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In vitro production of benzylisoquinoline from *Stephania tetrandra* through callus culture under the influence of different additives

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ABSTRACT. Plant secondary metabolites have enormous potential for research and new drug development. Many secondary metabolites have complex and unique structure and their production can be enhanced by introducing different types of additives into the basal media. An efficient *in vitro* callus induction system in *Stephania tetrandra* S. Moore (Fan fang ji, an important Chinese medicinal herb) was established on MS medium supplemented with 3% sucrose and different concentrations (0.5 to 2.0 mg/L) of plant growth regulators *i.e.* auxins (2,4D, IAA and NAA) and cytokinins (BA, kinetin, TDZ and zeatin) in the dark. The effects of various auxins and cytokinin on the growth and accumulation of benzylisoquinoline alkaloids *i.e.* Fangchinoline (Fan) and Tetrandrine (Tet) were investigated. MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L TDZ supported callus growth and its proliferation. A maximum amount of dry biomass (7.8 fold) was produced 45 days after culture. High Performance Liquid Chromatographic analysis of methanol extracts from callus revealed an accumulation of Fan and Tet alkaloids. The addition of casein hydrolysate (500 mg/L) and coconut milk (10%), respectively, enhanced Fan and Tet accumulation. The established *in vitro* callus induction system in *S. tetrandra* can be utilized for biomass production of pharmaceutically important alkaloids such as Fan and Tet.

Keywords: Casein hydrolysate (CH); Coconut milk (CM); Fangchinoline (Fan); Peptone; *Stephania tetrandra*; Tetrandrine (Tet).

INTRODUCTION

Stephania tetrandra S. Moore (Fan fang ji) belongs to family Menispermaceae. In traditional Chinese medicines *Stephania tetrandra* is prescribed for its diuretic, antiphlogistic and antirheumatic properties (Huang, 1999). In the Chinese Pharmacopoeia, the herb is recommended for treating general symptoms such as inflammation, fever, pain, edema, stagnant bronchial mucous and for detoxification. The use of Fan fang ji roots has been limited to China. For the past 50 years, animal studies on *Stephania* have been conducted in laboratories throughout the world. Many of its pharmacological properties are relatively well described including: its hypotensive activity (Kawashima et al., 1990), its effect on chronic inflammation by suppressing elevated interleukin-6, a pro-inflammatory cytokine (Kang et al., 1996), its use as a vasodilator (Kim, 1997), an anti-fibrotic (Nan et al., 2000), its anti-proliferative effect on vascular smooth muscle cells (Wang et al., 2000), its use in coronary disease (Yu et al., 2001), retinopathy (Liang et al., 2002), as an anti-hemolytic (Sekiya et al., 2005) and for the reduction of fibrosis-related gene transcription (Hsu et al., 2006).

About 10,000 plant alkaloids have been identified in *Stephania*, many of these pharmacologically active alkaloids are mostly involved in plant defense against pathogens, insects, and herbivores. Their potent toxicity makes alkaloids "privileged" structures for drug development (Leonard et al., 2009). Fan and Tet accumulate in the root of *Stephania tetrandra* and these alkaloids are the dimer of two benzylisoquinoline subunits condensed in a head-to-head, tail-to-tail fashion, with α, β stereochemistry, at chiral isoquinoline carbons through ether linkage (Figure 1a & b). The analyses of these alkaloids are important due to their potentially useful pharma-

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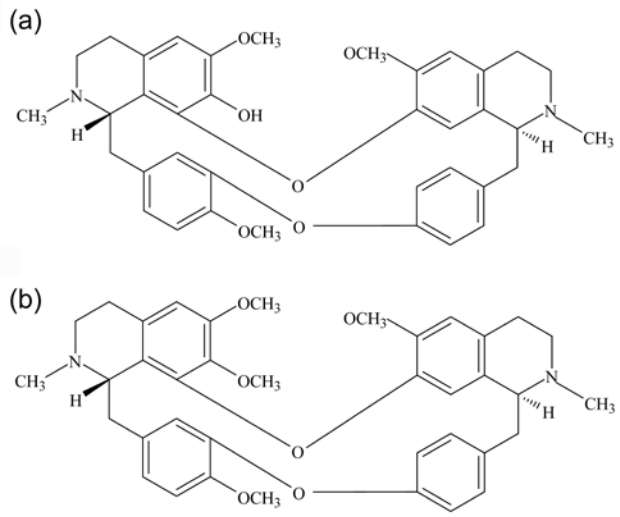

Figure 1. Chemical structure of (a) fangochinoline (b) tetrandrine.

cological activities (Ou, 1992) and methods such as high-performance liquid chromatography (HPLC) (Huang and Hong, 1998), thin-layer chromatography (TLC) (Lin et al., 1993), HPTLC (Blatter and Reich, 2004), capillary electrophoresis (CE) (Yang et al., 1998) non-aqueous CE (Li et al., 2004), flow injection-micellar electrokinetic capillary chromatography (FI-MEKC, Liu et al., 2005) and mass spectrometry (Koh et al., 2006) have been are used to detect the presence of Fan and Tet.

