Efficient Somatic Embryogenesis and Plant Regeneration from Immature Embryos of *Tapiscia sinensis* Oliv., an Endemic and Endangered Species in China

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Abstract. High-frequency somatic embryogenesis and plant regeneration were achieved from immature cotyledonary-stage embryos in the endangered plant, *Tapiscia sinensis* Oliv. Plant growth regulators with different concentrations and combinations on embryogenesis capacity were studied. The optimal explants for in vitro somatic embryogenesis were immature embryos in *T. sinensis*. A high callus induction rate of 100% was achieved on Murashige and Skoog (MS) basal medium supplemented with 1.0 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5% (w/v) activated charcoal. Alternatively, a high induction rate (96.16%) of somatic embryogenesis was obtained on MS basal medium supplemented with the combination of 0.05 mg·L⁻¹ α -naphthaleneacetic acid (NAA) and 0.2 mg·L⁻¹ 6-benzylaminopurine (6-BA), and somatic embryos proliferated fastest on the mentioned medium supplemented with 0.5% (w/v) activated charcoal and 3% (w/v) sucrose, inoculation of explants proliferating 21 times in the 23-day subculture. Of the 100 plantlets transferred to field after the acclimation, 95 (95%) survived. Based on the histocytological observations, the development of somatic embryos was similar to that of zygotic embryos. There were two accumulation peaks of starch grains in the embryogenic calli and in the globular-stage embryos, both closely related to the energy supply, and the embryoids were of multicellular origin.

Tapiscia sinensis Oliv., one of the rare species in China, belongs to the genus *Staphylea*, family Staphyleaceae. It has not only significant scientific value for the investigation of the origin of the semitropical flora of China, and the phylogenesis of the family Staphyleaceae for its characteristics of the ancient origin, but also a promising ornamental species because of its beautiful flowers and tree form. It is also an excellent plant in afforestation because of its rapid growth habit. This species has a very narrow distribution range in southwestern China and regenerate natural community by seedling. However, natural regeneration capacity of the species is weak because of its low seed setting rate and poor seedling viability under natural conditions (Sun and Liu, 2004). The species takes 17 months to complete the sexual reproductive cycle from zygote initiation to

fruit maturation (Liu et al., 2008). Moreover, it has been subject to tremendous human disturbance and thus has become an endangered species. Considering its rarity and important value, it has been listed in the Chinese Plants Red Cover Book as a priority plant to promote its conservation in China (Fu, 1992). Some researches were also performed on seedling and the process of seed germination of *Tapiscia sinensis* (Han, 2010; Zhou and Duan, 2008). However, no efficient in vitro plant regeneration system of *T. sinensis* has been reported. Hence, there is a demand for the development of a conservation strategy through micro-propagation to prevent extinction. Somatic embryogenesis is an alternative method for mass propagation and production of synthetic seeds (Cheruvathur et al., 2013; Levin et al., 1988; Tejavathi et al., 2007). Therefore, the aim of the present investigation was to establish a reproducible, affordable, and efficient in vitro plant regeneration protocol from immature embryos of *T. sinensis* through somatic embryogenesis.

Materials and Methods

Materials and culture conditions. Immature seeds of *T. sinensis* were collected in late May 2008 from the Shennongjia Nature

Reserve in Hubei Province, China (Seed of the species mature in September in Hubei Province.). The immature seeds were washed in $\approx$ 250 mL water containing two drops of Tween-20 and then surface-disinfected in 75% (v/v) ethanol for 1 min. They were soaked in 0.1% (w/v) HgCl₂ solution plus two drops of Tween-20 per 100 mL for 10 min followed by washing five times with sterile water and soaked in sterile water overnight at 4 °C to soften the seedcoats. The hard seedcoats were removed mechanically. The decoated seeds were rinsed five times with sterile water then disinfected with 0.1% (w/v) HgCl₂ solution for 5 min and rinsed with sterile water five times, and then the immature embryo of the decoated seeds was placed on the culture medium. The cultures were incubated at 25 $\pm$ 2 °C under a 16/8-h (light/dark) photoperiod with illumination by white fluorescent light with an intensity of 50 μ mol·m⁻²·s⁻¹.

