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PLANTLET REGENERATION THROUGH SOMATIC EMBRYOGENESIS IN *SCHISANDRA CHINENSIS* (TURCZ.) BAILL. AND ANALYSIS OF GENETIC STABILITY OF REGENERATED PLANTS BY SRAP MARKERS

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Abstract

To study the efficient plant regeneration system of *Schisandra chinensis* (Turcz.) Baill. via somatic embryogenesis and evaluated the genetic stability of regenerated plants was carried out. Dormant buds were cultured on Murashige and Skoog (MS) medium supplemented with 2,4-D and TDZ for induction of callus. The callus induction rate was highest on MS medium with 3.0 mg/l 2,4-D and 0.2 mg/l TDZ and MS medium supplemented with 1.0 mg/l TDZ and 0.2 mg/l zeatin (Zt) was beneficial to embryonic callus induction. The globular embryo formation was improved when embryonic calli were cultured in liquid half-strength MS medium than semi-solid medium. MS supplemented with 0.2 mg/l indole-3-butytric acid (IBA) was most favorable for root formation of regenerated plants. SRAP primer were used for PCR amplification on parental and regenerated plants, and there were no specific bands produced, confirming that the plant regeneration system was stable and reliable. The result of this study will be beneficial for genetic transformation and mass clonal propagation.

Introduction

Schisandra chinensis (Turcz.) Baill. is a woody, deciduous vine mainly found in Northeast China, Korea, Japan, and the Far East of Russia (Hancke *et al.* 1999). The fruits are used in traditional Chinese medicine and are also widely used in the pharmaceutical, wine, beverage, cosmetics and healthcare industries. The biological active compounds in the fruits of *S. chinensis* are lignans with a dibenzo [a,c] cyclooctadiene skeleton. The lignans of *S. chinensis* were found to prevent liver injuries, stimulate liver regeneration, and inhibit hepatocarcinogenesis and lipid peroxidation in rats (Kubo *et al.* 1992, Hikino *et al.* 1984). However, years of excessive exploitation and habitat losses have led to a rapid decline of *S. chinensis* in the wild. Currently, *S. chinensis* is mainly propagated by seed, but cultivated seed populations are rarely homogeneous and do not ensure good fruit quality and high yield. Vegetative propagation of *S. chinensis* through conventional methods is difficult because adventitious roots fail to form from cuttings. Micropropagation techniques may provide an alternative means for the rapid propagation of good quality clones.

Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological embryo stages generally encountered in zygotic embryogenesis. Somatic embryogenesis is not only a valid propagation procedure (Dodeman *et al.* 1997), but it is also an important route for the recovery of transgenic plants, as somatic embryos (SE) and embryogenic tissues are preferred targets for genetic transformation (Kantharajah and Golegaonkar 2004). Clonal propagation through somatic embryogenesis can shorten the time

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needed for breeding and can improve the uniformity and quality of nursery stock (Stasolla and Yeung 2003). Secondary somatic embryogenesis (SSE) is a phenomenon whereby new SE are initiated from other somatic embryos, and regeneration frequency could be improved by SSE. Secondary somatic embryogenesis has been reported in diverse plant species such as *Manihot esculenta* (Raemakers *et al.* 1993), *Medicago trunculata* (Neves *et al.* 1999) and *Calliandra tweedii* (Heikrujam *et al.* 2014).

There have been a few reports on the primary somatic embryogenesis in *S. chinensis* (Kim *et al.* 2005, Smiskova *et al.* 2005, Yang *et al.* 2011). However, the capacity of somatic embryogenesis and number of somatic embryos per explant were still low. Furthermore, secondary somatic embryogenesis in *S. chinensis* has not been reported yet. Therefore, the main objective of the present study was to describe optimal factors that would influence high-frequency primary and secondary somatic embryogenesis from dormant buds of *S. chinense*. The genetic stability of plants regenerated was evaluated by sequence-related amplified polymorphism (SRAP) markers.

