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Optimization of Major Culture Elements on Growth and Shikonin Production in the *Lithospermum erythrorhizon* Hairy Root Culture

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ABSTRACT : The effects of basal media, carbon, nitrogen, phosphate and some major macro elements on growth and shikonin production in *Lithospermum erythrorhizon* hairy root culture were studied. Among examined media, growth of hairy root cultured in B5 liquid medium was rapid, whereas shikonin production was high in MS liquid medium. Under B5 basal medium, sucrose concentration for optimal growth and shikonin production was 9% and 4% respectively. The growth and shikonin production on pH changes in B5 medium resulted little effect in pH 5.8 to pH 8.8 ranges, whereas growth was decreased dramatically in both above 8.8 and under 5.8. Nitrogen source and concentration effected on the growth and shikonin production. The highest growth rate was in B5 medium (50 mM KNO_3 and 1 mM NaH_2PO_4), whereas the highest shikonin production was in the condition supplemented with 5 mM KNO_3 and 10 mM NaH_2PO_4.

Key words : shikonin, basal media, sucrose, pH, nitrogen, phosphate, major macro elememnts

INTRODUCTION

Lithospermum erythrorhizon Sieb. et. Zucc. is a perennial herbaceous plant distributed in Hainan, Heilongjiang, Yanbian, Hunan, Guangxi, Guizhou of China, the Amur area, and Japan. For Korea, it used to be distributed in the wild life of entire nation, but the number has conspicuously reduced that it has become the rare plant now.

The root of *L. erythrorhizon* contains the red pigment and this red pigment is a quinones that is comprised with the shikonin, a naphthoquinone type, and shikonin derivatives. The red pigment has long been used in Japan for a pharmaceutical product such as injured part, burns and others or used for painting material as well as the drug for constipation. For medicinal action of shikonin, it has the effect of birth control, anti-biosis, anti-inflammation action, and anti-cancer action by controlling the formation of pituitary hormone. In particular, it strengthens the heart and relaxes the liver functions. Also, it removes the extravasated blood and helps the blood circulation as well as treating the constipation and hematria. The folk medicine uses it for burns, frostbite, blister and others as well as fever, diuresis, and birth control substance.

In Korea, the *L. erythrorhizon* root is used as the edible coloring substance. The distilled alcohol by using the *L. erythrorhizon*, Jindo Red Wine, has been widely known as the traditional alcohol in Korea for its existence of several hundred years. Currently, the wild *L. erythrorhizon* is very difficult to find and it has different formation and content of its pigment depending on the acidity of soil and others (Cho *et al.*, 1999) that it is hard to provide

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the consistent quality of raw materials. Currently, the *L. erythrorhizon* is cultivated in the Jecheon area in a small area, but the production volume in Korea is now known. It is estimated to consume approximately 29 tons per year as the material for Jindo Red Wine. The production volume of red wine is 57.7 *kl*/year (based on distribution volume with the available data) that the required volume for *L. erythrorhizon* for that amount is figured to be 29 tone (based on 1 of red wine/500g of raw root) that the expected cultivation area for the independent product is estimated to reach approximately 11 *ha*/year.

Studies on shikonin production of *L. erythrorhizon* to this point are reported on cell culture (Gaissers *et al.*, 1996; Heide *et al.*, 1989), shoot culture (Touno *et al.*, 2000), hair root culture (Shimomura *et al.* ; 1991) and several others. In Japan, the edible color substance, shikonin, has been used as the coloring for cosmetic products by using the cell culture from 1985 (Touno *et al.*, 2000; Shimomura *et al.*, 1991). In the event of the *L. erythrorhizon*, shikonin is produced mostly from the root, particularly it is accumulated only in the external skin in the full, root hair and other areas of the most outside tissues of the root. In the event of hair root culture, the surface area of each unit weight is broader than the wild or cultivated one that the shikonin production through the hair root culture is clearly more advantageous than any other culture method in the aspect of efficiency. (Shimomura *et al.*, 1991).

This study is aimed at examination of optimal culture requirement of hair root culture of *L. erythrorhizon* to enhance the shikonin productivity.

