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Full Length Research Paper

Induction of callus and extraction of alkaloid from Yi Mu Cao (*Leonurus heterophylus* Sw.) culture

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High efficient callus was induced from cotyledon and hypocotyls of *Leonurus heterophyllus* Sw. on Murashige and Skoog (MS) medium containing 2 mg/l 6-benzylaminopurine (6-BA) and 0.5 mg/l 2,4-dichlorophenxyacetic acid (2,4-D). The induction rate reached up to 94.2% in cotyledon explants and 99.2% in hypocotyls. They formed yellowish loose callus when they were subcultured on MS medium with 2 mg/l 6-BA and 0.5 mg/l naphtaleneacetic acid (NAA). Suspension culture system was established as the callus transferred to MS liquid medium supplemented with 2 mg/l 6-BA and 0.5 mg/l NAA, and the maximum biomass is obtained on the 16th day. The content of alkaloid extracted from suspension culture callus was only 0.081%, but the addition of 0.03 mg/l L-proline (L-Pro) could increase the content up to 2.1%.

Key words: *Leonurus* heterophyllus, Callus, suspension culture, alkaloid.

INTRODUCTION

Leonurus heterophyllus Sw. is a native (annual or perennial) herb in China, belonging to Labiatae family (Wu and Li, 1977). This plant is known as Yi Mu Cao in Chinese traditional medicine, and contains many alkaloid and diterpenes (Savona et al., 1982; Hon et al., 1991, 1993; Li and Ca, 2002). The dried aerial parts of this plant are applicable in the treatment of acute nephritis, coronary heart disease, high blood pressure, blood stasis, shrinking and exciting womb (Chinese Materia Medical, 1999), and anti inflammation (Li and Ca, 2002).

Usually, the secondary metabolites were extracted from natural grown plants; however, the content of alkaloid in wild plant is only 0.4% (Chinese Pharmacopoeia Part I, 2000). It is necessary to find an alternative method for alkaloid production and content improvement.

Plant cell suspension culture is an effective alternative way that could be used for large scale culturing of plant cells for secondary metabolites production. This method has been successfully used for secondary metabolites production such as alkaloids, flavanoids and diterpenoids in *Ailanthus altissima*, *Tabernaemontana divaricata*, and *Torreya nucifera* (Anderson, 1987; Sierra et al., 1992; Orihara et al., 2002). So far as we know, there is no report about the tissue culture and suspension culture about *L. heterophyllus* in the literature.

The conditions of callus induction of *L. heterophyllus* were investigated and suspension culture was established. Subsequently, alkaloids were extracted from the suspension cultures and addend was used to test its promotion effect on alkaloid production.

MATERIAL AND METHODS

Materials and culture conditions

Seeds of *L. heterophyllus* were collected from the country of Zhongxian in Chongqing, and cultured on Petri dish for germination with high moisture conditions at 25 ± 1°C. The seedlings germinated from the seeds were soaked in 0.1% $HgCl_2$ for 8 min then in 75% alcohol for 10 s, followed by three sequential rinses for 1 min in sterile distilled water. The cotyledons and hypocotyls were cut into 5 ± 1 mm segments, and used as explants.

The basal medium used was MS (Murashige and Skoog, 1962) medium, supplemented with 3% sucrose and solidified with 0.6% agar. They were placed into conical flasks, each containing 25 ml basal medium with exogenous hormone. The pH of the medium was adjusted to 5.8 prior to autoclaving at 0.15 MPa 121°C for 15 min. The cultures were incubated under florescent lights with 1500 - 2000 lux for 12 h photoperiod at 25 ± 1°C and 80 ± 10 relative humidity.

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Callus induction and subculture

NAA and 2,4-D as auxins and 6-BA as cytokinins were added into MS medium to test their effects on callus formation. The concentration of phytohormone varied from 0.5 - 2.0 mg/l. Each experiment contained at least 40 replicates and the experiments were repeated three times. Data were documented up to 5 weeks of culture.

Well grown callus induced from explants were selected to transfer to MS medium with appropriate hormones for subculture. Cultures were subcultured to the same medium every 15 days. After three times of subculture, the growing states of callus were compared.

