Effects of Media on the Callogenensis and Cell Mass Production in Cell Cultures of *Panax vietnamensis*

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Abstract This study was initiated to investigate the impacts of media types and other components on the callogenensis and cell mass production of *Panax vietnamensis* in the first step of the cell biomass procedure. Four media were checked: Murashige-Skoog (MS), White, Gamborg and Nitch-All. All the four media were shown potential media for *Panax vietnamensis* callogenensis and cell mass production, in which the MS medium showed the best results: the successful callogenensis ratio and cell mass formation were 30% and 62,93 ± 3,63 mg (DW) respectively, the Nitch medium showed the lowest results: the successful callogenensis ratio and cell mass formation were 15% and 27,10 ± 2,24 mg (DW) respectively. The results showed that the MS medium is the most suitable medium for *Panax vietnamensis* callogenensis and cell mass production.

Keywords: callogenensis, cell mass, *Panax vietnamensis*, media, callus.

Introduction

Vietnamese ginseng (*Panax vietnamensis* Ha *et* Grushv., *Araliaceae*) is a wild Panax species that was discovered in the mountains of Central Vietnamin 1973. It is used by ethnic minorities for treatment of many serious diseases and for enhancement of physical strength. Previous studies have demonstrated that Vietnamese ginseng is an important medicinal plant used in the cultural traditional medicine. This ginseng contains a large quantity of ocotillol-type saponins, e.g., majonoside R_2, that are not present in P. ginseng(1-3). In addition, Vietnamese ginseng was reported to exhibit interesting pharmacological effects such as stimulatory and suppressive effects on the central nervous system and anti tumor-promoting and hepatoprotective activity(4).

Panax vietnamensis is one of the most valued and rare plant in Vietnam(2). Nowadays,the supply of Vietnamese ginseng is very limited, as the wild ginseng plant is rare and difficult to find and the preservation of *Panax vietnamensis* has not been very successful.

Mass-production of embryogenic callus in *Panax vietnamensis*is of special importance because the cell and callus materials may be used as alternatives to naturally-grown ginseng roots. In vitro culture is an advantageous alternative of ginseng material production since it had no seasonal or regional restrictions. There is no clear difference in saponin production between naturally-grown roots and in vitro-produced cells in Panax ginseng (Furuya et al., 1983; Asaka et al., 1993a,b; Choi et al., 2000).

Callus production of ginseng with saponin content comparable with natural roots (Furuya et al., 1983, Asaka et al., 1993b; Zhong et al., 1996) has also been reported extensively. Application of biotechnology to increase cell biomass of *Panax vietnamensis* is studying in our lab. In this study, we reported the effects of media on the callogenensis and cell mass production in cell culture of *Panax vietnamensis*.

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Material and methods

Media preparation : The four media were prepared with

the basic inorganic medium supplemented with the same plant growth regulators in both type and concentration and 6.5% agar, 3% sucrose (Sigma). Media were then put into test tubes with equal number for each medium, sterilized at 121℃ for 20 minutes.

Plant material and callus induction

Five-year-old roots of *Panax vietnamensis*Ha et Grushv. were collected from Ngoc Linh mountains in central highland of Viet nam. Roots were washed first with running tap water and detergent solution for 10 min. Roots surface was sterilized with 70% ethanol for 30 s, immersed in a 2% NaClO solution containing two drops of tween-20 for 30 min and rinsed several time with sterile distilled water. The sterilized roots were cut into small pieces (1.0 × 0.5 × 0.3cm) and put onto four prepared media. Cultures were kept at 25℃ in the dark in order to form calli, which were subculturedevery month, for 6 months to obtain homogeneous and undifferentiated tissue cultures. Successful callogenensis was considered that the calli formed without organogenensis, slightly yellow and evenly callus. Then, the calli were counted on different media to estimate the successful callogenensis ratio for each medium. Each medium was conducted with 100 explants. Results were recorded after 4 weeks of culture.

Cell mass production evaluation

After successful callogenensis, the induced callus (homogeneous and undifferentiated callus) were subcultured onto the four prepared media. After 30 days of culture the callus were harvested and weighed, both dry and wet cell mass. The best condition of medium for the cell mass production is the medium that give the highest results.

Growth kinetic observation

After choosing the best medium for cell mass production, we evaluated the growth kinetic on that medium. The callus were subcultured onto the best medium. After 1, 20, 25, 30, 35, 40, 45 days, we harvested the callus and weighed, both fresh and dry cell mass.

