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Micropropagation of *Schizandra chinensis* BAILLON Using Glucose from Cotyledonary Nodes

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A protocol for the micropropagation of *Schizandra chinensis* has been developed using regenerated shoots from axillary bud explants. In preparing to do so, we found that seed type (i.e., mature vs. pre-mature) significantly influenced the rate of germination. The Woody Plant (WP) medium proved to be superior to the Murashige and Skoog (MS) medium for germination purposes. Multiple shoots were induced from cotyledonary nodes of axenic seedlings on WP media containing 6-benzylaminopurine (BA) alone or in combination with 1-napthaleneacetic acid (NAA). High frequencies of shoot proliferation and the greatest number of shoots per explant (11.6) were observed with the use of 1.0 mg L^{-1} BA. We also established a culture method for proliferating shoots by repeatedly subculturing the original cotyledonary nodes on a shoot multiplication medium each time newly formed shoots were harvested. To induce root formation, glucose was supplied as a carbon-source substitution for sucrose. The best rooting rate was obtained from a WP medium supplemented with 3% glucose and 0.5 mg L^{-1} NAA. Following transplantation in the field, 82% of the plantlets survived.

Keywords: cotyledonary node, glucose, micropropagation, *Schizandra chinensis*

S. chinensis Baillon (Magnoliaceae) is a broad-leaved vine whose red fruits have been used for drugs and food materials. Plants are found in North America, the Tropics, subtropical Asia, and East Asia, especially Korea (Baily and Baily, 1976). This species, which is characterized by its peculiar aroma and five-flavored fruits (sweet, bitter, salty, pungent, and sour), provides a warm sensation and is commonly used in traditional Chinese medicines for the lung, kidney, cardiovascular system, stomach, and intestinal tract (Tang and Eisenbrand, 1992). Both its fruits and seeds possess important hepatoprotective qualities (Hikino et al., 1984), as well as antioxidative properties (Toda et al., 1988; Huang et al., 1990), anti-inflammatory relief, and an antibiotic against *Listeria monocytogenes* (An et al., 1997). The biologically active components of *S. chinensis* include lignans, i.e., compounds with a dibenzo [a, c] cycluuctadiene skeleton (Ikeya et al., 1995). Chief among these are schizandrin, gomisin A, deoxyschizandrin, gomisin N, and wuweizisu C (Ikeya et al., 1979; Song et al., 1989).

A normally slow growth rate makes conventional vegetative methods, such as cutting, grafting, and layering, unsuitable for its propagation. Therefore, *in vitro* tissue culture would be a valuable tool for multiplying plant stocks. Such regeneration of this species has not yet been reported, but micropropagation of this economically important forest plant could overcome its historic limitations. For example, the use of nodal segments or axillary buds (Pradhan et al., 1998) from mature trees and seeds (Kaveriappa et al., 1997) has been very successful for rapid and large-scale propagation of a number of woody plant species (Perinet and Lalonde, 1983; Dos et al., 1996; Bhuyan et al., 1997).

Here, we present for the first time the manipulation of cotyledonary nodes for *in vitro* micropropagation of the tree species *S. chinensis*.

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MATERIALS AND METHODS

Plant Material and Germination Conditions

Mature and pre-mature seeds of *S. chinensis* were obtained from the Muju Agricultural Cooperative Union, Jeonbuk, Korea, in 2001. They were stored at 4°C for three months. Afterward, the seed coats were removed and the surfaces were sterilized with 2% sodium hypochlorite for 10 min before being rinsed five times with sterile distilled water. To determine the most effective germination conditions for disinfected seeds, we tested three types of media: MS (Murashige and

Skoog, 1962), half-strength MS, and WP (Woody Plant; Lloyd and McGown, 1980), all of which were supplemented with 3% sucrose and 0.7% agar and adjusted to a pH of 5.7 with 1 N NaOH. After the media were autoclaved, gibberellic acid (GA_3; 0.1 to 2.0 mg L^{-1}) was added and the seeds were applied to the plates. Cultures were maintained at 25±1°C in the dark.

