Establishment of Callus and Cell Suspension Cultures of *Corydalis saxicola* Bunting, a Rare Medicinal Plant

Hua Cheng[a], Long-Jiang Yu[a,*], Qiong-Yue Hu[a], Shan-Cai Chen[b], and You-Ping Sun[c]

[a] College of Life Science & Technology, Huazhong University of Science & Technology, Wuhan 430074, China. Fax: +86 27 87792265. E-mail: hustylj@yahoo.com.cn
[b] Zhejiang Hisoar Pharmaceutical Company, Ltd, Taizhou 318000, China
[c] Hunan Forestry Academy, Changsha 410004, China

* Author for correspondence and reprint requests

Z. Naturforsch. **61c,** 251–256 (2006); received August 11/September 9, 2005

An efficient procedure has been developed for callus induction and cell suspension cultures of *C. saxicola* for the first time. Explant selection was carried out among leaf, stem and root to select a suitable type of explants capable of higher callus formation. Leaf explants thus selected showed maximum response to callus induction (67.1%). Modified B_5 medium supplemented with 0.5 mg l^{-1} 2,4-D plus 2 mg l^{-1} BA was the most favorable medium for callus formation with the highest induction rate (94.8%) and greatest fresh weight of callus (1.7 g per explant). Cell suspension cultures were established by transferring 2–8 g fresh callus to 80 ml liquid B_5 medium. An inoculum size of 8 g produced the greatest biomass accumulation, dehydrocavidine and berberine productions, which was 13.1 g l^{-1}, 8.0 mg l^{-1} and 4.1 mg l^{-1}, respectively. In response to various sucrose concentrations from 10 g l^{-1} to 80 g l^{-1}, cultures with 60 g sucrose l^{-1} not only produced the highest dry biomass (18.5 g l^{-1}) but also the highest formation of dehydrocavidine (11.6 mg l^{-1}) and berberine (7.6 mg l^{-1}). These prepared cell suspension cultures provided a useful material for further regulation of alkaloid biosynthesis and for enhanced production of valuable alkaloids on a large scale.

Key words: Callus, Cell Suspension Cultures, *Corydalis saxicola* Bunting

Introduction

Corydalis saxicola Bunting, with the local name yan-huang-lian, is a perennial herbaceous plant which belongs to the Fumariaceae family and only grows in limestone hills, distributed in Southern China (Wen *et al.*, 1993). According to traditional Chinese medicine or folk medicine, it is beneficial in antibacterial, acesodyne, detumescence and antipsychotic. It is also applied in the treatment of hepatitis and hepatocirrhosis. Dehydrocavidine and berberine are the major bioactive components in *C. saxicola* (Ke *et al.*, 1982). In the pharmaceutical industry, alkaloids extracted from *C. saxicola* are utilized in the production of the Yan-Huang-Lian Injection to treat hepatitis, hepatocirrhosis and liver cancer (Jiang *et al.*, 2002).

With an increasing demand for those products, the plant cell culture provides an attractive alternative source that could overcome the limitations of extracting useful metabolites from limited natural resources. And it is now widely being employed as a model system to investigate the production of specific secondary metabolites because it offers experimental advantages both to basic and applied research and to the development of models with scale-up potential (Buitelaar and Trapmer, 1992; Chang and Sim, 1995; Mukherjee *et al.*, 2000). Additionally, cell cultures exhibit advantages for studies of elicitor-induced responses (Honee *et al.*, 1998; Steven *et al.*, 2000; Stella and Braga, 2002; Lu and Mei, 2003), which are otherwise difficult to be carried out in the intact plant. Although generation of callus and suspension cultures of some *Corydalis* species have been described (Iwasa and Kamigauchi, 1996; Sagare *et al.*, 2000; Nalawade *et al.*, 2003; Nalawade and Tsay, 2004), there is little information available in literature with respect to *C. saxicola*. Realizing the importance of *C. saxicola* and its extracts in clinical medicine, we attempted to apply this strategy to establish callus and cell suspension cultures of *C. saxicola* acting as source for the production of valuable secondary

Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; d, day; h, hour; FW, fresh weight; DW, dry weight; B_5, Gamborg's medium (Gamborg *et al.*, 1968).

0939–5075/2006/0300–0251 $ 06.00

metabolites. In the present paper, the initiation of callus and cell suspension cultures of *C. saxicola* and their characteristics are described. Moreover, special alkaloid productions are also investigated by manipulating inoculum size and sucrose concentration in 250 ml flasks.