One of the reasons for research using *in vitro* cultures of various plant cells, tissues or organs is their ability to synthesize metabolites in higher concentration under the influence of some additives as compared to whole plants (Panda et al., 1991; Fennell et al., 2003). This becomes an alternative for obtaining products that are difficult to obtain by conventional methods or are not economically viable. Furthermore, plant metabolites can vary due to climatic and seasonal conditions. Thus, it becomes imperative to grow the plants in a controlled environment. The aim of the present work was to establish the mass production of callus and analysis of pharmaceutically important alkaloids such as Fan and Tet under the influence of different additives.

MATERIALS AND METHODS

Plant material and sterilization

Plants of *Stephania tetrandra* S Moore (Han fang ji, Figure 2) were collected from Da-Du Mountain in Taichung County (Taiwan). The explants were washed with running tap water followed by surface sterilization with 70% v/v ethanol for 30‘s’. Explants were then washed three times with sterile distilled water to remove traces of ethanol and sterilized with 0.5% (w/v) sodium hypochlorite for 7 min in an ultrasonic oscillator (15 amplitude). Explants were rinsed five times with sterilized water to remove traces of hypochlorite.

Culture conditions and data analysis

Uniform culture conditions were applied in all experiments. The pH of the media was adjusted to 5.7 before autoclaving. The media was autoclaved for 15 min at 1.05 kg/cm^2 pressure at 121°C. Cultures were incubated at 25±1°C in the dark. All experiments were repeated three times with 20 replicates each time. All data were analyzed using standard applied method.

Callus induction from different explants

Different plant parts (leaf, stem, petiole and root) were used as explants for the callus induction. The explants were cut into small pieces and inoculated into different media *viz*., MS (Murashige and Skoog, 1962), N_6 (Chu et al., 1975), woody plant (Lloyd and McCown, 1980) and B_5 (Gamborg et al., 1966). These media were further supplemented with 3% sucrose and different concentrations (0.5 to 2.0 mg/L) of plant growth regulators: auxins (2,4 diphenoxy acetic acid {2,4-D}, indole acetic acid {IAA}, naphthalene acetic acid {NAA}) and cytokinins (6-Benzyladenine {BA}, kinetin, thiodiazuran {TDZ} and zeatin). The callus formation was observed from the cut surface 10-15 days after culture.

Callus growth determination

Leaf callus stocks were used to determine the growth curves. Pieces of friable callus (~0.2 gm) were inoculated in the MS medium supplemented with 1 mg/L of BA, 0.5 mg/L TDZ and 3% sucrose in the dark at 28±1°C. Weight of growing callus was recorded at an interval of four days for 45 days and a growth curve was plotted based on the fresh weight of callus over the respective time period.

Enhanced callus formation

The growth of callus was further enhanced by supplementing the previous media with 250-750 mg/L casein hydrolasate (Fluka analytical. USA), 5-20% coconut milk (Vegetable market, Taichung) and 1.0-4.0 g/L peptone

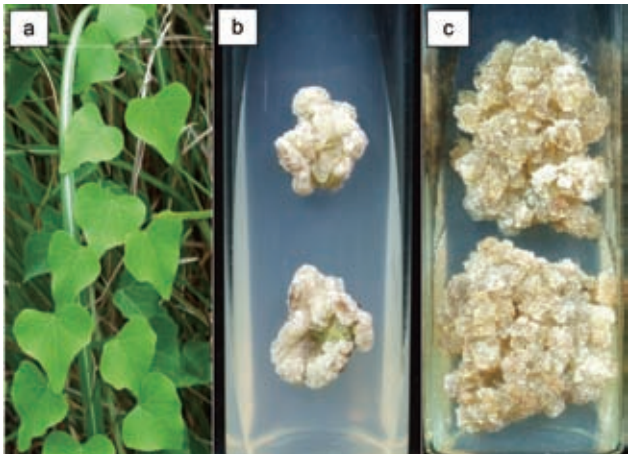

Figure 2. Callus induction and proliferation in *Stephania tetrandra.* (a): Plant in natural habitat; (b): Callus induction from leaf; (c): Proliferated callus.

extract (Amresco, USA). Enhanced callus growth was recorded six weeks after culture. The callus was maintained by sub-culturing every four weeks.

