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Optimization of ginseng hairy roots culture and its ginsenoside analysis

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Abstract : Hairy root culture of ginseng is industrially prospected because the cultivation period of ginseng is relatively long. In this study, the effect of medium concentration and sucrose concentration on hairy root culture of ginseng was evaluated. The optimization of ginseng hairy roots transformed by *Agrobacterium rhizogene* were performed liquid medium. The MS(Murashinge & Skoog basal medium) concentration was selected with 1/2 strength MS and the optimal sucrose concentration was determined at 2-3%(w/v). At the optimum culture condition, The yield (the ratio of weight of grown hairy root cultures to weight of fresh ginseng hairy roots) and production rate of ginseng root were 19.42 times and 5.73 g/l-day. The major ginsenosides were Rb group, Re and Rg1. The produced total ginsenoside content in the solid medium was 9.87 (mg/g) and increased 1.34 times in the liquid medium (13.23 mg/g). In solid culture, the contents of ginsenosides Rb, Re and Rg1 were 2.14, 3.65 and 1.87 mg/g, respectively. In liquid culture, the contents of ginsenosides Rb, Re and Rg1 were 3.54, 4.12 and 2.63 mg/g, respectively.

Keywords : ginseng hairy root, optimization, Agrobacterium rhizogene, ginsenosides, culture

1. Introduction

The ginseng is the root of plants in the genus *Panax,* such as Korean ginseng *(P. ginseng*), South China ginseng (*P. notoginseng*), and American ginseng (*P. quinquefolius*), typically characterized by the presence of ginsenosides[1,2]. Ingredients of ginseng, including ginsenosides, have been reported to have pharmacological effects such as anticancer, antioxidant activity, anti-diabetic activity, immunity, neuroprotective and anti-inflammation[3-5].

The roots formed from the differentiated cells are called adventitious roots. The roots develop into a wide range of branches and can produce high biomass. When *Agrobacterium rhizogene*, a Gram-negative soil bacterium, infects plants, adventitious roots called 'hairy roots' are induced from the infected site. In the lab. hairy roots can be obtained directly from the cut edges of the petioles of leaf explants or via callus two-three weeks after inoculation with A. rhizogenes They show the prominent advantages such as high genetic stability,

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hormone independent growth, and relatively fast growth[6-8]. Because of that, hairy roots have been developed for many plants to date.

Hairy root culture of ginseng is industrially prospected because the cultivation period of ginseng is relatively long. The root culture of ginseng started in the 1980s but did not develop industrially[9]. Recently, ginseng root culture has been studied to increase the content of certain ginsenosides (Rb1, Re, and Rg1). In addition, studies on the incorporation of functional substances such as antimicrobial and antioxidant through the root culture of ginseng have been advanced[10-13]. Ginsenosides are triterpene saponins that are the main pharmacologically active ingredients of ginseng which is a perennial herbaceous plant belonging to the family Araliaceae. The major groups of ginsenosides are the Rb group and the Rg group derived from the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol structures, respectively[14-15].

Ginsenosides Rb1, Rb2, Rc, and Rd belong to the Rb group. The Rg group includes saponins Re and Rg1. A lot of in vivo and in vitro tests have indicated that the ginsenosides act on the immune, cardiovascular and nervous systems and that they have antistress, anticancer and neuroprotective activities [16-19]. In this study, the effect of pH, medium concentration and carbon source concentration on hairy root culture of ginseng was evaluated. The ginsenosides isolated from hairy root and ginsenosides were compared and evaluated.

2. Meterials and Method

2.1. Materials

Ginseng (6 years old) was purchased at local market (Geumsan, Korea). Solvents such as methanol, ethanol, n-butanol, chloroform, and H_2SO_4 used for the extraction and the material separation of this study were first-class or special reagents. MS (Murashinge & Skoog basal medium) medium used for culture of ginseng root was obtained from Sigma Company (ST. Louis, U.S.A). Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 were purchased from Chromadex (Seoul, Korea). Ginsensides separation was performed at TLC silica gel 60 F_{254} pre-coated aluminum sheet (Merck Co., Darmstadt, Germany). The hairy roots transformed by *Agrobacterium rhizogens* was supplied from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

2.2. Mineral medium preparation

MS (Murashinge & Skoog basal medium, Sigma) medium, the basic medium for tissue culture, was used in this study. Since the concentration of salt in the MS medium was high, a medium diluted 1/2 times was prepared and tested. MS solid medium was prepared by dissolving 2.2 g of MS powder in 1 L of distilled water, adding 2% sucrose and 0.8% agar, and stirring with a stirrer. The pH of the medium was fixed to 5.8 using 1N-HCl and 1N-NaOH. This was sterilized by autoclave at 121° C for 15 min. The composition of the liquid medium was the same except for the agar in the solid medium.