Materials and Methods

Dormant buds of *S. chinensis* were collected from Fengjia *Schisandra* sub-base, Xinbin, Liaoning, China in December, 2010. The buds were rinsed in running tap water for 12 hrs, soaked in 70% (v/v) ethanol for 30 sec, surface disinfested with 0.1% (w/v) $HgCl_2$ solution for 30 min, and then rinsed five times with sterile distilled water. Excised buds from which three or four outer scales had been removed were placed on Murashige and Skoog (1962) MS basal medium supplemented with 1.0, 3.0 or 5.0 mg/l 2,4-D and 0.1, 0.2 or 0.3 mg/l TDZ and 3% (w/v) sucrose for callus induction. Rates of callus induction were calculated after 2 months.

After 2 months in culture, calli were then cut into 0.5 × 0.5 cm pieces and transferred to embryogenic callus induction medium which consisted of MS medium supplemented with 1.0 mg/l TDZ in combination with 1.0, 2.0, 3.0 or 5.0 mg/l 2,4-D, 0.1, 0.2, 0.5 or 1.0 mg/l zeatin (Zt) or 0.5, 1.0, 1.5 or 2.0 mg/l BA, individually. MS medium with 1.0 mg/l TDZ was used as the control.

Light-yellow embryogenic calli with loose structure were selected and transferred to various strengths of MS medium (one-quarter, half-strength, three-quarter or full strength) containing 3% sucrose without plant growth regulators (PGR) for embryo induction. To produce uniform plants rapidly and easily, a cell suspension system was investigated. One, three or five gram embryogenic calli were transferred to each 150 ml Erlenmeyer flask containing 50 ml liquid half-strength MS medium with 3% sucrose. Flasks were maintained on the orbital incubator at 60 rpm. Number of SE that formed was calculated after one month.

To promote further maturation and germination of SE and SSE and the development of roots, SE and SSE were transferred to MS medium supplied with 0, 0.2, 0.5 or 1 mg/l IBA. After one month, shoots from SE and SSE were transferred to MS medium supplied with 0.2 mg/l IBA and 0.1% actived charcoal.

In vitro raised plantlets with three to four leaves were rinsed in running tap water to remove agar medium, transplanted into pots containing sterile perlite and sand (1 : 3 mixture). Pots were covered with perforated polythene bags to maintain high humidity. The covers were removed after four weeks when new leaves developed. The greenhouse was kept at 20 ± 5°C. The survival rate was calculated after two months of hardening.

The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from leaves of seven parental plants (used as the source of explants for callus induction) and 35 regenerated plants. Genomic DNA was then polymerase chain reaction (PCR)-amplified using SRAP primers.

For PCR amplification, each 20 µl PCR-mixture consisted of 80 ng genomic DNA, 2 µl 10 × PCR buffer, 2.5 mM $MgCl_2$, 200 mM dNTPs, 0.6 µM each primer and 1.0 U Taq DNA polymerase. Amplification was performed in a PTC-100 thermo cycler (MJ Research, Inc, Watertown, Massachusetts) with the following PCR program: 5 min at 94°C, then 5 cycles of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C followed by another 35 cycles of 1 min at 94°C, 1 min at 49.5°C and 1 min at 72°C, with final extension at 72°C for 5 min. The PCR products were stored at 4°C till electrophoretic separation on 2% (w/v) agarose gels with ethidium bromide (EtBr) staining, in 0.5 × TBE (Tris-borate-EDTA, pH 8.3) at 150 V and room temperature. All chemicals used were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri).

All media were adjusted to pH 5.8 before adding 6 g/l (w/v) plant agar and were then autoclaved at 1.1 kg/cm (121°C) for 20 min. Cultures were grown in 150 ml triangular flasks containing 30 ml medium and maintained under a 16 hrs photoperiod at light intensity of 50 µmol/m/s provided by cool-white fluorescent lamps at 25 ± 2°C.

In this study, a completely random design was used. Each treatment consisted of 10 triangular flasks containing five explants, and experiments were repeated three times. The data variance (analysis of variance, ANOVA) was analyzed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) for Windows. Significantly differing means were compared using Duncan's multiple-range test (DMRT) at 5% probability level.

Results and Discussion

Dormant buds were cultured on MS medium supplemented with different concentrations of 2,4-D and TDZ to induce callus. Calli formed on the cut surfaces after 4 weeks of culture. The highest rate of callus formation was found on MS medium containing 3.0 mg/l 2,4-D and 0.2 mg/l TDZ, and the calli were light-green, friable (Fig.1a) and had strong regeneration ability (Table 1). On medium with higher concentrations of 2,4-D, explants produced soft, slow growing callus, which eventually turned brown.

