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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (3): 127-134, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.127.134

An Improve Method for Somatic Embryogenesis of *Schisandra chinensis* (Turcz.) Baillon

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Abstract: A somatic embryogenesis protocol for plant regeneration of *Schisandra chinensis* (Turcz.) Baillon was established. Increased callus induction was obtained from mature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} *N*-phenyl-1,2,3-thidiazol-5-yl-urea (TDZ) or 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Addition of Zeatin (Zt) promoted the formation of embryogenic calli. To induce somatic embryogenesis, 2,4-D, TDZ and Zt were incorporated in the medium alone or in combination. Development of the maximum number of somatic embryos (81 globular, 37 heart, 52 torpedo and 37 cotyledon-stage) and germination of the highest number of embryos (50%) was observed on 1/2 MS medium supplemented with 1.0 mg L^{-1} TDZ and 0.2 mg L^{-1} Zt. Further development of somatic embryos into plantlets was completed in 1/2 MS medium free of plant growth regulators.

Key words: Zygotic embryo, embryogenic calli, somatic embryos, N-phenyl-1,2,3-thidiazol-5-yl-urea

INTRODUCTION

Schisandra chinensis (Turcz.) Baillon is a monoecious liana with a woody stem and grows wild in most eastern regions of Russia, the Kuril Islands, southern Sakhalin, northeastern China, Korea and Japan (Weinberg *et al*., 1999). As an important medicinal plant, it is increasingly cultivated in northeastern China. The fruits of *S. chinensis*, referred to as 'Wu-Wei-Zi' in traditional Chinese medicine, contain many dibenzo[*a,c*]cyclooctene derivatives, lignins and other compounds including tannins, essential oils, organic acids and vitamins C and E (Hikino and Kiso, 1988). Many lignans from *S. chinensis* have been demonstrated to exhibit potent antihepatotoxic, antioxidant (Liu and Xiao, 1994) and anticancer effects (Ohtaki *et al*., 1996).

Presently, propagation of *Schisandra chinensis* is primarily reliant on seed propagation. The germination of *S. chinensis* seeds requires cyclic stratification to break dormancy and the frequency of germination is low (Saunders, 2000). As an outcrossing plant, its desirable phenotypic traits are difficult to control and are not reliably inherited by offspring. Conventional vegetative propagation methods, including air layering, grafting and cutting, are not sufficiently rapid to meet the demand for elite cultivars. Plant regeneration via somatic embryogenesis is non-chimeric and offers greater genetic uniformity and clonal fidelity (Trolinder and Xhixian, 1989). A somatic embryogenesis system has extensive practical and commercial applications, particularly for *in vitro* clonal micropropagation (Bornman, 1993). Of special interest for agroforestry is the use of somatic embryos as synthetic seeds (Litz and Gray, 1995) and their upscaled production in bioreactors (Ohishi *et al*., 1994). However, somatic embryogenesis has other practical applications in agroforestry, including crop improvement (Maluszynski *et al*., 1995), germplasm preservation (Kartha, 1987), virus elimination (Goussard and Wiid, 1992), *in vitro* metabolite production (Paiva and Janick, 1983) and *in vitro* mycorrhizal initiation (Redenbaugh *et al*., 1986).

Plant regeneration via somatic embryogenesis has been reported in a variety of explants, plant genera and species, such as cotton (Price and Smith, 1979), North American oaks (Merkle and Nairn, 2005) and sorghum (Harshavardhan *et al*., 2002). Somatic embryogenesis of *S. chinensis* has been achieved from both mature seeds (Kim *et al*., 2005; Chen *et al*., 2010) and immature seeds. (Smiskova *et al*., 2005) reported that induction of embryogenic callus in *S. chinensis* occured over 1 year on WV5 medium and synchronization of the development of somatic embryos to the globular stage was significantly improved by addition of 7.9 mg L^{-1} abscisic acid (ABA) and 3% polyethyleneglycol, but ABA was harmful to

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plant morphology and resulted in a high percentage of malformed somatic embryos with multiple cotyledons (Smiskova *et al*., 2005). With regard to *S. chinensis* regeneration, several problems such as a prolonged culture period, low frequency of somatic embryo induction and high frequency of abnormal embryo development exist.