Evaluation of plant growth regulators' effects on *Panax vietnamensis* cell mass production

The callus were subcultured on the MS medium supplemented with the same concentrations of different plant growth regulators. After 35 days, the callus wereharvested and weighed, both dry and fresh cell mass and compared between the different plant growth regulators.

In order to check the effects of different NAA concentrations on cell mass production, the callus were subcultured on the MS basic medium supplemented with 3% sucrose, 0.65% agar, 1 mg/L kinetin and 10, 20, 30, 40, 50 M NAA. After 35 days, the callus were harvested and weighed, both fresh and dry cell mass.

To check the effects of medium pH on cell mass production, the basic MS medium was supplemented with 3% sucrose, 30 M NAA, 1 mg/Lkinetin, 0.65% agar and adjusted pH to 5, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6. Callus were subcultured on the medium at different pH, after 35 days, the cell mass was harvested and weighed both dry and fresh cell mass.

To evaluate the effects of temperature on cell mass production, we adjusted the culture room temperature at 22, 23, 24, 25, 26, 27, 28℃. After 35 days the cell mass was collected and weighed both fresh and dry cell mass.

Statistical analysis

Data were analyzed using analysis of variance with mean separation based on Statview 501 E.

Results and discussion.

Effects of media on Panax vietnamensis callogenensis

The influences of four different media on the *Panax vietnamensis* callogenensis results (after 30 days of cultivation) are presented in the following table:

Table 1. The media influences on the callogenensis ratio

Medium	The number of samples	The number of calli	The successful callogenensis ratio (%)
MS	100	30	30%
White	100	25	25%
Gamborg	100	13	13%
Nitsch	100	15	15%

Table 1 showed that the successful callogenensis ratio with MS medium was the highest up to 40%. Most of

the calli grew equally, slightly yellow. Meanwhile, on the other media, the successful callogenensis ratios were significantly lower.

The influences of the media on the Panax vietnamensis cell mass formation results (after 30 days of cultivation) are present in the following table:

Table 2. The media influences on the cell mass formation

Medium	n	Callus cell mass (X SD)	
		Fresh weight (mg)	Dry weight (mg)
MS (1)	40	290,47 ± 20,10	62,93 ± 3,62
White (2)	25	145,76 ± 14,42	26,34 ± 2,57
Gamborg (3)	13	179,86 ± 23,25	33,29 ± 2,78
Nitsch (4)	15	150,87 ± 12,79	27,10 ± 2,24
P		$p_{2-1} < 0,05$; $p_{3-1} < 0,05$; $p_{4-1} < 0,05$	

Table 2 shows that, the weight (dry and fresh) of callus on MS medium was higher than other media, $p < 0.05$.

The cell mass changes versus time

The cell mass changes versus time result on MS medium are present in the following figure.

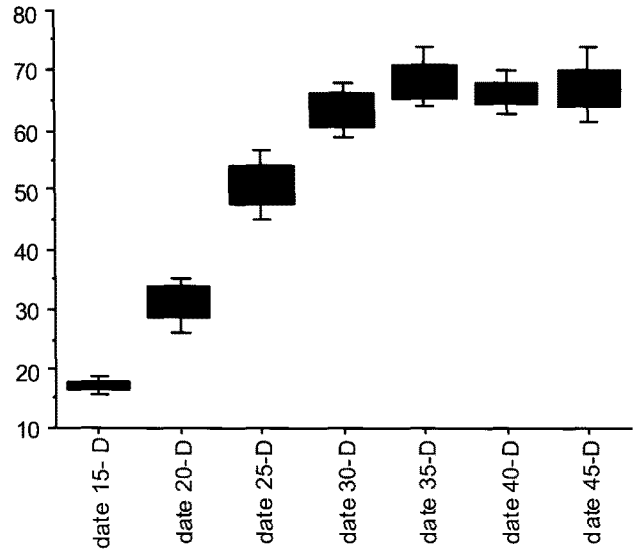

Fig. 1. The cell mass changes versus time.

In figure 1, after the 20th day, the callus mass increased rapidly, after the 35th day, the growth speed became lower. After 40 days, callus mass almost didn't change over time.So the best time to harvest callus mass is 35th day.

The effects of the plant growth regulators to cell mass production.

The influential investigation results of the plant growth regulators on cell mass production on 35th day are present in the following figure.