Suspension culture

MS basal medium containing 2.0 mg/l 6-BA and 0.5 mg/l NAA without agar were used for suspension culture. About 30 mg callus tissues were transferred from solidified medium into 150 ml conical flasks containing 50 ml of liquid medium. The cultures were incubated on a rotary shaker operated at 120 rpm.

At 2, 4, 6, 8, 10, 12, 14, 16 and 18 days, the suspension cultured callus was harvested by filter and their fresh weights were determined respectively. Then they were dried to constant weight at 60°C for 24 h, and their weights were recorded. Each experiment was conducted three times.

Extraction of alkaloid

Prior to extraction, calli achieved from the suspension culture were dried at 60°C for 24 h, and were ground into powder. The extraction method was modified based on that in Chinese Pharmacopoeia (2000, part I). Precise 3 g callus powder were weighed and put into acute 50 ml 95% alcohol for dipping (Wang et al., 2003). After ultrasonic (350 KW, 35 KHz) treatment for 30 min, they were cooled into room temperature, and their loss weight was made for by 95% alcohol. After filtration, 25 ml filtrate were assimilated and vaporized to dry, and then dissolved by 10 ml 0.1 mol/l HCl. After which, 0.5 g activate carbon was put into the solution for absorption, then they were filtered out. The filtrate was diluted to 50 ml by 0.1mol/l HCL.

The 10 ml extracted solution from callus and 3 ml 2% Reinecks salt were put together and diluted to 25 ml by 0.1 mol/l HCL. After 1 h of incubation on ice, they were filtered and the value of absorbance was determined by ultra spectrophotometer in 520 nm wavelength. All the experiments were conducted 3 times.

Stachydrine sample was used for standard curve test according to Chinese Pharmacopoeia (2000, part I). Extraction from wild plants was tested as the method mentioned above for comparison. 0.01 - 0.05 mg/l lactalbumin hydrolysate (LH) and L-proline (L-Pro) were supplemented to liquid MS medium to test their prompt effects on alkaloid production.

The extraction rate of alkaloid was calculated by the following formula:

$$R = \frac{A \times 50 \times 25 \times 50}{1.209 \times 10 \times 25 \times 3000};$$

or

$$R = \frac{A}{14.508}$$

A is the absorbance, and R is content of alkaloid.

The line type equation of the curve is A = 1.209×C (C = concentration of Stachydrine). All the data were analyzed statistically using SPSS software 11.0.

RESULTS

Callus induction and subculture

The seeds of *L. heterophyllus* began to germinate after 7 days of culture, and elongated to about 5 cm 15 days later. As cotyledons or hypocotyls were cultured on MS medium free of growth regulators, no calli were induced (Table 1). When 2,4-D was used into MS medium, all genotype explants formed white callus (Figure 1a), and induction rates increased with the increase of concentration of 2,4-D. It was found that the addition of high concentration of 6-BA into the MS medium in combination with 2,4-D had prompt effect on callus formation; meanwhile, the callus became pale green in color (Figure 1b). The maximum induction rate was recorded as 94.2% in cotyledon and 99.2% in hypocotyls in MS medium with 0.5 mg/l 2,4-D and 2 mg/l 6-BA. However, MS medium with single 6-BA induced no callus in either cotyledon or hypocotyls explants. The supplement of single NAA could induce callus formation only in hypocotyls explants, but not in cotyledon explants. Although the combination of NAA and 6-BA could induce callus formation in both types of explants, the callus turned brown (Figure 1c).

As previous primary experiments indicated that only high concentration of BA in combination with 2,4-D or NAA in medium was relatively more suitable for subculture (data not shown), the well grown callus were selected for subculture on MS medium with 2.0 mg/l 6-BA in combination with 0.5 - 2 mg/l 2,4-D or with 0.5 - 2.0 mg/l NAA to test their growing state. 45 days later after the callus were subcultured on the MS with 6-BA and 2,4-D, they turned from green to white and became compact (Figure 1d), while callus cultured on MS medium with 6-BA and NAA became yellowish, loose, and spongy (Figure 1e), which was more suitable for continuous subculture and suspension culture. Therefore, MS medium supplemented with 0.5 - 2.0 mg/l NAA and 2.0 mg/l 6-BA was used for subsequent experiments. Through testing, it was found that the MS medium containing 2 mg/l 6-BA and 0.5 mg/l NAA had the maximum effect (769.5%) (Figure 2), and was decided for subculture and cell suspension culture.

