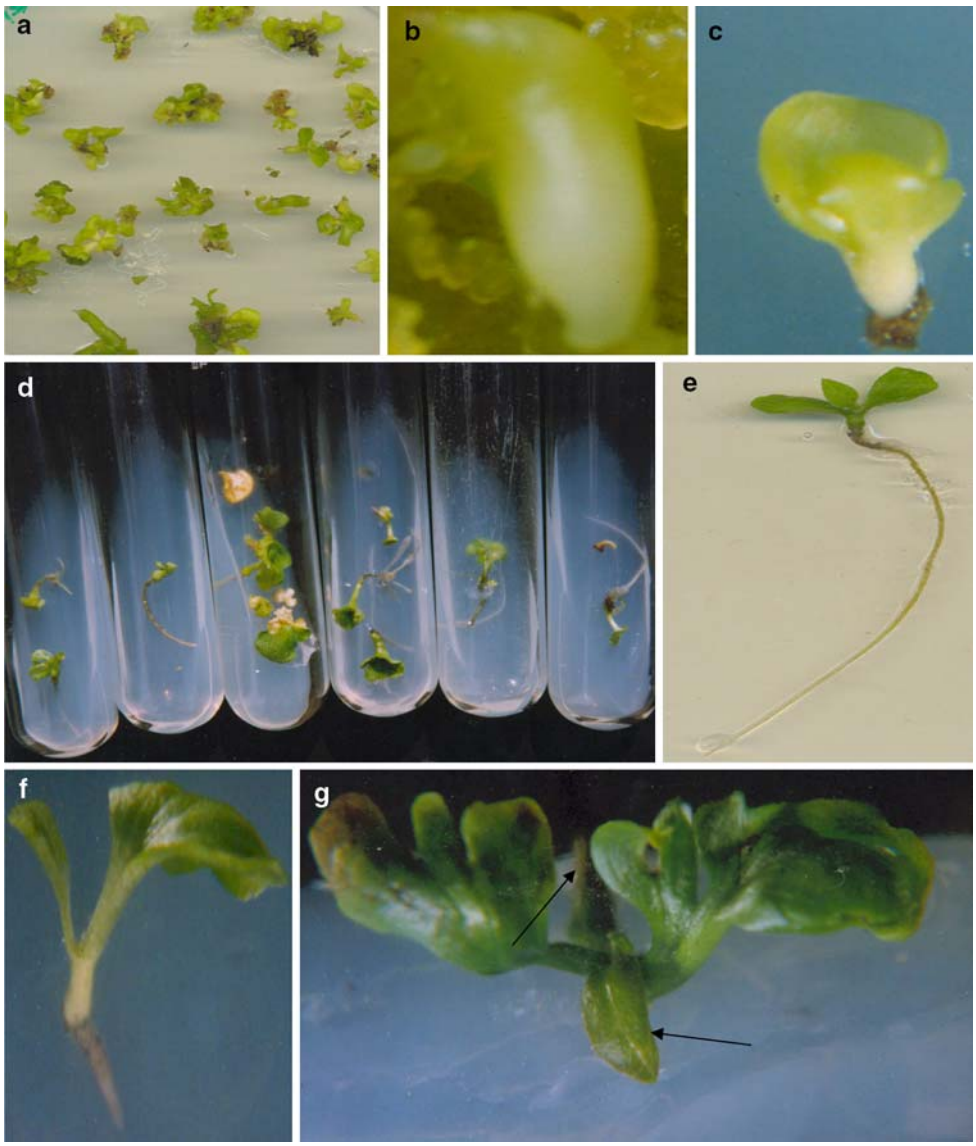

Fig. 1 Maturation and high frequency germination of somatic embryo to plantlets in *Terminalia chebula*. **a** Large number of somatic embryos on maturation medium. **b** Maturation of somatic embryo (late heart stage). **c** Maturation of somatic embryo (late cotyledon stage). **d** High frequency germination of somatic embryos. **e**, **f** View of the germinated somatic embryos to plantlets at different growth stages. **g** Conversion of somatic embryos to plants, growth of cotyledons and clear projecting epicotyls (*arrows*)

