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Optimal Culture Conditions for *in vitro* Propagation of *Orostachys japonicus* and Enhancement of Polysaccharide Production

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ABSTRACT : Optimal culture conditions for efficient *in vitro* propagation and polysaccharide production of *Orostachys japonicus* were established. The highest growth yield was achieved in 1/2 MS medium, while the lowest growth yield was obtained in 4 MS medium. The patterns of polysaccharide formation were a little similar in all cases, but on MB5 medium, the polysaccharide contents of plant were higher than others. Among the modified nitrate levels, effective growth level were obtained in 1/4 N and 1/2 N. High contents of polysaccharide were obtained in 4 N. Different concentration of potassium and calcium did not improve the growth and polysaccharide production. The micropropagated shoots were successfully acclimatized artificial soils.

Key words : *Orostachys japonicus*, polysaccharides, regeneration, macro nutrient

Abbreviations : IAA ; 3-Indole-acetic acid, NAA ; Naphthalene acetic acid, 2,4-D ; 2,4 dichlorophenoxy acetic acid, BA ; 6-benzyladenine

INTRODUCTION

Plant cell and tissues culture was considered a potential source of commercial products such as pharmaceuticals, flavors, fragrance agents, agrochemicals and oils; however, its large-scale cultivation has been hampered by a slow growth rate, sensitivity to shear stress, surface adhesion and cell aggregation. Even though there were many of the pharmaceuticals of interest, the production was too low or even zero in the cultured cells and tissues, despite extensive studies on the optimization of growth and production media and cell line selection for high producing strains (Verpoorte *et al.*, 2002).

Orostachys japonicus, which belongs to the Crassulaceae family, is one of the most famous medicinal plants in Korea, China and Japan (Shin *et al.*, 1994). The whole plants of *O. japonicus* have oxalic acid, sedoheptulose alkaloid, fatty acid ester, seco-α-triterpene, glutinone, friedeli, β-amyrin, glutinol, epi-fridelanol, 1-hexatriacontanol, and sterols (Park *et al.*, 1991).

Immunostimulating polysaccharides from natural products have widely been developed to treat cancer, immunodeficient syndrome such as AIDS, and chronic infections (Ahn *et al.*, 1998). Previously, polysaccharide fractions from the root of *Angelica gigas* Nakai have been reported to have an immunostimulating activity related with T-lymphocyte activation (Ahn *et al.*, 1996). The physico-chemical alterations of the main type of polysaccharides from *Aloe vera* parenchyma observed during dehydration may have important

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implications on the physiological activities attributed to the *Aloe vera* plant (Antoni *et al.*, 2003). Production of these biologically active chemicals by plant cell cultures may be a cost-effective approach to meet popular market demand. There have been few papers concerning *O. japonicus cultures* and numerous publications regarding polysaccharides contents by most plant cell and tissue cultures (Yang & Choi, 1992; Choi *et al.*, 1994; Zhong & Wang, 1998).

Thus, this study was conducted to determine the optimal culture conditions on *in vitro* propagation and polysaccharide production.

MATERIALS AND METHODS

Plant material

Two-year-old *O. japonicus* plants were collected in 2002 from cultivation of Jungchon, Jin-ju in Korea. The plants were handpicked, washed with distilled water, and surface sterilized by treating with 70% (v/v) ethanol for 1 min., 3% (v/v) sodium hypochlorite (NaOCl) solution for 10 min., followed by three times rinses with sterile distilled water. The plants were cut with a sterile knife to obtain the stems. All these operations were carried out in laminar flow hood. Shoot segments were then transferred to MS (Murashige & Skoog, 1962) basal medium containing 3% sucrose and adjusted to pH 5.7±0.1 before autoclaving at 121℃ for 15 min. and cultures were incubated under light condition at 25±2℃.

