J. Plant Biotechnology(2003) Vol. 5(2). pp. 131~135

Journal of Plant Biotechnology

Selection of Cell Source and the Effect of pH and MS Macronutrients on Biomass Production in Cell Cultures of Tongkat Ali (*Eurycoma longifolia* Jack)

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Abstract

Callus and cell suspension cultures of *Eurycoma longifolia* Jack were initiated from leaves of different trees. The leaf explant of tree Eu9 produced the most calli and also induced high cell biomass in the cell suspension culture. Optimum production of cell biomass could be initiated in proliferating culture medium with a pH of 5.75 prior to autoclaving. The effects of macronutrient inorganic salts of Murashige and Skoog (MS) liquid medium supplemented with X on production of cell biomass of *Eurycoma longifolia* were also investigated. The highest cell biomass was produced in MS medium containing macronutrients of 21 mM NH_4NO_3, 12.25 mM KNO_3, 3.00 mM $CaCl_2.2H_2O$, 0.575 mM $MgSO_4.7H_2O$, and 1.83 mM KH_2PO_4. A new medium labeled as TAM was formulated for the production of *Eurycoma longifolia* cell biomass in the cell suspension culture.

Key words: Cell suspension, Cell Biomass, *Eurycoma longifolia*, Macronutrients, Tongkat Ali

Introduction

Eurycoma longifolia Jack, a medicinal plant belonging to the Simaroubaceae family, is cultivated widely in Malaysia, Indonesia, Thailand and Vietnam. In Malaysia, *Eurycoma longifolia* is commonly known as 'Tongkat Ali'. It has been used as a traditional medicine for a long time (Nooteboom 1972). This plant produces quassinoids, canthin-6-one and its derivatives, squalane derivatives, tirucallane-type triterpenes as the primary and secondary metabolites (Chan et al. 1986; Chan et al. 1989; Darise et al. 1982; Kardono et al. 1991; Itokawa et al. 1991; Itokawa et al. 1992; Morita et al. 1993). These active compounds were reported to possess anti-malaria, cytotoxic, aphrodisiac and anti-ulcer activities (Ang et al. 1997, Chan et al. 1986, Kardono et al. 1991, Tada et al. 1991).

Limited supply of plant materials such as root and bark for the recovery of active compounds has stimulated researchers to develop alternative *in vitro* methods for the production of the active compounds. Studies on the production of active compounds from cell suspensions culture of *Eurycoma longifolia* have been carried out in Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, USM since 1996. Luthfi (2000) and Ong (1999) reported those alkaloids and others active compounds of Tongkat Ali could be detected in callus, root tissues and cell culture of *Eurycoma longifolia*.

In the present study, elite lines were selected to produce high yield of cell biomass that will be used as the cell source for secondary metabolites production in further research in the near future. A detailed examination of the Murashige and Skoog (MS) (1962) macronutrients constituents was conducted to optimize the concentration of the inorganic salt that could affect the production of cell biomass of *Eurycoma longifolia* Jack. The effect of culture medium pH on *Eurycoma longifolia* cell suspension cultures was also investigated.

Materials and Methods

Callus culture for selection of cell source

Callus tissues were initiated from leaves of nine different trees that were named as Eu1, Eu2, Eu4, Eu5, Eu6, Eu7, Eu8, Eu9 and Eu12. The leaves were collected randomly from each

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Received Mar. 5, 2003; accepted Apr. 13, 2003

of the 2 year-old trees that were planted in the campus of Universiti Sains Malaysia. The leaves were sterilized in 13 % Clorox® solution with continuous agitation for 15 minutes. After rinsing three times with sterile distilled water, the leaves were then sterilized again with 5 % Clorox® for 5 minutes followed by rinsing three times with sterile distilled water. The cleansed leaf explants were cut into 0.7 cm × 0.7 cm under aseptic condition and then inoculated into test tubes containing MS (Murashige and Skoog 1962) medium supplemented with 30 g/L sucrose and 10 mg/L NAA (1-naphthaleneacetic acid).

After ten weeks of culture, calli induced from the leaves of each tree were subcultured and maintained in 25 ml Erlenmeyer flask of same fresh medium every four week. Growth of callus from different tree sources (lines) was monitored based on the fresh weight and dry weight of the callus after 4 weeks of culture. Ten samples were used for each line. The data was analyzed by two-way analysis of variances (ANOVA) and comparisons of the mean weight were carried out by the Tukey's HSD Test at p=0.05 for selection of the best explant sources for callus production of Tongkat Ali. The induced callus was used as material for the preparation of Tongkat Ali cell culture.