root ends were scored to obtain the percentage of maturation of somatic embryos (Fig. 1a–c). The lowest frequency of somatic embryo maturation (28.87 ± 2.29) and the highest frequency of somatic embryo maturation (57.22 ± 2.02) were observed on maturation medium supplemented with 30 and 50 g/l sucrose, respectively (Table 1). Sucrose concentration above 50 g/l in the maturation medium showed reduction in the number of mature somatic embryos (43.33 ± 0.88). In the present study, MS medium supplemented with different concentrations of sucrose was evaluated for somatic embryo maturation. Fully developed mature embryos were obtained on MS medium containing 50 g/l sucrose (Fig. 1c). In this maturation medium, few embryos showed elongation of cotyledons, but no root end initiation was observed. MS and half-strength MS medium was used for maturation of somatic embryos in tree species (Yu et al. 2000; Xiao et al. 2004; Anjaneyulu et al. 2004; Indieka et al. 2007).

Sucrose concentration at 50 g/l was found to be critical for maturation of somatic embryos in the present study. Sucrose at a concentration of 60 g/l did not improve somatic embryo maturation further compared to 50 g/l sucrose. Currently, a study with *Psidium guajava* has also revealed that 5% (w/v) sucrose was the most favorable for maturation of somatic embryos (Rai et al. 2007). High sucrose concentration in the maturation medium may have resulted in high osmotic environment of the cells and tissues, which has been beneficial to prevent precocious embryo conversion in one hand and enhance embryo maturation in other plant species.

In earlier studies, higher concentration of sucrose was also used for somatic embryo induction and maturation in

Table 1 Effect of various concentrations of sucrose on maturation of somatic embryo in *Terminalia chebula*

MS media sucrose (g/l)	No. of somatic embryos inoculated	Percentage mature somatic embryos (mean ± SE)
30	28	28.87 ± 2.29
40	28	37.66 ± 0.90
50	30	57.22 ± 2.02
60	33	43.33 ± 0.88

Mean ± SE of three repeated experiments; MS medium was used for the study; 28–35 somatic embryos per treatment; observations were recorded after 4 weeks

Table 2 Effect of various concentrations of abscisic acid (ABA) on maturation of somatic embryo in *T. chebula*

MS media ABA (mg/l)	No. of somatic embryos inoculated	Percentage mature somatic embryos (mean ± SE)
0.1	34	11.08 ± 0.90
0.2	32	10.57 ± 2.42
0.5	35	11.66 ± 1.09
1.0	28	12.83 ± 0.82
2.0	28	15.75 ± 1.28
4.0	29	14.87 ± 1.21

Mean ± SE of three repeated experiments; MS medium was used for the study; 28–35 somatic embryos per treatment; observations were recorded after 4 weeks

Mangifera indica, *Litchi chinensis* and *Morus alba* (Laxmi et al. 1999; Yu et al. 2000; Agarwal et al. 2004). This type of variable response may be attributed to genotype, in vitro chemical and physical factors as well as to the endogenous level of plant growth regulators.

Maturation of somatic embryos was also studied using MS medium supplemented with various concentrations of ABA. MS medium containing 2.0 mg/l ABA and 30 g/l sucrose showed better somatic embryo maturation response compared to other concentrations of ABA (Table 2). A maximum of mature somatic embryos (15.75 ± 1.28) was obtained on medium supplemented with 2.0 mg/l ABA. A less frequency of mature somatic embryos (14.87 ± 1.21) obtained on maturation medium containing 4.0 mg/l ABA compared to 2.0 mg/l ABA. A minimum of mature somatic embryos (10.57 ± 2.42) was obtained on maturation medium containing 0.2 mg/l ABA. It revealed that somatic embryo maturation was best on MS medium with 50 g/l sucrose compared to all ABA concentrations evaluated. A similar response was observed in a related species *T. arjuna* with MS medium containing ABA (Kumari et al. 1998). ABA has been used in the past for embryo development and maturation media to improve synchronization of development and conversion of somatic embryos into plantlets. ABA has been used routinely for maturation of somatic embryos in a number of tree species (Naidu and Sreenivasan 2004; Pullman et al. 2005; Garcia-Martin et al. 2005; Kim and Moon 2007).