Plant culture conditions

A segment (1~2 ㎝) of stems (1.0 g fresh weight) was cultured on several basal media; 1/4 MS, 1/2 MS, MS, 2 MS, 4 MS , 1/4 B5, 1/2 B5, B5 (Gamborg *et al.*, 1968), 2 B5, MB5 (MS and B5 medium mixture), 2 MB5 (2MS and B5 medium mixture), WPM (Lloyd & McCown, 1980), SH (Schenk & Hildebrandt, 1972), LP (Quoirin & Lepoivre, 1977), NN (Nitsch & Nitsch, 1969) and White (White, 1963). All media contained 3% sucrose and adjusted pH 5.7 before autoclaving.

They were cultured under light condition at 25±2℃ for 4 weeks. All treatments were determined fresh weight (F.W.) on each growth of shoot. Each dish was inoculated with three stems and incubated in a growth chamber at 25±2℃.

Macro nutrients

A segment of stems was cultured in several macro nutrients (N, K, and Ca) on MS basal media. The macro nutrient solutions were purchased from Duchefa and filtered via a syringe filter (25 ㎜, Gelman sciences). For feeding, the solution was dissolved in distilled water and autoclaved at 121℃ for 15 min. Then, sterilized nutrients were added at various concentrations of 1/4, 1/2, 2 and 4 into MS basal medium and cultivated for 4 weeks. The concentration of N, K and Ca on the culture medium was regulated with NO_3^- and NH_4^+ as nitrate source, KI and KH_2PO_4 as potassium source, and $CaCl_2 \cdot 2H_2O$ as calcium source, respectively. MS basal medium was referred to as the control.

In the aim of monitoring the kinetics of growth and polysaccharide productivity, stems were cultured for 5, 10, 15, 20, 25 and 30 days. At each harvest time, stems were collected and measured growth and production.

Cultured shoots for 4 weeks were used in the experiments and initial inoculum weight (1.0 g F.W.) was compared with treatment each other. For determination of fresh weight, in vitro plants were separated from the medium and weighed in the all of experiments.

Acclimatization of plant

The rooted shoots on MS basal medium were collected from culture bottles, washed to remove gelrite and transferred to small plastic pots containing nutrient solution (MS basal liquid medium) or artificial soils which are not treated nutrient with peatmoss, perlite, vermiculite and sand, respectively. They were incubated in the growth chamber for 4 weeks. And then, these were grown for 8 weeks in a greenhouse maintained 70% relative humidity (RH) and a temperature of 26℃ with 16/8 h (light/dark) photoperiod.

Extraction and determination of polysaccharide

Extraction and determination of polysaccharide were operated by modifying the method of Dubois *et*

al. (1956). Harvested tissues were washed to remove the remained gelrite and then the whole plants were extracted for determination of polysaccharide. A segment of plants (1.0 g F.W.) was homogenated at 20,000 rpm for 2 min., ultra sonicated for 30 min. and centrifuged at 6,000 rpm for 10 min. The resulting supernatant was added with 0.5 ㎖ of sulphuric acid and placed at room temperature for 10 min. aiming at cleaving the polysaccharide bond. Thereafter the supernatant was boiled at constant 100℃ for 20 min., cooled, and filtered through a syringe filter (Sartorius, 0.20 ㎛, German).

A sample (0.1 ㎖) taken from the above supernatant was mixed with 1 ㎖ distilled water and then added with 5 ㎖ of concentrated sulphuric acid, held in a boiling water bath for 20 min., and cooled. Then, a sample was added with 1 ㎖ phenol (pure grade, Merck) and mixed vigorously. After 2 h of a reaction time in darkness at room temperature, a purplish red color developed and absorbance was measured at 490 ㎚ by ELISA leader (Bio-Rad, USA).

Statistical analysis

For each set of experiments, 15 replicates were taken and the experiment was repeated twice, thus giving 30 replicates for each treatment. Duncan's Multiple Range Test (DMRT) and LSD (Least Significant Difference) were used to analyze the variations. Values were represented as mean±standard deviation (SD).