Callus induction. The immature embryo explants were cultured on MS basal medium supplemented with 2,4-D and in combination with activated charcoal to induce embryogenic calli. Different concentrations of 2,4-D (0.1, 0.5, 1.0, and 2.0 mg·L⁻¹) and activated charcoal (0%, 0.05%, 0.1%, 0.5%, and 1.0% w/v) were tested for their effect on the initial callus induction. The rate of callus induction was measured after 20 d. Eight petri dishes for each treatment combination were prepared. On average, 30 explants were cultured to each petri dish, and each treatment was replicated three times.

Differentiation and induction of somatic embryos. Calli, induced from explants after cultured for 20 d, were transferred to new MS culture medium with 6-BA (0.1, 0.2, 0.5, and 1.0 mg·L⁻¹) and NAA (0, 0.01, 0.05, and 0.1 mg·L⁻¹) and different concentrations of sucrose (0.00%, 1.50%, 3.00%, 6.00%, and 9.00%) to develop somatic embryogenesis. Each treatment consisted of five petri dishes and 20 calli were inoculated to each petri dish, and all treatments were repeated three times. After 19-d culture, somatic embryos emerged and the embryogenesis rate was counted. Then, the somatic embryos were cultured on the growth regulator-free MS culture medium to allow the development of plantlets. The induction frequency of somatic embryogenesis was calculated as follows: the number of explants that induced somatic embryos divided by the total number of explants.

The growth of embryogenic callus was also evaluated. After weighing, the calli was cultured on embryo induction medium (which was screened out from the previous step) supplemented with different concentrations of glucose, and the inoculum was weighted again after 23-d culture. Multiplication rate was counted and presented as the mean $\pm$ SD. Each treatment consisted of five petri dishes and 0.85 to 0.90 g calli were inoculated to each petri dish, and all experiments were repeated three times.

The basal culture medium used for somatic embryogenesis induction was MS medium (Murashige and Skoog, 1962) supplemented

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with 3% (w/v) sucrose and solidified by 0.8% (w/v) agar (National Pharmaceutical Group Corporation, Shanghai, China). The medium was adjusted to pH 6.0 before autoclaving at 0.11 MPa and 121 °C for 20 min.

Histology. Embryogenic tissues were collected during embryonic development of somatic embryos and were fixed in FAA solution (formalin:acetic acid:absolute ethanol:distilled water of 5:5:45:45, v/v/v/v) for 24 h at room temperature for fixation, then dehydrated in a graded ethanol series (70%, 85%, 95%, and 100%, v/v) and embedded in paraffin. Sections were cut 6 to 8 µm thin with a rotary microtome, mounted onto glass slides, and then stained with Periodic acid-Schiff. At the end, they were observed and photographed with a photomicroscope (Nikon 80i, Japan).

Statistical analysis. The induction percentages were transformed into arcsine values before analysis, and then data were analyzed by analysis of variance to detect significant differences between means using SPSS V11.5 software (IBM, Chicago, IL). Means differing significantly were compared using Duncan's multiple range test at the 5% probability level. Variability around the mean was represented as $\pm$ SD.

Results

Callus induction. Explants of immature embryos, cultured on the growth regulator-free culture medium or the medium containing 0.1 mg·L⁻¹ 2, 4-D, largely formed seedlings rather than calli. Calli were induced from the explants of immature embryos on MS medium with 2,4-D (0.5, 1.0, 2.0 mg·L⁻¹). On the second day after inoculation, the folded cotyledons of immature embryos enlarged and their hypocotyls expanded. The expansion continued until white calli appeared on the seventh day (Fig. 1A). The calli turned from white to black after 10 d (Fig. 1B). These black calli were fragile and grew slowly. The induction frequency of calli varied with changes in the 2,4-D concentration. There were statistically significant differences among those experimental treatments; the treatment with 2,4-D (1.0 mg·L⁻¹) resulted in the highest induction rate, up to 64.67% (Table 1). However, there were no large differences in morphology and mass growth of calli formed on different culture media.

