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Somatic Embryogenesis and Plant Regeneration from Embryogenic cell Suspension Cultures of *Schisandra chinensis* Baill

Cheng Hao LI*, YudA Niu*, Bo Zhao*, Bimal Kumar Ghimire, Hyun Young Kil*, Kwon Heo**, Myong Jo Kim**, Seok Hyun Eom**, Dong Ha Cho**, Chang Yeon Yu**†**

**Key Laboratory of Forest Genetics and Tree Breeding, Northeast Forestry University, Harbin 150040, China*
***BioHerb Research Institute, Kangwon National University, Chunchon 200-701, Korea.*

ABSTRACT : An efficient somatic embryogenesis and plant regeneration protocol was developed for *Schisandra chinensis* Baill, using embryogenic cell suspensions and optimized media conditions. Friable embryogenic callus was induced from cotyledonary leaf and hypocotyl explants of 7 days old seedlings on MS agar medium supplemented with 1.0 to 4.0 ㎎ l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). Fast growing and well dispersed embryogenic cell suspensions were developed within two months when embryogenic calli were transferred to MS liquid medium containing 1.0 ㎎ l^{-1} 2,4-D. One third strength of MS medium was the best for both overall growth and development of somatic embryos in liquid culture. Over 3400 viable somatic embryos were produced from each 150 ㎖ flask with an initial cell density of 30 ㎎ in 30 ㎖ medium. Germinated somatic embryos developed in liquid medium converted into plantlets after transferred to half-strength MS semi-solid medium. Approximately 90% of the converted plantlets were successfully transplanted to soil and grew into fertile plants.

Key words : Plant regeneration, somatic embryogenesis; embryogenic cell suspension culture.

INTRODUCTION

Schisandra chinensis Baill. (Magnoliaceae) is a woody, deciduous vine, mainly distributed in the north-eastern China, most eastern parts of Russia, Korea and Japan (Hancke *et al.*, 1999). This plant species contains copious amounts of dibenzo[a,c]cyclooctadiene lignans. Extensive pharmacological and chemical studies have showed that these compounds have anti-hepatotoxic (gomicin A), anti-pyretic (gomisin A, H, J, N and schisandrin), sedative (schizandrol A), and analgetic (gomisin A and predominantly schisandrin) properties. This group of compounds can also function as muscle relaxant (gomisin A, schisandrin) and exert a negative effect on the human immunodeficiency virus activities (gomisin J) (Opletal *et al.*, 2004). The fruits of *S. chinensis* have long been used as traditional Chinese medicine. Recently, its demands increased dramatically for employing in pharmaceutical and industries. As a result, over exploitation and a lack of organized cultivation has led to a rapid decline of *S. chinensis* in nature.

S. chinensis is commonly propagated by seeds, as vegetative propagation through conventional methods is difficult as adventitious roots fail to form from cuttings. Consequently, micropropagation techniques may provide alternative means for rapid propagation of elite clones. Somatic embryogenesis is considered by many as a cost-efficient method for producing uniform plants, especially for those species which are difficult to propagate by conventional cuttings (Stasolla & Yeung, 2003). Totipotent embryogenic cell suspension cultures could also facilitate the generation of mutant or variant cell lines, isolation of protoplasts, genetic transformation, and physiological and biochemical studies (Mythili *et al.*, 1999). Micropropagation of *S. chinensis* by somatic embryogenesis from immature zygotic embryos has been reported (Kim *et al.*, 2005; Smiskova *et al.*, 2005). However, the culture procedure is too complex and multiplication efficiency is still low. Further more, there are no published reports on somatic embryogenesis and plant regeneration from vegetative tissues or through embryogenic cell suspension cultures of *S. chinensis*. In this study, we describe culture conditions for high frequency somatic embryogenesis from cotyledonary leaf and hypocotyl explant-derived embryogenic cell suspension cultures of *S. chinensis*.