Materials and Methods

Plant material and culture conditions for callus induction

Materials of *C. saxicola* were collected from Dongnan, Guangxi Province, China in November 2002. Fresh, healthy and mature explants obtained from the plant were cleaned under running tap water and surface-sterilized by placing them in 70% ethanol for 1 min, followed by two treatments of 10 min rinse in 0.1% mercuric chloride solution and five times wash with sterile distilled water. After surface sterilization, explants were cut into about 1 cm^2 for leaves and 1 cm length for stems and roots. Then they were placed separately on 30 ml B_5 basal medium in 150 ml flasks supplemented with 3% sucrose and different cytokinin/auxin combinations solidified with 1% agar.

The media used for all the experiments were autoclaved at 108 kPa, 121 °C for 20 min. The pH value of the media was adjusted to 6.0 prior to autoclaving. All the cultures were maintained under 16-h photoperiod illumination. The incubation temperature was kept at (25 ± 1) °C.

Suspension cultures and cell growth determination

Suspension cultures were developed by transfer of 2–8 g fresh weight (FW) friable calli into 250 ml flasks containing 80 ml B_5 liquid medium supplemented with 0.5 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and 2.0 mg l^{-1} BA (6-benzyladenine), the best exogenous hormone combination determined for callus formation. All the cell suspension cultures were placed on a rotary shaker with a speed of (130 ± 5) rpm at (25 ± 1) °C in the dark. The cells were harvested from suspension cultures by filtration via a Buchner funnel with a filter paper, washed with distilled water to remove residual medium, and then filtrated again under vacuum. After filtration the cells were dried at 50 °C to constant dry weight (DW). Cell growth was measured on DW basis.

Assay of alkaloids

Dried samples (100 mg) were pulverized and extracted with 5 ml methanol/HCl (99: 1, v/v), then ultrasonicated three times for 1 h at room temperature. After combining them and filtering through a No.1 filter paper, the resultant solution was adjusted to pH 9 using ammonia and then extracted three times with chloroform. A sample of 5 ml cell-free liquid medium was also adjusted to pH 9 and extracted with chloroform for three times. The combined organic phases were dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure, and then dissolved in 0.1 ml methanol. These solutions were passed through a 0.45 μm filter prior to HPLC analysis. A reverse-phase C18 column (Dikma Diamonsil, 5 μm, 250 mm × 4.6 mm) was used. The mobile phase consisted of acetonitrile and water (40:60, v/v) supplemented with 3.4 g l^{-1} KH_2PO_4 and 1.7 g l^{-1} sodium dodecyl sulfate. The mobile phase was filtered through a 0.45 μm filter membrane and then degassed. The flow rate was 1 ml min^{-1} and the column temperature was kept at 25 °C. The chromatogram was monitored at 345 nm. All injection volumes were 10 μl.

Alkaloids were identified by TLC, HPLC-diode array detection of spectra and MS compared with standards (Agilent 1100 LC/MSD Trap, Palo Alto, CA, USA). Authentic berberine was purchased from Sigma (St. Louis, MO, USA). Dehydrocavidine was obtained from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products), Beijing, China. Alkaloid concentrations in the samples were the combination of alkaloids in cells and media.

Results and Discussion

Effects of different types of explants on callus induction

Different types of explants were placed on basal B_5 medium containing 1 mg l^{-1} 2,4-D, 1 mg l^{-1} BA and 1 mg l^{-1} kinetin for 2 months of cultivation. Leaf explants exhibited highest frequency of callus formation (67.1%), which was almost 2- and 12-fold greater than that obtained in stem and root explants, respectively. These results obtained here indicated that leaf and stem explants were suitable for callus induction of *C. saxicola* rather than root explants. Similar results were reported in callus induction of *Orthosiphon stamineus*, in which it was described that callus could be induced successfully

from leaf, petiole, stem but not root (Lee and Chan, 2004). Owing to the capacity of higher callus formation, hence, leaf explants were used for further experiments.