MATERIALS AND METHODS

Plant material

Hairy root of Lithospermum erythrorhizon was obtained from Dr. Hwang Sung Jin(Dongsin university). For the hairy root induction, *A. rhizogenes* ATCC 15834 was used for transformation. Transformed hairy roots were culcured on B5 liquid medium containing 3% sucrose(pH 5.8 before autoclaving). Before this study, the hairy roots(five root tips per a flask) were maintained for 2 weeks on hormone-free B5 liquid media. The cultures were incubated on a rotary shaker(100rpm/min) at 25℃ in the dark.

Batch experiment procedure

B5, S-H, MS, 1/2 MS, White, RCM and M-9 media were used to investigate the growth of hairy roots and shikonin content of roots on each medium. In addition, the growth of hairy roots and shikonin content of roots on B5 medium under various conditions was compared. The investigated conditions were sugar concentration, initial pH and nitrogen, phosphate, calcium and magnesium concentration. To study, five root tips per a flask were inoculated into a 100mL flask containing 30ml of hormone-free B5 liquid medium with 30g/L of sucrose and cultured on a rotary shaker(100 rpm/min) at 25℃ in the dark conditions for four weeks.

Analysis of shikonin

The shikonin content in culture media and hairy roots was determined by shimomura *et al.*, (1991) method. Shikonin derivatives were converted into decarboxylated shikonin by 2.5% NaOH and its absorbance was measured at 620nm using UV spectrometer(Hanson Technology Co.).

RESULTS AND DISCUSSION

1. Changes in shikonin productivity depending on various culture media

In order to achieve mass multiplication through the culture of a plant, the study to optimize the culture element or culture condition that effects on the growth of the plant and secondary metabolism synthesis. Accordingly, for the purpose of selecting the most appropriate basic culture in the content increase of shikonin that contains the hair root and growth of the *L. erythrorhizon* hair root, 3% sucrose is added to the B5, S-H, MS, 1/2MS, White, RCM, M-9 medium to adjust it for pH 5.8. Then these are cultured in the dark for 4 weeks to have the best growth rate in the B5 medium (Fig 1). In the result of having the macro element density for 1/4. 1/2. 1. 2. and 4 times of B5, again B5 showed the best growth that B5 medium was selected as the basic

medium. In the event of *L. erythrorhizon* hair root that was induced by Shimomura *et al.*, (1991), the RCM medium was selected as the basic medium, and this is attributable to have a difference in the characteristics in the optimal culture condition depending on the strain even from the inducement of the same species. In particular, according to the experiment reported earlier, the MS medium showed a fine growth but no formation of coloring substance (Shimomura *et al.*, 1991), but in this experiment, the MS medium was shown to have higher coloring substance formation.

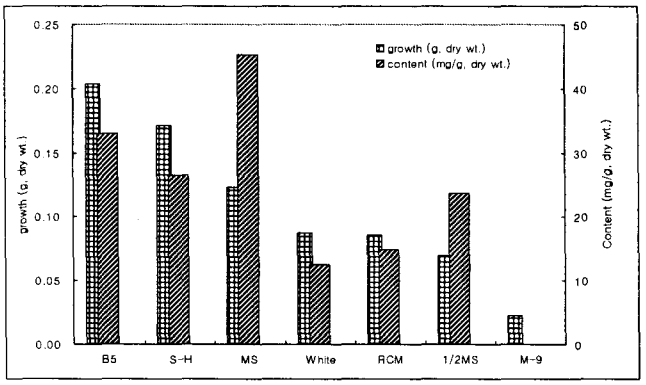

Fig. 1. Effect of various liquid medium on the growth and shikonin derivative formation of *L. erythrorhizon* hairy roots cultured in liquid B5 medium (30 ml medium/100 ml flask) on a rotary shaker (100 rpm) at 25 for 4 weeks in the dark.

2. Changes in shikonin productivity depending on various sucrose concentration

The B5 medium was fixed as the basic medium and the sucrose concentration was changed to 1~10% for the survey of growth and shikonin productivity. As the result, the growth showed the increase in proportion to the sucrose concentration, and the dried weight was shown to be highest at 0.48 g/flask at 9% sucrose, and it reduced at the higher concentration. On the other hand, the highest value was shown at 4% in the shikonin content (Fig 2). Such a result showed a slightly different result from the report that had the shikonin production promoted when the sucrose concentration of 5% or more at the cell culture of *L. erythrorhizon* (Mizukami *et al.*, 1977, 1978).