MATERIALS AND METHODS

Embryogenic callus induction

Mature seeds of *S. chinensis* were collected from the Langxiang, Heilongjiang province, China. Dehusked seeds were

† Corresponding author: (Phone) +82-33-250-6411 (E-mail) cyyu@kangwon.ac.kr
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immersed in 70% ethanol for 1 min followed by sterilized in 1% sodium hypochlorite containing 0.05% Tween 20 for 10 min with gentle agitation, and rinsed four times with sterile distilled water. Surface sterilized seeds with 3/4 part of endosperms removed by cross section, were then placed on hormone-free Murashige and Skoog (1962) medium with 3% sucrose, and 5.5 g l^{-1} Agar (Sanland, USA). Seeds began to germinate approximately after two weeks of culture. Cotyledonary leaf and hypocotyls explants from 7 days old seedlings were excised and cultured on MS agar medium with different concentrations of 2,4-dichlorophenoxyacetic acid (0.1, 0.5, 1, 2, 4, 6 and 10 mg l^{-1}) and 30 g l^{-1} sucrose. To test the effects of temperature on induction of embryogenic callus, explants were cultured at 25 ± 1 ℃ and 30 ± 1 ℃ in dark, respectively. All media were adjusted to pH 5.8 and autoclaved at 1.1 kg cm^2 (121 ℃) for 20 min. Ten explants were placed in each 10 × 2 cm (diameter × height) plastic Petri dish. Each experimental unit was consisted of twenty explants with three replicates. The percentages of embryogenic callus induction were calculated after eight weeks of culture.

Establishment of embryogenic cell suspension cultures

For the establishment of rapidly growing and finely dispersed cell suspension cultures, approximately 30 mg (fresh weight) of embryogenic callus was transferred to 150 ml Erlenmeyer flasks containing 30 mℓ of MS liquid media supplemented with 1.0 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. After two months in liquid culture, well dispersed and fast growing suspensions were passed through a 200 mesh sieve before embryogenesis experiments were initiated. To determine the inoculum density on suspension cell growth, 0.12 mℓ (approx. 3 mg fresh weight), 1.2 mℓ (30 mg), 3.6 mℓ (90 mg) of suspension aliquots were transferred to MS liquid media, and the growth was measured with five to ten days intervals. To obtain the fresh weights of suspension cells, 3 mℓ of suspension were passed through a preweighed filter paper which was followed by reweighing. Embryogenic cell suspensions were subcultured every two weeks and maintained at 25 ℃ on a gyratory shaker (100 rpm) in the dark.

Somatic embryo differentiation

For induction of somatic embryos, embryogenic cell suspensions were passed through a 200 mesh sieve and larger clump portions were collected and transfered to hormone-free MS medium. To optimize basal salt for somatic embryo development, 1.2 mℓ (about 30 mg in fresh weight) of aliquots were rinsed three times with MS free media and transferring to 150 mℓ Erlenmeyer flasks containing 30 mℓ of various concentrations of hormone-free MS medium (1/4X-, 1/3X-, 1/2X-, 1X-, or 2X-) with 30 g l^{-1} sucrose. The cultures were agitated at 100 rpm on a gyratory shaker and subcultured at a two week intervals. The culture room was maintained at 21 ℃ under cool-white fluorescent light with a flux rate of 35 mol s^{-1} m^{-2} and a 16 h/8 h (light/dark) photoperiod. After six weeks of culture, a 1.5 mℓ aliquot of the embryo suspension from each treatment was removed using a modified pipette tip (i.e., 5 mm was cut from the end of a standard 1 mℓ pipette tip to widen the aperture to prevent clogging) and somatic embryos were calculated. The overall growth of somatic embryos was then determined by measuring their fresh weight in a flask. For each treatment, five flasks were sampled in triplicate and the data combined, unless stated otherwise.

Somatic embryo conversion and transplantation

When most somatic embryos reached globular and heart stage, the density of suspensions were reduced by a 10 fold dilution and cultured in the same medium for further 4 weeks to promote their further development. Somatic embryos germinated in liquid medium were selected and transferred to 150 mℓ Erlenmeyer flasks containing 30 mℓ of hormone-free, half strength MS medium with 10 g l^{-1} sucrose and 5.5 g l^{-1} Agar (Sanland, USA). After five weeks of culture in semisolid medium, regenerated plantlets with well developed leaf and root systems were transferred to pots containing autoclaved sand and soil (1 : 3). Potted plants were covered with transparent plastic to maintain a high humidity, kept in a growth chamber at 21 ℃ and a 16 h photoperiod for six weeks, then the cover was removed. The survival rate was recorded three months after transplantation.

Results and Discussion

Embryogenic callus induction

All cultured explants formed callus within eight weeks when supplemented with various concentrations of 2,4-D. Two types of callus were readily observed, where one type of callus had a rigid, compact and nodular appearance and the other form had a soft and friable appearance. When transferred to growth regulator-free medium, somatic embryos only differentiated only from friable calluses, therefore, the friable type of callus was considered as embryogenic callus.

