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Effects of Macronutrients on Anthocyanin Production in Roselle (*Hibiscus sabdariffa* L.) Callus Cultures

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The effects of various macronutrients on growth and anthocyanin formation in callus cultures of roselle (*Hibiscus sabdariffa* L.) were investigated. Of the nutritional factors examined the type and concentration of carbon and nitrogen sources and phosphate concentration showed marked effects on the growth and anthocyanin production. Utilization of an optimized medium based on the results obtained in the present investigation resulted in a 2. 5 fold increase in the anthocyanin content. Potential exists for application of a two-stage culture method for the production of anthocyanin pigment.

Introduction

Calyces of roselle (*Hibiscus sabdariffa* L.) contain cyanidin and delphinidin glycosides[1] and have been used for making jelly, jams, beverages and food colorants[2]. Cultured roselle cells might potentially be a suitable source for large scale production of anthocyanin pigments. In a previous paper[3] we reported that callus tissues derived from seedlings of roselle could accumulate anthocyanin pigments tentatively identified as cyanidin-3-monoglucoside (major pigment) and cyanidin-3-xylosylglucoside. Anthocyanin formation in the callus markedly enhanced by 2, 4-D and inhibited by gibberelic acid.

The present paper describes the effects of macronutrients on cell growth and anthocyanin production of roselle callus as well as an optimized growth and production medium based on the results obtained in this study.

Materials and Methods

Plant material and culture method. Callus tissues derived from seedlings of roselle were subcultured at 1-month intervals on Linsmaier and Skoog (LS) basal agar medium[4] supplemented with 1 μM 2, 4-D and 1 μM kinetin at 25° under 3, 000 lux illumination (16 hr/day). For investigating the infiuence of various macronutrients, callus tissues (ca 0. 2 g) were transferred onto 20 ml of test medium in 50 ml Erlenmeyer flasks and incubated at 25° under illumination for four weeks before harvest. All the test media contained both 2, 4-D and kinetin at 1 μM level.

Extraction and quantitative analysis of anthocyanin. Fresh callus tissues were homogenized with 1% methanolic HCl in a mixer. The homogenate was allowed to stand overnight at 4° and then filtered. Absorbance of the filtrate was measured at 530 nm and the anthocyanin content was calculated as a percentage of fresh weight of callus using the molecular extinction coefficient (log ε 4. 47) for cyanidin-3-monoglucoside[5].

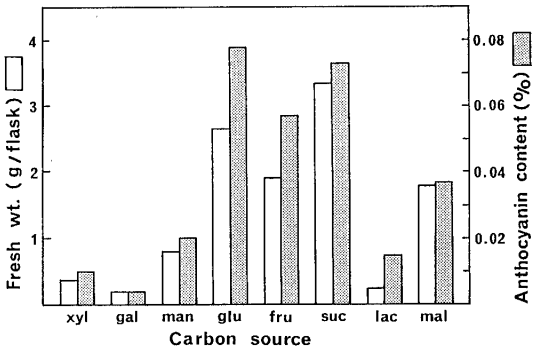

Fig. 1 Effects of various carbon sources on growth and anthocyanin production of roselle callus cultures. All the carbon sources were added to the medium at 3% concentration. Each column indicates the average from triplicate measurements. Abbreviations=xyl, xylose; gal, galactose; man, mannose; glu, glucose; fru, fructose; suc, sucrose; lac, lactose; mal, maltose.

Results and Discussion

Effects of carbon source

Although sucrose as a carbon source can support both growth and secondary metabolite production in callus and suspension cultures of plant cells, other carbon sources can also be effective. The effects of different carbon sources at 3% concentration on the growth and anthocyanin production of roselle callus are shown in **Fig. 1**. As regards anthocyanin formation glucose was as effective as sucrose, whereas fructose and maltose could support anthocyanin formation to a limited extent. In contrast, sucrose was most effective for cell growth; glucose, fructose and maltose were much inferior to sucrose. Other carbon sources such as xylose, galactose, mannose and lactose could support neither cell growth nor anthocyanin formation.