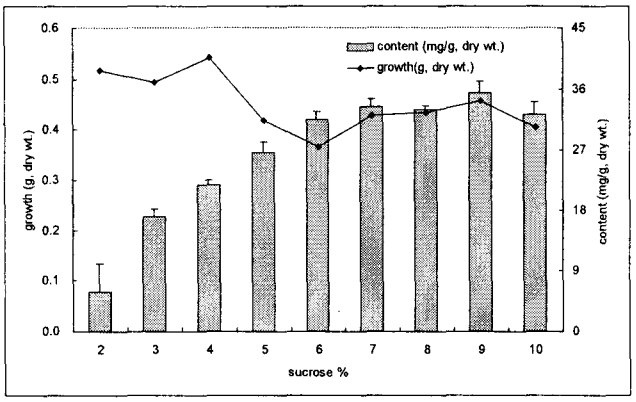

Fig. 2. Effect of sucrose concentration (2~10%) on the growth B5 and shikonin content of *L. erythrorhizon* hairy roots cultured in liquid medium (30 ml medium/100 ml flask) on a rotary shaker (100 rpm) at 25 for 4 weeks in the dark.

3. Changes in shikonin productivity depending on pH variation

pH of B5 medium (3% sucrose) was adjusted for 3.8~8.8 and culture for 4 weeks, then the growth rate and the shikonin content were investigated. As the result, the pH range of 5.8~8.8 showed not much difference in the growth rate, and showed a rapid growth reduction effect at pH 5.3 or less (Fig 3). Another word, it was observed that it has wide pH tolerance. In the meantime, enzyme related to the shikonin synthesis was reported to have the optimal pH of broad scope in between pH 7.1 and 9.3

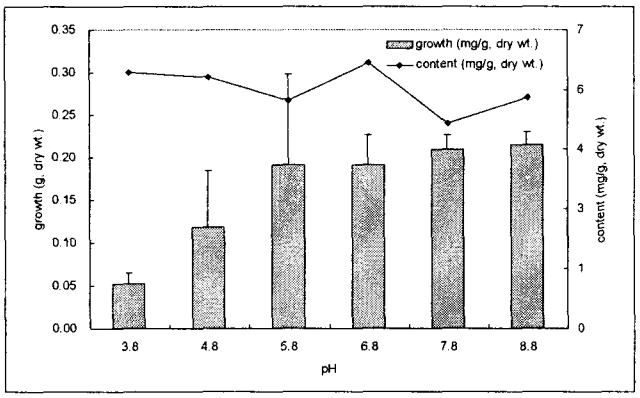

Fig. 3. Effect of pH on the growth and the shikonin content of *L. erythrorhizon* hairy roots cultured in B5 liquid medium (30 ml medium/100 ml flask) on a rotary shaker (100 rpm) at 25 for 4 weeks in the dark.

(Heide and Tabata, 1987). The pigmentation showed even contents regardless of pH, but the color change depending pH changes was observed. When pH was acid, it showed red color. In neutral, it showed the violet to gradually changed to deep blue that it is consistent to the result in the characteristics to vary the color following the pH changes that showed the red color for acid, violet for neutral, blue for alkali (Cho *et al.*, 1999). However, the change of pH does not effect greatly on the shikonin production.

4. Changes in shikonin productivity depending on Macro Elements Concentration

Macro element has the B5 liquid medium as the basic medium to measure the change of concentration of mass composition element from the medium composition element effecting on the shikonin production and the growth of the *L. erythrorhizon* hairy root.

1) Nitrogen

The nitrogen for plants is mainly NH_4^+ and NO_3^-. Plants have different absorption capability of nitrogen and formation to use depending mainly on then species and development stage. The absorbed nitrogen goes through the assimilation to impact heavily on the plant growth with its major component of protein, nucleic acid, amines, chlorophyll and enzyme. Also, the NH_4^+/NO_3^- ratio in medium is known to effect in the production of useful substance in addition to the growth of plants that the understanding of the types and density of appropriate nitrogen is critical not only for growth but also for increase of useful substance synthesis.