Friable, white embryogenic callus was initiated mainly from the cut end of explants when cultured at 30 ℃ (Fig. 1A). In contrast, no embryogenic callus was induced from the explants cultured with different concentrations of 2,4-D at 25 ℃, which was also reported by Kim *et al.* (2005) using globular stage zygotic embryos as a material. These above results showed that elevated culture temperature exerts stimulating effect on

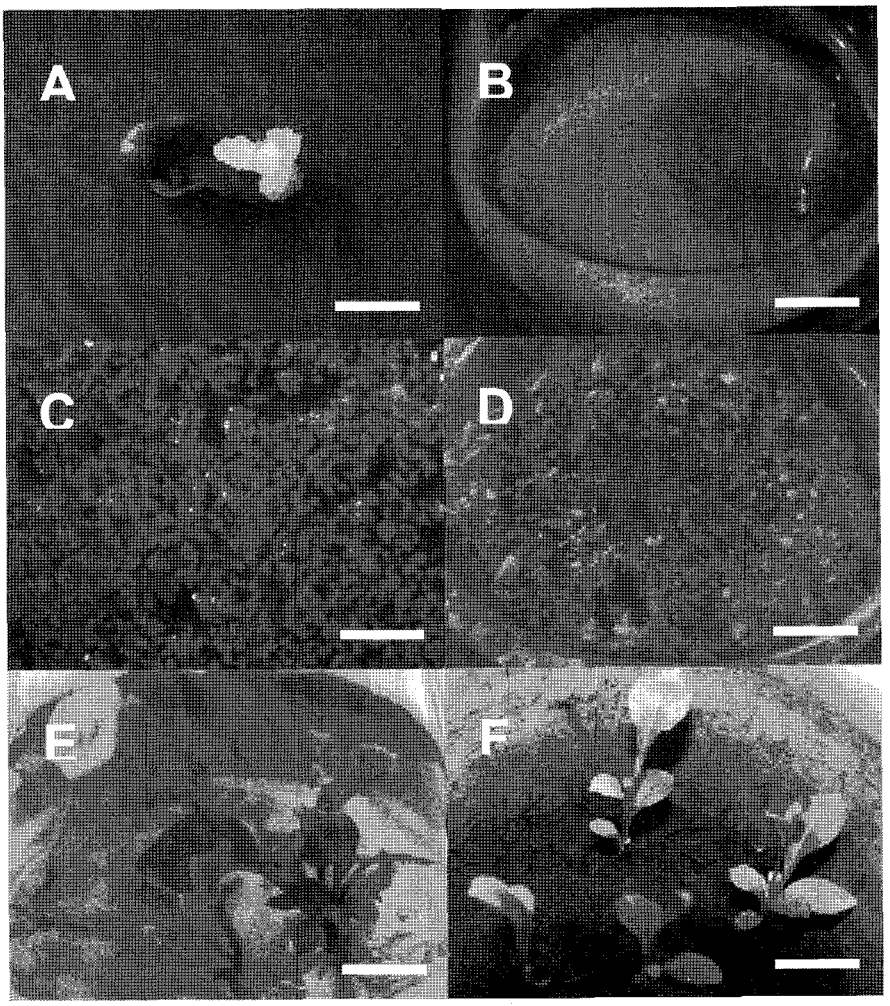

Fig. 1. A-F Somatic embryogenesis and plant regeneration from cell suspensions of *Schisandra chinensis*. A, Embryogenic callus originated from cotyledonary leaf explants (bar = 2.5 ㎜). B, Embryogenic cell clumps in liquid medium (bar = 10 ㎜). C, Numorous somatic embryos developed in liquid medium (bar = 4 ㎜). D, Germinated somatic embryos in liquid medium (bar = 12 ㎜). E, Plantlets grown in a semisolid medium after 2 months (bar = 15 ㎜). F, Plantlets growing in the greenhouse 2 months after acclimatization (bar = 25 ㎜).

embryogenic callus induction. The promoting effects of high temeperature on induction of somatic embryogenesis have been previously reported in alfalfa (Gyorgyey *et al.*, 1991), carrot (Kamada *et al.*, 1989, 1994) and chicory (Decout *et al.*, 1994).