Increase in the sucrose concentration above the 2-3% normally used in the basal medium has been reported to enhance the production of various phenolic compounds, including anthocyanin[6-8]. The optimal concentration of sucrose for the growth and anthocyanin production of roselle callus cultures was examined by varying the sucrose concentration from 1% to 8% in LS medium. Average callus fresh weight per flask was highest with 3% sucrose concentration. Above or below this concentration cell growth tended to decrease rather markedly. In contrast, the anthocyanin content was highest with 3-5% sucrose concentrations (data not shown). Therefore, the optimal sucrose concentration was concluded to be 3% for both growth and anthocyanin production.

Effects of nitrogen sucroses

It has been shown that both type and amount of nitrogen sources in the basal medium affect the biosynthesis of various phenolics in cultured plant cells. *Lithospermum* cells cultured in suspension could only accumulate shikonin derivatives with NO_3^- as the sole nitrogen source[9] and produced rosmarinic acid and lithospermic acid instead of shikonin when cultured in LS medium which contains about 20 mM NH_4^+ in addition to 40 mM NO_3^-[10]. With respect to anthocyanin biosynthesis, cultured cells of *Vitis* showed the highest anthocyanin accumulation when cultured in a medium containing NH_4^+ and NO_3^- in a 1 : 1 ratio[8].

The effects of the composition of nitrogen sources on roselle callus cultures were examined by varying the ratio of NH_4^+ to NO_3^- without changing total nitrogen concentration (**Fig. 2**). Ammonium chloride and potassium nitrate were used as sources of NH_4^+ and NO_3^-, respectively. Cell growth was highest when NO_3^- was uted as sole nitrogen source and decreased with increasing

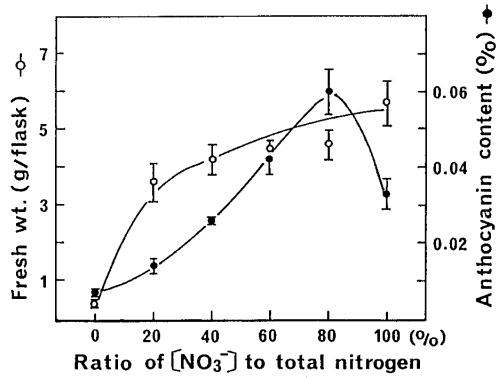

Fig. 2 Effects of altering the ratio of nitrate to total nitrogen concentration on growth and anthocyanin production of roselle callus cultures. Nitrate was added as KNO_3 and the balance was added as NH_4Cl. Total nitrogen concentration was 60 mM. Vertical lines represent standard errors (n= 5).

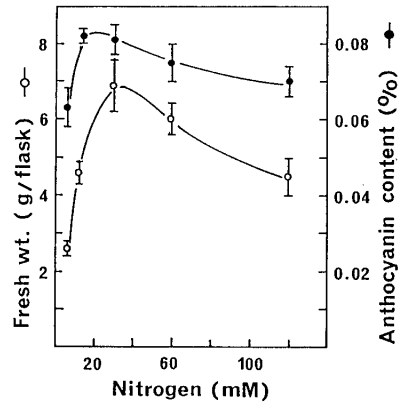

Fig. 3 Effects of varying the initial total nitrogen concentration on growth and anthocyanin production of roselle callus cultures. Initial NH_4^+ to NO_3^- was 1 : 4. Ammonium and nitrate were added as NH_4Cl and KNO_3, respectively. Vertical lines represent standard errors (n=5).

NH_4^+ concentration. In contrast, medium containing both NH_4^+ and NO_3^- at a molar ratio of 1 : 4 was most effective for anthocyanin production of roselle callus.