Explants of immature embryos were cultured on MS basal medium supplemented with 2,4-D (1.0 mg·L⁻¹) and different concentrations of activated charcoal (0.05%, 0.1%, 0.5%, and 1.0%). After 20 d of culture, the results revealed that activated charcoal had a marked effect on callus induction. The 0.5% activated charcoal sharply increased the induction rate to almost 100% (Table 2), whereas it was only 64.67% without activated charcoal.

Somatic embryogenesis. The calli developed for 20 d from the explants of immature embryos were subcultured on MS basal medium supplemented with 2,4-D. It was found that their color turned to black and

Fig. 1. Various calli from immature embryos of *Tapiscia sinensis*. (A) Callus from immature embryos. (B) Callus turned from white to black ($\times 8$).

Table 1. Effect of 2,4-D concentrations on callus induction in *Tapiscia sinensis*.^z

Concentrations of 2,4-D (mg·L ⁻¹)	Induction frequency of callus (%)	Transformation the percentages in arcsin values
0.00	0.00	0.00 $\pm$ 0.00
0.10	0.00	0.00 $\pm$ 0.00
0.50	33.34	0.34 $\pm$ 0.03 c
1.00	64.67	0.70 $\pm$ 0.02 a
2.00	50.20	0.53 $\pm$ 0.01 b

^zExplants of immature embryos were cultured on Murashige and Skoog basal medium supplemented with the different concentrations of 2,4-D. Data generated after 20 d in culture and were presented as the mean $\pm$ SD. Different letters indicated that the values were significantly different at $P < 0.05$ according to Duncan's multiple range test.

2,4-D = 2,4-dichlorophenoxyacetic acid.

Table 2. Effect of active charcoal on callus induction from explants of immature embryos of *Tapiscia sinensis*.^z

Concn of 2,4-D (mg·L ⁻¹)	Concentrations of active charcoal (%)	Induction frequency of callus (%)	Transformation the percentages in arcsin values
1.00	0.05	61.29	0.67 $\pm$ 0.04 d
1.00	0.10	83.52	0.99 $\pm$ 0.03 c
1.00	0.50	100.00	1.57 $\pm$ 0.00 a
1.00	1.00	91.88	1.17 $\pm$ 0.08 b

^zExplants of immature embryos were cultured on Murashige and Skoog basal medium supplemented with the different concentrations of active charcoal. Data generated after 20 d in culture and were presented as the mean $\pm$ SD. Different letters indicated that the values were significantly different at $P < 0.05$ according to Duncan's multiple range test.

2,4-D = 2,4-dichlorophenoxyacetic acid.

their growth progressively stopped; there was not somatic embryogenesis after 40 d culture. However, the calli that had grown for 20 d were transferred to MS basal medium with different combinations of 6-BA and NAA, their color turned black, but after a growth pause of ≈ 13 d, yellow somatic embryos emerged on the surface of the callus (Fig. 2A). After 19 d, globular-, heart-, torpedo-, and cotyledonary-stage embryos appeared (Fig. 2B–E). After 32 d growth, plantlets regenerated from these somatic embryos (Fig. 2F).

The effect of different combinations of 6-BA and NAA on embryogenesis in *T. sinensis* is shown in Table 3. When 6-BA was maintained at 0.2 mg·L⁻¹, the embryogenesis rate rose first and then declined along with the NAA within a certain concentration range. The embryogenesis rate rose to 96.16% on the MS medium with 6-BA (0.2 mg·L⁻¹) and NAA (0.05 mg·L⁻¹). After subculture, somatic embryos grew quickly with a normal morphology. When only 6-BA was added

to the medium, the embryogenesis rate was sharply reduced.

For callus multiplication, calli were transferred to solid MS basal medium supplemented with 6-BA (0.2 mg·L⁻¹), NAA (0.05 mg·L⁻¹), activated charcoal (0.5% w/v), and different concentrations of sucrose. The results showed the sucrose had a significant impact on callus proliferation according to the observation after growing for 23 d. At a concentration of 3% (w/v) sucrose, a large number of embryos developed and the mass growth increased by 21 times in the 23-d subculture (Table 4). It was noted that embryos could not be differentiated on the sucrose-free culture medium, whereas higher concentrations were unfavorable for embryogenesis in *T. sinensis*. For example, on a medium with 9% (w/v) sucrose, calli failed to differentiate.