Table 3 Effect of in vitro passage on somatic embryo maturation using mature zygotic embryo-derived cultures of *T. chebula*

In vitro passage		Percentage mature somatic embryos (mean ± SE)
Days	Weeks	
45	0	18.69 ± 2.89
52	1	33.50 ± 2.68
59	2	57.22 ± 2.02
66	3	28.49 ± 3.49
73	4	11.87 ± 2.75
80	5	4.52 ± 1.32
87	6	–

Mean ± SE of three repeated experiments; MS medium supplemented with 50 g/l sucrose used for the experiment; – no response; in vitro passage referred to the maintenance of embryogenic callus on 2,4-D 1.0 mg/l and KN 0.01 mg/l + 30 g/l sucrose for different time durations; observations were recorded after 4 weeks

Effect of in vitro passage duration on somatic embryo maturation using MZE-derived embryogenic cultures

In vitro passage duration of embryogenic cultures for somatic embryo maturation was investigated. The choice of embryonic tissue culture age on optimum somatic embryo maturation frequency was found to be critical. The embryogenic tissue 45 days after culture initiation was considered as zero week. Embryogenic calli of 45 and 52 days after in vitro passage showed a somatic embryo maturation frequency of up to 18.69 ± 2.89 and 33.50 ± 2.68, respectively. The highest frequency (57.22 ± 2.02) of somatic embryo maturation was observed when the embryogenic cultures were maintained on induction medium for 59 days (Table 3). A frequency (28.49 ± 3.49) of mature somatic embryos was obtained after 66 days of culture. Further maintenance of embryogenic cultures beyond 66 days on induction medium showed a decline in the frequency of maturation of somatic embryos. Maturation of somatic embryos was not observed when 87-day-old in vitro cultures were used for embryo maturation. Maintenance of the embryogenic calluses after somatic embryo induction on induction medium for 1–2 weeks was found useful for somatic embryo maturation. A greater number of globular embryos was observed and had no adverse effect on maturation. Continued presence of 2,4-D plus kinetin was found to be inhibitory for further development of somatic embryos in prolonged cultures. The mature zygotic embryo derived callus remained embryogenic with globular structures up to 55–60 days. Following further maintenance of callus on

Table 4 Effect of different strengths of MS nutrients and different media additives on conversion of somatic embryos in *T. chebula*

Media composition	No. of somatic embryos inoculated	Percentage somatic embryos germinated (mean ± SE)
MS	23	19.43 ± 1.66
1/2 MS	30	17.60 ± 1.71
1/4 MS	27	14.87 ± 2.08
MS + media additives (g/l)		
Maltose		
10	22	10.35 ± 1.65
20	26	8.43 ± 1.16
40	25	8.50 ± 2.17
Glutamine		
0.05	29	11.43 ± 2.94
0.1	25	12.73 ± 3.45
0.2	29	10.77 ± 1.91
0.4	30	9.46 ± 2.07
Mannitol		
10	32	7.75 ± 1.30
20	36	4.55 ± 0.57
40	29	3.54 ± 0.33
Sorbitol		
10	25	7.75 ± 1.30
20	28	5.15 ± 0.96
40	32	5.32 ± 1.17
PEG		
10	28	8.00 ± 1.30
20	25	8.35 ± 0.60
40	22	5.71 ± 0.67

Mean ± SE of three repeated experiments; 22–36 somatic embryos per treatment; full strength MS medium with 3% sucrose was used for the study; observations were recorded after 4 weeks

induction medium, 30% of cultures developed brown sectors in the callus mass and the growth rate was reduced.

Effect of different strengths of MS nutrients on conversion of somatic embryos into plantlets

Different strengths of MS nutrients were evaluated for conversion of somatic embryos. Fully developed somatic embryos from MZE-derived embryogenic cultures were used for our study on conversion. Somatic embryos with fully developed cotyledons and initiating root ends were considered for scoring percentage of germination (Fig. 1c). Among different strengths of MS nutrients used for conversion of somatic embryos, full-strength MS basal medium showed best response compared to half-strength MS and quarter-strength MS nutrients (Table 4). The highest frequency of somatic embryo conversion (19.43 ± 1.66) was observed on full-strength MS nutrients, followed by conversion (17.60 ± 1.71) of somatic embryos on half-strength MS nutrients. The least frequency of somatic embryo conversion (14.87 ± 2.08) was observed on quarter-strength MS nutrients. The fully developed root and green shoot emerged on MS basal medium containing reduced concentration of sucrose, i.e., 30 g/l upon transfer of mature somatic embryos from maturation medium. MS medium was used for conversion of somatic embryos in the tree species and half-strength MS and full-MS nutrients promoted conversion of somatic embryos (Moon et al. 2005). This finding showed a positive role of nutrients besides plant growth regulators on somatic embryo conversion.