Suspension culture

Well-grown callus was subjected to MS liquid medium containing 2 mg/l 6-BA and 0.5 mg/l NAA where it formed loose yellowish callus (Figure 1f), with the biomass examined at 2 days interval. The results showed that the biomass increased with culture prolonging, and reached the optimum value of accumulation, 1509% of the inoculated weight, on the 16th day (Figure 3). Therefore, callus cultured just for 16 days was harvested for alkaloid extraction.

Table 1. The induction of callus from cotyledon and hypocotyls.

No.	Plant growth regulators (mg/l)			Induction rate (% ± SD)[a]		Growing state
	2,4-D	NAA	6-BA	Cotyledon	Hypocotyls	
1	0	0	0.5	0	0	-
2	0	0	1.0	0	0	
3	0	0	2.0	0	0	
4	0.5	0	0	13.3±3.8	13.3±1.3	Pale, compact, hard, granular
5	1.0	0	0	30.8±3.8	20.2±2.5	
6	2.0	0	0	63.3±2.0	39.2±1.3	
7	0	0.5	0	0	5.8±1.2	Yellowish, loose, spongy
8	0	1.0	0	0	15±2.0	
9	0	2.0	0	0	28.3±3.9	
10	0.5	0	0.5	9.2±1.3	35.8±1.4	Pale, compact, hard, granular
11	1.0	0	0.5	15.8±1.2	30.8±3.8	
12	2.0	0	0.5	21.6±1.2	24.2±1.2	
13	0.5	0	1.0	55.8±5.0	50.8±3.8	
14	1.0	0	1.0	50.0±2.5	28.3±2.5	
15	2.0	0	1.0	74.2±3.0	35.0±3.8	
16	0.5	0	2.0	94.2±6.2	99.2±0.2	Pale green, light compact
17	1.0	0	2.0	86.7±2.5	94.2±1.8	
18	2.0	0	2.0	85.8±3.0	90.8±2.5	
19	0	0.5	0.5	60.0±6.2	85.0±2.4	Yellowish, loose, spongy
20	0	1.0	0.5	61.7±2.5	80.8±1.9	
21	0	2.0	0.5	40.0±1.8	70.8±2.0	
22	0	0.5	1.0	77.5±1.7	87.5±1.7	
23	0	1.0	1.0	67.5±2.0	75.8±3.2	
24	0	2.0	1.0	37.5±5.3	71.7±3.1	
25	0	0.5	2.0	87.5±3.9	99.2±1.6	
26	0	1.0	2.0	86.7±1.4	94.2±2.8	
27	0	2.0	2.0	76.7±2.5	90.8±2.5	
28	0	0	0	0	0	-

Extraction for alkaloid

Alkaloid extracted from 16 day suspension cultured callus was 0.081%, much lower than that from the wild plants (0.301%) tested in this experiment and the record (0.4%) in Chinese Pharmacopoeia. Single LH or L-Pro was added into MS liquid medium to test their effects on alkaloid accumulation. It was found that LH and L-Pro had different effects (Table 2). Low concentration of L-Pro gave rise to biomass and alkaloid accumulation, and the concentration of alkaloid reached 2.100% when 0.03 mg/l L-Pro supplemented in medium, while high concentration of L-Pro decrease their accumulation. However, LH had no obvious effects on either biomass or alkaloid formation.

DISCUSSION

The precondition of the suspension culture is to obtain well grown callus. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and usually used together (Gang et al., 2003). Subsequently plant growth regulators were added into MS medium to test their effects on callus formation of cotyledon and hypocotyl explants of *L. heterophyllus*. The results revealed that auxins play an import role in the callus induction (Skoog and Armstrong, 1970) and different types of auxins had various effects (Baskaran et al., 2006). 2,4-D is superior to NAA in callus induction of *L. heterophyllus*. Furthermore, the cytokinins facilitated the effect of auxin in callus induction (Rao et al., 2006). The cotyledon and hypocotyls responded differently to auxins, especially to single NAA supplemented medium of *L. heterophyllus*.

To establish a system of suspension culture, it is necessary to obtain well growing callus. In this work, the callus induced from medium with 2,4-D and 6-BA was light compact, while that from NAA and 6-BA readily turn brown. Wang and Fang (2002) reported that this problem could be resolved via continuous subculture or supplementation with antioxidants. In this study, this problem is solved through transferring the callus to the new medium.

