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Effects of Growth Regulators on Shoot Regeneration and Polysaccharide Production of *Orostachys japonicus Berger*

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ABSTRACT : Optimal culture conditions for efficient *in vitro* propagation and polysaccharide production of *Orostachys japonicus* were established. *O. japonicus* was cultured in media containing various growth regulators and carbon sources. The highest regeneration rate was achieved in 1.0 and 3.0 mg ℓ^{-1} of 2,4-D concentration, while the lowest was obtained in 10.0 mg ℓ^{-1} 2,4-D concentration. When different carbone sources were added in the culture medium, plant growth was high in 3% sucrose treatment. The micropropagated shoots were successfully acclimatized in artificial soils and produced comparable amont of polysaccharide compred to parent cultivated plants.

Key words : *Orostachys japonicus*, polysaccharides, tissue culture, carbon source, regeneration

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

INTRODUCTION

Utilization of phytochemicals, obtained from various kinds of plant, has increased all over the world. In East Asian countries, many researches have been conducted to find useful products, such as anticancer agents from traditional medicines. Compared to chemical syntheses, screening of natural plants has many advantages due to the long history of oriental medical treatments.

A diverse range of important pharmaceuticals are produced by higher plants. There is still a continuing interest in the use of plant cell suspension or organ cultures as alternative sources for the field cultivation of plant derived chemical products (Rao & Ravishanker, 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-β-triterpene, glutinone, friedeli, -amyrin, glutinol, epi-fridelanol, 1-hexatriacontanol, sterols and polysacchaides (Park *et al.*, 1991).

Polysaccharide, in the whole plant of *O. japonicus*, is a well-known crude drug, and a traditional Korean herbal medicine, used in treatment of cancer. Immunostimulating polysaccharides from natural products have been widely developed to treat cancer, immunodeficient syndrome such as AIDS and chronic infections (Ahn *et al.*, 1998). In the case of oriental medicinal plants, the polysaccharide fraction was obtained from *Angelica acutiloba*. There have been reported to have a mitogenic activity on β-lymphocyte and interferon inducing activity (Kojima *et al.*, 1980), antitumor activity against *Erlich ascites* cells (Kumazawa *et al.*, 1982) and anti-complementary activity (Yamada *et al.*, 1984a, b). Previously, polysaccharide

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fractions from the root of *A. gigas* Nakai have been reported to have an immunostimulating activity related with T-lymphocyte activation (Ahn *et al.*, 1996). Production of these biologically active chemicals by plant cell cultures may be a cost-effective approach to meet popular market demand. Although numerous publications have been reported regarding polysaccharides contents by most plant cell and tissue cultures, there have been few papers concerning *O. japonicus* cultures (Yang & Choi, 1992; Choi *et al.*, 1994; Zhong & Wang, 1998; Kim *et al.*, 2004). Thus, this study was conducted to determine the optimal culture conditions on *in vitro* propagation and/or polysaccharide production.

MATERIAL AND METHODS

Plant material

Two-year-old *O. japonicus* plants were collected in 2002 from cultivation of Jungchon, Jinju, 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 cultures were incubated under light condition at 25±2℃.

Carbon sources

Sucrose, fructose and glucose were added at concentrations of 1, 3 and 5% (w/v) into medium. All media were adjusted to pH 5.7 and sterilized by autoclaving at 121℃ for 15 min. The cultures were maintained under the 16/8 hour (light/dark) photoperiod at 25±1℃. All treatments were determined as fresh weight (F.W.) on each growth of shoot. Three stems were inoculated on each plate and incubated at 25±2℃ in a growth chamber. Cultured shoots for 4 weeks were used in the experiments and the growth was compared with treatment each other. For determination of fresh weight, *in vitro* plants were separated from the medium and weighed in all of the 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 (MS medium) solution or nutrient not treated. The nutrient solution was MS medium. 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).

Sample of 0.1 ㎖ taken from the above supernatant was mixed with 1 ㎖ distilled water and then added with 5 ㎖ of sulphuric acid, held in a boiling water bath for 20 min, and cooled. Then, the sample was added with 1 ㎖ phenol (pure grade, Merck) and mixed vigorously. After 2 hours of a reaction time in darkness at room temperature, a purplish red color developed and absorbance was measured at 490 ㎚ by ELISA reader (Bio-Rad, model 550, 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) was used to analyze the variance. Values were represented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Effect of various growth regulators on shoot regeneration

Table 1 indicates that the addition of various growth regulators improved the regeneration rate of *O. japonicus*. In leaf part, the highest root regeneration rate was achieved in MS medium with 3.0 mg ℓ^{-1} of IAA, while the highest shoot regeneration rate was achieved in MS medium with 3.0 mg ℓ^{-1} of 2.4-D (Fig. 1a); each values were 83 and 57%. But shoot regeneration rate was worse than in stem part. Relatively, in a stem part, the highest shoot regeneration rate was achieved in MS supplemented medium with 1.0 and 3.0 mg ℓ^{-1} 2,4-D (Fig. 1b), while the lowest shoot regeneration rate was obtained in 5.0 mg ℓ^{-1} NAA; each values were 97 and 50%, respectively.