Previous investigators typically used 2,4-dichlorophenoxyacetlcacid (2,4-D) in combination with 6-benzyladenine (BA) and zeatin (Zt) to induce somatic embryogenesis (Fei *et al*., 2002; Zhang *et al*., 2001). N-phenyl-1,2,3-thidiazol-5-yl-urea (TDZ) is considered to be the most potent cytokinin currently available (Huetteman and Preece, 1993). TDZ has been used at concentrations of 0.1~2.0 mg L^{-1} to stimulate somatic embryogenesis in chickpeas (Murthy *et al*., 1995, 1996), white ash (Preece and Bates, 1990; Bates *et al*., 1992), eastern black walnut (Neuman *et al*., 1988, 1993), *Rubus* (Fiola *et al*., 1990) and *Vitis vinifera* (Matsuta and Hirabayashi, 1989). TDZ can stimulate the production of both somatic embryos and adventitious shoots in the same species (Fiola *et al*., 1990; Bates *et al*., 1992).

However, to date, no studies have investigated somatic embryogenesis of *S. chinensis* induced by TDZ and Zt. The purpose of the present study was to establish a protocol for high-efficiency induction and development of somatic embryos and conversion of embryos into normal plantlets within 5-6 months on Murashige and Skoog (MS) medium that contained TDZ and Zt.

MATERIALS AND METHODS

Callus induction of different explants: Mature *S. chinensis* seeds were collected from a local farm in Xinbin, Liaoning, China and treated by sand stratification at 4°C for 3 months. The seeds were washed in running tap water for 4 h and then soaked in Tween-20-containing distilled water for 10 min. Surface sterilization was carried out using 70% ethyl alcohol for 30 sec, followed by 0.1% mercury bichloride solution for 8 min and then rinsing three to four times with sterile distilled water. The seed coat was removed and the seeds were germinated on MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose. In order to select appropriate explants for callus induction, the cotyledons and hypocotyls from germinated seeds and zygotic embryos from mature seeds were excised and cultured on MS solid medium containing 3% (w/v) sucrose, 1.0 mg L^{-1} N^6-benzyladenine (BA) and 0.2 mg L^{-1} naphthaleneacetic acid. Cultures were incubated at 25±2°C under dark conditions for callus induction.

Induction of somatic embryo: Mature zygotic embryos from surface-sterilized seeds (described above) were isolated using forceps and a surgical scalpel under a stereomicroscope. Embryos were placed on the surface of MS basal medium supplemented with 3% (w/v) sucrose and different concentrations of 2,4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 or 11.0 mg L^{-1}) and TDZ (0.0, 0.1, 0.5, 1.0 or 2.0 mg L^{-1}). The medium pH was adjusted to 5.8 using 1 N NaOH or HCl before addition of 0.7% (w/v) agar. The media was sterilized at 121°C for 15 min. Cultures were incubated at 25±2°C under dark conditions for callus induction.

After 4 weeks in culture, callus tissues were transferred to embryogenic callus initiation medium comprised of full-strength MS salts and vitamins supplemented with 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} TDZ, either alone or in combination with Zt (0.1, 0.2 or 0.3 mg L^{-1}). The embryogenic calli were subcultured on the same medium every 2 weeks. Embryogenic calli were cultured under a 16/8 h light/dark photoperiod at a light intensity of 50 μmol $m^{-2}sec^{-1}$ provided by cool white fluorescent lamps at 25±2°C. For further embryo development and maturation, embryogenic calli were cultured under identical conditions, except that the medium contained 1/2 MS salts and vitamins and was supplemented with 3% sucrose.

Germination of somatic embryos: To promote further maturation and germination of somatic embryos and the development of plantlets, cotyledonary-stage embryos were separated and cultured on medium supplemented with 1/2 MS, 2.0 mg L^{-1} 2,4-D, 3% sucrose, 0.7% agar and 0.1, 0.2 or 0.3 mg L^{-1} Zt. Plantlets that developed from somatic embryos were transferred to 1/2 MS medium free of Plant Growth Regulators (PGRs). Cultures were maintained under a 16/8 h photoperiod at a light intensity of 50 μmol m^{-2} sec^{-1} provided by cool white fluorescent lamps at 25±2°C.

Experimental design and data analysis: Each treatment consisted of 10 Erlenmeyer flasks that contained five explants and the treatments were repeated three times. Data were analyzed using one-way ANOVA and the differences between treatment means were compared with Duncan's multiple range test at the 5% significance level using SPSS software (SPSS, Inc., Chicago, USA).