Both explant types and 2,4-D concentration significantly affect the percentage of explants producing embryogenic callus. Hypocotyl explants produced more embryogenic callus than cotyledonary leaf explants. Among the different 2,4-D concentration tested, 4 ㎎ l^{-1} 2,4-D showed better responses than the other treatments, with an average of 18.3% of the hypocotyl explants produced embryogenic calluses after 2 month (Table 1). Such phenomenon was also reported in *Eleutherococcus senticosus* (Choi *et al.*, 1999) and *Gymnema sylvestre* (Ashok Kumar *et al.*, 2002). In *S. chinensis*,

Table 1. Effect of explants type and 2,4-D concentration on embryogenic callus induction from 7days old seedlings of *S. chinensis* after 8 week cultured in 30℃

2,4-D (㎎ L^{-1})	Embryogenic callus induction frequency (%)	
	Cotyledonary leaf[z]	Hypocotyl[z]
0	0^{d}	0^{d}
0.1	0^{d}	0^{d}
0.5	0^{d}	1.7^{cd}
1.0	0^{d}	5.0^{c}
2.0	1.7^{cd}	11.7^{b}
4.0	3.3^{cd}	18.3^{a}
6.0	1.7^{cd}	15.0^{ab}
10.0	1.7^{cd}	3.3^{cd}

Z; Values with the different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

formation of more embryogenic callus might be due to the higher embryogenic potential of hypocotyl explant than cotyledonary leaf explant, though these two types of explants have same callus induction capacity.

Establishment of embryogenic cell suspension cultures

Though it could separate into small pieces when transferred to liquid medium containing 1.0 mg l^{-1} 2,4-D, the embryogenic callus remained intact within first four weeks even with continuous shaking. After three times subculture to fresh medium, small cell aggregates initiated and proliferated. A fine embryogenic cell suspensions established after a further two or three subculture period. The cell suspensions were well dispersed and contained small groups of cells which were small and actively dividing (Fig. 1B). Similar observation have been reported in wheat (Redway *et al.*, 1990) and palmarosa grass (Patnaik *et al.*, 1997), where authors explained slow dissociation of the cell aggregates in suspensions was due to the compact and highly organized nature of the embryogenic callus that cultured in solid medium. In this study, a comparatively dispersed and fast growing embryogenic cell suspensions was achieved in the MS liquid medium supplemented with 1 mg l^{-1} 2,4-D. Similar observation have been reported in many suspension culture studies such as in *E. senticosus* (Choi *et al.*, 1999) and *Sorghum dimidiatum* (Mythili *et al.*, 1999), which is essential for the development and maintenance of embryogenic suspension cultures.

The inoculation densities affected the growth of the cell suspensions (Fig. 2). When initial density was 3 mg in 30 mℓ medium, the proliferation were almost ceased, showing that too low initial density is not suitable for proliferation of embryogenic suspensions in *S. chinensis*. With initial cell density of 30 mg or 90 mg in 30 mℓ medium, cell clumps proliferated rapidly and no marked lag phase were observed. The stationary growth phase was reached earlier with higher inoculum densities (15-25 days). Embryogenic cell suspensions can be maintained over two years by regular transfer to fresh liquid proliferation medium with two to four week subculture period.

It has been previously reported in carrots cell suspension cultures that low density cells could develop into globular embryos even in the presence of 2,4-D, which usually inhibits somatic embryogenesis, and suppression of somatic embryogenesis required both 2,4-D and high cell density (Higashi *et al.*,1998; Sung & Okimoto,1981). In our experiment, cell growth and somatic embryo formation were both strongly inhibitory in the low-density cell cultures in medium containing 2,4-D, indicating 2,4-D supplement plays an inhibition effect on somatic embryo formation of *S. chinensis*.

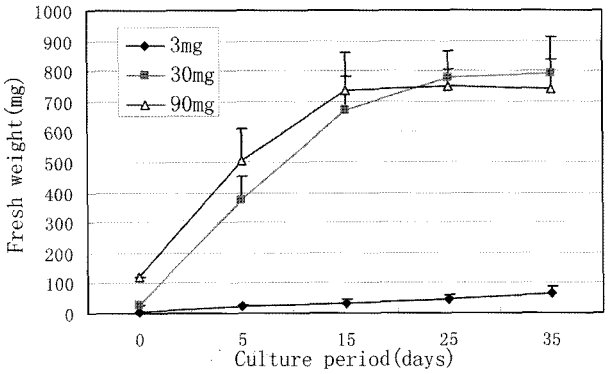

Fig. 2. Effect of inoculum density on embryogenic cell growth in suspension culture of *S. chinensis*.