① Nitrate ion : The KNO_3 concentration of B5 medium was adjusted for 0, 5, 25, 50 and 100 mM each and investigated the growth and shikonin content. The shikonin formation was shown to be best for 5 mM, but the growth was better for about 5 times when comparing from 50 mM to 5 mM. In the productivity aspect considering the growth and pigment volume, the KNO_3 of 50 mM was deemed as the most appropriate (Fig 4).

② Ammonium ion : $(NH_4)_2SO_4$ concentration of B5 medium was adjusted for each 0, 1, 5, 10 and 50 mM each and investigated the growth and shikonin content. When the KNO_3 was 0 mM, there was no

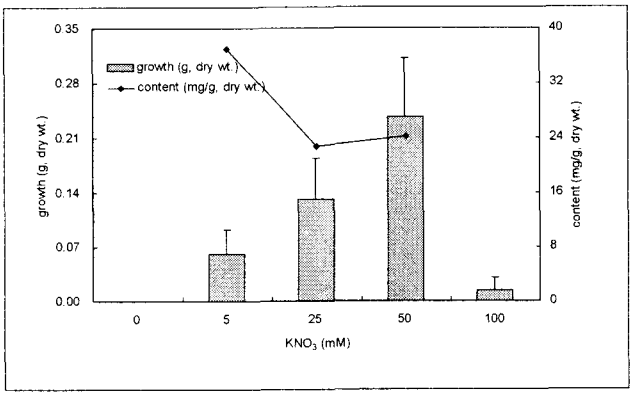

Fig. 4. Effect of nitrate ion (KNO_3) concentration on the growth and the shikonin content of *L. erythrorhizon* hairy roots cultured in B5 liquid medium (30 ml medium/100 ml flask) containing 3% sucrose at 25 for 4 weeks in the dark.

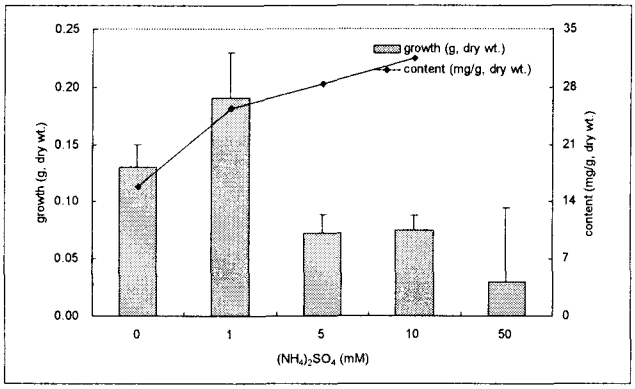

Fig. 5. Effect of ammonium ion [$(NH_4)_2SO_4$] concentration on the growth and the shikonin content of *L. erythrorhizon* hairy roots in B5 liquid medium containing 3% sucrose at 25 in the dark.

showing of growth at all, on the other hand, NH_4^+ showed a certain level of growth even when it is not contained in the medium, but the best growth was shown for 1 mM $(NH_4)_2SO_4$ and the shikonin formation was best at 5 mM $(NH_4)_2SO_4$ (Fig 5). This result was slightly different from the report of Fujita and others (1981, a, b) that NH_4^+ interferes tnhibits formation of shikonin. However, under the density of 5 mM or more, the growth interference was shown that, when considering the growth and shikonin production, the $(NH_4)_2SO_4$ of 1 mM is deemed to be most appropriate.

2) Phosphate

Phosphate is a structure element of molecules in the cell, and is the prodromal substance for synthesis of nucleic metabolism and phosphatide. It is also known to adjust the numerous metabolism activation and path in the body that it is critical to understand the appropriate phosphate density. Accordingly, as the result of experimenting with NaH_2PO_4 of 0, 1, 50, 10 and 100 mM concentration for phosphate on the B5 medium, 1 mM showed the best shikonin formation, but the growth was highest at the 10 mM (Fig 6). However, when considering all growth and pigmentation volume, NaH_2PO_4 of 10 mM is shown to be most appropriate.