Table 1. Effects of 2,4-D and TDZ on the induction of callus of *S. chinensis.*

2,4-D (mg/l)	TDZ) (mg/l	Induction rate of callus (%)	Callus morphology
0	--	--	--
1.0	0.1	54.3h	Green, compact
1.0	0.2	72.7g	Green, compact
1.0	0.3	87.7d	Green, compact
3.0	0.1	90.3c	Light green, friable
3.0	0.2	95.0a	Light green, friable
3.0	0.3	92.7b	Yellowish green, friable
5.0	0.1	85.0e	Light brown, fragile watery
5.0	0.2	76.3f	Light brown, fragile watery
5.0	0.3	74.3g	Brown, fragile watery

Different letters in a column indicate a significant difference ($p < 0.05$) according to DMRT.

The frenquency of embryogenic callus increased with TDZ plus 2,4-D, Zt or BA compared to TDZ alone (Table 2). The highest frequency of embryogenic callus occurred on MS medium supplemented with 1.0 mg/l TDZ and 0.2 mg/l Zt. The percentage of embryogenic callus production was significantly higher in medium containing TDZ and Zt, which ranged from 26.67

to 49.67%. The effect of BA proved better than that of 2,4-D when combined with TDZ on embryogenic callus induction.

Table 2. Effects of combanitions and concentrations of TDZ and 2,4-D, Zt or BA on embryogenic callus induction.

TDZ (mg/l)	2,4-D (mg/l)	ZT (mg/l)	BA (mg/l)	Embryogenic callus induction renquency (%)
1	--	--	--	4.67i
1	1.0	--	--	7.33h
1	2.0	--	--	11.33g
1	3.0	--	--	13.33g
1	5.0	--	--	9.00h
1	--	0.1	--	26.67d
1	--	0.2	--	49.67a
1	--	0.5	--	33.00b
1	--	1.0	--	30.33c
1	--	--	0.5	21.67e
1	--	--	1.0	25.67d
1	--	--	1.5	16.00f
1	--	--	2.0	13.33g

Different letters in a column indicate a significant difference ($p < 0.05$) according to DMRT.

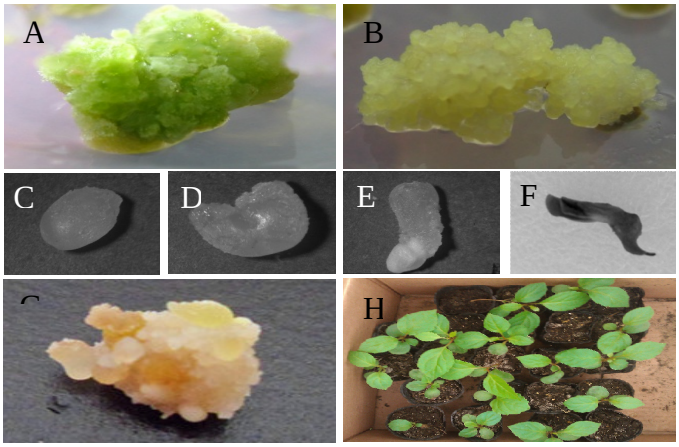

Fig.1. Different types of callus of *Schisandra chinensis.*

A: Callus in light green; B: Embryonic callus; C: Globular embryo; D: Heart-shaped embryo; E: torpedo-shaped embryo; F: cotyledonary embryo; G: secondary somatic embryos; H: Regenerated plants transplanted to matrix.

After one month of culture, globular embryos formed and the number of SE induced by each gram of embryogenic callus were collected at one week intervals. The strength of MS medium significantly influenced the formation and development of SE (Fig. 2). A larger number of SE emerged on half-strength MS medium within 4 weeks and 95.3 globular embryos differentiated into 69.7 cotyledon embryos, in contrast, embryogenic calli cultured on 1/4 MS and MS showed a poor response.