Preparation of cell culture

Cell suspension culture of Tongkat Ali was initiated by transferring onegram friable callus of each selected line into 100 mL Erlenmeyer flask containing 20 mL MS liquid medium + X which was previously formulated (X was not revealed for possible future patent purpose). After 3 weeks one-gram of cell was collected from each cell line and transferred into the same liquid medium. Six samples were used for each cell line. Cell biomass was determined after 14 days of culture. The data were analyzed by two-way ANOVA followed by comparisons of the mean with the Tukey's HSD Test at p=0.05 for determining the best cell line for cell biomass production of Tongkat Ali.

Effect of pH of culture medium on cell biomass production

Cell line Eu9 of *Eurycoma longifolia* used in this experiment was having the most rapid growth. One-gram cell biomass of Eu9 was cultured in 100 mL Erlenmeyer flask containing 20 mL MS liquid medium + X with different pH. The pH of culture medium was adjusted with 0.1 N NaOH and 0.1 N HCl until the desired pH was obtained. The cultures were maintained on a gyratory shaker (G10 Gyrotory Shaker®, New Brunswick Scientific, N.J. U.S.A.) at 130 rpm at 25±2°C with 24 hour photoperiods of 1500 lux. Five different pH (4.75, 5.25, 5.75, 6.25 and 6.75) of the culture medium were used in this study. Cell biomass was determined after 14 days of culture. Five samples were used for each pH study and the experiment was repeated three times. The data were analyzed using two-way ANOVA. The optimal pH of the culture medium was selected after the Tukey's HSD Test at p=0.05.

Effect of MS macronutrients on cell biomass

One-gram cell from line Eu9 was cultured into 100 mL flask containing 20 mL MS liquid medium + X with modified MS macronutrients. The macronutrients were NH_4NO_3 (0, 5.25, 10.5, 15.75, 21, 31.5, 42 and 52.5 mM), KNO_3 (0. 4.75, 9.50, 14.25, 19, 28.5 and 38 mM), $CaCl_2.2H_20$ (0, 0.75, 1.50, 2.25, 3, 4.50, 6 and 7.50 mM), $MgSO_47H_2O$ (0,38, 0.75, 1.13, 1.5, 2.25, 3 and 3.75 mM) and KH_2PO_4 (0, 0.31, 0.63, 0.94, 1.25, 1.88, 2.5 and 3.13 mM). Six replicates were used for each modified medium. Cell biomass was determined after 14 days culture. The cells were dried in room temperature for obtaining the dried weight of cells. The curve fitting and optimum concentration of each macronutrient were determined by Sigmastat® Statistical Software.

Results and Discussion

The amount of calli initiated from different trees was not significantly different. More calli (3.7-3.8 g) were induced within 4 weeks from the leaf explants of Eu8, Eu9 and Eu12 as compared with leaf explant of other trees. The leaf explant of Eu7 produced the least callus within the same duration. The dried weight of calli was correlated with their fresh weight (Table 1).

The amount of calli produced from a leaf explant was not correlated to the cell biomass in the cell suspension culture except the leaf explant of tree Eu9 which produced more calli and also induced a high cell biomass in the cell suspension

Table 1. Production of callus from leaf explants of different *E. longifolia* trees in solid MS medium+10 NAA after 4 weeks of culture

Leaf Explant from 2-year old trees	Increased fresh weight[a] (g)	Dry weight±s.e (g)
Eu1	3.101 ab	0.120±0.008
Eu2	3.442 ab	0.130±0.004
Eu4	2.982 ab	0.114±0.005
Eu5	2.832 ab	0.113±0.008
Eu6	3.216 ab	0.122±0.006
Eu7	2.723 b	0.106±0.009
Eu8	3.734 a	0.123±0.007
Eu9	3.816 a	0.141±0.005
Eu12	3.752 a	0.135±0.004

[a]Means followed by same alphabet were not significantly different based on Tukey's HSD Test (p=0.05)

culture. Leaf explant from tree Eu8 produced high amount of callus. However, after it was used for the preparation in cell suspension culture, only 3.137 g of cell biomass was produced. (Table 2). This clearly showed that growing callus was not correlated with cell biomass. Ketchum et al. (1995) also found that cell line from different trees of pacific yew produced different growth characteristics. In the cell cultures, there are many factors involved in the process, beginning with the source of plant material. For higher active compounds, the screening and induction of productive cell line should be considered as another important step. Choi et al. (1994) reported that cell line screening of ginseng was achieved by comparative studies of different cell lines on the growth and saponin yields. In addition to a high and stable secondary metabolite content, the most desirable characteristics of a cell line for large-scale production should include rapid growth and sufficient tolerance to agitation and mixing stress. The stability of the tissue and cell strains with respect to culture life may be a major concern in the industrial application of the cell culture process.