Effect of different media additives on germination of somatic embryos to plantlets

The effect of different media additives such as maltose, glutamine, mannitol, sorbitol, polyethylene glycol (PEG) was evaluated for conversion of somatic embryos. MS medium supplemented with 0.1 g/l glutamine showed highest frequency of somatic embryo conversion (12.73 ± 3.45) when compared to MS medium supplemented with all other media additives (Table 4). Among maltose concentrations used for conversion of somatic embryos, 10 g/l maltose promoted the best response, i.e., 10.35 ± 1.65. In the case of media additive mannitol, frequency of conversion ranged from as low as 3.54 ± 0.33 to as high as 7.75 ± 1.30 when concentrations of 40 and 10 g/l were used, respectively. The highest frequency of conversion (7.75 ± 1.30) of somatic embryos was observed with MS medium containing 10 g/l compared to other sorbitol concentrations. The maximum conversion frequency (8.35 ± 0.60) was observed with medium containing 20 g/l polyethylene glycol compared to other concentrations (Table 4).

In the present study, the frequency of conversion of somatic embryos to plantlets could not be increased further with media additives such as maltose, glutamine, mannitol, sorbitol and PEG when added to the conversion media. The concentrations of 10 g/l maltose; 0.1 g/l glutamine; 10 g/l mannitol and sorbitol and 20 g/l PEG showed comparable responses for somatic embryo conversion. In previous studies, the addition of sorbitol had enhanced the conversion capacity of oak somatic embryos from juvenile origin (Sánchez et al. 2003), whereas sorbitol-supplemented maturation medium may not be the most appropriate treatment for maturation of embryos derived from certain mature trees evaluated in tissues of mature oak trees (Valladares et al. 2006). Currently, in a study with *Myrciaria aureana* (Brazilian grape tree), light, activated charcoal, and polyethylene glycol (PEG) were tested for the regeneration and maturation of somatic embryos. The combination of light and PEG provided the highest number

of mature embryos and also conversion of somatic embryos (Motoike et al. 2007).

Media additives are occasionally used for somatic embryo conversion in tree species. In an earlier report, media additive maltose in the case of *Abies alba* and glutamine in case of *Picea mariana* and *Santalum spicatum* was found suitable for conversion of somatic embryos (Giri et al. 2004). In a recent report, media additive polyethylene glycol was used for maturation and conversion of somatic embryos (Motoike et al. 2007).

Full strength MS, half-strength MS, full-strength MS + 5% sucrose, supplemented with 0.1–4.0 mg/l GA_3 was also evaluated for conversion of somatic embryos. The maximum number of somatic embryo conversion (12.22 ± 1.12) was obtained with MS medium containing 1.0 mg/l GA_3 compared to other GA_3 concentrations. In *Santalum* sp. and *M. indica*, GA_3 was found suitable for conversion of somatic embryos (Rugkhla and Jones 1998; Ara et al. 2000). Somatic embryo conversion up to 100% was obtained on MS basal medium, and addition of GA_3 lowered the percentage of conversion of somatic embryos in *T. arjuna*. However, fused cotyledonary embryos showed maximum conversion on GA_3 containing medium in *T. arjuna* (Kumari et al. 1998). Based on our observations, the addition of GA_3, in the conversion medium inhibited embryo conversion compared to MS basal medium alone.

Effect of different concentrations and combinations of BA, IBA and GA_3 on conversion of somatic embryos

MS medium supplemented with BA, IBA and GA_3 combinations was evaluated for conversion of somatic embryos. Among media combinations, MS supplemented with 0.5 mg/l BA produced the maximum number of somatic embryos (58.80 ± 1.47) (Table 5). In earlier studies, BA was also found to promote similar conversion response of somatic embryos in *Castanea dentata* (American chestnut), *Pistacia vera*, and *Abies alba* × *A. cephalonica* (Xing et al. 1999; Onay et al. 2000; Salaj and Salaj 2003). The concentration of 1.0 mg/l IBA showed the highest frequency (8.49 ± 1.34) of conversion somatic embryo compared to other concentrations. The highest frequency of somatic embryo conversion (12.22 ± 1.12) was obtained with MS medium containing 1.0 mg/l GA_3 compared to other GA_3 concentrations. Among the concentrations and combinations of BA and IBA, 0.5 mg/l BA, 0.1 mg/l IBA showed best response of somatic embryo conversion (29.94 ± 4.85). The combination of 0.5 mg/l BA and 0.1 mg/l GA_3 promoted a maximum (30.39 ± 4.73) of somatic embryo germination compared to other concentrations and combinations of BA and GA_3. The highest frequency conversion (25.01 ± 6.42) of somatic embryos was obtained with a combination of BA, IBA and GA_3.