RESULTS AND DISCUSSION

Effects of explants on callus induction: The callus induction frequency differed significantly among the explant types used (Table 1). Zygotic embryos showed

the highest callus induction frequency (95%), followed by hypocotyls (88%) and cotyledons (78%). After 7 days in culture, zygotic embryos began to swell and form callus, whereas the formation of callus was observed after 14 and 20 days in hypocotyl and cotyledon cultures, respectively.

Chen *et al*. (2010) reported that the highest embryogenic callus induction frequency of 56.7% was obtained from shoot apical meristem-containing hypocotyl explants of *S. chinensis*. In our experiment, a better somatic embryogenesis system was established, with a higher embryonic callus induction rate (95%) than that report. Therefore, zygotic embryos of *S. chinensis* were considered to be the most effective materials for callus induction and were selected for embryogenic callus induction.

Table 1: Effects of explant type on callus induction from mature zygotic embryos and 1-month-old germinated seeds of *S. chinensis* after 40 days of culture

Explant	Callus induction frequency (%)	Time to induction (days)	Callus characteristics
Cotyledon	78.00±1.00^{c}	20	Green and hard
Hypocotyl	88.33±1.53^{b}	14	Reseda and looser
Zygotic embryo	95.33±1.53^{a}	7	Reseda and crisper

Different letters within a column indicate a significant difference ($p<0.05$) according to Duncan's multiple range test

Callus induction: Zygotic embryos (Fig. 1a) from mature seeds of *S. chinensis* produced callus on MS medium supplemented with different concentrations of 2,4-D and TDZ (Table 2). Zygotic embryos began to swell and form calli (Fig. 1b) within 7 days of culture on MS medium with 1.0 mg L^{-1} TDZ. Media supplemented with 1.0 mg L^{-1} TDZ showed the highest frequency of callus induction, followed by 2.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} TDZ. The callus obtained with all concentrations of 2,4-D and TDZ was green or light green.

Kim *et al*. (2005) reported that 2.0 mg L^{-1} 2,4-D alone induced greenish white and less friable callus with a low

Table 2: Effects of 2,4-D and TDZ on callus induction of *S. chinensis*

2,4-D (mg L^{-1})	TDZ (mg L^{-1})	Callus induction frequency (%)	Callus characteristics
-	-	-	-
0.5	-	62.00±1.00^{f}	Light green and loose
1.0	-	67.33±1.53^{e}	Green and loose
1.5	-	83.00±1.00^{c}	Green and crisp
2.0	-	91.67±0.58^{ab}	Green and crisp
2.5	-	83.67±1.53^{c}	Light green and loose
3.0	-	74.00±1.00^{d}	Green and hard
11.0	-	50.33±1.15^{g}	Green and solid
-	0.1	84.00±1.00^{c}	Green and loose
-	0.5	88.00±1.00^{b}	Green and crisp
-	1.0	94.67±1.53^{a}	Light green and loose
-	2.0	91.00±1.00^{ab}	Light green and crisp

Different letters within a column indicate a significant difference ($p<0.05$) according to Duncan's multiple range test