HPLC Analysis

Reagents and materials. HPLC-grade methanol, acetonitrile, diethylamine and the reversed-phase column (Lichrospher 100 RP-18, 5 µm, 250×4.6 mm were purchased from Merck (Germany).) Purified water was obtained from Milli Q system from Millipore (Milford, MA, USA).

Apparatus and conditions. HPLC was performed on a Waters Model 2695 equipped with an auto sampler 717+ (injection volume 20 µl) and 2996 Photodiode Array Detector (Waters). Detection wavelength was set at 241.4 nm and 281.4 nm for Fan and Tet. The separations were obtained with a reversed-phase column Lichrospher 100 RP-18, 5 µm, 250×4.6 mm (Merck, Germany). The samples were eluted at the rate of 1 ml/min using mobile phase (methanol: acetonitrile: diethylamine 3:1:1).

Preparation of standard solution and calibration. Standards Fan (98%) and Tet (98%) were purchased from Jiuding company (Shijiazhuang, China) and Sigma (St. Louis, Madison, USA) respectively. Stock standard solutions of Fan and Tet was prepared in methanol to the final concentration of 1 mg/ml. Calibration curves were established based on five points covering a concentration range of 12.5-250 µg/ml both for Fan and Tet. The standard solution (20 µl) was used for HPLC injections (n = 5). Calibration graphs were plotted based on linear regression analyses of the responses in peak areas in response to concentrations of standards injected. The repeatability of the migration time and peak area of Fan and Tet in the experiment was determined by repeated injection (n = 5) of a standard mixture solution of 12.5-250 µg/ml under the optimum conditions. The limit of detection (LOD) for Fan and Tet were 0.02 and 0.025 µg/ml respectively, whereas the limit of quantification (LOQ) for Fan and Tet were 1.0 and 1.4 µg/ml respectively. Their correlation coefficients of regression were 0.983 and 0.997.

Table 1. Callus induction efficiency in different explants of *Stephania tetrandra*.

Explants*	No. of explants cultured	No. of plants induced callus	% Efficiency**
Leaf	30	30	100[a] ***
Petiole	30	27	90[b]
Root	30	04	13.3[d]
Stem	30	26	86.7[c]

*Basal medium: MS salts with 1.0 mg/L BA, 0.5 mg/L TDZ and 3% sucrose pH=5.7 ± 0.1.
**Data collected from explants were cultured for 45 days.
***Different letter within a column indicate significant differences at 5% level by LSD (least significant difference) test.

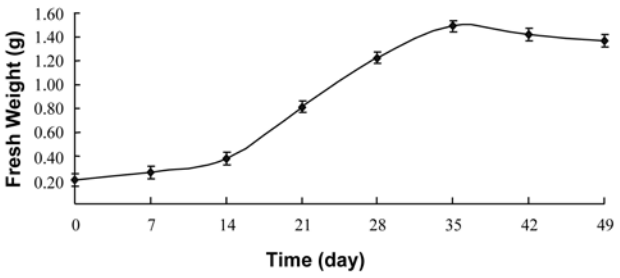

Figure 3. Growth curve of *S. tetrandra* callus cultures. Fresh weight (g). Vertical bars denote ±S.E.

Sample preparation from callus for HPLC. The callus was collected from the culture bottle and their fresh weight was recorded. The callus was freeze-dried for dry weight determination. The weight of dried callus was recorded later and crushed into fine powder. About 1.0 g of powdered callus was accurately weighed and ultra-sonicated with 10 ml methanol for 15 min. The process was repeated three times for each sample. After filtration, the combined methanol extracts were evaporated to dryness by a rotary evaporator. The residue was dissolved in 10 ml methanol and filtered by 0.45 µm (Nalgene®, New York, USA) membrane before analysis.

RESULTS

Different plant parts (leaf, stem, petiole and root) were cut (measuring 0.5 cm in length) and used as explants for the callus induction. In the responding medium, leaf disk explants hardened and turned bright green, and compact calluses were developed from the leaf disk margins two weeks after culture.

Our preliminary results showed that among leaves, stems, petioles and roots, the callus induction frequency was 100% in leaves (Table 1). A growth curve (Figure 3) was established between days of proliferation and total callus mass gain with 20 replicates. Callus induction occured after 14 days (Figure 2-b) and it grew well for up to 37 days (Figure 2-c). Hence, in subsequent experiments, leaves were selected as explants for further callus induction and callus were harvested after 45 days. All experiments were repeated three times with 20 replicates and the data evaluated. Methanol extracts of dried callus were analyzed using HPLC (Figure 4) and their alkaloid contents were calculated with the help of a graph plotted for standard.

