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Micropropagation and Pharmacological Analysis of a Medicinal Herb *Sarcandra glabra*

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ABSTRACT

An efficient sterilization and micropropagation protocol for a medicinal herb, *Sarcandra glabra*, was established. An optimal surface sterilization protocol involved explants being shaken in 120 mg l^{-1} rifampicin for 2-4 days between 2X 0.1% $HgCl_2$ treatments for 10 min. The most effective medium for bud induction was Murashige and Skoog (MS) medium supplemented with 4.0 mg l^{-1} 6-benzylaminopurine (BA); highest bud proliferation was observed on MS medium containing 4.0 mg l^{-1} BA and 0.2 mg l^{-1} α-naphthaleneacetic acid (NAA); optimal rooting was observed on ½- or ¼-strength MS medium with 0.2 mg l^{-1} indole-3-butyric acid (IBA) and 30 g l^{-1} sucrose. The thin layer chromatography (TLC) fingerprints and bacteriostatic activity of the *in vitro* plants showed no obvious differences with wild plants.

Keywords: *in vitro*, propagation, surface sterilization
Abbreviations: BA, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **KT**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, α-naphthaleneacetic acid; **TDZ**, thidiazuron; **TLC**, thin layer chromatography

INTRODUCTION

Sarcandra glabra (Thunberg) Nakai is an evergreen subshrub, 50-150 cm tall. The stem of *S. glabra* (2n=30) is cylindric, erect, and glabrous with swollen nodes. *S. glabra* are distributed in forests, valleys, ravines, slopes, roadsides, trailsides, grasslands, swamps, streamsides, sandy soil, near sea level to 2000 m, from South East Asia to India (Xian and Jeremie 1999; Eklund *et al.* 2004; Pan *et al.* 2004). It is a traditional Chinese medicine, which is widely used to relieve cancer, pneumonia, appendicitis, gastritis, enteritis, diarrhea, rheumatism and injuries (Cheung 1991; Xian and Jeremie 1999; Graham *et al.* 2000). It is also used to prevent and treat thrombocytopenia after chemotherapy (Zhong *et al.* 2005). Several natural pharmaceutical components have been identified from this plant, including isofraxidin, fumaric acid, chloranthalactone and sesquiterpene lactones (Wang *et al.* 1988; Tsui and Brown 1996; Zheng *et al.* 2003). Isofraxidin has good anticancer and cholagogic effects; fumaric acid has antibiosis, antalgic and antitumor effects (Pharmacopoeia Committee of People's Republic of China 2000). Pollen germinability and the development of embryos have been described in *S. glabra* (Yamazaki 1998; Hristova 2005). Pollen germinability of *S. glabra* was low and embryonic development was confined to the Polygonad type (Hristova 2005). When pollen grains germinate, a continuous extracellular matrix (ECM), the esterase-positive cuticle proper, exists on the stigma; since pollen tubes are unable to track the surface of the stigma or adaxial epidermal cells at the site of angiospermy because of the presence of the ECM, this might prevent seed from pollen tube setting (Hristova 2005).

During the past 10 years, several modern Chinese medicines have been developed from *S. glabra*, so wild *S. glabra* can not satisfy commercial demand according to Chinese herb pharmacy. A protocol for the *in vitro* mass propagation of *S. glabra* should be developed to meet these commercial needs since the survival rate by stem cuttings is low (about 40%; Tu *et al.* 1989) as is the seed germination rate (only 10-20%, unpublished data). However, to data there are no reports on the micropropagation of *S. glabra* except for one report on callus formation (Tu *et al.* 1994). The present study demonstrates a high frequency *in vitro* proliferation system for *S. glabra via* bud tip culture. Chemical components and pharmacological characteristics of tissue-cultured plants were also analyzed and compared with non-tissue-cultured plants. Furthermore, an effective explant surface sterilization protocol suitable for *S. glabra* micropropagation is described.

MATERIALS AND METHODS

Chemicals and reagents

Rifampicin (Rif), 6-benzylaminopurine (BA), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), kinetin (KT) and thidiazuron (TDZ), were purchased from Sigma-Aldrich (Whigan, China). Other chemical reagents, all of analytial grade, such as $HgCl_2$, sucrose, etc., were purchased from Guangzhou Chemical Ltd., China.