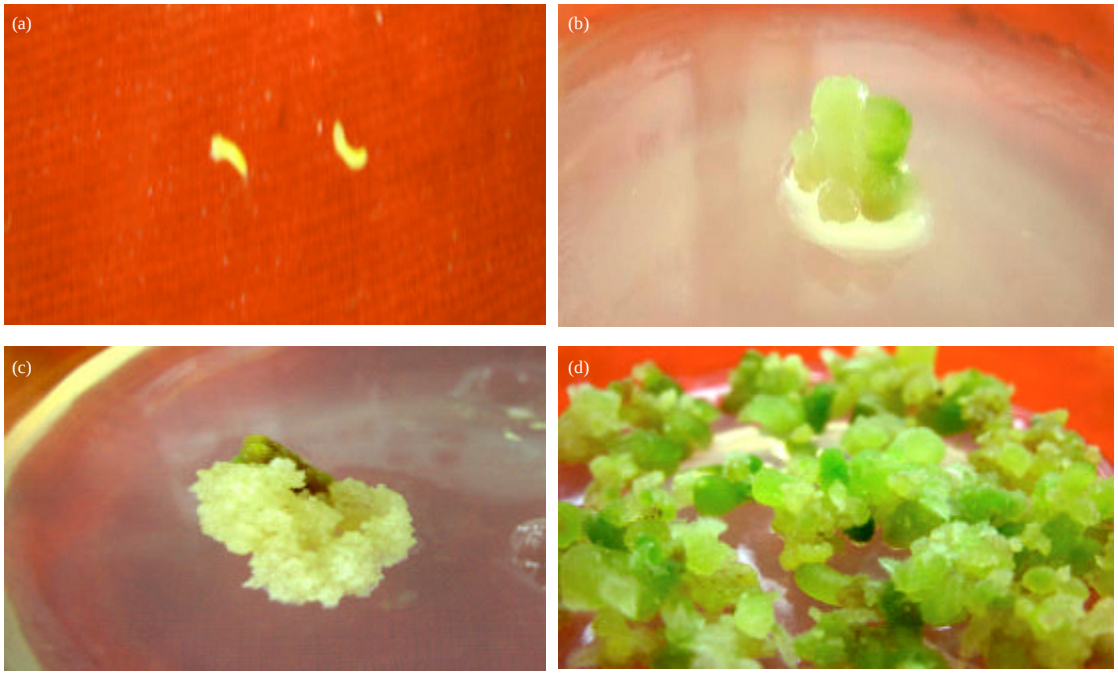

Fig. 1(a-d): Somatic embryo development from zygotic embryos of *S. chinensis*, (a) Zygotic embryo explants, (b) Callus from zygotic embryo cultured on callus induction medium for 4 weeks, (c) Actively growing embryogenic callus cultured on embryogenic callus induction medium for 4 weeks and (d) Somatic embryos cultured on somatic embryo induction medium at different stages of development after 5 weeks of culture

embryogenic nature and a 2,4-D concentration less than 2.0 mg L^{-1} induced yellowish white and less friable nonembryogenic callus, whereas a 2,4-D concentration that exceeded 2.0 mg L^{-1} induced brownish compact and nonembryogenic callus. These findings differed from our results and might reflect differences in the genotypes used and culture conditions, such as humidity and medium pH. Thidiazuron is a potent cytokinin for promotion of callus formation from woody explants, especially when used at ≥0.02 mg L^{-1} (Huetteman and Preece, 1993). Capelle *et al.* (1983) showed that *Phaseolus lunatus* L. callus became cytokinin autonomous when cultured on media containing a variety of concentrations of TDZ. In this study, either TDZ or 2,4-D were effective for callus induction.

Embryogenic callus induction: Of the different concentrations of 2,4-D and TDZ tested either alone or in combination with Zt, all PGRs were effective for induction of embryogenic callus formation (Fig. 1c), but with varied responses (Table 3). Medium supplemented with 1.0 mg L^{-1} TDZ in combination with 0.2 mg L^{-1} Zt induced the highest frequency of embryogenic callus formation (54.3%), followed by medium with 2.0 mg L^{-1} 2,4-D and 0.2 mg L^{-1} Zt (40.3%). Kim *et al.* (2005) reported that the maximum frequency of embryogenic callus induction (32%) from mature zygotic embryos of *S. chinensis* was obtained on MS medium that contained 2.0 mg L^{-1} 2,4-D and 0.02 mg L^{-1} Zt. Smiskova *et al.* (2005) reported that cream-colored embryogenic calli from immature zygotic embryos of *S. chinensis* developed on the basal regions of zygotic embryos (11.6%) on WV5 medium that contained 11 mg L^{-1} 2,4-D. In the present study, mature *S. chinensis* zygotic embryos cultured on MS medium that contained TDZ and Zt showed a markedly higher frequency of embryogenic callus induction. Thus, our results indicate that TDZ is a potent cytokinin for induction of somatic embryogenesis in *S. chinensis*.

Kim *et al.* (2005) reported that only globular somatic embryos developed from embryogenic callus on medium supplemented with 2,4-D 2.0 mg L^{-1} alone and that subsequent stages of somatic embryo development were arrested, whereas the embryogenic callus induced by 1.0 mg L^{-1} TDZ did not develop further. In the present study, either 2,4-D or TDZ in combination with ZT were effective for induction of embryogenic callus, which indicated that Zt could stimulate further development of somatic embryos in *S. chinensis*. In accordance with our results, Bonneau *et al.* (1994) and Das *et al.* (1997) reported that a combination of auxin and cytokinin are essential to induce somatic embryogenesis in *Euonymus europaeus* and *Dalbergia sissoo*, respectively.