Effects of exogenous plant growth regulators on callus formation

Calli were successfully generated in all combinations of 2,4-D and BA two months after inoculation. But no callus was induced by basal B_5 medium only (Table I). This indicated that exogenous hormone was essential to callus formation of *C. saxicola*. The exclusive presence of BA in the medium, regardless of its concentration, was less satisfactory for callus initiation. While in the absence of BA, the cultures containing corresponding concentrations of 2,4-D showed higher callus weight in a range from 0.25 g to 0.63 g FW per explant and higher response from 27.7% to 68.3% (Table I). This phenomenon suggests that 2,4-D played a more important role in callus formation from leaf explants compared to BA. However, the combinations of auxin and cytokinin were found to produce more callus than auxin or cytokinin alone. One combination containing 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} BA, was proved to be the most efficient in promoting callus development (1.68 g) from leaf explants with 94.8% response, followed by 1 mg l^{-1} 2,4-D + 2 mg l^{-1} BA (1.40 g) and then 0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} BA (1.35 g). In addition, most of the cultures containing 2,4-D (0.5–1 mg l^{-1}) and BA (0–2 mg l^{-1}) produced friable yellowish calli, which initiated at cut surfaces of the explants about 20 d after inoculation and proliferated quickly. However, cultures with BA only resulted in less friable calli and white roots germinated from those compact calli after 3 months of cultivation. This observation was similar to callus development of *Holostemma adakodien* (Martin, 2003).

Table I. Effects of exogenous plant growth regulators on callus formation from leaf explants of *C. saxicola.*

2,4-D [mg l^{-1}]	BA [mg l^{-1}]	Induction rate (%)	Fresh weight [g]
0	0	0^{e}	0^{d}
	0.5	18.2 ± 6.5^{d}	0.22 ± 0.05^{c}
	1	16.8 ± 5.2^{d}	0.26 ± 0.04^{c}
	2	24.5 ± 0.5^{c}	0.32 ± 0.23^{c}
0.5	0	61.3 ± 10.5^{b}	0.62 ± 0.11^{b}
	0.5	77.0 ± 1.8^{a}	0.89 ± 0.15^{b}
	1	86.2 ± 5.2^{a}	1.35 ± 0.26^{a}
	2	94.8 ± 8.1^{a}	1.68 ± 0.13^{a}
1	0	68.3 ± 5.2^{a}	0.63 ± 0.15^{b}
	0.5	71.2 ± 10.5^{a}	0.82 ± 0.07^{b}
	1	69.5 ± 7.3^{a}	0.67 ± 0.06^{b}
	2	87.6 ± 6.5^{a}	1.40 ± 0.07^{a}
2	0	27.7 ± 8.1^{c}	0.25 ± 0.11^{c}
	0.5	50.1 ± 10.5^{b}	0.45 ± 0.15^{c}
	1	27.5 ± 8.1^{c}	0.29 ± 0.07^{c}
	2	28.7 ± 5.2^{c}	0.25 ± 0.05^{c}

Percentages of callus formation were scored on each explant after 8 weeks of cultivation. Data present the average of triplicate experiments (means ± S.D.). Means followed by the same letters are not significantly different at $p < 0.05$ using Tukey's test.

A plant growth regulator is a key factor responsible for callus initiation and development in plant cell cultures. Our results indicate that the combination of 2,4-D and BA was effective to satisfactorily induce calli from leaf explants of *C. saxicola*. The medium used for callus formation of *C. platycarpa* Makino and *C. ochotensis* var. *raddeana*, which are also relatives of *Corydalis*, contained a low level of 2,4-D (1 mg l^{-1}) (Iwasa and Kamigauchi, 1996; Iwasa *et al.*, 2003). In *C. ambigua*, Hiraoka *et al*. (2001) described that the medium including 2,4-D and BA could induce callus on the explant surface. The stimulative effect of 2,4-D in combination with BA on callus formation has also been reported for *Oryza sativa* (An *et al.*, 2004).

During following subcultures, healthy and young calli cultured on B_5 + 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} BA showed a bright yellow color, but some of them turned brownish as they grew older. Those calli were also observed to favor light condition (16-h photoperiod) rather than complete darkness. Calli cultivated under complete dark condition more frequently turned into brown or dark in comparison to those calli under light condition. Therefore, the homogenous calli selected from cultures were maintained on B_5 medium containing 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} BA under light condition for a long repeated subculture process. Development of fast-growing homogeneous callus was achieved after about one year.