The primary goal of *in vitro* callus culturing was to achieve maximum alkaloid accumulation in the callus. Thus, different parameters were studied to enhance callus production and benzylisoquinoline accumulation (Fan and Tet). These parameters and their HPLC analysis were discussed simultaneously.

Influence of different parameters on callus production

Approximately 200 mg of fresh callus was used as the

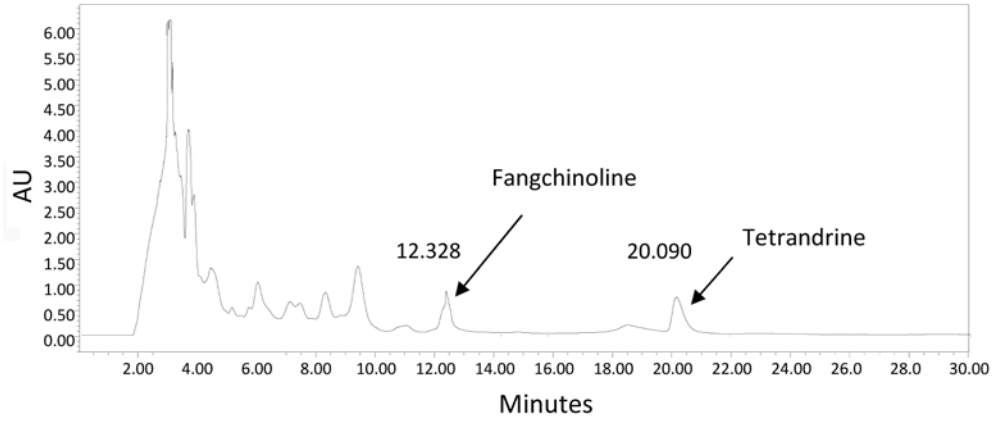

Figure 4. HPLC Chromatogram of fangchinoline and tetrandrine in callus cultures derived from the leaf of *Stephania tetrandra.*

Table 2. Individual effect of different growth parameters on callus culture in *Stephania tetrandra*.

Different parameter*	Different constituent	Weight of the callus (g)
Physical condition	Light	1.449[b]
	Dark	1.562[a]
Media	MS	1.572[a]
	B_5	1.457[b]
	N_6	1.260[c]
	WPM	1.260[c]
MS strength	½	1.214[b]
	1	1.563[a]
	2	0.734[c]
Sucrose	1%	1.008[c]
	3%	1.571[a]
	5%	1.154[b]
	7%	0.679[d]
Auxin (1 mg/L)	2-4D (4.12 µM)	1.595[c]
	IAA (5.71 µM)	1.645[b]
	NAA (5.37 µM)	1.733[a]
Cytokinins (1 mg/L)	BA (4.44 µM)	1.119[b]
	Kinetin (4.44 µM)	0.409[e]
	TDZ (4.54 µM)	1.315[a]
	Zeatin (4.56 µM)	0.859[c]
TDZ (mg/L)	0.5 (2.27 µM)	1.459[a]
	1.0 (4.54 µM)	1.349[b]
	2.0 (9.08 µM)	1.056[c]

*Data collected from the 200 mg of the callus were cultured for 45 days with varying concentrations of different constituents.
**Different letter within a column indicate significant differences at 5% level by LSD (least significant difference) test.

initial material and transferred to MS media supplemented with 3% sucrose, 0.9% agar, 4.44 µM BA and 2.27 µM TDZ. Half of the replicates were grown under continuous cool white fluorescence lamps (100 µE/m^2s), the other half of the callus were grown in complete darkness at 25 ±1°C. The average weight gain of callus grown in light and dark was 7.25 and 7.80 fold respectively. The Fan content was double (0.203 mg/g) in dark-grown callus compared to light-grown (0.1 mg/g), whereas 0.090 mg/g Tet was detected in dark grown callus and no Tet was detected in light grown callus. Thus we concluded that a dark environment favored callus formation and promoted Fan and Tet accumulation.

Four different media (MS, B5, N6 and WPM) were used to grow the callus and their fresh weight was measured 45 days after culture. The average weight gain was 7.75 fold on 1X MS, whereas, it was 6.3, 7.3 and 6.9 fold on B5, N6 and WPM media respectively. The accumulation of Fan and Tet was 0.203 and 0.101 mg/g respectively in 1X MS media which was comparatively higher than in the other media (Table 2). Thus, MS was the most suitable basal salt media for callus multiplication and Fan and Tet accumulation.

Callus was grown in different MS strengths (½, 1 and 2X), and 1X MS was the most suitable for callus proliferation (7.75 fold) compared to ½ X and 2X MS, where the average weight gains were 6.0 and 3.7 fold respectively. The callus grown on 1X MS accumulated higher amounts of Fan (0.203 mg/g) and Tet (0.097 mg/g), which was comparatively higher than the other MS strengths (Table 2).