2.3. Hairy roots culture inoculum preparation

In this study, we performed aseptic and proliferative cultures in order to obtain excellent ginseng hairy roots which is fast growing. After incubation at 25℃ for 30 days, excellent ginseng hairy roots as inoculum were obtained by subculture in fresh medium. The selected hairy roots were transplanted in a 250 mL flask containing 50 mL of the same liquid medium without agar, and cultured in shaking incubator at 60 rpm, 25℃ in dark condition. The hairy roots were transplanted into new medium at intervals of 30 days.

2.4. The effect of MS and sucrose concentration on ginseng hairy roots growth

Cultivation of hairy roots according to the concentration of MS medium was carried out in a 250 mL flask containing 50 mL of MS medium. Cultivation of hairy roots according to the concentration of MS medium was carried out in a 250 mL flask containing 50 mL of MS medium. The concentration of MS medium for culture was 1/4, 1/2, 1, 2, and 3 times strength of original MS concentration, and 0.5-0.7 g of Petri-dish-grown hairy root was inoculated. The ginseng hairy roots culture was performed at pH 5.8 and 25℃ for 5 weeks. Cultivation of hairy roots according to the sucrose concentration was carried out in a 250 mL flask containing 50 mL of MS medium. Cultivation of hairy roots according to the concentration of sucrose was carried out in a 250 mL flask containing 50 mL of 1/2 strength of MS medium. The concentration of sucrose for culture was 1, 2, 3, 4, and 5% (w/v). and 0.5-0.7 g of Petri-dish-grown hairy root was inoculated. The ginseng hairy roots culture was performed at pH 5.8 and 25℃ for 5 weeks.

2.5. Analysis

In this study, the solvent extraction of ginsenosides from each sample was extracted and separated using a water saturated n-butanol extraction method. The dried sample powder was placed in an Erlenmeyer flask, and water-saturated n-buOH was added and extracted in a constant temperature water bath. The butanol extract was washed twice with distilled water, concentrated under reduced pressure, ether was added, and the mixture was refluxed for cooling to remove lipids and the like, followed by concentration under reduced pressure and analysis by TLC.

The 1 mg each of 7 kinds of ginsenoside standard substances Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 and 1 mg of each sample concentrate were dissolved in 50% methanol. 1 uL each was taken and loaded onto a silica gel TLC plate. The developing solvent was CHCl3: CH3OH: H2O (65: 35: 10, v/v). The developed TLC plate was dried at room temperature, immersed in a 5% H2SO4-EtOH solution for 30 seconds, developed in a 105℃ dry oven for 10 minutes, and developed. Quantification of ginsenoside was performed by using a Scion Image program (Scion Image, Scion Corp., Maryland, U.S.A), which was a digital densitometry.

3. Results and Discussion

3.1. Ginseng hairy root cultures transformed by *Agrobacterium rhizogene*

Growth of ginseng root in solid medium and growth of ginseng root in liquid medium are shown in Fig. 1. Selected ginseng hairy root cultures were performed in 1/2 strength of MS solid medium. The initial ginseng cultivar was light yellow, but its color became

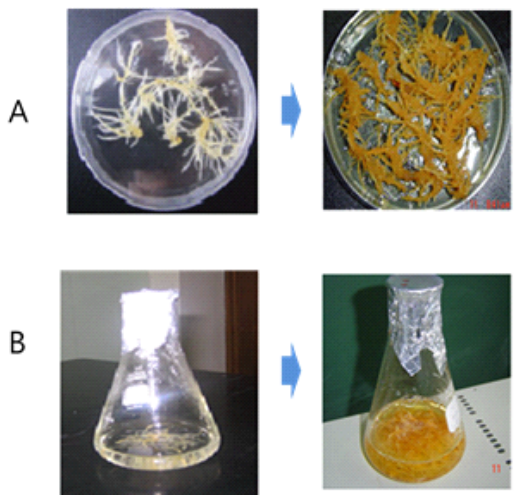

Fig. 1. Ginseng hairy root cultures (A; solid culture and B; liquid culture) transformed by *Agrobacterium rhizogene*. Hairy roots grew at 25℃, pH 5.8, 3%(w/v) sucrose, and 1/2 strength of MS medium for 5 weeks.

similar to cultivated ginseng as it grew. The color of mature ginseng hairy roots seemed to be a mixture of orange and brown as the color of cultivated ginseng, and the surface was rough. Newly growing areas are thin and light yellow or white, but as the cultivating muscle grows, the cultivating muscle becomes thicker and the color becomes darker. In the liquid medium, The ginseng roots grew dark brown in color, as grown in solid culture. The ginseng hairy roots grown as naturally cultivated ginseng formed hairs around the body and smell like typical ginseng favour.