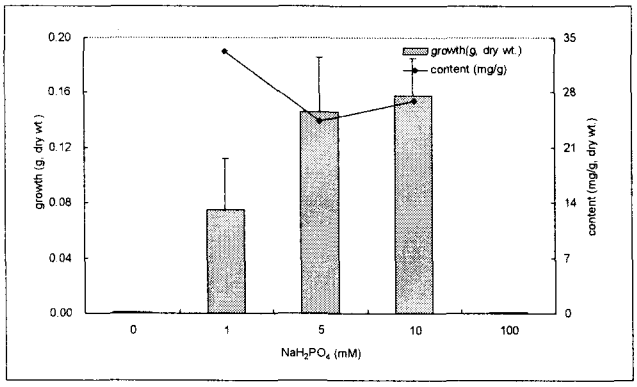

Fig. 6. Effect of phosphate ion (NaH_2PO_4) concentration on the growth and the shikonin content of *L. erythrorhizon* hairy roots in B5 liquid medium containing 3% sucrose at 25 in the dark.

3) Calcium

Calcium works as a cofactor of many enzyme with the important function in the synthesis of cell wall that, if the calcium is deficient, there will be necrosis in the shoot tips (Aimin *et al.*, 1999). On the B5 medium, $CaCl_2$ was adjusted for 0, 0.5, 1, 10 and 50 mM density as the calcium source, and measured the growth and shikonin contents. The growth showed the highest at the high density of 50 mM, but the shikonin content was highest when 1 mM $CaCl_2$, the B5 medium content, was added (Fig 7).

4) Magnesium

Magnesium is not only the chlorophyll structure element, but also, it takes an important role in the enzyme function, and it has the function to control the ion balance as the positive ion in the plants. In particular, magnesium is reported to work as the activator of geranyltiansferase, an important regulating enzyme of shikonin synthesis. (Heide and Tabata, 1987). Therefore, to find out the optimal density of magnesium in the hairy root of *L. erythrorhizon*, the density of $MgSO_4$ is adjusted for 0, 0.5, 1, 10 and 50 mM on the B5 medium, the growth and shikonin content are measured. As the result, when $MgSO_4$ is 10 mM, it shows the highest growth in two times than the basal concentration of B5 medium, 1 mM, and when $MgSO_4$ is 0.5 mM, it shows the best shikonin formation (Fig 8). However, considering the growth and pigmentation, it is most appropriate when $MgSO_4$ has the density of 10 mM.

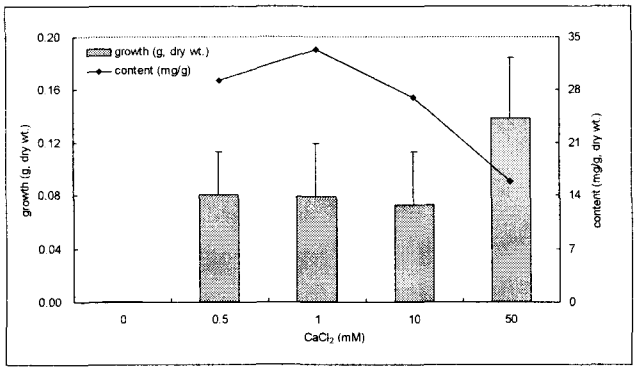

Fig. 7. Effect of calcium ($CaCl_2$) concentration on the growth and the shikonin content of *L. erythrorhizon* hairy roots in B5 liquid medium containing 3% sucrose at 25 in the dark.

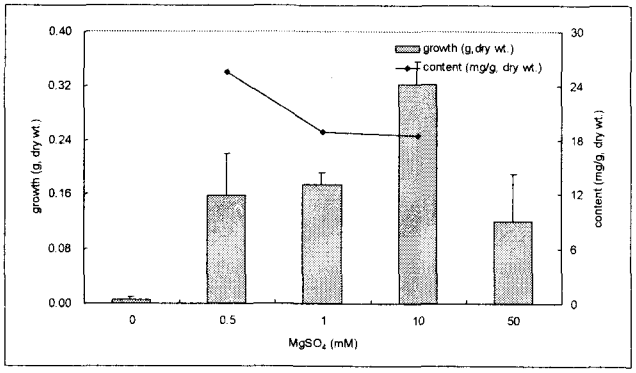

Fig. 8. Effect of magnesium ($MgSO_4$) concentration on the growth and the shikonin content of *L. erythrorhizon* hairy roots in B5 liquid medium containing 3% sucrose at 25 in the dark.

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