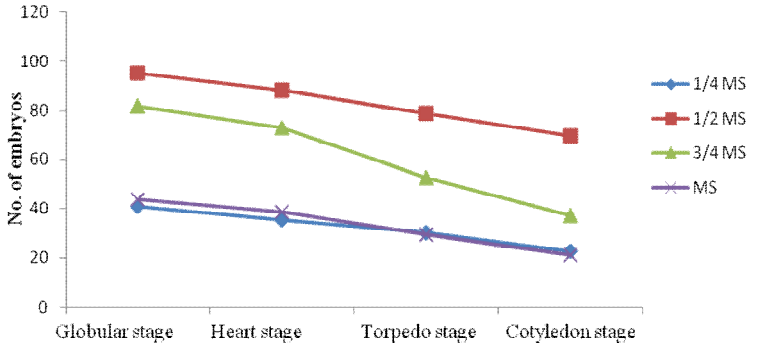

Fig. 2. Production of different stages of somatic embryo after 4 weeks of culture on different media.

In liquid culture medium, inoculum density was shown to be significant in inducing somatic embryogenesis (Fig. 3). With the initial density of 50 ml medium containing 5 g embryogenic callus, globular embryos differentiated much faster after one week. However, poor SE development and cells browning maybe because of insufficient nutrients during late stage in liquid culture. The initial density of 3 g embryogenic calli produced the largest number of SE, which indicated that a suitable inoculum density could improve SE growth and development (Fig.1c - f). Cultures inducing somatic embryogenesis in semi-solid or liquid media strongly affected the number of SE. Compared to cultured on semi-solid media, embryogenic calli formed much more somatic embryos with uniform dispersion in liquid culture and the plantlets originated from liquid culture were shown to be better growing and developing consistently as compared to plants obtained via semi-solid media. IBA treatments had a significant effect on rooting percentage and MS without PGR showed low rooting capacity (Table 3). The highest rooting percentage was obtained on MS medium with 0.5 mg/l IBA, however, high concentration of IBA resulted in 50.67% malformed seedlings. MS medium with 1.0 mg/l IBA showed 80.33% rooting and 89% abnormal seedlings. Therefore, on the basis of rooting and abnormal seedling formation, 0.2 mg/l IBA treatments was suitable for plantlet regeneration of *S. chinensis.*

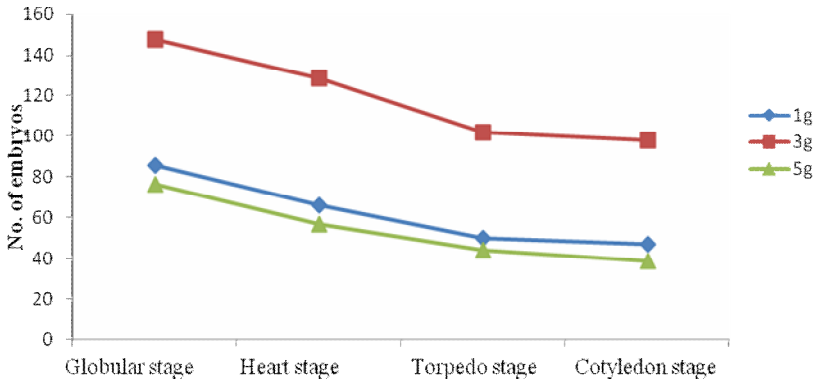

Fig. 3. Effect of inoculum density on somatic embryo production after four weeks of culture in liquid media induction of secondary somatic embryos.

Plantlets with well-developed root systems were transferred to small containers containing sterile perlite and soil (1 : 3 mixture) (Fig.1h). During the early transplantation period, a transparent plastic membrane was used to cover the containers to maintain high humidity. The covers were removed after four weeks when new leaves developed. The greenhouse was kept at 20 ± 5°C. The regenerated small plants were transplanted to field, and about 90% developed normally after two months.

Table 3. Effect of IBA concentration on rooting of plantlets regeneration.

IBA concentration (mg/l)	Rooting rate (%)	Rate of abnormal seedlings (%)
0	23.33d	12.00d
0.2	74.00c	18.33c
0.5	93.67a	50.67b
1.0	80.33b	89.00a

Different letters in a column indicate a significant difference ($p < 0.05$) according to DMRT.