These result have shown that regeneration rate of the *O. japonicus* was dependent on the type and concentration of growth regulators used in the medium. In particular, the best regeneration rate was shown on the treatment of 2,4-D compared to other auxins and produced the highest average number of somatic embryos per culture, also 2,4-D has been widely used for somatic embryogenesis in oil plants such as peanut (Eapen & George, 1993) and soybean (Lazzeri *et al.*, 1987).

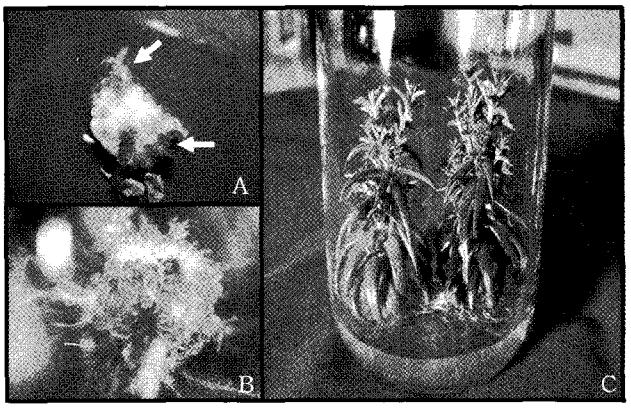

Fig. 1. *In vitro* propagation of *O. japonicus*. (A) Shoot regeneration from leaf. (B) Shoot regeneration from stem. (C) Shoot elongation on MS medium without growth regulators.

Table 1. Effect of various growth regulators on shoot regeneration rate from leaf and stem tissues of *O. japonicus*.

Growth regulators (mg ℓ^{-1})			Leaf		Stem	
			Rooting (%)	Shooting (%)	Rooting (%)	Shooting (%)
Control†			45[hi§]	-	50[f]	-
IAA	1.0‡		80[a]	29[b]	65[bc]	80[bc]
	3.0	+0.5 mg ℓ^{-1} BA	83[a]	-	65[bc]	67[d]
	5.0		59[ef]	29[b]	62[bdc]	78[bc]
	10.0		72[b]	-	68[a]	75[c]
NAA	1.0		59[ef]	-	63[bda]	58[e]
	3.0	+0.5 mg ℓ^{-1} BA	63[de]	-	64[bc]	59[e]
	5.0		55[fg]	-	60[dc]	50[f]
	10.0		58[ef]	-	67[ba]	68[d]
2.4-D	1.0		38[i]	14[c]	65[bc]	97[a]
	3.0	+0.5 mg ℓ^{-1} BA	69[bc]	57[a]	53[ef]	97[a]
	5.0		66[cd]	33[b]	53[ef]	82[b]
	10.0		50[gh]	-	58[de]	50[f]

† Control represents growth regulators free treatments.
‡ All cultures except control treatment were cultured on MS medium with 0.5 mg ℓ^{-1} BA.
§ 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 test and DMRT (Duncan's multiple range test).

Effect of various growth regulators on growth

In order to investigate optimal culture conditions for *in vitro* propagation, a regenerated shoots were cultured in three kinds of growth regulators (IAA, NAA and 2,4-D) on each MS basal medium. IAA, NAA and 2,4-D were used as optimal condition in O. japonicus tissue culture (Table 2). The effective growth level of 6.0 g F.W. were obtained in MS medium with 1.0 mg ℓ^{-1} of IAA concentration.

Auxins are a group of natural and synthetic plant growth regulators that affect cell growth and division (Taiz & Zeiger, 1991). For example, the application of the natural auxin IAA to shoots stimulates cell elongation, whereas its application to roots inhibits elongation and promotes adventitious root formation.

At the cellular level, one of the earliest responses in pea stem epidermal cells to IAA treatment is a transient increase in the percentage volume fraction of Golgi stacks in the cytoplasm, but this increase lasts for less than 90 min. (Cunninghame & Hall, 1985). A more sustained increase in the amount of Golgi material, in parallel with increased rates of cell elongation, has been noted in IAA-treated oat coleoptiles (Quaite *et al.*, 1983).

Table 2. Effect of various growth regulators on plant growth regenerated from *O. japonicus*.