Plant materials and culture conditions

Nodal explants with axillary buds were collected from 2-3-years-old *S. glabra* growing in the forest at the JingXiuTang GAP (Good Agricultural Practice for Traditional Chinese Medicine) Station at MaoFengShan Mountain in Guangzhou, China. Sterilization of bud explants involved testing combinations of $HgCl_2$ and Rif, and is described in the results section because of the difficult disinfection in initial cultures. Surface-sterilized single nodal explants were cultured on induction medium consisting of MS (Murashige and Skoog 1962) salts with 30 g l^{-1} sugar and 0.8% agar, and

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maintained at 28°C and a 16-h photoperiod with 50 μmol m^{-2} s^{-1} light intensity (S_1, Fushan Light Ltd., China).

Regeneration, rooting and acclimatization experiments

Buds induced *in vitro* and grown for 2 weeks in culture were used as the starting materials. Buds with axillary buds were excised and cultured on bud multiplication medium: MS inorganic salts and vitamins with 30 g l^{-1} sugar and 0.8% agar. The type and concentration of plant growth regulator, i.e., BA, TDZ and NAA, subculture period (10-60 days) and subculture generations (5-20 cycles) were tested to ascertain which of these factors could best promote bud proliferation and development. Plant height, the number of leaves and the number of growing buds induced per explant (as an indicator of proliferation rate) were recorded after 35 d cultivation.

The proliferated buds (1-2 cm long) were used as explants for the rooting trial. To optimize root induction, two rooting agents (NAA, IBA), different MS medium concentrations and sucrose were tested. Plant height, root length, diameter and number were determined after 30 d.

Healthy plantlets (3-5 cm in height, 2-4 roots and 4-6 leaves) were transferred to a soil/perlite (3:1) mixture (Fushan Horticultue Co., China) in a 10-cm pot with 2 plantlets/pot. The pots were kept under shade. During the first week of transfer, the plantlets were covered with a transparent plastic film to maintain high humidity (about 80% outside) and then watered at weekly intervals. The plantlets were planted under 50% shade dark-nets (about 50% sunlight cut). The survival rate was examined 1 month after transfer.

Pharmacological analysis

The main natural medicinal component and bacteriostatic of the *in vitro* proliferated plantlets were analyzed by a TLC fingerprint and inhibition of the growth of two bacteria, *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 8739), which were obtained from the Guangdong Institute of Microbiology. Leaves (20 g) were collected from the middle part of plants at different developmental stages and were dried in a 60°C chamber for 2 days. The dry leaves were ground into a powder in liquid nitrogen and placed into 250 ml deionized water. Then the mixture was treated by ultrasonic (20 Khz, SCIENTZ-II D, Ningbo Scientz Biotechnology Co. Ltd, China) for 2 × 30 min, and centrifuged at 8000 rpm at 20°C for 15 min. The 200 ml supernatant was mixed vigorously with 100 ml of ethyl acetate for 30 min, and then the mixture was centrifuged at 1000 rpm at 20°C for 10 min. The supernatant was transferred into a new bottle, and concentrated to a dry residue in hot water. 0.5 g dry residue was re-dissolved in 1 ml of MeOH. TLC fingerprints were used to indicate the main chemical components of the plants. The 0.5 g extract residue per ml was further diluted to 1 μg/ml and 4 μg of the extracts were spotted onto silica TLC plates (Yantai, Shangdong, China) and run with a solvent system consisting of toluene: ethyl acetate: formic acid = 9: 4: 1. The components of the mixture that were separated were visualized with UV light (Fushan Ltd., Shanghai, China) at 365 nm, or color was developed on the TLC plates after steaming in 5% ammonia solution for 10 min and then in iodine for 5 min. The extracted residue was serially diluted into different concentrations with water to test its MIC (minimal inhibitory concentration). MIC was used to indicate the antibacterial activity, which showed that the minimal inhibitory concentration against *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 8739) (Pharmacopoeia Committee of People's Republic of China 2000).

Statistical analyses

The statistical analyses were performed with SPSS software using Duncan's multiple range tests. Data were from 30 samples.

RESULTS AND DISCUSSION

Surface sterilization

S. glabra grows in sub-shrub fields in which there may be a lot of fungal contamination of explants. However, fungal contamination could be effectively controlled by co-culture of explants in distilled water between two 0.1% (w/v) $HgCl_2$ treatments. The first 0.1% $HgCl_2$ sterilization could not completely remove fungal contamination, indicating that some fungal spores still survived. Co-culture in water for 2-4 days may have promoted fungal spore germination, and the second $HgCl_2$ treatment effectively sterilized the newly germinated spores. A single $HgCl_2$ treatment or $HgCl_2$ alone could not control bacterial contamination.