Evaluation of genetic stability of regenerants of *S. chinense* 35 well-developed healthy regenerated plants and 7 parental plants produced clear amplification profiles using four pairs of SRAP primers. Twenty-two unambiguous bands in the size range 100 - 1000 bp were scored from four primers, of which 22 were present in parental plants as well as in all the regenerated plants. The results showed that all regenerated plants showed 100% genetic similarity with the parent at the DNA level and plant regeneration through somatic embryogenesis was technically feasible. This study established a stable and reliable regeneration system, which could be applied to the mass production of plants of *S. chinensis.*

This study developed a high-frequency plant regeneration method through somatic embryogenesis using dormant buds of *S. chinensis* and the regenerated plants obtained were genetically identical with the parents. A combination of semi-solid and liquid culture was adopted for somatic embryogenesis and auxins and cytokinins were tested for induction of SSE.

From proliferative callus, embryogenic callus could be stimulated with the right type and concentration of PGR. The use of 2,4-D alone or in combination with other PGR has become almost routine in inducing somatic embryogenesis (Huang and Yeoman 1984, Mordhorst *et al.*1998). Huetteman and Preece (1993) reported that TDZ was a potent cytokinin for woody plant tissue culture. TDZ promotes somatic embryogenesis more effectively compared to BA in rice and *Bambusa edulis* (Gairi and Rashid 2004, Lin *et al.* 2004). In this study, the callus induction rate was highest when dormant buds of *S. chinensis* were cultured on MS medium with 3.0 mg/l 2,4-D and 0.2 mg/l TDZ. MS medium with 1.0 mg/l TDZ and 0.2 mg/l Zt was beneficial to embryogenic calli induction. This result was consistent with the results of above studies.

The osmotic pressure of the culture medium would be directly affected by changing the medium strength (Liao and Amerson 1995). A change in osmotic pressure directly affects the formation, development, and maturity of SE (Komatsuda *et al.*1992). In this study, a low concentration of inorganic salts was conducive to SE formation and development. This was probably because high concentrations of inorganic nutrients in the culture medium result in high osmotic pressure, inhibiting *S. chinensis* embryogenic callus differentiation. The inhibitory effects of high MS medium strength on SE development have been also reported for *Eleutherococcus senticosus* (Choi *et al.* 1999).

Cultures inducing somatic embryogenesis in semi-solid or liquid media strongly affected the number of SE. Choi *et al.* (2002) reported that somatic embryogenesis from callus or cell suspension culture was an efficient method offering a high frequency of plant regeneration utilizing liquid medium. In this study, embryogenic calli cultured in semi-solid medium formed fewer SE rather than in liquid medium, especially 3 g embryogenic callus cultured in a volume of 50 ml medium produced more globular embryos. Return to semi-solid medium during the maturation phase improved the yield of SE and their subsequent germination. Guo and Zhang (2005) reported that the inoculated amount of ginger suspension cultures greatly affected cell vitalities in further subcultures. Direct relationship between the growth of suspension cells and inoculation density in liquid medium was also discovered in our experiment.

As a growth regulator, the use of IBA for root formation has been reported in a number of studies. Treatment with IBA increased root formation by 25.6% as reported by Rugini *et al.* (1990) on olive. In the current experiments, we observed its positive effect on normal seedlings formation as well. Concentrations of IBA had significant effects on germination of somatic embryo and when MS supplied with 1.0 mg/l IBA, values of 89% abnormal seedlings formation frequency were observed. Because of relatively small amounts of abnormal seedlings, 0.2 mg/l IBA treatments was suitable for plantlets regeneration of *S. chinensis.*

Li *et al*. (2014) reported that the regenerated plants of *Distylium chinense* obtained via SE were genetically identical with the parents. In this study, the results obtained from the SRAP-PCR analysis of the plants regenerated via somatic embryogenesis revealed that regenerated plants were identical with the parent germplasm and showed no genetic variation. This study also indicated that SRAP-PCR could give rapid indication of the level of genetic stability in plants regenerated *in vitro*. It requires small amounts of plant material and can be used at any stage *in vitro*.

In conclusion, we report an efficient plant regeneration protocol *in vitro* of *S. chinense* via somatic embryogenesis for its germplasm conservation and commercial cultivation. Furthermore, the established regeneration protocol will be fruitful for genetic transformation and mass clonal propagation of *S. chinensis*.

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