RESULTS AND DISCUSSION

Effect of plant culture medium on plant growth and polysaccharide production

O. japonicus growing in cultivation of Jungchon, Jinju in Korea (Fig. 1A) were handpicked and stem are sterilized and there transferred on MS basal Medium without growth regulators (Fig. 1B). Shoots were induced from cutted stem tissues on MS basal medium after 4 weeks of culture (Fig. 1C). Shoot was formed multiple shoots after 4 weeks (Fig. 1D). Multiplied shoots were cut on basal shoots, and them transferred to dish. On fresh medium shoots developed further (Fig. 1E). The shoots elongated very well, and healthy roots induced from shoot base after 4 weeks (Fig. 1F).

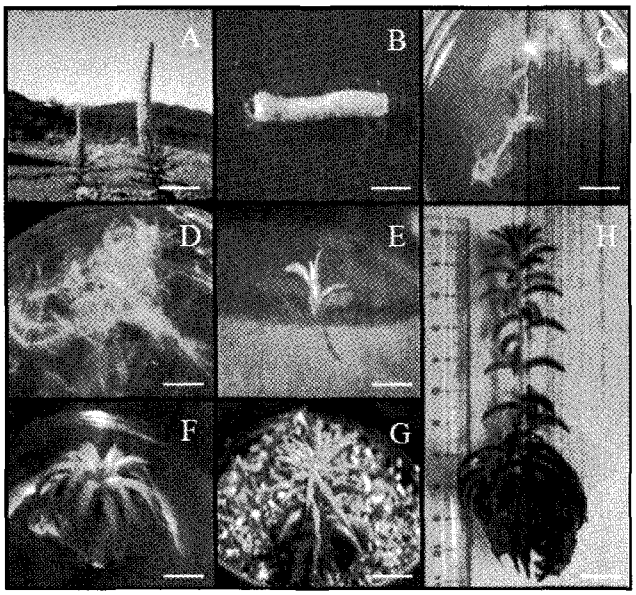

Fig. 1. ***In vitro*** **propagation of** ***O. japonicus.*** **(A)** ***O. japonicus*** **growing from cultivation of Jungchon, Jinju in Korea, Bar 5.29 ㎝. (B) Stem of shoot transferred on MS basal Medium, Bar 0.59 ㎝. (C) Propagation of shoot on MS medium without growth regulators after 4 weeks, Bar 0.48 ㎝. (D) Propagation of multiple shoot on the fraction after 4 weeks, Bar 0.90 ㎝. (E) Elongation of multiple shoots were transferred from dish to culture bottle after 4 weeks, Bar 0.43 ㎝. (F) Elongation of shoots on MS solid medium after 4 weeks, Bar 0.90 ㎝. (G) Plants transferred to pot after 4 weeks, Bar 1.13 ㎝. (H) Plants were acclimatized in a greenhouse after 8 weeks, Bar 2.68 ㎝.**

Table 1 indicates that addition of optimal medium improved the plant growth of *O. japonicus*. The highest growth yield was achieved in 1/2 MS medium, while the lowest growth yield was obtained in 4 MS medium; each values were 5.44 and 0.94 g F.W., respectively.

In addition, various culture medium influences on the content of polysaccharide in *O. japonicus*. The patterns of polysaccharide formation were no significant in all treatments, but on MB5 medium, the polysaccharide contents of plant (0.24×10^{3} ㎎ per g D.W.) were higher than others. The total production

of polysaccharide in MB5 medium was higher, which was 2-fold of the MS medium. Furthermore, the polysaccharide productivity in *O. Japonicus* was also conspicuously enhanced by MS and B5 medium combinations. A similar positive effect on combination of culture medium was reported in suspension cultures of strawberry cells for anthocyanin production (Mori *et al.*, 1994).

Table 1. Effects of culture media on plant growth and polysaccharide production of *O. japonicus*.