3.2. Effect of MS strength on hairy roots culture

The growth of ginseng cultured muscle was studied with different concentrations of MS medium. The results of the changes in weight after 5 weeks proliferation from the initial weight of ginseng hairy roots according to the concentration of MS medium are shown in Fig. 2. After 5 weeks, we weighed the growth ginseng root. The weight of ginseng hairy roots was significantly different according to the concentration of MS medium. The average weight of ginseng root was about 14 g/L at the early stage of culture. The growth was about 60 to 170 g/L at the end of culture regardless of MS concentration. The highest proliferation was observed at 1/2 MS, and the weight was 169.8 g. And then decreased as the concentration increased. The weight of ginseng hairy roots after 5 weeks of propagation at 1, 2, and 3 strength MS concentrations was 130.9, 92.2, and 63.2 g/L, respectively. Therefore, the optimum MS concentration of cultured ginseng root was determined at 1/2 strength MS.

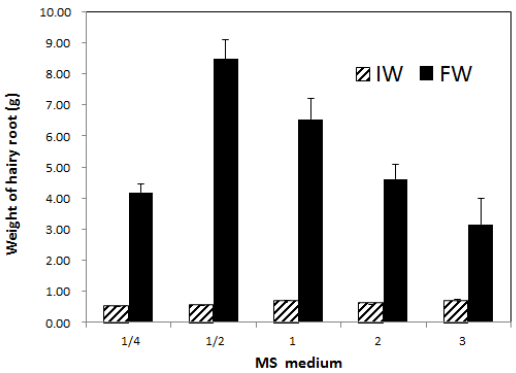

Fig. 2. Ginseng hairy root cultures transformed by *Agrobacterium rhizogene.* Hairy roots grew at 25℃, pH 5.8, 2%(w/v) sucrose for 5 weeks. MS concentrations were 1/4, 1/2, 1, 2, and 3 strength of MS.

3.3. Effect of sucrose concentration on ginseng hairy roots culture

Ginseng roots cultures were studied in different concentrations of sucrose. The weight change of ginseng root after 5 weeks of propagation from the initial weight of ginseng hair root according to sucrose concentration is shown in Fig. 3, The growth of ginseng root hair was significantly different according to sucrose concentration. After 5 weeks of culture, the growth of ginseng roots was proliferated from about 74 to 232 g./L within sucrose concentration. The average weight of ginseng root was about 12 g/l at the early stage of culture. The highest growth was observed at 3(w/v) sucrose concentration, and the weight was 200.4 g. From 1 to 3% sucrose concentration, the growth of ginseng roots increased. In sucrose concentration more than that, the mass of ginseng hairy roots decreased. The optimum concentration of sucrose in cultured ginseng root was not significantly different from 2 to 3%. Therefore, the optimal sucrose concentration was determined to be 2.5%.

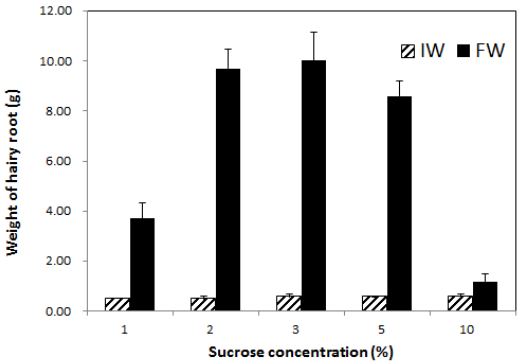

Fig. 3. Ginseng hairy root cultures transformed by *Agrobacterium rhizogene*. Hairy roots grew at 25℃, pH 5.8, 1/2 strength of MS for 5 weeks. The sucrose concentrations were 1, 2, 3, 5, and 10% (w/v).

3.4. Yield and productivity of ginseng hairy roots culture

Table 1 showed the changes in yield and production rate for the growth of ginseng root according to MS medium concentration and sucrose concentration. The yield was calculated by dividing the weight of grown ginseng hairy root after 5 weeks by the weight of the initial root. The production rate was evaluated as the amount of ginseng hairy roots that can be produced in a day in the incubator. The yield and production rate of ginseng root were 19.42 times and 5.73 g/L at the optimum condition.