Sucrose in culture medium functions both as a carbon source and as an osmotic regulator. Both functions are critical for embryoid and callus formation (Last and Brettell, 1990). Different concentrations of sucrose 1, 3, 5 and 7% were used to grow the callus. The maximum average weight gain was 7.85 fold with 3% sucrose. Whereas, the average weight gain was 5.0, 5.8 and 3.5 fold respectively with 1, 5 and 7% of sucrose (Table 2). Fan and Tet production was 0.203 and 0.087 mg/g respectively with 3% of

sucrose, which was comparatively higher than the other sucrose concentrations (Table 2). Thus, 3% sucrose favored callus formation and Fan and Tet accumulation.

Different plant growth regulators i.e. auxins and cytokinins, were used to study their effect on callus growth, the maximum weight gain was 8.7, 8.3 and 7.9 fold respectively with 5.37 µM NAA, 4.12 µM 2, 4-D and 4.90 µM IBA. In different cytokinins, maximum callus was obtained with 4.54 µM TDZ followed by 4.44 µM BA, 4.56 µM zeatin and 4.65 µM kinetin, the average weight gains were 6.5, 6.0, 4.3 and 2.0 fold respectively. No Fan and Tet were detected in auxin-derived callus, whereas cytokinin-derived callus favored Fan and Tet accumulation; 4.54 µM TDZ and 4.44 µM BA accumulated 0.191, 0.192 mg/g Fan and 0.083, 0.087 mg/g Tet respectively. This was comparatively higher than in the other cytokinins used (Table 2). Thus, the best plant regulator for callus production and alkaloid accumulation were the cytokinins (BA and TDZ).

In further experiments three different concentrations (2.27 µM, 4.54 µM and 9.08 µM) of TDZ was used to grow the callus. Maximum callus was obtained using 2.27 µM TDZ, followed by 4.54 µM and 9.08 µM of TDZ and their average weight gains were 7.3, 6.75 and 5.0 fold respectively. The 2.27 µM TDZ favored Fan (0.199 mg/g) and Tet (0.085 mg/g) accumulation over other concentrations of TDZ (Table 2).

The different parameters which could influence callus growth were determined one by one. The 1X MS media supplemented with 3% of sucrose, 1.0 mg/L BA (4.64 µM) and 0.5 mg/L TDZ (2.27 µM) grown in the dark produced maximum callus and accumulated higher Fan (0.203 mg/g) and Tet (0.090 mg/g). Callus formation and higher Fan and Tet yields were further enhanced by the addition of different additives, *i.e.* peptone, casein hydrolysate (CH) and coconut milk (CM). The addition of 1, 2 and 4 g/L peptone into media yielded maximum callus with 2 g/L (0.2%), followed by 4 and 1 g/L of peptone. Their average weight gains were 13.5, 13.0 and 10.6 fold respectively. The higher concentration of peptone (4 g/L) promoted Fan accumulation (2.27 mg/g), whereas, the lower concentration (1 g/L) promoted Tet accumulation (0.220 mg/g, Figure 5). The addition of 250, 500 and 750 mg/L CH into media yielded maximum callus with 500 mg/L (0.05%) of CH, followed 750 and 250 mg/L. Their average weight gains were 9.7, 9.5 and 8.25 fold respectively. The higher concentration of CH (750 mg/L) promoted Fan accumulation (0.660 mg/g), whereas, the lower concentration of CH (250 mg/L) favored Tet (0.230 mg/g) accumulation (Figure 6). The addition of 5, 10 and 20% CM into media yielded maximum callus with 10% of CM, followed by 20% and 5%. Their average weight gains were 9.35, 8.9 and 8.6 fold respectively. HPLC analysis indicated that as the concentration of CM increased from 5, 10 to 20%, Fan content also increased 0.152, 0.274 to 0.399 mg/g, respectively. The maximum Tet (0.392 mg/g) accumulation was recorded with 10% CM (Figure 7).

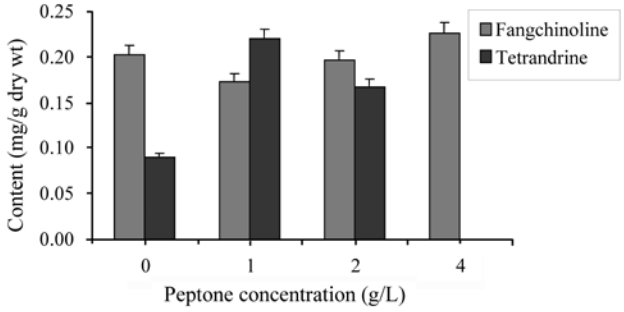

Figure 5. Influence of peptone concentration on the accumulation of fangchinoline and tetrandrine in *S. tetrandra* callus. X-axis: Concentration of peptone (g/L); Y-axis: Alkaloid content (mg/g). Experiments were replicated three times. Vertical bars denote ±S.E.