Despite the several disinfection treatments that were used to increase the number of aseptic cultures, explants were heavily contaminated and showed bacterial contamination within 12 days of initiation of culture in the fungus-controlled explants. About eight types of bacterial contamination could be distinguished by the shape and color of bacteria in the fungal-disinfection culture (data not show). These bacterial clones were isolated and used to test their sensitivity to antibiotics. Among 10 antibiotics tested, only Rif could inhibit the growth of all isolated clones (data not show). 120 mg l^{-1} of Rif could effectively control bacterial contamination when the explants were cultured in Rif for 2-4 days. This might be caused by the inhibition of fungi to $HgCl_2$ and of bacteria to Rif. Since Rif inhibited bud growth when added into media (data not show), it was thus not directly added into media.

Therefore, the most suitable protocol for sterilization of *S. glabra* explants takes place in 5 steps: (1) Leaves are removed from bud explants and placed under flowing tap water for 10 min. (2) Nodal explants are excised (1-2 cm long with a single node in the center of the explant) and wiped with 75% ethanol for 30 s and rinsed 3 times with sterile distilled water (SDW). (3) Explants are immersed in 0.1% (w/v) $HgCl_2$ with 7-8 drops of Tween-80 for 10 min with gentle agitation, and then rinsed 3 times with SDW. (4) Explants are cultured in 120 mg l^{-1} Rif for 2-4 days with gentle agitation, and rinsed 3 times with SDW. (5) Explants are immersed in 0.1% (w/v) $HgCl_2$ with 7-8 drops of Tween-80 for 10 min with gentle agitation again, and rinsed 3 times with SDW. This protocol could result in ~56% sterilized explants from plants grown in the field compared to 100% contamination when only $HgCl_2$ or Rif were used to sterilize the explants.

Bud initiation, multiplication and rooting

1. Induction of growth and development of axillary buds

The sterilized explants were used to induce the growth of axillary buds. The induction of axillary buds from *S. glabra* nodal explants cultured on MS basal and MS medium supplemented with various concentrations of BA alone or in combination with NAA is summarized in **Table 1**. The induction rate of axillary buds revealed a negative effect of a higher concentration of NAA (0.5 mg l^{-1}), which probably inhibited the development of axillary buds. However, BA increased the axillary bud induction rate. An orthogonal table $L_9(3^3)$ with three factors at three levels was selected for studying the effects of MS basal medium, BA and NAA

Table 1 Influence of various medium combinations on shoot induction of *S. glabra*.

Combination	Concentration of MS medium *	6-BA (mg l^{-1})	NAA (mg l^{-1})	Survival explants	Shoot induction rate (%)
A	1/2	0.0	0.0	34	11.76
		2.0	0.5	32	0.00
		4.0	1.0	29	0.00
B	1	0.0	0.5	33	0.00
		2.0	1.0	29	0.00
		4.0	0.0	31	100.00
C	3/2	0.0	1.0	32	0.00
		2.0	0.0	34	76.47
		4.0	0.5	34	0.00

*Concentration strength of the inorganic salts and vitamins in MS media

Table 2 Influence of 6-BA, TDZ, KT and NAA on shoot proliferation of *S. glabra*.