Table 5 Effect of different concentrations and combinations of BA, IBA and GA_3 on conversion of somatic embryos in *T. chebula*

BA	IBA (mg/l)	GA_3	No. of somatic embryos inoculated	Percentage somatic embryos germinated (mean ± SE)
0.1	–	–	31	18.37 ± 1.48
0.2	–	–	29	28.87 ± 2.29
0.5	–	–	30	58.80 ± 1.47
1.0	–	–	28	30.99 ± 3.15
2.0	–	–	29	12.19 ± 2.84
4.0	–	–	30	9.00 ± 2.64
–	0.1	–	28	5.81 ± 1.20
–	0.2	–	27	6.49 ± 1.13
–	0.5	–	30	7.14 ± 0.29
–	1.0	–	27	8.49 ± 1.34
–	2.0	–	29	6.97 ± 1.68
–	4.0	–	24	5.33 ± 0.85
–	–	0.1	28	4.52 ± 1.32
–	–	0.2	30	5.06 ± 0.80
–	–	0.5	31	8.38 ± 0.73
–	–	1.0	22	12.22 ± 1.12
–	–	2.0	26	11.14 ± 2.78
–	–	4.0	22	7.44 ± 3.09
0.5	0.1	0.0	35	29.94 ± 4.85
0.5	0.2	0.0	34	27.77 ± 7.76
0.5	0.5	0.0	31	28.51 ± 6.36
0.5	0.0	0.1	31	30.39 ± 4.73
0.5	0.0	0.2	28	27.01 ± 2.01
0.5	0.0	0.5	29	24.32 ± 1.82
0.5	0.1	0.1	28	21.22 ± 2.65
0.5	0.1	0.2	24	24.42 ± 3.08
0.5	0.1	0.5	32	23.56 ± 4.36
0.5	0.2	0.1	29	24.49 ± 3.54
0.5	0.2	0.2	31	25.01 ± 6.42
0.5	0.2	0.5	35	22.42 ± 3.94
0.5	0.5	0.1	27	23.09 ± 4.58
0.5	0.5	0.2	33	21.50 ± 3.90
0.5	0.5	0.5	38	16.56 ± 7.91

Mean ± SE of three repeated experiments; 22–38 somatic embryos per treatment; MS medium was used for the study; observations were recorded after 4 weeks

The effect of BA, IBA, GA_3 on conversion of somatic embryos was analyzed by one-way ANOVA and multiple comparisons. One-way ANOVA analysis showed that all effects are significant at the 0.05% level. This analysis showed that the interaction within BA, IBA, GA_3 produced different frequencies of somatic embryo conversion and were found to be significant (Table 6). Multiple comparison analysis showed that all effects are significant at the 0.05% level except for a few interactions (Table 7). This analysis revealed that the multiple interactions of BA, IBA,

Table 6 One-way ANOVA for the effect of BA, IBA, GA_3 on conversion of somatic embryos in *T. chebula*

Source	Sum of squares	*df*	Mean square	*F*	*P* value
Interaction between BA, IBA, GA_3	6,731.572	5	1,346.314	18.557	0.00
Interaction with in BA, IBA, GA_3	6,747.128	93	72.550		
Total	13,478.701	98			

All the effects are significant at the 0.05% level

Table 7 Multiple comparisons for the effect of GA_3, BA, IBA on somatic embryo conversion in *T. chebula*

Interaction	Mean difference	*P* value
GA_3		
BA	−14.035482[a]	0.00
IBA	1.4779764	0.604
BA + IBA	−20.613522[a]	0.00
BA + GA_3	−19.110623[a]	0.00
BA + IBA + GA_3	−14.345655[a]	0.00
BA		
IBA	15.5134586[a]	0.00
BA + IBA	−6.5780398	0.062
BA + GA_3	−5.0751411	0.148
BA + IBA + GA_3	−0.3101725	0.905
IBA		
BA + IBA	−22.091498[a]	0.00
BA + GA_3	−20.588600[a]	0.00
BA + IBA + GA_3	−15.823631[a]	0.00
BA + IBA		
BA + GA_3	1.5028987	0.709
BA + IBA + GA_3	6.2678673[a]	0.059
BA + GA_3		
BA + IBA + GA_3	4.7649686	0.149