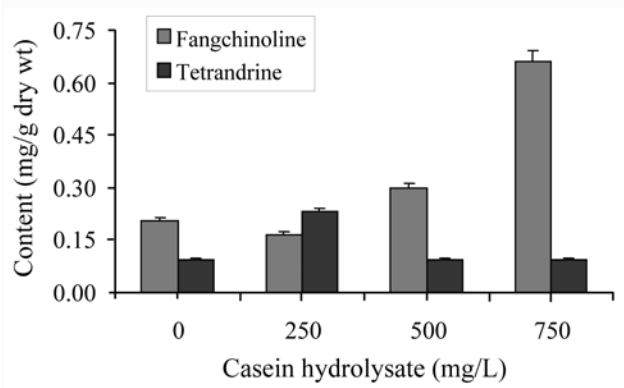

Figure 6. Influence of casein hydrolysate concentration on the accumulation of fangchinoline and tetrandrine in *S. tetrandra* callus. X-axis: Concentration of casein hydrolysate (g/L); Y-axis: Alkaloid content (mg/g). Experiments were replicated three times. Vertical bars denote ±S.E.

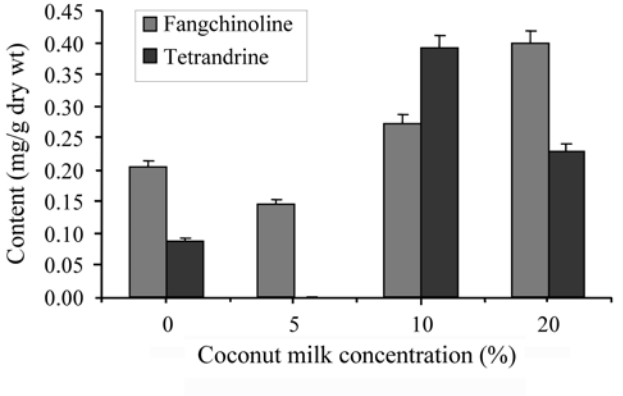

Figure 7. Influence of coconut milk concentration on the accumulation of fangchinoline and tetrandrine in *S. tetrandra* callus. X-axis: Percentage of coconut milk; Y-axis: Alkaloid content (mg/g). Experiments were replicated three times. Vertical bars denote ±S.E.

DISCUSSION

The aim of the present work was to optimize the callus culture of *S. tetrandra* for biomass production and to maximize alkaloid content in callus. These alkaloids have potential pharmacological importance (Kang et al., 1996; Nan et al., 2000; Wong et al., 2000; Liang et al., 2002; Sekiya et al., 2005 and Hsu et al., 2006). Different parameters were tested one by one and the best media and additives were selected to promote maximum callus growth and alkaloid accumulation. The yield of many secondary products in plant tissue cultures are influenced by the composition of the nutrient medium, particularly the type and concentration of the plant growth regulators, the physical conditions under which the cultures have grown and the physical state of the culture itself. In *in vitro* culture, quantitative and qualitative nutritive requirements may vary with different plant materials and in some instances with different tissues of the same species. Explants type and anatomical structure seems to play significant role in *Stephania* callus initiation. Variations in the callus forming ability of different explant types, has been reported in many plants (Ishii et al., 2004; Zouine and Hadrami, 2004). Different explants (stem, root, leaf and shoot apex) were placed in the medium to compare their growth responses. The leaf segments responded better to callogenesis than the other selected explants (Table 1). Callus from leaf segments showed initiation of vigorous, proliferating, soft and green colored tissue. Similar response has been reported for *Nigella sativa* (Chand and Roy, 1980). Callus induction favored a dark environment rather than the light environment favored in tobacco (Lance et al., 1976). Like *Stephania*, dark has induced callus in tomato anther (Jaramillo and Summers, 1991) and have higher fresh weight gain in the dark than in light. Although there was no remarkable difference in the fresh weight gain, but darkness induced rapid callus growth in *Stephania*. In some plants, such as *Macuna pruriens*, light has favored culture growth (Brain, 1976) or, have an effect on morphogenic capacity (Segura and Calvo, 1991). Light can also have an inhibitory effect on growth eg. *Solanum eleagnifolium* (Guilietti et al., 1991).