Somatic embryo induction: Embryogenic callus developed into somatic embryos after transfer to 1/2 MS medium supplemented with different combinations of PGRs and culture for 5 weeks (Table 3). In the control group, embryogenic calli and somatic embryos did not develop further. Of the different concentrations of PGRs tested, higher numbers of globular (Fig. 2a), heart-shaped (Fig. 2b), torpedo-shaped (Fig. 2c) and cotyledonary somatic embryos (Fig. 2d) were observed on 1/2 MS medium that contained 1.0 mg L^{-1} TDZ in combination with 0.2 mg L^{-1} Zt. In the presence of 1.0 mg L^{-1} TDZ and 0.2 mg L^{-1} Zt, each gram of embryogenic calli yielded 81 globular somatic embryos. These globular embryos developed into heart-stage embryos (73±2.08) and these embryos further developed into torpedo-stage embryos (52±3.79) and finally into cotyledon-stage embryos (37±1.52). A significantly lower number of somatic embryos developed from the embryogenic callus that formed on medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.2 mg L^{-1} Zt and an average of 70 globular somatic embryos were produced per gram of embryogenic callus, but only an average of 30.0 embryos progressed to the cotyledonary stage. Increasing or decreasing concentrations of TDZ and 2,4-D resulted in a decline in the number of embryos per gram of embryogenic calli.

Kim *et al.* (2005) reported that each gram of embryogenic callus gave rise to an average of

Table 3: Effects of treatment with plant growth regulators (PGRs) on the number of somatic embryos that developed from zygotic embryos of *S. chinensis* on somatic embryo induction medium after 5 weeks of culture

PGRs (mg L^{-1})				Mean No. of somatic embryos per gram embryogenic callus			
2,4-D	TDZ	Zt	Zygotic embryos forming embryogenic callus (%)	Globular stage	Heart stage	Torpedo stage	Cotyledon stage
0.0	-	-	-	-	-	-	-
2.0	-	-	4.00±1.00^{f}	10.00±2.00^{f}	-	-	-
2.0	-	0.1	11.67±2.08^{e}	58.67±1.53^{c}	38.33±1.53^{d}	19.00±2.00^{e}	10.33±1.53^{e}
2.0	-	0.2	40.30±1.53^{b}	70.00±1.00^{b}	63.33±2.08^{b}	41.33±2.08^{b}	30.00±1.00^{b}
2.0	-	0.3	6.67±2.08^{d}	35.30±1.53^{e}	30.00±1.00^{e}	18.30±2.08^{e}	10.00±2.65^{e}
-	1.0	-	1.70±0.58^{g}	-	-	-	-
-	1.0	0.1	14.00±2.00^{e}	60.70±1.53^{c}	49.00±2.00^{c}	36.00±1.00^{c}	22.67±2.52^{c}
-	1.0	0.2	54.30±4.16^{a}	81.67±2.08^{a}	73.67±1.53^{a}	52.67±3.79^{a}	37.70±1.52^{a}
-	1.0	0.3	8.70±2.08^{d}	42.70±2.08^{d}	38.30±2.08^{d}	29.30±2.08^{d}	18.67±1.52^{d}

Different letters within a column indicate a significant difference ($p<0.05$) according to Duncan's multiple range test