Effects of inoculum size on suspension cultures

Suspension cultures were initiated by transferring 2–8 g fresh friable calli into 80 ml B_5 liquid medium in 250 ml flasks. In the case of 8 g, cell growth increased in the initial 12 d followed by a remarkable decrease. This biomass decrease could be caused by some cells which couldn't adapt to

the change from solid to liquid medium and gradually went to death. After that, biomass increased rapidly and reached the highest value (13.1 g l^{-1}) on day 21, with about 6 times increase compared to that at the beginning (2.1 g l^{-1}). Similar development of 6 g callus was demonstrated and the stationary phase was reached after 21 d. As to 4 g, a lag phase of about 12 d was observed, followed by a slower growth. However, there was no marked growth for 2 g during the whole cultivation and an appreciable amount of cell debris accumulated in the media in 30 d. For alkaloid production, it was enhanced remarkably with the increase of inoculum size (Table II). Dehydrocavidine production reached the lowest value of 0.51 mg l^{-1} at the inoculum size of 2 g, and increased to the highest value of 8.02 mg l^{-1} at 8 g. Berberine production also increased from 0.28 mg l^{-1} to 4.13 mg l^{-1} when inoculum size rose from 2 g to 8 g.

The results show that cell growth requires a certain initial density of cells and lower inoculum size is inhibitory to growth of suspension cultures. Similar growth inhibition has also been reported for transformed cell suspension cultures of *Coleus forskohlii* (Mukherjee *et al.*, 2000). The inoculum size had a positive effect on biomass and metabolite production up to an optimum concentration (Gorret *et al.*, 2004). In this study, the optimal inoculum size for biomass accumulation and the production of alkaloids was 10% (FW, w/v), which is comparable to those obtained by Chiou *et al.* (2001) for production of polyunsaturated fatty acids in cell suspension cultures of *Marchantia polymorpha*.

Time course of cell growth and alkaloid production in suspension cultures

These suspension cultures showed continuous and stable accumulation of biomass after 4–5 subcultures by transferring 10% (FW, w/v) cells to fresh medium. The time course of cell growth and alkaloid production has been studied by growing suspension cells in 250 ml flasks, containing 80 ml liquid B_5 medium (30 g l^{-1} sucrose). As shown in Fig. 1, cell growth was slow during initial 6 d of cultivation. From day 6, biomass accumulated rapidly and reached the greatest value on day 18, with 14.1 g DW l^{-1}. Then a slow decrease of biomass was observed in the later stage of cultivation. Some cultures continued to grow for up to 30 d, but invariably darkened and appeared less healthy. For alkaloid production, dehydrocavidine production increased stably 6 d after inoculation and resulted in a peak of 8.22 mg l^{-1} on day 18. The time profile of berberine production was similar to that of dehydrocavidine production. The highest pro-

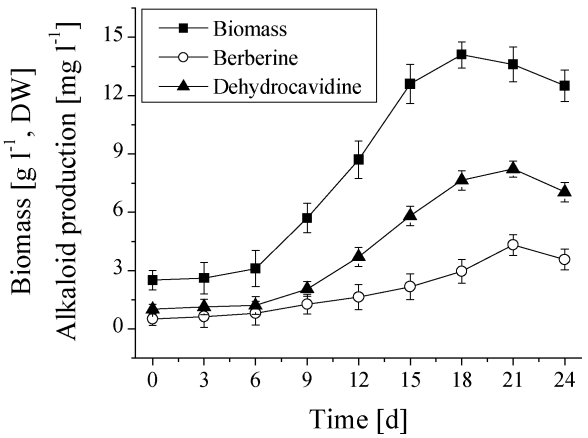

Fig. 1. Time courses of cell growth and alkaloid production in *C. saxicola* suspension cultures. The suspension cells were grown in liquid B_5 medium containing 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} BA and incubated on a rotary shaker at (25 ± 1) °C in the dark. Data indicate means of three independent experiments (means ± S.D.).

Table II. Effects of inoculum size on dry biomass and alkaloid production in *C. saxicola* suspension cultures.