One of the most important factors governing callogenesis is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Different basal media respond differently to callogenesis due to differences in their nutrient composition (Mandal and Gupta, 1997; Obert et al., 2004). Several media formulations have been commonly used for the majority of cell and tissue culture work. These media formulations include those described by Murashige and Skoog (1962), Gamborg et al. (1966), McCown (Lloyd and McCown, 1980). Murashige and Skoog's medium (MS) and Gamborg's medium (B5) are all highly concentrated in macronutrients. There was significant difference between calli formed among media. MS medium showed the highest percentage and dry weight of the callus followed by B5 medium and WPM medium (Table 2). This basic formulation was suitable to obtain vigorous callus. This reactivity difference of MS medium was seems to be in relation to the calcium and nitrogen concentrations. In effect, the MS have eight and four fold higher calcium and nitrogen levels than the B5 medium (Zouzou et al., 2000). Inorganic nitrogen has a determining action on callogenesis (Trolinder and Goodin, 1987; Grimes and Hodges, 1990) and this probably explains the differences of callus dry weight on MS and other media. Consequently, MS medium was used for the following studies. Khatun et al., 2003 have reported that MS was the most suitable media for callus induction on different varieties of rice. *S. tetrandra* grown on different basal salt media obtained maximum callogenesis using lX MS. 2X MS have favored callogenesis in *Psidium guajava* L. (Yang and Lu, 2006). The nutrient composition of MS favored more callus growth, but if the nutrient content varied from optimal, it inhibited growth. In *S. tetrandra* callus growth was most robust with 1X MS, as MS strengths were adjusted to lower (½X) or higher (2X) level, growth was inhibited.

Sugars have influenced cellular proliferation and differentiation according to Swankar et al. (1986). The effect of sucrose concentration on the callus induction frequency might be due to its contribution to the osmotic potential of the medium rather than its utilization as a carbon source. Sucrose in culture medium functions both as a carbon source and as an osmotic regulator. Both the functions are critical for embryoids and callus formation (Last and Brettell, 1990). Sucrose rapidly hydrolyzed to glucose and fructose, nearly doubling the osmolality of the medium. Sucrose has been used as major carbohydrate source in the induction medium. In rice anther culture, higher sucrose levels have not only promoted the induction and growth of callus but also useful in organogenesis (Chang, 1978). Higher callogenesis have been reported in the rice cultivar, where, callogenesis was more in response to lower concentrations of the sucrose than the higher concentrations, where it shows an inhibitory effect (Al-Khayri and Al-Bahrany, 2002). *S. tetrandra* callus showed similar response with sucrose, it was more responsive at lower concentration (3%) but inhibited at higher concentration (Table 2).

A range of plant growth regulators were tested for callus initiation using leaf, petiole, root and stem segments. The results indicated that all treatments induced callus (Table 1). However, differences were observed on the basis of hormone concentration and nature of the explants. The induction percentage of callus initiation, their dry weight and alkaloids accumulation were increased with 4.44 μM BA + 2.27 μM TDZ. Although 5.71 μM IAA and 5.37 μM NAA induced callus in a much more robust way, callus could not accumulate any alkaloids under the influence of these plant growth regulators (Table 2). Auxins and cytokinins are the most widely used plant growth regulators and have been used together (Gang et al., 2003). The current investigation suggests that only auxin (Trolinder and Goodin, 1987) or cytokinin (Zhang et al., 2001) was necessary

in obtaining callus but cytokinins favored alkaloid accumulation (Garnier et al., 1996).

Peptides and amino acids could be more efficient sources of nitrogen for sustaining *in vitro* callus proliferation than the usual inorganic nitrate and ammonium salts. At an efficient concentration, organic and inorganic nitrogen sources have promoted the growth of explants (Chen and Chang, 2002). A 0.2% concentration of peptone, as an inorganic nitrogen source has promoted both shoots and root regeneration in *Persea americana* (Nhut et al., 2008). Addition of peptone into the culture medium has strongly improved the growth of tobacco callus (Parc et al., 2007). In this study, the addition of peptone (1, 2 and 4 g/L) promoted callus growth, but had not affected Fan and Tet (Figure 5) accumulation when added at higher concentration. However, lower concentration (1g/L) promoted Tet accumulation (0.220 mg/g, i.e. 2.5 fold, Figure 5).

Casein is a milk protein and a rich source of amino nitrogen. Casein acid hydrolysate, a hydrochloric acid hydrolysate of casein has added to media primarily because of the organic nitrogen and growth factor components. The use of CH as an organic nitrogen source in the culture medium has been found to improve the growth of some tissues, such as *Taxus* spp. (Gibson et al., 1993). Addition of CH to basal media has increased the callogenesis frequency in deepwater rice (Khaleda and Al-Forkan, 2006). The addition of CH has promoted both callogenesis and alkaloid accumulation in *Catharanthus roseus* (Ahmed et al., 2000). Similar response was recorded in *S. tetrandra* callus. At higher concentration (750 mg/L) more Fan (3.25 fold), whereas, at lower concentration (250 mg/L) more Tet (2.55 fold) was accumulated (Figure 6).