Shoot Multiplication

The seeds were germinated for six weeks, after which they were transferred to a hormone-free WP medium and maintained at 25±1°C under a 16-h photoperiod (light intensity of 30 µM m^{-2} s^{-1} from cool-white fluorescent tubes; Philips, USA). After the seedlings had grown to 4 or 5 cm, about eight weeks after transplanting to WP, their cotyledonary nodes were harvested. The radicles were removed from the seedlings and the cotyledonary nodes were transplanted into a 100-mL Erlenmeyer flask containing a WP medium with 3% sucrose. To this medium was added, individually or in combination, 0.1 to 5.0 mg L^{-1} BA and 0.1 to 1.0 mg L^{-1} NAA. The number and lengths of the developing shoots were recorded following 8 weeks of cultivation. After each harvesting of newly formed shoots, the original cotyledonary nodes were subcultured on fresh shoot multiplication media containing BA.

Rooting of Multiplicated Shoots

Compared with other woody species, techniques for improving the rooting efficiency of *S. chinensis* are not well developed. Therefore, we examined the production of roots from proliferated shoots on WP media supplemented with auxins and two types of carbon sources. Because sucrose is a less effective source than glucose, we tested both compounds. Here, we used microshoots (at least 1.0 cm long) that were excised from cultures on WP media containing 1.0 mg L^{-1} BA. These shoots were transferred to 100-mL Erlenmeyer flasks containing 30 mL of the WP medium supplemented with either 3% glucose or 3% sucrose and solidified with 0.7% (w/v) agar. For all media, 0.1 to 2.0 mg L^{-1} NAA and 0.5 to 1.0 mg L^{-1} IBA were added, either separately or in combination.

Acclimatization

Rooted shoots were removed from their culture flasks, washed with sterile distilled water, and transplanted to 8-cm-diameter pots containing autoclaved vermiculite. High humidity was maintained by covering the pots with plastic sheets. The potted plants were kept for three weeks in a culture room (25±1°C) and watered with sterile WP salts. for 3 weeks. Established plants were then re-potted in a 1:1 (v:v) mixture of soil and sand.

Statistical Analysis

To test germination, 90 seeds were used per experiment. The multi-shoot-induction experiments required 40 plants per treatment; the rooting trials, 30 micropropagated shoots each. All experiments were repeated three times, and the data were analyzed by one-way ANOVA.

RESULTS AND DISCUSSION

Seed Germination and Shoot Multiplication

To examine the effect of seed sources on germination rates, we cultured seeds on MS basal medium containing 3% sucrose. After 6 weeks of cultivation, the germination rate of premature seeds (38%) was 19-fold greater than that of mature seeds (Fig. 1A). The highest germination rate of premature seeds was obtained from WP medium containing 1.0 mg L^{-1} GA_3 (Table 1). This ability to germinate depended on media type and GA_3 concentration. The germinated seeds were then cultured on a hormone-free WP medium and the cotyledonary nodes were excised from eight-week-old plantlets. Although these nodes failed to respond morphologically without supplemental growth regulators, the addition of cytokinins to the media prompted the formation of new shoots. Therefore, we examined the effect of BA, kinetin, and thidiazuron on shoot multiplication, and achieved the best results from a WP medium supplemented with BA (data not shown). To determine the optimum BA concentration, we cultured three cotyledonary nodes per flask. Increasing the concentration of BA from 0.1 to 1.0 mg L^{-1} enhanced the number as well as the length of the shoots (Table 2, Fig. 1B). In particular, 8 weeks of cultivation and a concentration of 1.0 mg L^{-1} BA induced the greatest shoot formation; each explant had approximately 11.6 shoots, with an average length of 4.2 cm. In contrast, media supplemented with 2.0 mg L^{-1} BA suppressed this pattern of proliferation, and the resulting shoots had a rosette appearance with small, green leaves.