Effect of various growth egulators (mg ℓ^{-1})			Growth of plant F. W. (g)
Control†			4.28 ± 0.49d§
IAA	0.5†	+0.5 mg ℓ^{-1} BA	4.42 ± 0.16d
	1.0		6.00 ± 0.35a
	3.0		5.68 ± 0.14ab
	5.0		2.53 ± 0.12f
	7.0		4.80 ± 0.17cd
NAA	0.5	+0.5 mg ℓ^{-1} BA	5.05 ± 0.31c
	1.0		5.49 ± 0.34b
	3.0		4.42 ± 0.16d
	5.0		2.72 ± 0.11f
	7.0		3.28 ± 0.14e
2.4-D	0.5	+0.5 mg ℓ^{-1} BA	3.47 ± 0.15e
	1.0		5.68 ± 0.24ab
	3.0		4.93 ± 0.18c
	5.0		5.05 ± 0.28c
	7.0		5.05 ± 0.23c

† Control represents growth regulators free treatments.
‡ All cultures except control treatment were cultured on MS medium with 0.5 mg ℓ^{-1} BA.
§ 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 test and DMRT (Duncan's multiple range test).

Effect of carbon sources

Fig. 1 showed the effects carbon source on plant growth and polysaccharide production of *O. japonicus*. When 3% sucrose was included in MS medium, it was indicate the largest growth yield was achieved. A like instance, Francisco *et al.* (2000) reported that sucrose and glucose were both good carbon sources for berry growth and hexose accumulation in *in vitro* cultured berries.

The polysaccharides were produced by *O. japonicus* in media containing different cabon sources, such as sucrose, fructose and glucose. Among them, the polysaccharide contents of plant (0.24×10^3 mg per g D.W.) was the highest in treatment of glucose. Similar results have been reported that the carbon source and growth conditions interacted with the biosynthetic systems of the organisms to produce polymers with a range of different sugars and sugar profiles (Proma Khondkar *et al.*, 2002).

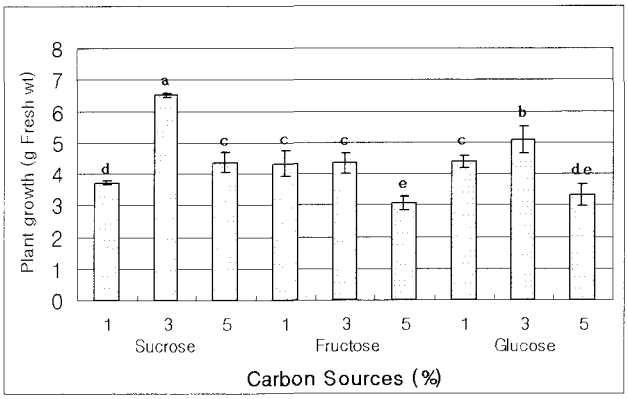

Fig. 2. Effect of carbon sources on plant growth of *O. japonicus*. All cultures were cultured on MS medium without growth regulators. Each numerical value represents the mean and standard deviation from 3 replications after 4 weeks. Means follow by different letters are at 5% level by LSD test and DMRT (Duncan's multiple range test).

In plant cell cultures, the carbon sources may significantly affected growth and metabolites formation. This work focuses on a systematic investigation of the effects of carbon source on *in vitro* propagation of *O. japonicus* plants for the simultaneous shoot growth and polysaccharide production.

Acclimation of micropropagated 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. Each treatment was composed of peatmoss, perlite, sand and vermiculite and treatment of nutrient solution was added into MS basal liquid medium on each treatment. Peatmoss of nutrient solution were better effect on polysaccharide content compared to all treatments. But it was worst about plant growth. Relatively, vermiculite of non-nutrient solution was better effect on plant growth compared to all treatments.

Table 3. Effect of acclimated soils on plant growth and polysaccharide production of *O. japonicus*.

Acclimate medium		Growth of plant F. W. (g)	Contents of polysaccharide† (mg×10³ g⁻¹ D.W.)
Nutrient Solution	Peatmoss	0.91±0.10^{d}	1.89±0.05^{a}*
	Perlite	2.12±0.19^{b}	1.10±0.06^{b}
	Vermiculite	1.74±0.28^{bc}	0.11±0.08^{e}
	Sand	1.62±0.18^{bc}	0.92±0.05^{c}
Non-nutrient Solution	Peatmoss	1.34±0.10^{cd}	0.89±0.07^{c}
	Perlite	1.56±0.20^{c}	0.49±0.02^{d}
	Vermiculite	3.03±0.34^{a}	0.41±0.08^{d}
	Sand	1.71±0.17^{b}	0.87±0.01^{c}

† Contents of polysaccharide measured by Dubois *et al.* (1956).
* Each numerical value represents the mean and standard deviationfrom 3 replications after 4 weeks in culture. Means follow by different letters are at 5% level test and DMRT (Duncan's multiple range test).

This study was done for the optimization of culture conditions such as growth regulators and the carbon sources for *O. japonicus*. Optimal condition of *O. japonicus in vitro* culture could achieve the 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 the enhancement of polysaccharides production.

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