Once embryos reached the cotyledonary stage, they were transferred to a growth regulator-free medium for 4 weeks to allow them to further develop into plantlets. After

Fig. 2. Generation of somatic embryos and plant regeneration of *Tapiscia sinensis*. (A) Yellow embryogenic callus emerged from black callus. (B) Globular-stage embryos ($\times 63$). (C) Heart-stage embryos ($\times 32$). (D) Torpedo-stage embryos ($\times 32$). (E) Cotyledonary-stage embryo ($\times 32$). (F) Plants from somatic embryos.

Table 3. Effect of different combinations of growth regulators on somatic embryogenesis in *Tapiscia sinensis*.^a

Concn of growth regulators (mg·L ⁻¹)		Frequency of somatic embryogenesis (%)	Transformation the percentages in arcsin values
6-BA	NAA		
0.10	0.00	16.51	0.17 $\pm$ 0.18 g
0.10	0.01	46.46	0.48 $\pm$ 0.09 f
0.10	0.05	49.54	0.52 $\pm$ 0.14 f
0.10	0.10	40.66	0.42 $\pm$ 0.11 f
0.10	0.50	45.37	0.47 $\pm$ 0.07 f
0.20	0.00	19.33	0.19 $\pm$ 0.04 g
0.20	0.01	70.60	0.78 $\pm$ 0.05 de
0.20	0.05	96.16	1.34 $\pm$ 0.18 a
0.20	0.10	89.08	1.10 $\pm$ 0.03 b
0.20	0.50	81.17	0.95 $\pm$ 0.06 bcd
0.50	0.00	17.33	0.17 $\pm$ 0.03 g
0.50	0.01	64.12	0.70 $\pm$ 0.03 e
0.50	0.05	85.44	1.03 $\pm$ 0.04 b
0.50	0.10	82.48	0.97 $\pm$ 0.04 bc
0.50	0.50	66.83	0.73 $\pm$ 0.07 e
1.00	0.00	18.75	0.19 $\pm$ 0.03 g
1.00	0.01	73.83	0.83 $\pm$ 0.05 cde
1.00	0.05	84.57	1.02 $\pm$ 0.17 b
1.00	0.10	70.67	0.79 $\pm$ 0.05 de
1.00	0.50	81.30	0.96 $\pm$ 0.16 bcd

^aBlack calli were subcultured on Murashige and Skoog basal medium supplemented with the different combination of plant growth regulators. Data generated after 19 d in culture and were presented as the mean $\pm$ SD. Different letters indicated that the values were significantly different at $P < 0.05$ according to Duncan's multiple range test.

6-BA = 6-benzylaminopurine; NAA = naphthaleneacetic acid.

growing to a height of 10 cm, the plantlets were removed from the culture flask and carefully washed in tap water to remove the traces of agar. These plantlets were planted in plastic cups in the greenhouse at $25 \pm 2^\circ\text{C}$ (relative humidity 60%). When the plantlets grew to a height of 20 cm for 4 weeks, the seedlings were transferred to the field. Among the 100 seedlings developed from somatic embryos transferred to the field, 95 survived.

Histological observation. Cytohistological observations showed that the proembryos originated in two different ways in somatic embryogenesis of *T. sinensis*, one of which developed directly from the surface layer of explants of the immature embryos. There were many embryonic tissue protuberances (the red arrow labeling) under the epidermis (Fig. 3A). At the same time, some cells at the sub-epidermis divided and formed embryonic

callus tissue after 5 d of culture with a large amount of starch grain (Fig. 3A). In another case, proembryos (the red arrow labeling) initiated from the surface layer or internally from the embryonic calli, which had abundant starch granules (Fig. 3B–C). Somatic embryos could not be initiated from the division of one single cell but were able to develop from a group of meristematic cells (Fig. 3D, the red arrow labeling).