Combination	Plant growth regulators (mg l^{-1})		No. of explants	Shoots per explant*	Height of shoots (cm)*
A	6-BA	NAA			
	0	0	30	1.07 ± 0.07 f	2.05 ± 0.07 a
	1	0	30	3.40 ± 0.44 d	1.81 ± 0.06 b
	2	0	30	3.77 ± 0.51 cd	1.69 ± 0.09 c
	4	0	30	3.95 ± 0.24 bc	1.66 ± 0.06 c
	8	0	30	3.72 ± 0.31 cd	1.65 ± 0.08 c
	1	0.2	30	2.29 ± 0.11 e	2.10 ± 0.10 a
	2	0.2	30	2.36 ± 0.39 e	2.03 ± 0.08 a
	4	0.2	30	4.48 ± 0.33 a	2.01 ± 0.10 a
	8	0.2	30	4.22 ± 0.29 ab	1.84 ± 0.05 b
B	TDZ	NAA			
	0	0	30	1.08 ± 0.05 g	2.19 ± 0.12 a
	0.05	0	30	1.47 ± 0.12 ef	1.97 ± 0.12 b
	0.1	0	30	1.78 ± 0.17 d	1.95 ± 0.19 b
	0.2	0	30	2.85 ± 0.30 b	1.69 ± 0.11 c
	0.4	0	30	3.47 ± 0.20 a	1.59 ± 0.06 c
	1.0	0	30	3.44 ± 0.28 a	1.60 ± 0.05 c
	0.05	0.2	30	1.27 ± 0.10 f	2.17 ± 0.24 a
	0.1	0.2	30	1.66 ± 0.23 de	2.10 ± 0.12 ab
	0.2	0.2	30	2.10 ± 0.13 c	2.09 ± 0.11 ab
	0.4	0.2	30	3.35 ± 0.25 a	1.75 ± 0.07 c
	1.0	0.2	30	3.48 ± 0.30 a	1.66 ± 0.05 c
C	KT	NAA			
	0	0	30	1.07 ± 0.07 e	2.11 ± 0.07 a
	0.5	0	30	1.52 ± 0.24 c	1.99 ± 0.06 b
	1.0	0	30	2.47 ± 0.26 a	1.79 ± 0.05 c
	3.0	0	30	2.51 ± 0.33 a	1.63 ± 0.07 d
	6.0	0	30	2.58 ± 0.41 a	1.60 ± 0.08 d
	0.5	0.2	30	1.23 ± 0.12 d	2.13 ± 0.08 a
	1.0	0.2	30	2.06 ± 0.21 b	1.93 ± 0.11 b
	3.0	0.2	30	2.02 ± 0.18 b	1.65 ± 0.10 d
	6.0	0.2	30	2.12 ± 0.27 b	1.64 ± 0.06 d

*Within each combination treatment, values with the same letter are similar ($P < 0.05$) according to Duncan's multiple range tests for each clone.

on axillary bud induction. The tests showed that MS basal medium supplemented with 4.0 mg l^{-1} BA alone was the most suitable medium. This medium combination (MS + 4.0 mg l^{-1} BA) could result in 100% bud induction, and the growth of auxillary buds could be observed after 10 days' culture.

2. Bud multiplication

The *in vitro* propagation of buds is strongly influenced by the plant regulator(s) for *Cordyline terminalis* and banana (Evaldsson *et al.* 1985; Wong 1986). *S. glabra* buds could grow on MS basal medium, but axillary buds could not develop. Cytokintins have a positive effect on bud proliferation and promote axillary bud development. The effect of three cytokinins (BA, KT and TDZ), and their combination with NAA in MS medium were examined during bud multiplication. Nodal cuttings cultured on MS basal medium did not show any axillary bud growth, while those cultured on MS media containing BA, KT or TDZ alone or in combination with NAA, showed axillary bud proliferation (**Table 2**). The proliferation rates (number of buds/explant) of the three cytokinins were BA > TDZ > KT, with BA being the most efficient among the three cytokinins tested. Buds cultured in medium with cytokinins combined with a low concentration of NAA (0.2 mg l^{-1}) grew better than those only on medium with cytokinin alone, while 0.2 mg l^{-1} NAA alone did not significantly increase bud number.

The most suitable period of subculture was 25-35 days, and the highest tested subculture generation of 12 cycles has no significant effect on the proliferation rate.

3. Rooting and acclimatization

Adventitious root formation is a key step in micropropagation (De Klerk *et al.* 1997). The efficiency of two auxins (NAA and IBA), the concentration of MS and sucrose on root responses of *S. glabra* was tested. The influence of these factors on the number, length and diameter of adventitious roots is shown in **Fig. 1**. In most combinations of MS basal salts and auxins (NAA and IBA), root initiation was observed on the 12th day after the transfer of buds to rooting medium. 0.05-0.5 mg l^{-1} of auxins (NAA and IBA) in ½- or full-strength MS medium increased the number of roots. However, NAA induced callus formation at the base of microcuttings and tufted roots at a concentration > 0.1 mg l^{-1} (**Fig. 1A, 1B**). However, 0.05-0.5 mg l^{-1} IBA increased the number of roots and had no significant effect on callus induction (**Fig. 1**). *S. glabra* could not root in the absence of sucrose, which increased the rooting efficiency and root number when increased from 20 to 40 g l^{-1} (data not show). The optimal rooting medium was ½-MS + 0.2 mg l^{-1} IBA + 30 g l^{-1} sucrose. 95% of the *in vitro*-rooted *S. glabra* plantlets with well-developed roots could survive after being