CM is an undefined nutrient media which has been used as an additive to produce callus mass in *Gynura aurantiaca* (Roche et al., 1986). Total biomass and alkaloid contents have increased in *Trigonella foenum-graecum* with the addition of CM (Oncina et al., 2000). Coconut-containing media have accumulated higher alkaloids (Barberine) in *Arcangelisia flava* callus (Yanpaisan, 1989). Similar response was recorded with the addition of CM in the callus media, it promoted both, callus growth as well as alkaloid accumulation. About 20% CM promoted Fan accumulation (0.399 mg/g), whereas, 10% CM favored Tet (0.392 mg/g) accumulation (Figure 7).

CONCLUSION

The higher accumulations of active metabolites in cultured cells than in native plants could be acquired through the optimization of cultural conditions, for e.g. anthraquinone from *Ophiorrhiza pumila* (Kitajima et al., 1998), harringtonine and homoharringtonine from *Cephalotaxus fortune* (Zhang, 1998), rosmarinic acid from *Coleus blumei* (Ulbrich et al., 1985) and ubiquinone -10 from *Nicotiana tabaccum* (Matsumoto et al., 1981). The results obtained from additives on benzylisoquinoline accumulation showed peptone (a source of inorganic nitrogen), CH (a source of organic nitrogen) and CM (a source of sugars and fat) enhanced biomass production and alkaloid accumulation. Benzylisoquinoline (Fan and Tet) accumulation was greater with an organic nitrogen supplement than with an inorganic one in the media, however, the undefined saturated fat and sugar content of CM promoted Tet accumulation (5 fold) at moderate concentration and accumulated both Fan and Tet (2.0 and 2.8 fold) at higher concentrations. Leaves were the most suitable explants for callus induction in *S. tetrandra*. Callus grew well in the dark on 1X MS media supplemented with 3% sucrose, 0.9% agar and different cytokinins (4.44 µM of BA and 2.27 µM TDZ). Since the demand for plant based raw materials for pharmaceuticals is ever increasing, the present study can be used as a guide for the *in vitro* production of the active alkaloids in *S. tetrandra*. So far, there is no known report of *S. tetrandra* alkaloid production using callus. This is the first successful attempt to enhance the secondary metabolites content into the callus of *S. tetrandra* where the production level could be manipulated by the use of different additives.

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利用粉防己癒合組織在不同添加物作用之下生產生物鹼

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植物二次代謝物在新藥研究及開發上有極大的潛力。很多植物二次代謝物具有複雜和獨特的結構，可經由組織培養方法，添加不同種類物質於 MS 基礎鹽類培養基中以提高其產率。粉防己 (*Stephania tetrandra* S. Moore) 為常用且重要的中草藥，本研究利用植物組織培養技術，建立粉防己癒合組織之誘導及繼代培養系統，結果顯示在 MS 基礎鹽類培養基中添加 3% 蔗糖和不同濃度 (0.5 to 2.0 mg/L) 的植物生長調節劑，如生長素 (2,4-D, IAA and NAA) 和細胞分裂素 (BA, kinetin, TDZ and zeatin)）於暗培養中，可建立其高效之癒合組織培養系統。探討各種生長素和細胞分裂素對癒合組織的生長和二次代謝產物基異喹啉 (benzylisoquinoline) 生物鹼，如防己諾林鹼 (fangchinoline) 及粉防己鹼 (tetrandrine) 的累積之影響，結果顯示在 MS 基礎鹽類中添加 1.0 mg/L BA 及 0.5 mg/L TDZ 之固體培養基，最有利於癒合組織增殖；暗培養 45 天，可得最高乾重生物產量 (7.8 倍)。以高效液相層析儀分析粉防已癒合組織的甲醇提取物之防己諾林鹼 (fangchinoline) 及粉防己鹼 (tetrandrine) 的含量，結果顯示培養在 MS 基礎鹽類中添加 500 mg/L 水解酪蛋白及 10% 椰子汁，最有利於癒合組織生物鹼的生成；培養基中分別添加水解酪蛋白及椰子汁，可得最大量的防己諾林鹼 (fangchinoline) 及粉防己鹼 (tetrandrine)。本研究所建立粉防己癒合組織之誘導及培養系統，可用來量產藥用上重要之生物鹼，如防己諾林鹼 (fangchinoline) 及粉防己鹼 (tetrandrine) 等。

關鍵詞：粉防己；粉防己鹼；防己諾林鹼；水解酪蛋白；椰子汁。