Embryonic cells were always smaller in size than those of the parenchymatous cells with bigger nuclear and thicker plasma, some of which contained an abundance of starch grains. Given the morphological development of somatic embryos, which emerged from proembryos through globular-stage embryos, heart-stage embryos, and torpedo-stage embryos, they finally developed into cotyledonary-stage embryos with dramatic changes in synthesis ability and distribution of starch grains. At the early stages of embryonic calli, starch grains in cells began to accumulate. In some globular-stage embryos, all cells were full of starch grains (Fig. 3E, dye spot). However, this occurred only in cells of the surface layers rather than that of the entire body (Fig. 3F). At the later stage of globular-stage embryos, starch grains (the red arrow labeling dye spot) had a polarized distribution in the embryo bodies (Fig. 3G). The ability to synthesize starch grains sharply decreased from the heart-stage embryos. At the period of torpedo-stage embryos and the early phase of the cotyledonary-stage embryos, starch grains only occurred in some cells of the peripheral region and provascular tissues (Fig. 3H–K, dye spot). Some secondary embryos (the red arrow labeling) formed on the surface of the heart-stage embryos (Fig. 3L).

Discussion

Compared with mature embryos, immature embryos could induce callus and differentiate into somatic embryos. This result indicated that the type and physiological status of the explants were important factors affecting somatic embryogenesis in *T. sinensis*. Jain et al. (1995) also reported some difficulties in induction of calli from mature tissue of *Picea abies*, whereas juvenile tissues seemed more suitable. Other results indicated that zygotic embryos of cashew nut (*Anacardium occidentale* L.) at different developmental stages showed different potential for somatic embryogenesis (Gogate and Nadgauda, 2003). In further research, the relationship between the physiological–biochemical status of zygotic

embryos and the induction of somatic embryos

should be investigated.

The role of 2,4-D in the induction of somatic embryos in the various systems is well established (George and Sherrington, 1984; Guo and Zhang, 2005). In our previous experiments it was also found that 2,4-D was an important plant growth regulator for the induction of somatic embryos. Somatic embryos could not be induced on media containing only NAA or indole-3-acetic acid, which was consistent with a number of previous reports in other species (Eapen and George, 1993; Gu et al., 2004; Guo and Zhang, 2005). The induction of somatic embryos in some species could only be achieved on a medium containing 2,4-D (Pareek and Kothari, 2003). However, 2,4-D would antagonize development and regeneration of somatic

embryos (Mao et al., 2012; Murashige, 1974). In our investigation, somatic embryos could be effectively induced on the medium with 2,4-D, but higher concentrations of 2,4-D would inhibit growth of the embryo, which also appeared in other species (Bai et al., 1998), and the growth of embryogenic callus progressively stopped after 13 d in culture. In *Agave victoriae-reginae* and *A. vera-cruz*, the presence of 2,4-D was required in the medium for induction of somatic embryoids, but for their germination and development, a cytokinin was essential in place of 2,4-D (Martínez-Palacios et al., 2003; Tejavathi et al., 2007). Therefore, like in previous studies, a two-step culture was required for the completion of somatic embryogenesis in the present investigation. Although the presence of 2,4-D in the medium induced the embryogenic potential of the calli, cytokinin was required for the development of the somatic embryos in the subculture medium. In the present study, 6-BA was used to promote somatic embryogenesis. The embryogenesis rate rose to 96.16% on the conversion medium with 6-BA (0.2 mg·L⁻¹) combined with NAA (0.05 mg·L⁻¹).

Activated charcoal played a key role in embryogenesis of *T. sinensis* because it was capable of absorbing hazardous substances such as phenols, thus significantly increasing the induction rate and effectively preventing browning and stimulating the growth of the calli in *Torreya grandis* Fort. and *Citrus*

Table 4. Effect of sucrose concentrations on somatic embryogenesis in *Tapiscia sinensis*.^a

Concn of sucrose (%)	Inoculating volume (g)	Growth volume (g)	Multiplication rate
0.00	0.87 ± 0.06 a	1.07 ± 0.05 d	1.24 ± 0.13 d
1.50	0.88 ± 0.06 a	3.24 ± 0.21 cd	3.66 ± 0.16 c
3.00	0.88 ± 0.05 a	18.68 ± 0.49 a	21.33 ± 0.88 a
6.00	0.88 ± 0.15 a	4.17 ± 0.09 b	4.72 ± 0.18 b
9.00	0.89 ± 0.03 a	3.71 ± 0.09 c	4.19 ± 0.22 bc

^aBlack calli were subcultured on Murashige and Skoog basal medium supplemented with 6-BA (0.2 mg·L⁻¹), NAA (0.05 mg·L⁻¹), active charcoal (0.5%), and the different concentrations of sucrose. Different letters indicated that the values were significantly different at *P* < 0.05 according to Duncan's multiple range test.