[a] The mean difference is significant at the 0.05% level; other interactions are not significant

GA_3 produced different frequencies of somatic embryo conversions and were found significant except for a few interactions, i.e., GA_3 − IBA, BA − BA + IBA, BA − BA + GA_3, BA − BA + IBA + GA_3, BA + IBA − BA + GA_3, BA + GA_3 − BA + IBA + GA_3.

Effect of MS supplemented with charcoal, BA and IBA on conversion of somatic embryos

MS medium supplemented with various concentrations (0.2, 0.5, 1, 2%) of activated charcoal, 0.5 mg/l BA and 1 mg/l IBA was evaluated for conversion of somatic embryos. Among various concentrations of activated charcoal and 0.5 mg/l BA used for conversion of somatic embryos, 0.5 g/l charcoal concentration showed the best response of conversion frequency (34.25 ± 0.88). Activated charcoal 0.5 and 1 mg/l IBA combination produced a maximum (9.80 ± 1.45) of somatic embryo conversion compared to other combinations (Table 8). It was found that the frequency of conversion of somatic embryos was less compared to BA alone. It was reported that medium containing IBA and activated charcoal was found suitable for somatic embryo development and conversion (Vookova and Kormutak 2001; Pullman et al. 2005). Recently, it has been found that the combination of GA_3 and activated charcoal enhanced recovery of plants from somatic embryos in *Litchi sinensis* (Simon et al. 2007).

Table 8 Effect of MS supplemented with charcoal, BA and IBA on conversion of somatic embryos to plantlets in *T. chebula*

Concentration of charcoal (g/l)	Concentration of PGR (mg/l)		Percentage somatic embryos germinated (mean ± SE)
	BA	IBA	
0.2	0.5	–	29.82 ± 0.83
0.5	0.5	–	34.25 ± 0.88
1.0	0.5	–	30.70 ± 0.88
2.0	0.5	–	30.20 ± 0.84
0.2	–	1	7.89 ± 1.52
0.5	–	1	9.80 ± 1.45
1.0	–	1	7.01 ± 0.66
2.0	–	1	5.56 ± 0.57

Mean ± SE of three repeated experiments; in each treatment 32–38 somatic embryos used; observations were recorded after 4 weeks

PGR Plant growth regulators

Studies of different factors that influence somatic embryo maturation and conversion have been routinely investigated in the past. Besides the determining role of specific genotypes, the in vitro culture environment (both chemical and physical) has shown dramatic effects on somatic embryo growth and development (Giri et al. 2004). The findings in the present study have augmented the continuous need for the study of factors that influence somatic embryo development until efficient germination is achieved (Agarwal et al. 2004). In the present study, an attempt is made for the first time to assess some defining probable factors and their influence on somatic embryo maturation and high frequency conversion in *T. chebula*. A recent study has revealed an interesting finding on how the influence of smoke-saturated-water could influence somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana*,

strengthening the role of external factors on somatic embryo growth and development (Malabadi and Nataraja 2007). In our study, we also found variation in the growth and morphology of somatic embryos as has been reported in *T. arjuna*. Similar to *T. arjuna*, we observed that some of the somatic embryos showed high frequency germination, although the cotyledons were fused to give a funnel-like structure (Fig. 1d). However, the frequency of these abnormal was not very high and the percentage was 23.43 ± 2.9. The germination and plantlet formation was better with somatic embryos with clear separate normal cotyledons. Keeping in view the high frequency somatic embryo induction, maturation and germination, the occurrence of so-called abnormal somatic embryos may not be an impediment for its exploitation for plant propagation.

Somatic embryo maturation and high frequency germination of plantlets achieved in the present study can be useful as an alternative pathway for propagation of *T. chebula*. Furthermore, the outcome can be exploited for genetic transformation studies and germplasm conservation of this important medicinal tree.