Inoculum mass [g]	Harvest day	Dry biomass [g l^{-1}]	Alkaloid production	
			Dehydrocavidine [mg l^{-1}]	Berberine [mg l^{-1}]
2	24	2.5 ± 0.25	0.51 ± 0.18	0.28 ± 0.07
4	24	5.9 ± 0.37	2.62 ± 0.24	1.50 ± 0.30
6	21	10.8 ± 0.41	5.93 ± 1.18	3.38 ± 0.77
8	21	13.1 ± 0.77	8.02 ± 0.74	4.13 ± 0.35

The suspension cultures were cultivated in B_5 medium containing 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} BA on a rotary shaker. Data are expressed as means ± S.D. ($n = 3$).

duction (4.31 mg l^{-1}) was also produced on day 18. Those alkaloid productions were found to be slightly greater than that in the first suspension cultures (Table II). The cause was the increase of biomass. The culture behavior demonstrated that suspension cells acclimatized themselves to liquid medium and exhibited stable growth. Results obtained here indicate that the favorable subculture method is the transfer of an inoculum size of 10% (FW, w/v) to fresh media every 21 d.

Effects of sucrose concentration on suspension cultures

High sucrose concentration usually resulted in high biomass accumulation and secondary metabolite production of plant cell cultures. In response to increased sucrose concentration from 10 g l^{-1} to 60 g l^{-1}, cultures not only accumulated more biomass but also produced more alkaloids (Table III). On the contrary, biomass accumulation and alkaloid production were significantly decreased when sucrose concentration increased from 60 g l^{-1} to 80 g l^{-1} (Table III). This phenomenon suggests that the cell growth was suppressed by relatively higher initial sucrose concentration, which contributed to higher osmotic pressure (Zhang *et al.*, 1996). Similar suppression was also reported in cell cultures of *Panax ginseng* (Akalezi *et al.*, 1999) and *Camptotheca acuminata* (Pasqua *et al.*, 2005). The optimal sucrose concentration for maximal biomass and alkaloid production was 60 g l^{-1}. In all cases of sucrose concentrations, the pH value of medium presented a similar profile to biomass, which coincided with alkaloid biosynthesis. The results demonstrate that manipulation of sucrose concentration is an effective method to improve alkaloid production in this cell culture, which is in agreement with Zhang *et al.* (1996) and Pasqua *et al.* (2005).

In conclusion, callus and cell suspension cultures of *C. saxicola* were achieved for the first time and provided a homogenous material for the production of dehydrocavidine and berberine. Remarkable improvements of both biomass accumulation and alkaloid production were successfully obtained by manipulating inoculum size and sucrose concentration. The optimal inoculum size and sucrose concentration were 10% (FW, w/v) and 60 g l^{-1}, respectively. This work may be beneficial for further regulation of alkaloid biosynthesis and for enhanced production of valuable alkaloids on a large scale.

Table III. Effects of various sucrose concentrations on suspension cultures.

Sucrose [g l^{-1}]	pH of medium	Dry biomass [g l^{-1}]	Alkaloid production	
			Dehydrocavidine [mg l^{-1}]	Berberine [mg l^{-1}]
10	5.83 ± 0.35	8.09 ± 0.27	5.91 ± 0.38	2.38 ± 0.45
30	6.25 ± 0.42	14.1 ± 0.67	8.22 ± 0.41	4.31 ± 0.53
60	6.53 ± 0.39	18.5 ± 1.52	11.6 ± 0.72	7.57 ± 0.52
80	5.85 ± 0.42	10.6 ± 1.10	6.84 ± 0.36	3.43 ± 0.58

The suspension cultures were cultivated in B_5 medium containing 0.5 mg l^{-1} 2,4-D and 2 mg l^{-1} BA on a rotary shaker and collected on day 21 of cultivation. Data indicate means of three independent experiments (means ± S.D.).

Akalezi C. O., Liu S., Li Q. S., Yu J. T., and Zhong J. J. (1999), Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*. Process Biochem. **34**, 639–642.

An Y. R., Li X. G., Su H. Y., and Zhang X. S. (2004), Pistil induction by hormones from callus of *Oryza sativa in vitro*. Plant Cell Rep. **23**, 448–452.

Buitelaar R. M. and Trapmer J. (1992), Strategies to improve the production of secondary metabolites with plant cell cultures: a literature review. J. Biotechnol. **23**, 111–141.

Chang H. N. and Sim S. J. (1995), Extractive plant cell culture. Curr. Opin. Biotechnol. **6**, 209–212.

Chiou S. Y., Su W. W., and Su Y. C. (2001), Optimizing production of polyunsaturated fatty acids in *Marchantia polymorpha* cell suspension culture. J. Biotechnol. **85**, 247–257.

Gamborg O. L., Miller R. A., and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158.