6-BA = 6-benzylaminopurine; NAA = naphthaleneacetic acid.

Fig. 3. Histological observation of somatic embryo development in *Tapiscia sinensis*. (A) Multicellular proembryos originated directly on the surface of explants of immature embryos (bar = 500 µm). (B) Multicellular proembryos formed on the surface of embryonic calli (bar = 200 µm). (C) Proembryos stemmed from internal embryonic calli (bar = 200 µm). (D) Multicellular proembryos inside embryonic calli (bar = 200 µm). (E–F) Globular-stage embryos full of starch (bar = 200 µm). (G) Globular-stage embryo with a polarized distribution of starch (bar = 200 µm). (H) Heart-stage embryo (bar = 500 µm). (I) Torpedo-stage embryo (bar = 200 µm). (J) Cotyledonary-stage embryo with two cotyledons (bar = 200 µm). (K) Cotyledonary-stage embryo with four cotyledons (bar = 500 µm). (L) Secondary embryos developed from a heart-stage embryo (bar = 200 µm).

sinensis Osbeck. (Jiang and Chen, 2004; Liu et al., 2004). Other research also showed some relation between activated charcoal and embryos, which were induced (Agarwal et al., 2004).

Sucrose was used as a carbon source during the process of embryo growth and could also regulate osmotic potential (Park and Ahn, 2005; Wang et al., 2004). Concentrations of sucrose imposed significant effects on differentiation and proliferation of somatic embryos. The fastest differentiation and proliferation rate appeared with a culture medium containing 3% (w/v) sucrose. Both higher concentrations (9% w/v) and insufficient (0% or 1.5% w/v) concentrations were not suitable for inducing somatic embryos. However, Lee et al.'s (2001) report showed that low concentrations (0% and 1.5% w/v) of sucrose showed a greater advantage in inducing somatic embryos of *Daucus carota*. However, Thomas' (2006) result showed that somatic embryos of *Tylophora indica* occurred more frequently under 200 mmol·L⁻¹ sucrose concentration (6.8%). Park and Ahn (2005) also found that higher sucrose concentrations (60 g·L⁻¹) were of benefit to embryogenesis in *Eleutherococcus koreanum*. Thus, we speculate that there are some differences in requirement of sugar concentration in different species' somatic embryogenesis.

Cytological observations illustrated that somatic embryos initiated from the surface layer of immature embryos. Division of epidermic cells of ground tissues at first formed calli, which appeared in various shapes with a large amount of starch granules, and then developed into somatic embryos. Somatic embryos could not be initiated from the division fission of one cell but only from a group of meristematic cells, which was in conformity with the report of Martínez-Palacios et al. (2003). However, others stated that embryoids arose from a single cell (Tejavathi et al., 2000; Wang et al., 1990). Michaux-Ferrière et al. (1992) thought that the culture conditions favor uni- or multicellular modes of embryogenesis in callus with embryogenic potential. As mentioned, the origin of somatic embryos is still highly debated and needs further studies. At the same time, during the growth of somatic embryos, the peak of the starch granule disposition appeared at the embryonic callus and globular embryo period; this phenomenon may be an adaption dictated by the demand for energy during early phases of embryogenesis (Lin et al., 2000; Liu et al., 2004).

Conclusion

In vitro somatic embryo induction in *T. sinensis* was developed, which is a viable

method for rapid propagation of this plant. The high somatic embryo induction and transplant survival rates suggest that this reproducible, affordable, and efficient in vitro plant regeneration protocol can be used for the conservation of this endemic and endangered plant.

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