Culture media that were too acidic or too alkaline would reduce the cell biomass production of *E. longifolia*. The best pH of proliferation culture media (MS + X) was found to be 5.75 and the *E. longifolia* cells cultured in this medium were able to produce an increased cell fresh weight of 5.035 g within 14 days from an initial weight of 1.0 g. The dried weight of cells corresponded with fresh cell biomass (Table 3). Several studies have been carried out on the effect of initial pH of the medium on the growth of plant cells, but in most cases, the development of the cultured cells altered and reduced the final pH of the medium (Gamborg et al. 1968). Thus it was difficult to estimate the effect of external pH on the metabolism of the cells. Nesius and Davies (1972) reported that fresh weight yields were maximum at pH 5.2 to 5.4 when the pH of suspension culture of rose was controlled with 2-(N-morpholine) ethane sulfonic acid (MES buffer). However, a somewhat greater production of fresh weight yields occurred when the pH of the culture increased or was adjusted from about 5 to 6 during the growth period. The growth and development of the cell culture of *Ipomoea* sp. was affected mainly through pH effect on the uptake or utilization of ammonium and nitrate (Martin and Rose 1975).

Based on cell biomass production of *Eurycoma longifolia*, the optimum concentration of NH_4NO_3 was 21.5 mM for fresh weight and 19.5 mM for dry weight (Figure 1), which was nearly equal to that contained in the MS medium (21 mM). Potassium nitrate (KNO_3) stimulated the production of biomass but the yield became constant at concentration higher than 30 mM in the MS medium. The optimum concentration was 12.25 mM for fresh weight and 11.25 mM for dry weight as compared to KNO_3 used in basic MS (19 mM) (Figure 2). However, the addition of more than 15 mM of KNO_3 caused a decreased in the growth rate. It was a general trend that a lower NH_4^+ to NO_3^- ratio was more favorable for plant tissue and cell growth (Franklin

Table 2. Effect of callus source on production of *E. longifolia* cell biomass in liquid MS+X medium

Callus source	Increased fresh weight[a] (g)	Dry weight ± s.e. (g)
Eu-1	3.481 d	0.278±0.013
Eu-2	4.016 c	0.290±0.010
Eu-4	2.920 e	0.227±0.028
Eu-5	4.121 c	0.281±0.009
Eu-6	5.138 b	0.329±0.006
Eu-7	3.246 de	0.265±0.006
Eu-8	3.137 de	0.223±0.027
Eu-9	6.161 a	0.383±0.004
Eu-12	4.832 b	0.315±0.009

[a]Means followed by same alphabet were not significantly different based on Tukey's HSD test (p=0.05)

Table 3. Effect of pH of culture medium MS+X for the biomass production of Eu-9, *E. longifolia* cell suspension culture

pH	Increased fresh weight (g/20 mL medium)[a]	Dry weighta (g/20 mL medium) ± s.e.
4.75	3.012 b	0.182±0.019
5.25	3.184 b	0.198±0.009
5.75	5.035 a	0.309±0.010
6.25	3.472 b	0.212±0.011
6.75	3.460 b	0.206±0.006

[a]Means followed by same alphabet were not significantly different based on Tukey's HSD test (p=0.05)