Media	Plant growth F. W. (g)	Contents of polysaccharide† (mg×10³ /g D.W.)
1/4B5	2.83 ± 0.32^{ce}‡	0.10 ± 0.01^{ef}
1/2B5	2.50 ± 0.36^{cf}	0.16 ± 0.03^{bc}
B5	4.78 ± 0.47^{ba}	0.04 ± 0.01^{ij}
2B5	2.33 ± 0.06^{de}	0.05 ± 0.01^{ih}
4B5	1.72 ± 0.04^{ef}	0.07 ± 0.01^{hi}
1/4MS	4.06 ± 0.56^{bd}	0.13 ± 0.01^{ce}
1/2MS	5.44 ± 0.59^{a}	0.13 ± 0.01^{cd}
MS	4.28 ± 0.49^{bc}	0.12 ± 0.01^{de}
2MS	3.89 ± 0.65^{bd}	0.08 ± 0.01^{fg}
4MS	0.94 ± 0.14^{f}	0.14 ± 0.01^{cb}
MB5 (1:1)	2.94 ± 0.06^{cd}	0.24 ± 0.03^{a}
MB5 (2:1)	3.17 ± 0.07^{cb}	0.08 ± 0.01^{gh}
LP	3.44 ± 0.43^{cb}	0.11 ± 0.01^{fe}
NN	2.67 ± 0.64^{ce}	0.22 ± 0.03^{a}
SH	3.61 ± 0.68^{ca}	0.03 ± 0.01^{ij}
WHITE	2.17 ± 0.81^{de}	0.06 ± 0.01^{hi}
WPM	4.00 ± 0.12^{bd}	0.17 ± 0.03^{b}

†Contents of polysaccharide measured by Dubois *et al.* (1956).
‡Each numerical value represents the mean and standard deviation from 3 replications after 4 weeks. Means follow by different letters are at 5% level by LSD and DMRT.

Effect of macro nutrients on plant growth and polysaccharide production

A segment of stems was cultured in several macro nutrients (N, K, and Ca) on each MS basal medium. Macro nutrients (N, K, and Ca) were used as optimal condition in *O. japonicus* tissue culture (Table 2).

Effective growth levels of 6.31 and 6.32 g F.W. were obtained in 1/4 N and 1/2 N, respectively. The growth yield in 1/2 N was higher than that in control. These results indicated that it was different with previous reports. For example, insufficient supply of nitrate will lead to a lack of intracellular phosphorus and finally limited the plant growth (Wen & Zhong, 1997).

High contents of polysaccharide were obtained in 4 N (0.95×10^3 mg per g D.W.). The growth yield in 4 N was higher than control, 2.5-fold of polysaccharide contents. In general, macro nutrients in medium were taken up completely by the plant during the first 4 days of cultivation in all the cases, and the intracellular phosphorus was then utilized for the further growth and metabolism of plant (Wen & Zhong, 1997). In plant cell cultures, the macro nutrient sources may significantly affect growth and metabolites formation. This work focuses on a

Table 2. Effect of macro nutrient on plant growth and polysaccharide production of *O. japonicus*.

Macro nurient	Plant growth F. W. (g)	Contents of polysaccharide† (mg×10³ /g D.W.)
Control‡	5.52 ± 0.08^{cd}§	0.32 ± 0.03^{c}
Nitrate		
1/4N	6.31 ± 0.01^{a}	0.28 ± 0.06^{c}
1/2N	6.32 ± 0.01^{a}	0.19 ± 0.03^{d}
2N	4.01 ± 0.03^{h}	0.29 ± 0.06^{c}
4N	2.44 ± 0.01^{i}	0.95 ± 0.08^{a}
Potassium		
1/4K	5.34 ± 0.03^{d}	0.07 ± 0.01^{e}
1/2K	4.21 ± 0.06^{g}	0.34 ± 0.04^{c}
2K	4.58 ± 0.03^{ef}	0.04 ± 0.01^{e}
4K	5.69 ± 0.01^{bc}	0.02 ± 0.01^{e}
Calcium		
1/4Ca	5.83 ± 0.05^{b}	0.09 ± 0.01^{e}
1/2Ca	4.08 ± 0.05^{g}	0.09 ± 0.01^{e}
2Ca	4.71 ± 0.02^{e}	0.51 ± 0.07^{b}
4Ca	4.32 ± 0.01^{fg}	0.32 ± 0.02^{c}

†Contents of polysaccharide measured by Dubois *et al.* (1956).
‡Control was cultured on MS basal medium.
§Each numerical value represents the mean and standard deviation from 3 replications after 4 weeks. Means follow by different letters are at 5% level by LSD and DMRT.

systematic investigation of the effects of N, Ca and K source on *in vitro* propagation of *O. japonicus* plants for the simultaneous shoot growth and polysaccharide production.