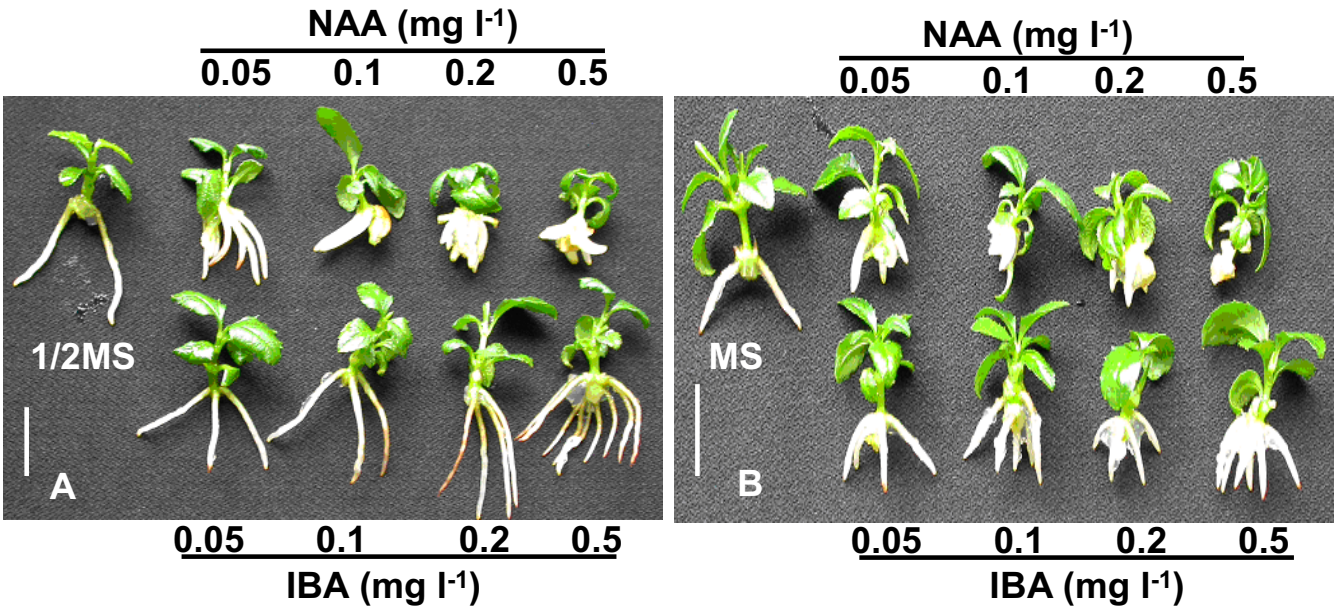

Fig. 1 Effects of NAA and IBA on *in vitro* rooting of *S. glabra*. The photographs show the *in vitro* rooting of *S. glabra* plantlets grown on ½-strength MS (**A**), and full-strength MS (**B**) supplemented with different concentrations of NAA or IBA. Bar = 1 cm.

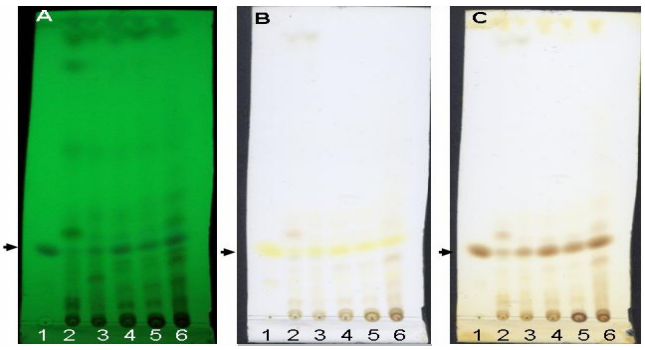

Fig. 2 TLC of tissue cultured plantlets and mature wild plants of *S. glabra*. Samples were spotted on TLC plates and run with the solvent system (toluene: ethyl acetate: formic acid = 9: 4: 1). The separated components of the mixture were visualized with UV at 365 nm (**A**), or colored by ammonia solution (**B**), and by iodine (**C**). The samples were spotted as isofraxitin standard (1), extracts from plantlets of *S. glabra* cultured on medium (2), *in vitro* regenerated plantlets cultivated in the field for 9 months (3) or for 18 months (4), mature wild plants (5), and solution of the capsule (6) from Guangzhou Jingxiutang Pharmaceutical Co. Ltd., China.

acclimatized in room conditions and transplanted to the field. About 5000 plantlets proliferated *in vitro* have been transplanted to MaoFengShan mountain GAP station of Guangzhou JingXiuTang Pharmaceutical Co. Ltd. The *in vitro* propagated plants displayed no somaclonal variation (regarding shape).