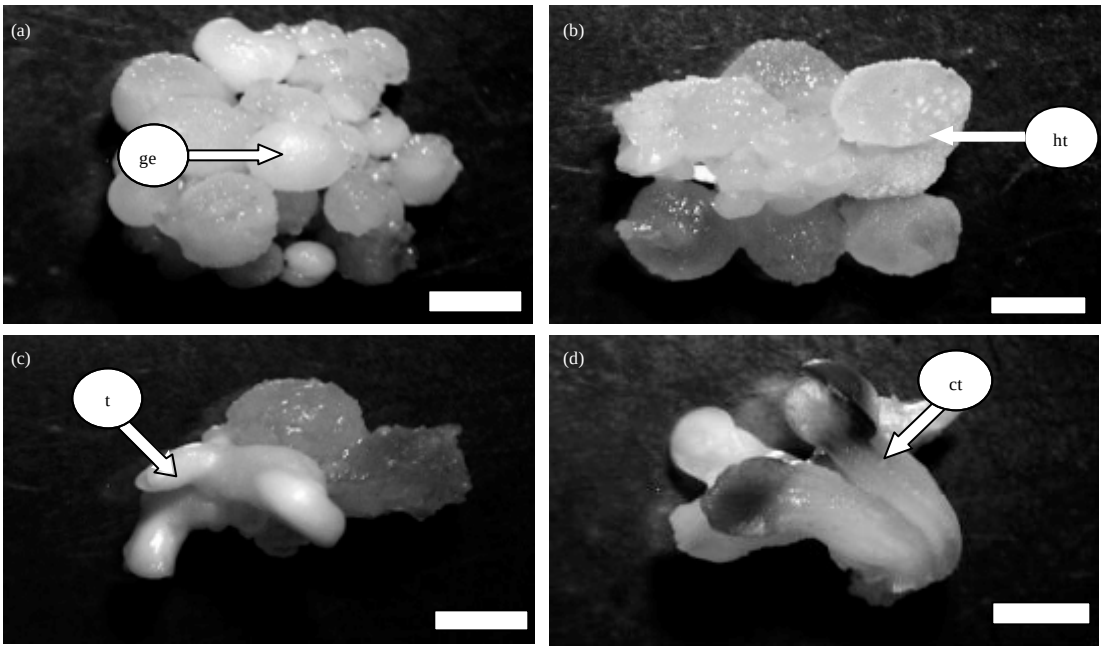

Fig. 2(a-d): Different stages of somatic embryogenesis from zygotic embryos of *S. chinensis*, (a) Globular embryos (ge) (bar = 1 cm), (b) Heart-shaped embryos (ht) (bar = 1cm), (c) Torpedo-shaped embryos (t) (bar = 1 cm) and (d) Cotyledonary embryos (ct) (bar = 1 cm)

61.7 globular somatic embryos, but only an average of 20.0 embryos progressed to the torpedo-shaped stage on MS medium free of PGRs. Our findings differ from those of Kim *et al*. (2005), which suggests that a suitable concentration of TDZ or 2,4-D in combination with Zt promotes somatic embryo development in *S. chinensis*. TDZ promotes somatic embryogenesis more effectively compared to BA in rice and *Bambusa edulis* (Gairi and Rashid, 2004; Lin *et al*., 2004). In addition, TDZ activity is much stronger than that of KT in somatic embryo induction in *Cayratia japonica* (Zhou *et al*., 1994). In the present study, application of TDZ alone did not induce development of somatic embryos. However, combination of Zt and TDZ markedly increased the frequency of somatic embryogenesis in *S. chinensis*. Therefore, Zt promoted somatic embryo development under low salt and vitamin conditions.

Somatic embryo germination: Cotyledonary somatic embryos germinated on medium supplemented with 2,4-D or TDZ in combination with Zt. After 1 week, the somatic embryos swelled and the cotyledons turned green. The cotyledons gradually increased in size and the hypocotyls elongated and developed white roots after 2 weeks. The roots elongated for approximately 4 weeks after which the roots were 0.5-1 cm in length (Fig. 3a-b). Maximum germination (50%) occurred on 1/2 MS medium that contained TDZ 1.0 mg L^{-1} and Zt 0.2 mg L^{-1} (Fig. 4).

Some previous studies reported that somatic embryos germinated on medium that lacked PGRs. In larch (*Larix×leptoeuropaea*), somatic embryos germinated on monosodium glutamate medium that lacked PGRs (Lelu *et al*., 1994). In *Areca catechu*, somatic embryos developed into plantlets from zygotic embryos after 10 weeks of culture on a medium free of PGRs (Wang *et al*., 2003). *S. chinensis* differed from these species in that somatic embryos germinated on MS medium that contained 2,4-D or TDZ in combination with Zt in the present study.

A protocol for the induction, maturation and germination of somatic embryos from zygotic embryos of *S. chinensis* is summarized in Fig. 5. Starting with mature zygotic embryos as the explant source, *S. chinensis* plantlets can be obtained within 21 weeks of culture (Kim *et al*., 2005). The procedure outlined in Fig. 5 is considerably faster and thus cheaper than traditional methods of culture. Furthermore, the shorter culture duration may be associated with a reduced occurrence of somaclonal variation.