3.5. Ginsenosides analysis of ginseng hairy roots culture

TLC chromatograms of ginsenosides of the grown hairy roots and standard ginsenosides Rb1, Rb2, Rc, Rd, Rf, Re, and Rg1 are shown in Fig 4. The distribution of ginsenosides in the ginseng hairy roots cultured in the solid medium (HRP) was similar to that of the ginsenoside cultured in the liquid medium (HRF). The main ginsenosides were Rbs (Rb1+Rb2+etc), Rc, Re and Rg1.

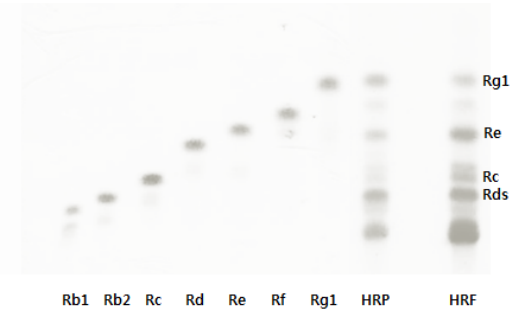

Fig. 4. The TLC chromatogram and analysis of ginsenoside produced from root culture. HRP and HRF represent the ginseng hairy root cultures grown at Petri-dish solid and flask liquid culture.

The total ginsenoside, Rb, Re, and Rg1 amount of ginseng hairy root grown for 5 weeks under optimal culture conditions was analyzed (Table 2). Production of ginsenoside was better in liquid medium than in solid

Table 1. Yield and productivity of ginseng hairy root cultures transformed by *Agrobacterium rhizogene*. Hairy roots grew at 25℃ and pH 5.8 for 5 weeks. MS concentrations were 1/4, 1/2, 1, 2, and 3 strengrt of MS. The sucrose concentrations were 1, 2, 3, 5, and 10% (w/v)

	MS strength (S)					Sucrose concentration (%)				
	0.25	0.5	1	2	3	1	2	3	5	10
Yield[1]	7.91	14.87	9.57	7.48	4.54	7.25	19.42	17.18	14.75	1.99
Productivity[2]	2.39	4.64	3.74	2.63	1.81	2.11	5.55	5.73	4.92	0.66

[1]Yield was calculated by the ratio of weight of grown hairy root cultures to weight of fresh ginseng hairy root cultures. [2]Productivity (g/l-day) was the amount of weight of grown hairy for 5 weeks.

Table 2. The amount of ginsenosides of ginseng hairy root cultures transformed by *Agrobacterium rhizogene* at the optimized conditions for 5 weeks

	Total ginsenosides	Rb	Re	Rg1
	mg/g dw	mg/g dw	mg/g dw	mg/g dw
HRP	9.87	2.14	3.65	1.87
HRF	13.23	3.54	4.12	2.63

HRP and HRF represent the ginseng hairy root cultures grown at Petri-dish solid and flask liquid culture.

medium. The total ginsenoside content in the solid medium was 9.87 (mg/g) and increased 1.34 times in the liquid medium (13.23 mg/g). In solid cuture, the contents of ginsenosides Rb, Re and Rg1 were 2.14, 3.65 and 1.87 mg/g, respectively. In liquid cuture, the contents of ginsenosides Rb, Re and Rg1 were 3.54, 4.12 and 2.63 mg/g, respectively. Perhaps it is because the nutrients in the medium are distributed in the liquid medium. In the previous other study, the most abundant ginsenosides of *P. quinquefolium* hariy roots were Rb1 and Re and was similar to this study[20]. It was reported that incase of *P. ginseng* hariy roots, the most abundant ginsenosides were Rb1 and Re[21].

4. Conclusion

The ginseng hairy roots transformed by *Agrobacterium rhizogene* were cultured in solid medium and growth of ginseng root in liquid medium. The grown ginseng roots showed the dark brown in color and smell like typical ginseng favour. The growth of ginseng hairy roots was studied with different concentrations of MS medium. The optimum MS concentration of cultured ginseng root was determined at 1/2 strength MS. Ginseng roots cultures were also studied in different concentrations of sucrose. The optimal sucrose concentration was determined to be 2-3%(w/v). At the optimum culture condition, The yield and production rate of ginseng root were 19.42 times and 5.73 g/L. The distribution of ginsenosides in the ginseng hairy roots cultured in the solid medium was similar to that of the ginsenoside cultured in the liquid medium. The main ginsenosides were Rb, Rc, Re and Rg1. The total ginsenoside content in the solid medium was 9.87 (mg/g) and increased 1.34 times in the liquid medium (13.23 mg/g). In solid culture, the contents of ginsenosides Rb, Re and Rg1 were 2.14, 3.65 and 1.87 mg/g, respectively. In liquid culture, the contents of ginsenosides Rb, Re and Rg1 were 3.54, 4.12 and 2.63 mg/g, respectively.

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