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References

Agarwal S, Kanwar K, Sharma DR (2004) Factors affecting secondary somatic embryogenesis and embryo maturation in *Morus alba* L. Sci Hortic 102:359–368

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

Ara H, Jaiswal U, Jaiswal VS (2000) Somatic embryogenesis and plantlet regeneration in Amarapali and chausa cultivars of mango (*Mangifera indica* L.). Curr Sci 78:164–169

Bhardwaj SD, Chakraborty AK (1994) Studies on time of seed collection, sowing and pre sowing seed treatments of *Terminalia bellirica* Roxb and *Terminalia chebula* Retz. Ind For 120:430–439

Bonjar GHS (2004) Evaluation of antibacterial properties of Iranian medicinal plants against *Micrococcus luteus*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Bordetella bronchoseptica*. Asian J Plant Sci 3:82–86

Chadha YR (1989) The wealth of India. Publication and Information Directorate, CSIR, New Delhi, pp 171–177

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228

Garcia-Martin G, Manzanera JA, González-Benito ME (2005) Effects of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. Plant Cell Tissue Organ Cult 80:171–177

Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F (2008) Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tissue Organ Cult 92:1–13

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

Indieka SA, Odee DW, Muluvi GM, Rao KN, Machuka J (2007) Regeneration of *Melia volkensii* Gürke (Meliaceae) through direct somatic embryogenesis. New Forests 34:73–81

Jain SM, Ishii K (eds) (2003) Micropropagation of woody trees and fruits. Kluwer, Dordrecht, Netherlands

Jaya PG, Rajeshkumar NV, Rajesh LT, Anuj S, James W, Steven RM, Anoop KS, Radha KM (2004) Protective effect of a polyherbal preparation, Brahma rasayana against tumor growth and lung metastasis in rat prostate model system. J Exp Ther Oncol 4:203–212

Khare CP (ed) (2004) Encyclopedia of Indian medicinal plants. Springer, Berlin Heidelberg New York

Kim YW, Moon HK (2007) Enhancement of somatic embryogenesis and plant regeneration in Japanese larch (*Larix leptolepis*). Plant Cell Tissue Organ Cult 88:241–245

Krishnamurthy KV (1999) On embryos and embryoids. Curr Sci 76:647–659

Kumari N, Jaiswal U, Jaiswal VS (1998) Induction of somatic embryogenesis and plant regeneration from leaf callus of *Terminalia arjuna* Bedd. Curr Sci 75:1052–1055

Laxmi DV, Sharma HC, Kirti PB, Mohan ML (1999) Somatic embryogenesis in mango (*Mangifera indica* L.) cv. Amarapali. Curr Sci 77:1355–1358

Lee SI, Hyun PM, Kim SH, Lee SK, Kim BS, Maeng PJ, Lim JS (2005) Suppression of the onset and progression of collagen induced arthritis by chebulagic acid screened from a natural product library. Arthritis Rheum 52:345–353

Lelu-Walter MA, Bernier-Cardou M, Klimaszewska K (2008) Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell Tissue Organ Cult 92:31–45

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

Naidu MM, Sreenivasan CS (2004) Effect of abscisic acid and cytokinin on cultured zygotic embryos of Coffea arabica cv. Cauvery (Catimor). Plant Cell Tissue Organ Cult 79:279–284

Malabadi RB, Nataraja K (2007) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* A.B. Jacks. J Plant Sci 2:45–53

Moon HK, Kim YW, Lee JS, Choi YE (2005) Micropropagation of *Kalopanax pictus* tree via somatic embryogenesis. In Vitro Cell Dev Biol Plant 41:303–306

Motoike SY, Saraiva ES, Ventrella MC, Silva CV, Salomão LCC (2007) Somatic embryogenesis of *Myrciaria aureana* (Brazilian grape tree). Plant Cell Tissue Organ Cult 89:75–81

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

Onay A (2000) Somatic embryogenesis from mature seed cultures of *Pistachia atlantica*. Turk J Agric 24:465–473

Perán-Quesada R, Sánchez-Romero C, Barceló-Muñoz A, Pliego-Alfaro F (2004) Factors affecting maturation of avocado somatic embryos. Sci Hortic 102:61–73

Prasanthi I, Perera P, Hocher V, Verdeil JL, Doulbeau S, Yakandawala DMD, Weerakoon LK (2007) Unfertilized ovary: a novel explant for coconut (*Cocus nucifera* L.) somatic embryogenesis. Plant Cell Rep 26:21–28

Pullman GS, Gupta PK, Timmis R, Carpenter C, Kreitinger M, Welty E (2005) Improved Norway spruce somatic embryo development through the use of abscisic acid combined with activated carbon. Plant Cell Rep 24:271–279