The concentration of total nitrogen sources in the based medium was then changed, keeping the ratio of NH_4^+ to NO_3^- as 1 : 4 (**Fig. 3**). The anthocyanin content was highest with 12-30 mM nitrogen concentration and decreased gradually with increasing nitrogen concentration. This seems to be consistent with the results in most other studies, which have found that decreasing the nitrogen level stimulated the production of phenolic compounds[11].

The highet cell growth was obtained when 80 mM potassium nitrate was added to the medium as sole nitrogen source (data not shown).

Effects of phosphate

Phosphate is known to be an important regulator of secondary metabolism in cultured plant cells. It has been reported that high phosphate levels decreased formation of various phenolics including coumarins[12], polyphenols[13] and anthocyanins[8,14-15]. In contrast, production of anthraqinones in *Morinda citrifolia*[15], rosmarinic acid in *Anchusa officinalis*[16] and betacyanin in *Phytolacca americana*[17] was improved by increasing the phosphate concentration.

As shown in **Fig. 4**, the anthocyanin content of roselle callus tissues decreased significantly with

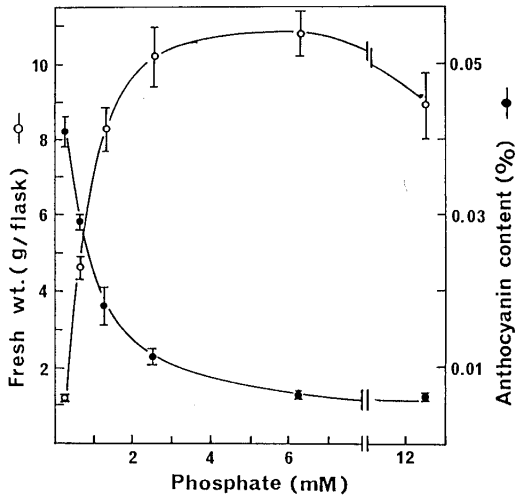

Fig. 4 Effects of varying the initial phosphate concentration on growth and anthocyanin production in roselle callus cultures. The initial phosphate concentration in LS basal medium is 1.25 mM as KH_2PO_4. Vertical lines represent standard errors (n=5).

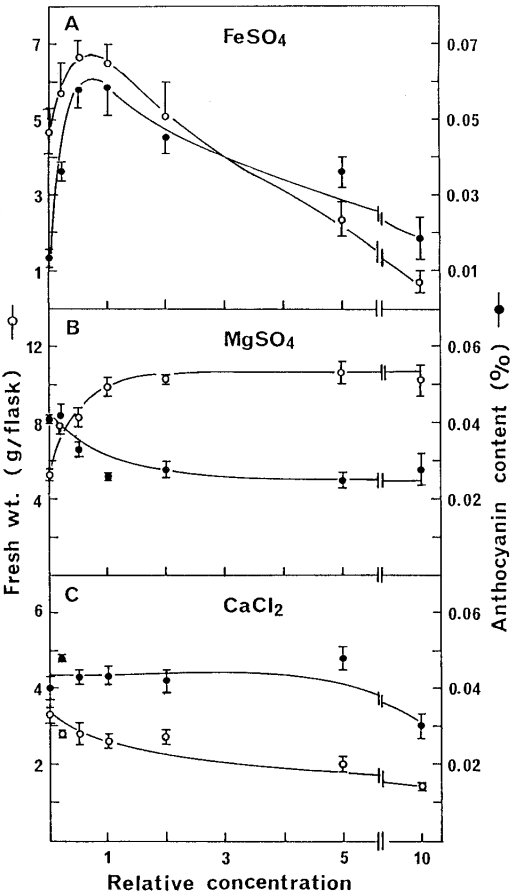

Fig. 5 Effects of (A) $FeSO_4$, (B) $MgSO_4$ and (C) $CaCl_2$ on growth and anthocyanin production of roselle callus cultures. The initial concentrations of $FeSO_4$, $MgSO_4$ and $CaCl_2$ in LS basal medium (relative concentration=1) were 0.1, 1.5 and 3.0 mM, respectively. Vertical lines represent standard errors (n=5).

increasing phosphate levels, while the cell growth increased. Therefore, the normal phosphate concentration in LS medium was found to be supraoptimal for the anthocyanin formation and suboptimal for the cell growth. This finding is in agreement with the previous reports on anthocyanin formation in cultured cells of *Catharanthus roseus*[6], *Vitis* hybrid[8] and *Daucus carota*[14]. However, the mechanism of inhibitory effect of phosphate on the anthocyanin formation remains to be elucidated.