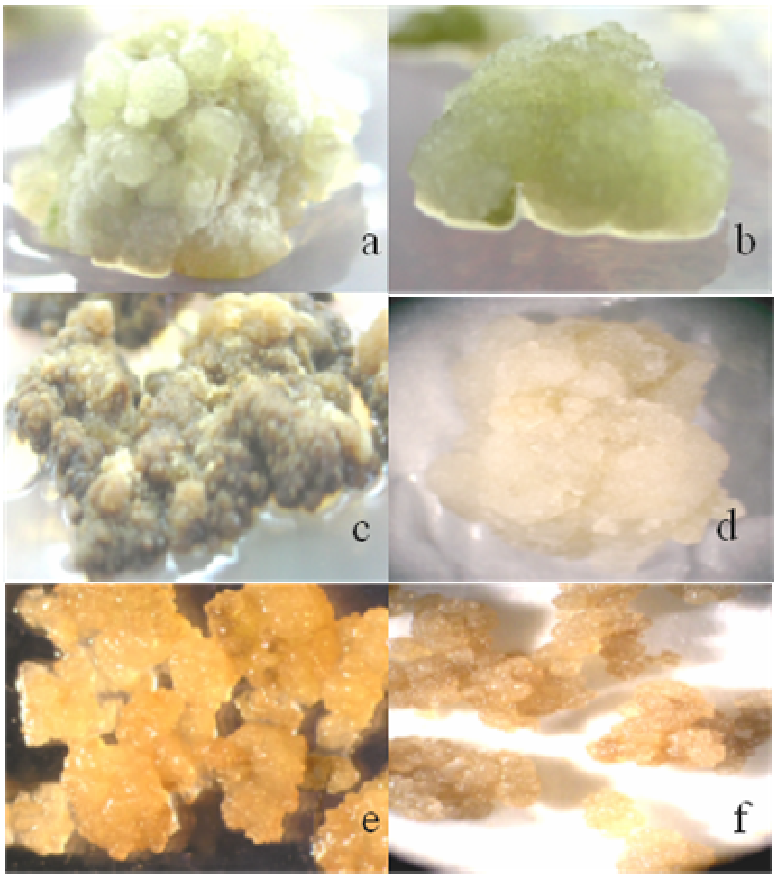

Figure 1. Callus of *L. heterophyllu*. **a.** Pale, compact, hard and granular callus induced with MS medium containing 0.5 mg/l 6-BA and 2 mg/l 2,4-D. **b.** Pale green and light compact callus induced with MS medium containing 2.0 mg/l 6-BA and 2 mg/l 2,4-D. **c.** Yellow and thanatoic callus induced with MS medium containing 1.0 mg/l 6-BA and 2 mg/l NAA. **d.** White and compact callus subcultured on MS medium containing 2 mg/l 6-BA and 2.0 mg/l 2,4-D. **e.** Yellow and loose callus subcultured on MS medium containing 2 mg/l 6-BA and 0.5 mg/l NAA. **f.** Callus cultured in MS liquid medium containing 2 mg/l 6-BA and 0.5 mg/l NAA.

Table 2. Effects of addition of addend to medium on biomass and alkaloid accumulation.

Source	**Concentration (mg/l)**	**FW (g)[a]**	**DW (g)**	**Alkaloid (%)**
W	-	-	-	0.301±0.011
MS	0	0.520±0.11	0.034±0.012	0.081±0.020
MS+LH	0.01	0.525±0.10	0.034±0.012	0.037±0.015
	0.03	0.560±0.13	0.036±0.015	0.072±0.003
	0.05	0.553±0.12	0.036±0.004	0.051±0.026
MS+L-Pro	0.01	0.595±0.016	0.039±0.021	1.127±0.014
	0.03	0.634±0.011	0.041±0.012	2.100±0.016
	0.05	0.351±0.015	0.023±0.016	1.072±0.017

W, Wild plantsl FW, fresh weight of callus; DW, the dry weight of callus.
[a]Each value represents mean±SE of 40 explants in three repeated experiments.

When callus induced by 2,4-D and BA subcultured to the same medium, they turned more compact, but when they were transferred to new medium with NAA and 6-BA, they became light yellowish, loose, and were more suitable for suspension culture.