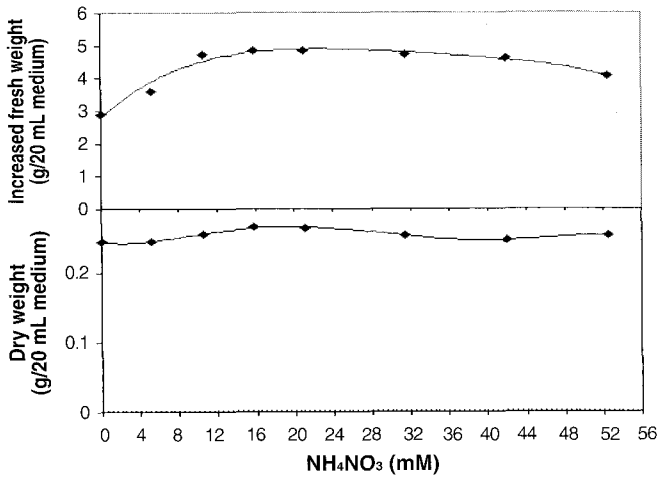

Figure 1. Effect of NH_4NO_3 concentration in liquid MS supplemented with X for biomass production of Eu-9 cell suspension culture of *Eurycoma longifolia*.

and Dixon 1994). For the *Panax ginseng* cells, Ushiyama (1991) also reported that lower NH_4^+/NO_3^- ratios in the medium were favorable for cell growth.

Result showed that $CaCl_2.2H_2O$ had no particular effect on the production of the cell biomass (Figure 3). It stimulated optimum biomass growth at 3.10 mM for fresh weight and 2.85 mM for dry weight. Fujita et al. (1981) reported that the concentration of calcium in the cell suspension culture of *Lithospermum erythrorhizon* was almost the same as in White's medium and it produced little change in the cell yield. Jung et al. (1994) found that increased concentrations of calcium did not affect the catharanthine content of *Catharanthus roseus* cell culture. But it greatly reduced the total catharanthine yield in the root cultures because of the decreased growth of hairy roots.

Magnesium is an essential component of nutrient medium for the culture of plant tissue and cells. In the present study, the optimum concentration of $MgSO_4.7H_2O$ was 0.575 mM for fresh weight and 0.555 mM for dry weight (Figure 4), which was only one-third the concentration used in MS medium (1.5 mM). The effect of magnesium on cell biomass production in *Ipomoea* sp. cell cultures had been studied, and the addition of 0.3 and 0.6 mM magnesium in culture medium were reported to elevate the cell biomass (Veliky et al. 1977).

The optimum concentration of KH_2PO_4 in the cell culture medium of Tongkat Ali was found to be 1.83 mM for fresh weight and 1.70 mM for dry weight (Figure 5). This was 1.5

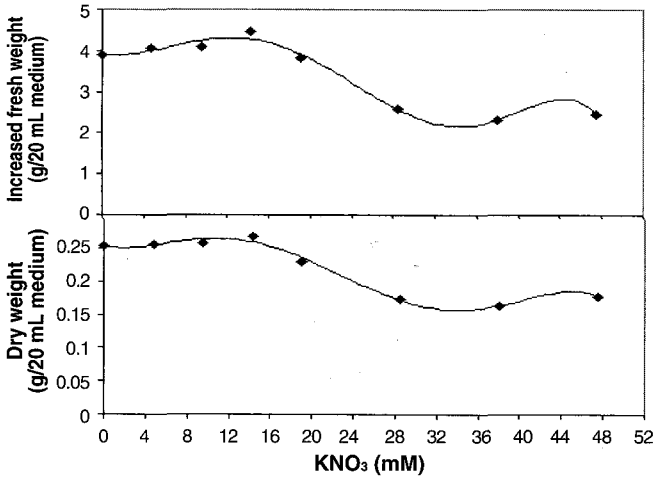

Figure 2. Effect of KNO_3 concentration in liquid MS supplemented with X for biomass production of Eu-9 cell suspension culture of *Eurycoma longifolia.*

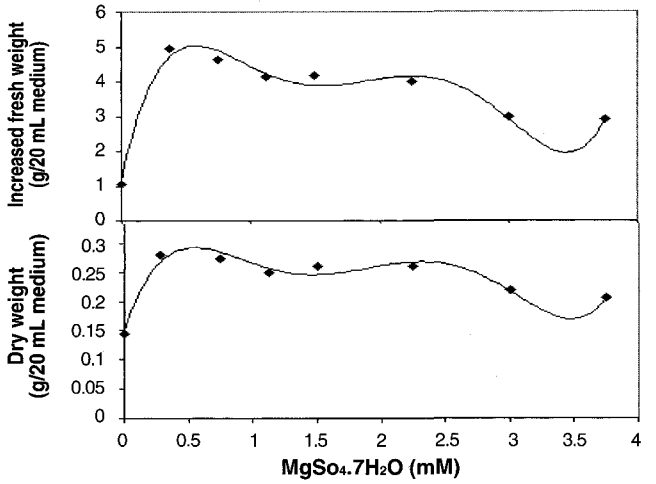

Figure 4. Effect of $MgSO_4.7H_2O$ concentration in liquid MS supplemented with X for biomass production of Eu-9 cell suspension culture of *Eurycoma longifolia*