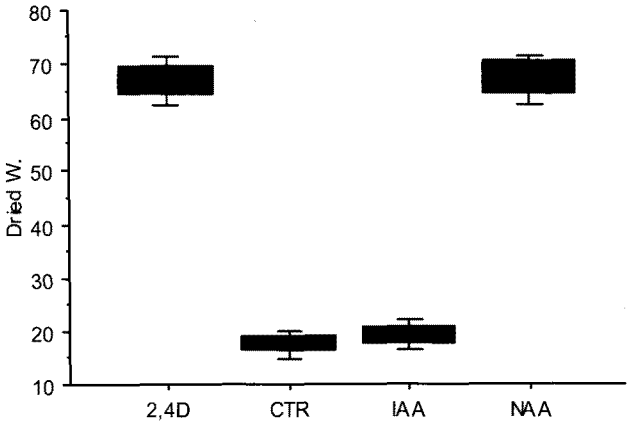

Fig. 2. The influences of plant growth regulators on the cell mass production.

In figure 2, we found out that the cell mass of *Panax vietnamensis* callus in the group used NAA and 2,4-D higher than others, was statistically significant with $p < 0.001$. Because 2,4-D is more toxic than NAA, so we choose NAA as the plant growth regulator for further investigation.

The effects of the NAA concentrations on cell mass productionresults

The influences of NAA concentrations on the cell mass production (in the MS medium, on the 35th day of cultivation) are presented in the following figure.

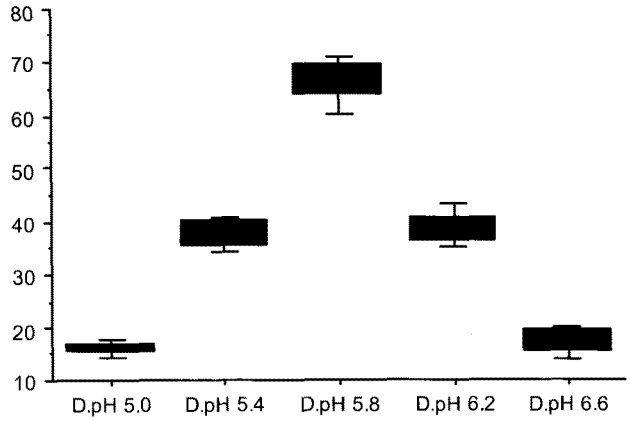

Fig. 3. The influences of NAA concentrations on the callus mass.

Through the figure 3, with the NAA level of 30 M, the cell mass of *Panax vietnamensis* callus was higher than other groups, the differences were statistically significant with $p < 0.001$. However, in the group NAA used at concentration from 40 and 50 M compared to the concentration 30 M was not higher, $p > 0.05$.

The effects of medium pH on the cell mass productionresults.

The influences of the medium pH on the cell mass production, supplemented with NAA concentration of 30 M after 35 days of cultivation in MS medium are presented in the following figure.

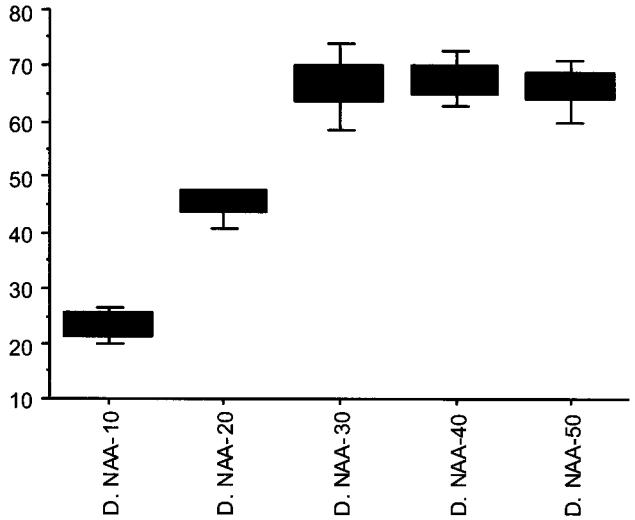

Fig. 4. The effect of medium pH on the cell mass production.

Through the figure 4, the cellmass of harvested callus in the group medium pH 5.8 was much higher than other groups, the difference was statistically significant, with $p < 0.001$

The effects of the culture room temperature on *Panax vietnamensis* callogenensis

The result about the influences of culture room temperature on the callogenensis, supplemented with NAA concentration of 30 M, pH = 5, 8, after 35 days of culture in MS medium are present in the following figure.