Gorret N., bin Rosli S. K., Oppenheim S. F., Willis L. B., Lessard P. A., Rha C., and Sinskey A. J. (2004), Bioreactor culture of oil palm (*Elaeis guineensis*) and effects of nitrogen source, inoculum size, and conditioned medium on biomass production. J. Biotechnol. **108**, 253–263.

Hiraoka N., Kato Y., Kawaguchi Y., and Chang J. I. (2001), Micropropagation of *Corydalis ambigua* through embryogenesis of tuber sections and chemical evaluation of the ramets. Plant Cell Tiss. Org. Cult. **67**, 243–249.

Honee G., Buitink J., Jabs T., De Kloe J., Sijbolts F., Apotheker M., Weide R., Sijen T., Stuiver M., and De Wit P. J. (1998), Induction of defense-related responses in Cf9 tomato cells by the AVR9 elicitor peptide of *Cladosporium fulvum* is developmentally regulated. Plant Physiol. **117**, 809–820.

Iwasa K. and Kamigauchi M. (1996), 13-Hydroxylation of tetrahydroberberine in cell suspension cultures of some *Corydalis* species. Phytochemistry **41**, 1511–1515.

Iwasa K., Kuribayashi A., Sugiura M., Moriyasu M., Lee D. U., and Wiegrebe W. (2003), LC-NMR and LC-MS analysis of 2,3,10,11-oxygenated protoberberine metabolites in *Corydalis* cell cultures. Phytochemistry **64**, 1229–1238.

Jiang S. Y., Hu X. H., and Zhao R. F. (2002), Study on the introduction and cultivation of *Corydalis saxicola* Bunting. Guihaia **22**, 469–473.

Ke M. M., Zhang X. D., and Wu L. Z. (1982), Studies on the active principles of *Corydalis saxicola* Bunting. Acta Bot. Sin. **24**, 289–292.

Lee W. L. and Chan L. K. (2004), Establishment of *Orthosiphon stamineus* cell suspension culture for cell growth. Plant Cell Tiss. Org. Cult. **78**, 101–106.

Lu C. T. and Mei X. G. (2003), Improvement of phenylethanoid glycosides production by a fungal elicitor in cell suspension culture of *Cistanche deserticola*. Biotechnol. Lett. **25**, 1437–1439.

Martin K. P. (2003), Plant regeneration through somatic embryogenesis on *Holostemma adakodien*, a rare medicinal plant. Plant Cell Tiss. Org. Cult. **72**, 79–82.

Mukherjee S., Ghosh B., and Jha S. (2000), Establishment of forskolin yielding transformed cell suspension cultures of *Coleus forskohlii* as controlled by different factors. J. Biotechnol. **76**, 73–81.

Nalawade S. M. and Tsay H. S. (2004), *In vitro* propagation of some important Chinese medicinal plants and their sustainable usage. *In Vitro* Cell. Dev. Biol. Plant **40**, 143–154.

Nalawade S. M., Sagare A. P., Lee C. Y., Kao C. L., and Tsay H. S. (2003), Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Bot. Bull. Acad. Sin. **44**, 79–98.

Pasqua G., Monacelli B., Mulinacci N., Rinaldi S., Giaccherini C., Innocenti M., and Vinceri F. F. (2005), The effect of growth regulators and sucrose on anthocyanin production in *Camptotheca acuminata* cell cultures. Plant Physiol. Biochem. **43**, 293–298.

Sagare A. P., Lee Y. L., Lin T. C., Chen C. C., and Tsay H. S. (2000), Cytokinin-induced somatic embryogenesis and plant regeneration in *Corydalis yanhusuo* (Fumariaceae) – a medicinal plant. Plant Sci. **160**, 139–147.

Stella A. and Braga M. R. (2002), Callus and cell suspension cultures of *Rudgea jasminoides*, a tropical woody Rubiaceae. Plant Cell Tiss. Org. Cult. **68**, 271–276.

Steven M. E., Joseph C. K., Vicki M. G., and Daniel A. Y. (2000), Establishment and characterization of American elm cell suspension cultures. Plant Cell Tiss. Org. Cult. **61**, 245–249.

Wen H. Q., Xu Z. R., Villa L. J., and Skog L. E. (1993), A list of threatened limestone plants in south China. Guihaia **13**, 110–127.

Zhang Y. H., Zhong J. J., and Yu J. T. (1996), Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng*: manipulation of medium sucrose. J. Biotechnol. **51**, 49–56.