Transferring the primary regenerated shoots into a

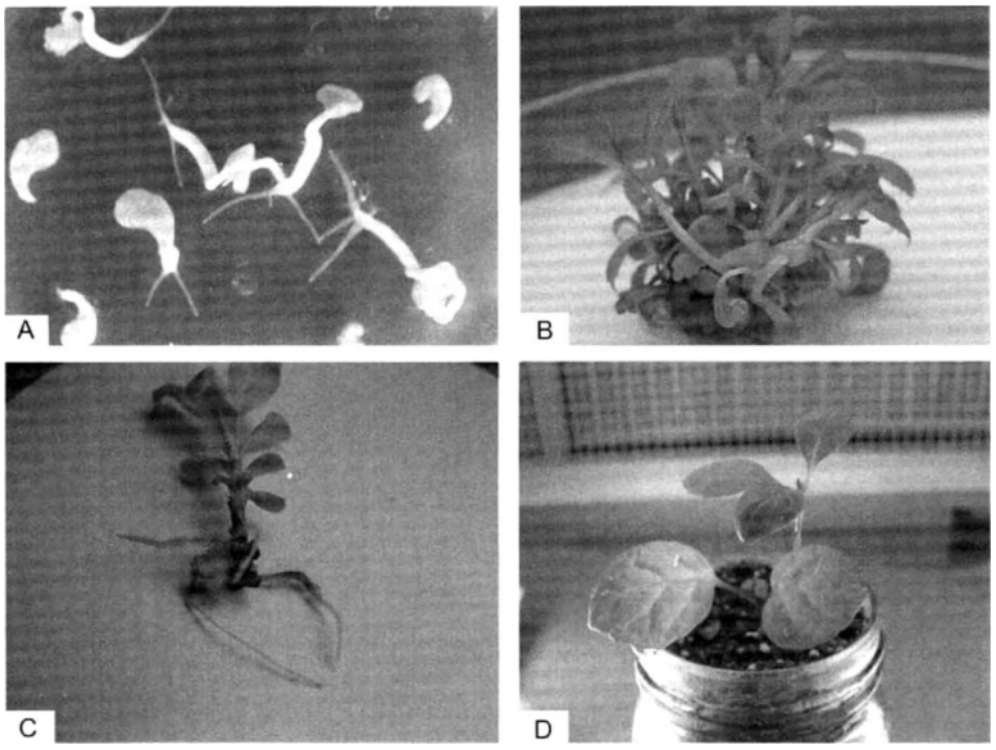

Figure 1. Micropropagation of *S. chinensis* from cotyledonary nodes. **A.** Pre-mature seeds germinated on WP medium supplemented with 1 mg L^{-1} GA_3. **B.** Multiple shoots developed from cotyledonary nodes on WP medium supplemented with 1.0 mg L^{-1} BA. **C.** Roots induced from multiple shoots on WP medium supplemented with 3% glucose and 0.5 mg L^{-1} NAA. **D.** Plantlet of *S. chinensis* two months after transplanting in mixture of soil and sand.

Table 1. Effects of GA_3 concentration and culture media (1/2 MS, MS, or WP) on germination of pre-mature seeds from *S. chinensis*.

GA_3 concentration (mg L^{-1})	Rates of seed germination (%)		
	1/2 MS	MS	WP
0.0	65±3.7	38±0.5	46±1.7
0.1	68±2.0	63±2.0	48±3.5
0.5	70±6.2	57±3.3	74±2.2
1.0	78±1.3	51±4.8	85±3.3
2.0	55±0.9	31±0.3	66±1.2

Datum represents mean value ± standard error from three experiments.

Table 2. Effects of BA concentration on shoot multiplication from cotyledonary nodes of *S. chinensis* on WP medium.

BA concentration (mg L^{-1})	Number of shoots after 8 weeks	Shoot length (cm)
0.0	NR	2.6
0.1	3.4 ±0.9	3.7
0.5	8.4 ± 1.5	4.5
1.0	11.6 ± 1.8	4.2
2.0	5.7 ± 0.4	3.4
5.0	2.8 ± 0.1	2.8

Datum represents mean value ± standard error from three experiments. NR, no response.

hormone-free WP medium did not lead to multiplication, but only to shoot elongation. This result is similar to that reported with *Syzygium alternifolium* (Sha Valli Khan et al., 1997). Therefore, our second set of proliferation experiments involved combining various concentrations of BA with 0.0 to 2.0 mg L^{-1} NAA. Media containing both BA and NAA induced our cotyledonary nodes to produce fewer shoots than the BA-only media (Table 3). Moreover, although supplementing NAA on BA-containing media increased the number of shoots over those from hormone-free media, these combinations suppressed shoot multiplication more than did BA alone. In addition, high concentrations of NAA (>1.0 mg L^{-1}) led to the formation of green-hard calli rather than shoot proliferation (data not shown). These shoots were carefully separated and again subcultured for shoot multiplication on a WP medium containing 1.0 mg L^{-1} BA.