Growth and polysaccharide production on culture periods

For the plant propagation, stems were cultured on MS medium for 5, 10, 15, 20, 25 and 30 days and monitored on plant growth such as root , shoot and leaf and polysaccharides production. The maximum growth was obtained at 25 days in all plant parts (root, shoot and leaf) (Fig. 2A). However, the production rate of polysaccharide decreased significantly after passed culture days. The difference between the levels of the polysaccharide contents was obtained in plant cultures (Fig. 2B).

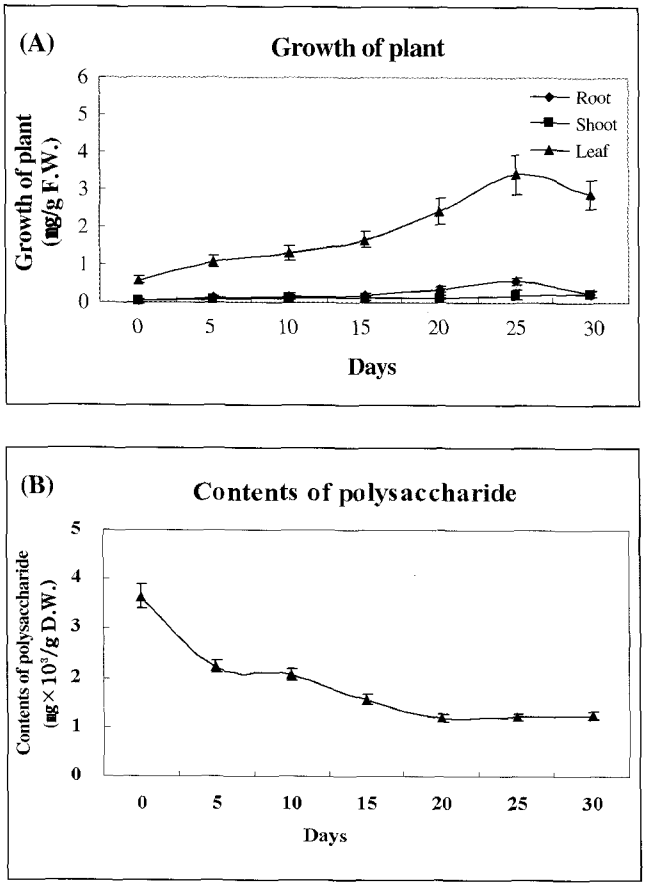

Fig. 2. Effects of culture period on plant growth and polysaccharide production of *O. japonicus*. (A) grow of plant on culture period (B) contents of polysaccharide on culture period.

Acclimation of micro propagated plants

The rooted shoots on MS basal medium were separated, washed for removing of gelrite and transferred to small plastic pots containing nutrient solution or nutrient not treated artificial soil, peatmoss, perlite, sand and vermiculite, for 4 weeks (Fig. 1G). These were grown for 8 weeks in a greenhouse, maintained 70 % relative humidity (RH) and a temperature of 26℃ (Fig. 1H). Artificial soils were better effect on plant growth and polysaccharide content compared to nutrient solution treatments. Regenerated plants were grown in plastic pot containing peatmoss for about 4 weeks.

In vitro plant was regenerated from the stem tissue of *O. japonicus*. This study was done for the optimization of culture conditions such as culture media and the macro nutrient for *O. japonicus*. Investigations on culture period were studied for shoot growth and polysaccharide production. Optimal condition of *O. japonicus in vitro* culture could be achieved highest shoot propagation instead of general callus and organ cultural propagation. The content of polysaccharide in *in vitro* propagated plants was higher than that of the native growing plants. These techniques could provide an efficient and rapid method for *in vitro* propagation of *O. japonicus* and enhancement of polysaccharides production.

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