Table 2. Effect of MS medium strength on the production of somatic embryos from embryogenic cell suspension cultures of *S. chinensis* after 6 week in culture

Medium Strength	Globular[xz]	Heart[xz]	Torpedo[xz]	FW (g)[yz]
1/4 MS	101.7 ± 8.9[b]	27.0 ± 3.3[b]	4.5 ± 3.2[a]	0.95 ± 0.15[c]
1/3 MS	119.1 ± 11.3[a]	41.4 ± 5.1[a]	2.6 ± 0.7[a]	1.42 ± 0.13[a]
1/2 MS	114.4 ± 9.2[a]	43.3 ± 4.6[a]	3.7 ± 4.2[a]	1.36 ± 0.12[a]
1 MS	87.3 ± 13.1[c]	11.0 ± 2.1[c]	0 ± 0[b]	1.19 ± 0.12[b]
2 MS	11.3 ± 3.1[d]	0 ± 0[d]	0 ± 0[b]	0.23 ± 0.07[d]

x Mean number ± SD of somatic embryos per 1.5 mL of embryogenic suspension culture from 150 mℓ Eranmayer flasks containing 30 mL liquid medium.
y Mean Fresh weight ± SD per 150 mℓ Erlenmeyer flasks containing 30 mL liquid media
z Values with the different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

Somatic embryo differentiation

No somatic embryos were induced in 2,4-D containing medium though the concentration was decreased to 0.1 mg l^{-1}. A multitude of globular-shaped embryos developed within four weeks when embryogenic suspensions were transferred to various strength of hormone free MS liquid medium (Fig. 1C).

The effect of different concentrations of MS medium in somatic embryo formation is summarized in Table 2. The yield of somatic embryos and overall growth were highest in 1/3 X- and 1/2 X-strength MS medium, where over 160 somatic embryos could developed from 1.5 mℓ of liquid medium. Somatic embryo could progressed to heart stage or a few to torpedo stage (Fig. 1C), but further development was mostly blocked. Numerous cotyledonary stage embryos achieved within two to four weeks when suspensions with globular to heart stages were divided into ten flasks for each subculture to decrease the culture density (Fig. 1D). After further four weeks of culture, somatic embryos could germinated

and reached 1-2 ㎝ in length. Primary root development also occurred from most embryos, but no shoot and leaf differentiation was observed during the suspension culture period. Cell density is thought to be an important factor affecting somatic embryogenesis in suspension culture. In most cases, high-cell-density culture causes strong inhibition of somatic embryo formation, whereas low density cells could develop into globular embryos even in the presence of 2,4-D (Higashi *et al.*, 1998; Sung & Okimoto 1981; Osuga *et al.*, 1993). Higashi *et al.* (1998) reported an initial cell density greater than 1.0 ㎖ packaged cell volume per liter showed strong inhibition of carrot somatic embryo formation as compared to that in 0.2 ㎖ $PCVl^{-1}$. However, in some cases high initial cell density in the medium did not influence the development of embryogenic cultures to globular and heart shaped embryos, but greatly suppress to torpedo stage embryos as described by Shigeta *et al.* (1996). Also in our present study, *S. chinensis* cell clumps could develop into early stage somatic embryos with high cell density of 30 ㎎ in 30 ㎖ medium (over 1.0 ㎖ $PCVl^{-1}$). This might be due to the fact that the cell clumps were sieved and only the larger size fractions were used for further development to somatic embryos.

Somatic embryo conversion and transplantation

Germinated somatic embryos developed in 1/3-strength MS liquid medium converted into plantlets at a low frequency (11.2%) within 5 weeks after transferred to hormone free, half strength MS semisolid medium with 10 g l^{-1} sucrose (Fig. 1E). Altered MS medium strength and sucrose concentration did not increase the conversion efficiency, but gibberellic acid could significantly diminish the conversion efficiency (data not shown). Somatic embryos and plantlets developed from suspension cultures frequently formed numerous secondary somatic embryos. Approximately 90% of the *S. chinensis* somatic embryos which converted to plantlets were successfully transferred to soil and grew into fertile plants (Fig. 1F).

In this study, we developed a culture method for induction of somatic embryogenesis from young seedling explants of *S. chinensis*. Our protocol for the production of *S. chinensis* somatic embryos from embryogenic cell suspension cultures represents a substantial improvement over previous method based on the use of embryogenic callus cultures (Kim *et al.*, 2005). In this report, over 3400 viable somatic embryos were produced per 30 ㎖ of embryogenic cell suspension culture. In contrast, only about 12 viable embryos were produced from approximately the same mass of embryogenic callus cultured on agar-solidified media (Kim *et al.*, 2005). The regeneration of plants from suspension cultured embryogenic cells offers an improved opportunity to develop a mass micropropagation and highly efficient genetic transformation protocol for *S. chinensis*.

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