Similar to previous reports (Khiet et al., 2006), the sus-

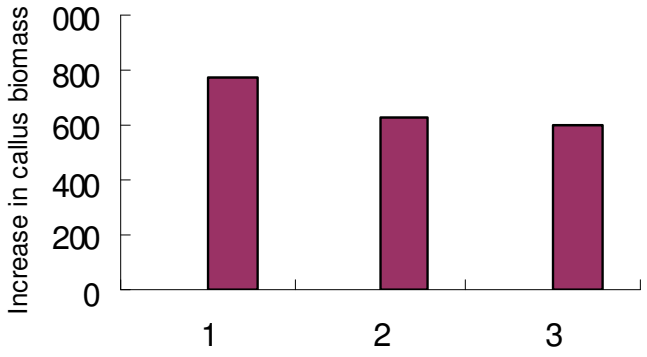

Figure 2. Increase in callus biomass subcultured on MS medium containing 6-BA and NAA. 1: Callus subcultured on MS medium with 2mg/l 6-BA and 0.5 mg/l NAA. 2: Callus subcultured on MS medium with 2mg/l 6-BA and 1.0 mg/l NAA. 3. Callus subcultured on MS medium with 2mg/l 6-BA and 2.0 mg/l NAA.

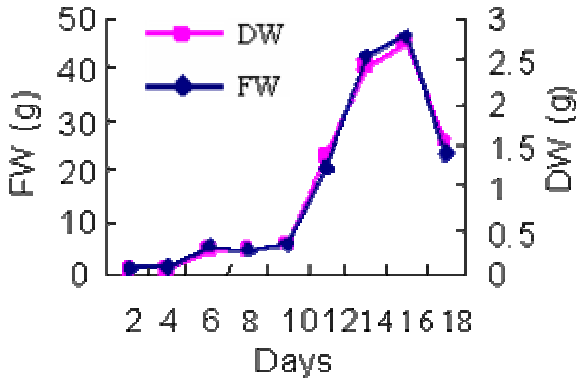

Figure 3. Biomass accumulation of callus culture on MS liquid medium with 2 mg/l 6-BA and 0.5 mg/l NAA. FW: fresh weight; DW: dry weight.

pension culture growth showed lag phase, followed by exponential stage and stationary phases. The lag phase (10 days) of *L. heterophyllus* last longer than that of *Passiflora edulis* (5 days), but its maximum biomass increase (1509%) is much higher than that of *P. edulis* (about 400%).

Numerous research reports exist in the literature about the effects of plant growth regulators on secondary metabolites of *in vitro* cultures. In certain cases, they enhance alkaloid production in several plant species, for example, berberine in *Coptis japonica* (Nakagawa et al., 1986; Ikuta and Itokawa, 1988) and sanguinarine in *papaver somniferum* (Tyler et al., 1989). However, in this study, it seems the plant growth regulators did not have effects on the accumulation of alkaloid, and the content of alkaloid in callus from suspension culture is even lower than that from wild plants.

Many researchers have increased the content of secondary metabolites through adding some addends to the medium. Ketchum (1996) reported that addition of carbohydrate during the growth cycle could increase the production rate of paclitaxel. It was reported that the addition of phenylalanine was found to assist in maximum taxol production in *Taxus cuspidata* cultures (Fett-Neto et al., 1994). In this work, the addition of LH had no effect on the alkaloid accumulation, while the addition of L-Pro increased the alkaloid content prominently. This could be explained by L-Pro being the precursor of stachydrine synthesis (He and Wang, 2005). However, the addition of large amounts of L-Pro could inhibit the accumulation of alkaloid, similar to previous report (Hernandez et al., 2005)

In summary, efficient callus was induced on MS medium with 2 mg/l 6-BA and 0.5 mg/l 2,4-D, and was subcultured on MS medium with 2 mg/l 6-BA and 0.5 mg/l NAA. Suspension culture was established when the was callus transferred to MS liquid medium containing 2 mg/l 6-BA and 0.5 mg/l NAA. The addition of 0.03 mg/l L-Pro promoted alkaloid accumulation in suspension-cultured callus. This work provides a basic information for massive production of alkaloid in *L. heterophyllus.*

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