Rai MK, Akhtar N, Jaiswal VS (2007) Somatic embryogenesis and plant regeneration in *Psidium guajava* L. cv. Banarasi local. Sci Hortic 113:129–133

Ramesh Singh DR, Dhir KK, Vij SP, Nayyar H, Kamaljit S (2003) Study of genetic improvement techniques of *Terminalia chebula* Retz: An important multipurpose medicinal tree species of India. Ind For 129:154–168

Rugkhla A, Jones M (1998) Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. J Exp Bot 49:563–571

Salaj T, Salaj J (2003) Somatic embryo formation on mature *Abies alba x Abies cephalonica* zygotic embryo explants. Biol Plant 47:7–11

Sánchez MC, Martínez MT, Valladares S, Ferro E, Viéitez AM (2003) Maturation and germination of oak somatic embryos originated from leaf and stem explants: RAPD markers for genetic analysis of regenerants. J Plant Physiol 160:699–707

Sandip B (2003) Medicinal plants with anti-HIV potential. J Med Aromatic Plant Sci 25:427–440

Shankar U (2001) A case of high tree diversity in a sal (*Shorea robusta*) dominated lowland forest of Eastern Himalaya: floristic composition, regeneration and conservation. Curr Sci 81:776–786

Sharry S, Ponce JLC, Estrella LH, 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. Electron J Biotech 9:188–194

Shyamkumar B, Anjaneyulu C, Giri CC (2003) Multiple shoot induction from cotyledonary node explants of *Terminalia chebula* Retz. Biol Plant 47(4):585–588

Silveira V, Floh EIS, Handro W, Guerra MP (2004) Effect of plant growth regulators on the cellular growth and levels of intra cellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell Tissue Organ Cult 76:53–60

Simon H, Raharjo T, Litz RE (2007) Somatic embryogenesis and plant regeneration of litchi (*Litchi chinensis* Sonn.) from leaves of mature phase trees. Plant Cell Tissue Organ Cult 89:113–119

Steinmacher DA, Cangahuala-Inocent GC, Clement CR, Guerra MP (2007) Somatic embryogenesis from peach palm zygotic embryos. In Vitro Cell Dev Biol Plant 43:124–132

Suchalatha S, Shyamala Devi CS (2004) Protective effect of *Terminalia chebula* against experimental myocardial injury induced by isoproterenol. Ind J Exp Biol 42:174–178

Suguna L, Singh S, Sivakumar P, Sampath P, Chandrakasan G (2002) Influence of *Terminalia chebula* on dermal wound healing in rats. Phytotherapy Res 16:227–231

Valladares S, Sánchez C, Martinéz MT, Ballester A, Vieitez AM (2006) Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RAPD analysis. Plant Cell Rep 25:879–886

Vooková B, Kormuták A (2001) Effect of sucrose concentration, charcoal, and indole-3-butyric acid on conversion of *Abies numidica* somatic embryos. Biol Plant 44:181–184

Wu YJ, Huang XL, Chen QZ, Li XJ, Engelmann F (2007) Induction and cryopreservation of embryogenic cultures from nucelli and immature cotyledon cuts of mango (*Mangifera indica* L. var Zihua). Plant Cell Rep 26:161–168

Xing Z, Powell WA, Maynard CA (1999) Development and germination of *American chestnut* somatic embryos. Plant Cell Tissue Organ Cult 57:47–55

Xiao JN, Huang XL, Wu YJ, Li XJ, Zhou MD, Engelmann F (2004) Direct somatic embryogenesis induced from cotyledons of mango immature zygotic embryos. In Vitro Cell Dev Biol Plant 40:196–199

Yi ZC, Wang Z, LI HX, Liu MJ, Wu RC, Wang HX (2004) Effects of chebulinic acid on differentiation of human leukemia K562 cells. Acta Pharmacol Sin 25:231–238

Yu C, Chen Z, Lu L, Lin J (2000) Somatic embryogenesis and plant regeneration from litchi protoplasts isolated from embryonic suspensions. Plant Cell Tissue Organ Cult 61:51–58

Zhang CX, Li Q, Kong L (2007) Induction, development and maturation of somatic embryos in Bunge's pine (*Pinus bungeana* Zucc. ex Endl.). Plant Cell Tissue Organ Cult 91:273–280