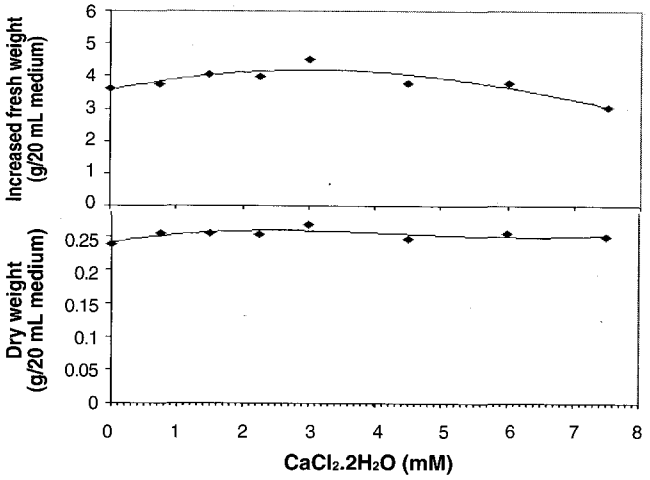

Figure 3. Effect of $CaCl_2.2H_2O$ concentration in liquid MS supplemented with X for biomass production of Eu-9 cell suspension culture of *Eurycoma longifolia*

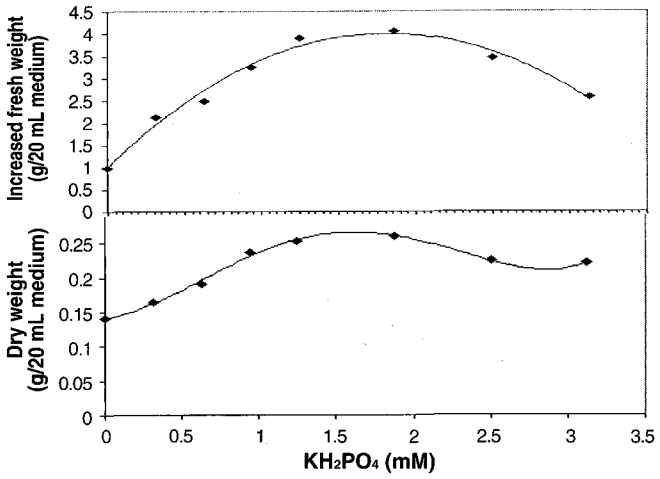

Figure 5. Effect of KH_2PO_4 concentration in liquid MS supplemented with X for biomass production of Eu-9 cell suspension culture of *Eurycoma longifolia*

times higher than that present in MS medium. The addition of more than 2 mM of KH_2PO_4 into the cell culture medium caused a decreased in the cell growth rate. Phosphorus is another key nutrient for plant cell growth. Zhang and Zhong (1997) found that an increase in initial phosphate from 1.25 to 3.75 mM enhanced both cell growth and saponin yield in suspension culture of *Panax notoginseng* cells. Enhancement of the growth rate of suspension cultures of *Cataharanthus roseus* was higher with 2.2 and 5.45 mM of PO_4^{3-} than with 1.1 mM of PO_4^{3-}, but an increase in the concentration of PO_4^{3-} to 10.9 mM caused a decrease in the cell growth (Carew and Kreuger 1977).

Based on these results, a modified MS medium was formulated for the production of *E. longifolia* cell biomass. The optimum culture medium for the production of *E. longifolia* cell biomass was liquid MS medium which contain a modified macronutrient of 21.5 mM NH_4N_3, 12.25 mM KNO_3, 3.10 mM $CaCl_2.2H_2O$, 0.575 mM $MgSO_4.7H_2O$ and 1.83 mM KH_2PO_4 with the addition of X and adjusted the pH of the medium to 5.75 before autoclaving at 1.05 kg/cm with a temperature of 121°C for 13 minutes.

Acknowledgement

We thank the Penang State Government for permission to use *Eurycoma longifolia* Jack (Tongkat Ali) plants for research and Universiti Sains Malaysia for research funding and facilities.

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