Pharmacological analysis

The TLC fingerprint and bacteriostatic effect of the plant extracts from micropropagation and mature wild plants were investigated. Specifically, 18 month-old wild plants, and *in vitro* proliferated plantlets grown in tissue culture for 2 months, or transferred to the field for 9 and 18 months were tested. The chemical components and pharmacological activity of the *in vitro* regenerated plantlets cultivated in the field for 18 months were similar to mature wild plants. Both of the extracts had a similar TLC fingerprint (**Fig. 2**) and same antibacterial activity against *S. aureus* and *E. coli*. Their MIC against *S. aureus* and *E. coli* was 62.5 and 125 mg ml^{-1}, respectively. From the fingerprints and bacteriostatic effect of the *in vitro*-grown field plants, no apparent mutation of the *in vitro* regenerated plantlets was observed.

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REFERENCES

* in Chinese with English abstract

Cheung SC (1991) *Chinese Medicinal Herbs of Hong Kong*, The Commercial Press, Hong Kong, 18 pp

De Klerk GJ (1997) Effectiveness of indolacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in *Malus* 'Jork 9'. *Plant Cell, Tissue and Organ Culture* **49**, 39-44

Eklund H, Doyle JA, Herendeen PS (2004) Morphological phylogenetic analysis of living and fossil Chloranthaceae. *International Journal of Plant Sciences* **165**, 107-151

Evaldsson IE, Welander NT (1985) The effects of medium composition on *in vitro* propagation and *in vitro* growth of *Cordyline terminalis* cv. Atoom. *The Journal of Horticultural Science and Biotechnology* **60**, 525-530

Graham JG, Quinn ML, Fabricant DS, Farnsworth NR (2000) Plants used against cancer – an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology* **73**, 347-377

Hristova K, Lam M, Field T, Sage T (2005) Transmitting tissue ECM distribution and composition, and pollen germinability in *Sarcandra glabra* and *Chloranthus japonicus* (Chloranthaceae). *Annals of Botany* **96**, 779-791

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Pan C, Xu H, Peng H, Ou W, Lu S (2004) Resource investigation and exploitable foreground of *Sarcandra glabra. Zhong Yao Cai* **27**, 556-557*

Pharmacopoeia Committee of People's Republic of China (2000) *Pharmacopoeia of People's Republic of China*. Chemistry Industry Publisher, Beijing, 179 pp (in Chinese)

Tsui WY, Brown GD (1996) Cycloeudesmanolides from *Sarcandra Glabra*. *Phytochemistry* **43**, 819-821

Tu Y, Jiang H, Wang B (1994) Studies on the suspension cell cultures of *Sarcandra glabra*. *Jiangxi Science* **12**, 162-166*

Tu YQ, Tu Y, Jiang HR (1989) Culture of seedlings of *Sarcandra glabra*. *Jiangxi Agricultural Science and Technology* **3**, 14-15 (in Chinese)

Wang AQ, Feng SC, He X, Xu RS (1988) A new sesquiterpene lactone from *Sarcandra glabra*. *Yao Xue Xue Bao* **23**, 64-66*

Wong WC (1986) *In vitro* propagation of banana (*Musa* spp.): Initiation, proliferation and development of bud-tip cultures on defined media. *Plant Cell, Tissue and Organ Culture* **6**, 159-166

Xian NH, Jeremie J (1999) Chloranthaceae. In: Wu ZY, Raven PH (Eds) *Flora of China* (Vol 4), Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis, pp 132-138

Yamazaki T (1998) Embryogeny of *Sarcandra glabra* (Thunb.) Nakai (Chloranthaceae) and phylogenetic position of the Chloranthaceae. *Journal of Japanese Botany* **73**, 22-25

Zheng WJ, Wang SF, Ceng XG, Hu ZD (2003) Analysis of *Sarcandra glabra* and its medicinal preparations by capillary electrophoresis. *Talanta* **60**, 955-960

Zhong L, Liu T, Ceng Y, Zhong X, Du X, Lu Z, Weng J, Wu S, Lin W (2005) The study on effect of *Sarcandra glabra* on prevention and treatment of thrombocytopenia by chemotherapy. *Zhong Yao Cai* **8**, 35-38*