Through the figure 5, the callus mass of Vietnamese ginseng in the group with temperature from 23-25℃ was much higher than other groups, the difference was statistically significant with $p < 0.001$.

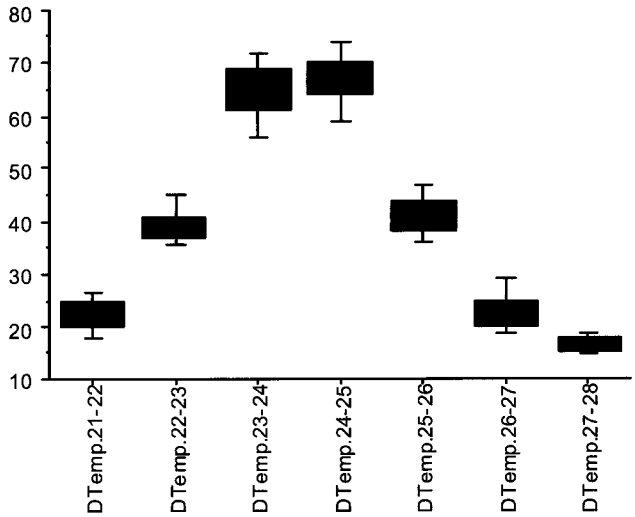

Fig. 5. The culture room temperature effects on cell mass production.

Discussion

Plant biomass technology is the technique to culture, maintain, and produce plant cell mass in the aseptic nutrient medium, without the need of planting the whole plant(1). Callus technique (callus : the group of undifferentiated plant cells created in the laboratory (in vitro), similar to the plant scar tissue created to heal the wound site of the tree)(1), (2). The efficiency, the yield and the quality of the callus induction depend on many factors. The factors have to be investigated to optimize the callus induction procedure include : the type of culture medium, medium pH, culture room temperature, the time to harvest, and plant growth regulators(3), (5).

Our investigation results showed that, MS medium was the most suitable medium for Vietnamese ginseng, up to 30% successful callus formation after 30 days of culturing, meanwhile the ratios on other media were lower. Our results fitted well with investigations of MS medium to be the best medium for Korean ginseng callus induction. When compared with the ratio of Korean ginseng, we found out that the ratio of Vietnamese ginseng callus induction was similar.

After selecting the most suitable medium, we investigated the callus growth versus time and determined the time in which the callus grew the most (the suitable time to harvest the largest amount of ginseng cell mass). This is also one of the important factors, because if we collect the cell mass sooner or later, the efficiency is not high. If we harvest the cells sooner, is wasteful

because ginseng callus still grow well, if we harvest the cells later, is also more expenses and don't save time, even the cells could die or change the color(6), (7). The investigation results showed that, the most suitable time to harvest was about 35 days, a little longer compared to the time to harvest the Korean calli(4).

The plant growth regulators of ginseng callus have very important roles in the callus induction and development. These substances are many, and the problem here is to select the most suitable one and its concentration. If we don't choose the suitable ones, the callus induction efficiency is low, and the unsuitable concentrations are very wasteful and low efficiency(4), (6). In this investigation we chose 3 kinds of plant growth regulators often used in ginseng callus induction: 2,4-D; IAA and NAA. The investigation results show that the callus mass of group used 2,4-D and NAA was higher than others, and the difference had no statistically significance with $p > 0.05$. We chose NAA to be the plant growth regulator for Vietnamese ginseng callus with concentration of 30 μM. This concentration was similar to some other investigation results used NAA with concentration from 0.1 to 50 μM.

Besides, the above factors, the culture room temperature and medium pH are also the important factors in the callogenensis. If pH is too low or too high, the callogenensis is not high, no callus formation or slow callogenensis. At the high temperature, calli can't grow or die, at the low temperature, callus grow very slowly(3), (5), (6). Our investigation results show that the culture room temperature was about 23-25℃ was the best. Medium pH about 5.8 was the most suitable.

In conclusion, callus culture was the first step of procedure of plant cell biomass technology. Cell culture protocol for production of *Panax Vietnamensis* callus was successfully established in our laboratory. The optimal conditions for callus formation included: The MS medium was the suitable medium. The optimal pH was 5.8. The temperature of 23-25℃ was the best for induction of callus. The suitable growth hormone was NAA with concentration of 30 μM. The good time to harvest callus was the 35th day after starting culture.

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