We also examined the role of a solidifying agent in the medium and its effect on shoot proliferation and

Table 3. Effects of auxin-cytokinin combination on shoot multiplication and elongation of *S. chinensis* cotyledonary nodes.

Hormones (mg L^{-1})		Number of shoots after 8 weeks	Shoot length (cm)
BA	NAA		
0.0	0.5	NR	2.1
0.5	0.5	3.5±0.7	3.8
1.0	0.5	6.3±0.9	5.1
0.0	1.0	NR	1.8
0.5	1.0	3.1±0.2	3.0
1.0	1.0	2.8±0.7	3.2

Datum represents mean value ± standard error from three experiments. NR, no response.

Table 4. Effects of IBA and NAA on root induction efficiency from micropropagated shoots of *S. chinensis* after 6 weeks of cultivation.

Auxins (mg L^{-1})		Rooting (%)	Number of roots	Root length (cm)
Control	0.0	NR	NR	NR
IBA	0.5	21±5.3	2.4	2.2
	1.0	25±5.8	2.8	2.9
NAA	0.5	62±4.4	5.6	3.7
	1.0	52±4.7	4.9	2.6
	2.0	45±5.2	4.6	2.6
IBA 0.5 NAA 0.5		43±3.8	4.2	2.8
IBA 0.5 NAA 1.0		40±3.9	3.9	2.2
IBA 1.0 NAA 0.5		49±3.1	4.5	3.3
IBA 1.0 NAA1.0		39±4.6	3.7	2.5

As a carbon source, 3% glucose was added to WP medium. Datum represents mean value ± standard error from three experiments. NR, no response.

elongation. Here, agar proved superior to gellan gums. However, that finding is the opposite of that reported in other studies (Hu and Wang, 1983; Sharma and Ramamurthy, 2000).

Effect of Glucose and NAA on Rooting

WP media without any supplemental plant growth regulators failed to induce root formation from the multiple shoots, even after 10 weeks. Nevertheless, well-developed shoots from hormone-enriched media were excised and inoculated individually on a rooting medium to which glucose had been added as a carbon source. Various combinations of IBA and NAA were tested in these rooting media, and the WP medium supplemented with 3% glucose and 0.5 mg L^{-1} NAA was the most effective after six weeks. Our substitution of glucose for sucrose increased not only the number of rooted shoots but also the number of roots per shoot (Fig. 1C). However, the simultaneous presence of both auxin types did not improve rooting of *S. chinensis* (Table 4). This result contrasts with the enhanced root induction reported when auxin combinations were used with other tree species, e.g., *Cinnamomum zeylanicum* Breyn. (RavishankarRai and Jagadishchandra, 1987), *Morus* sp. (Pattnaik and Chand, 1997), and *Dalbergia sisso* Robx. (Pradhan et al., 1998).

We successfully acclimated the rooted plantlets in our laboratory and under greenhouse conditions, attaining an overall survival rate of approximately 70%. After 10 weeks, they were then transferred to the field, where 82% of those plants survived (Fig. 1D).

In this study, we developed first-time micropropagation techniques for regenerating *S. chinensis*. By modifying the composition of the culture media, we were able to induce a large number of multiple shoots from cotyledonary nodes, which, subsequently, developed into normal plantlets. The optimum combination for shoot multiplication and elongation proved to be a WP medium supplemented with 3% sucrose and 1.0 mg L^{-1} BA. In addition, a WP medium containing 3% glucose and 0.5 mg L^{-1} NAA was the most effective for root induction, with the increased number of rooted shoots leading to the production of numerous plantlets. Therefore, these results support a promising method for efficient propagation on a commercial scale as well as for the conservation of superior genetic strains. Despite many troublesome aspects (e.g., difficulty in transplantation and rooting, physiological defects, and low germination rates), we have now demonstrated a valuable *in vitro* micropropagation procedure that relies on cotyledonary nodes from members of the Magnoliaceae family.

Micropropagation has been used extensively for rapid and large-scale production of a number of woody plant species. We believe that medicinal plants as well as other trees can be micropropagated from either the axillary or the apical buds that are harvested from mature, elite specimens for which their horticultural characteristics are stable and well-developed. The present work describes a simple and successful method for inducing shoot proliferation that could be useful in the clonal propagation and mass production of *S. chinensis* plants.

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