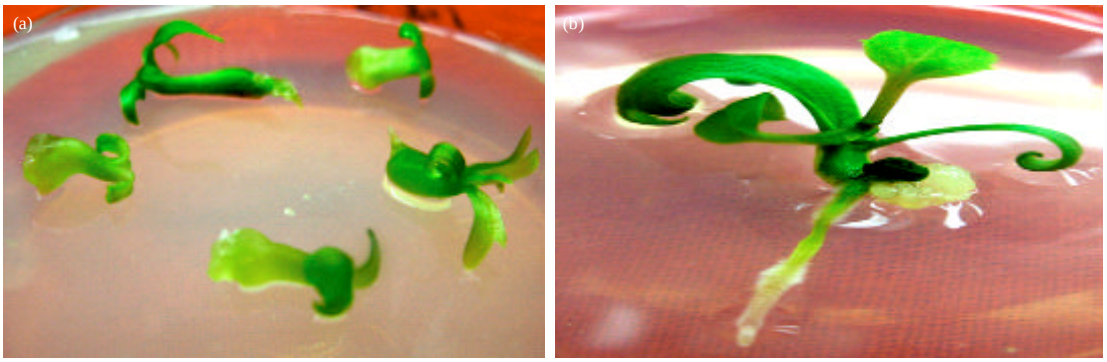

Fig. 3(a-b): Somatic embryo germination, (a) Germination and (b) Plantlet regeneration from *S. chinensis* somatic embryos

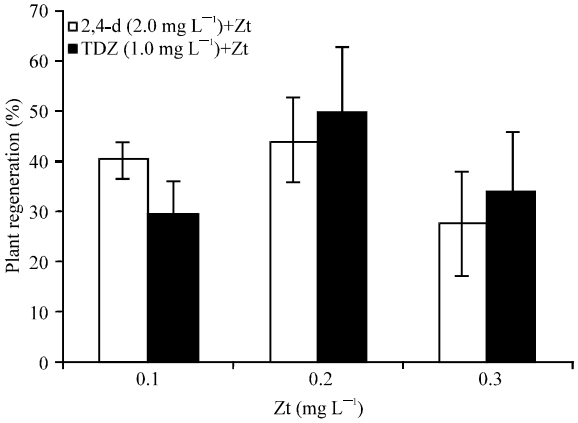

Fig. 4: Germination frequency of *S. chinensis* somatic embryos on germination medium after 4 weeks of culture

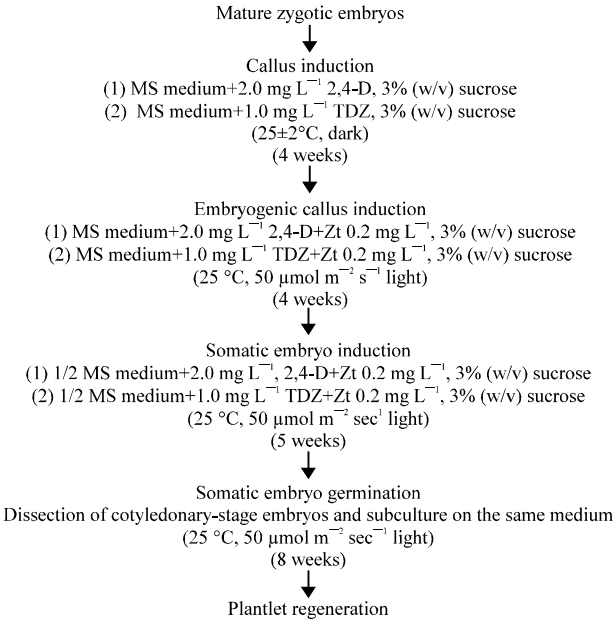

Fig. 5: Protocol for the induction, maturation and germination of somatic embryos from mature zygotic embryos of *S. chinensis*

In conclusion, our procedure resulted in an appreciable reduction in duration to plantlet development, a higher frequency of embryo formation and a lower incidence of abnormal embryos during maturation of somatic embryos. The efficient somatic embryogenesis system developed in the present study will provide a platform for regeneration of a large number of homogeneous plants in genetic transformation of *S. chinensis*.

ACKNOWLEDGEMENT

We thank the Special Fund for Agri-scientific Research in the Public Interest (201103037) for financial support of our research program.

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