Effects of other nutrients

There have been few studies on the role of nutrients other than carbon, nitrogen and phosphate in the regulation of secondary metabolism in cultured plant cells except for those reported with respect to production of anthraquinones[15], rosmarinic acid[16] and naphthoquinones[19].

In addition to carbon-and nitrogen-sources and phosphate we have investigated the effects of other macronutrients such as $FeSO_4$, $CaCl_2$, $MgSO_4$ and *myo*-inositol on roselle callus cultures. Anthocyanin production in roselle callus cultures was highly dependent on the addition of $FeSO_4$ (**Fig. 5A**); both growth and anthocyanin content were highest with 0.05 to 0.10 mM $FeSO_4$. The latter is the nomal concentration in LS basal medium. Below these concentrations, both anthocyanin content and callus fresh weight rapidly decreased, while above these concentrations they tended to decrease gradually.

The doso-effect relationship for $MgSO_4$ was quite different from that for $FeSO_4$ as shown in **Fig. 5B**. The cell yield increased rapidly with increasing $MgSO_4$ concentration up to 1.5 mM, the concentration in LS medium, and it remained constant above this concentration. In contrast, the anthocyanin content was highest at 0-0.30 mM $MgSO_4$ concentrations and then decreased with increasing $MgSO_4$ level.

Anthocyanin production in roselle callus cultures was little affected by changes of either $CaCl_2$ (**Fig. 5C**) or *myo*-inositol (data not shown) concentrations except that it was slightly inhibited by extremely high concentration (10 times higher than the normal level) of $CaCl_2$. Cell growth was slightly enhanced with 0.56 mM *myo*-inositol (the concentration in LS medium), whereas supplying additional $CaCl_2$ was unnecessary to support the cell growth. This is contrary to the results obtained for rosmarinic acid production in *Anchusa officinalis* cultured cellss, where $CaCl_2$, up to 0.25 mM, had marked stimulatory effects[17].

Cell growth and anthocyanin production in the revised medium

Based on the results obtained in the present investigation, revision of the medium optimized either for cell growth (growth medium) or for anthocyanin production (production medium) was attempted by combining the nitrogen source, phosphate, $MgCl_2$ and $CaCl_2$ levels determined individually to be

Table 1. Effects of revised medium on growth and anthocyanin content of roselle callus cultures

Medium	Fresh weight[1] (g/flask)	Anthocyanin[1] (% of fw)
Linsmaier and Skoog	7.3±0.5	0.046±0.005
Growth medium[2]	10.4±1.1	0.003±0.001
Production medium[3]	2.9±0.5	0.113±0.016

1) Values are means ± standard errors fom at least 5 replicates.
2) Growth medium contains 80 mM KNO_3, 6.25 mM KH_2PO_4, 0.6 mM $CaCl_2$ and 1 μM 2, 4-D and 1 μM kinetin. NH_4^+ is omitted. Other nutrients are the same as those in LS medium.
3) Production medium contains 6 mM NH_4Cl, 24 mM KNO_3, 0.25 mM KH_2PO_4, 0.6 mM $CaCl_2$, 1 μM 2, 4-D and 1 μM kinetin. $MgSO_4$ is omitted. Other nutrients are the same as those in LS medium.

optimal. In the growth medium, 80 mM KNO_3 was added to the medium as sole nitrogen source and phosphate and $CaCl_2$ were changed respectively to 5-fold and one-fifth of those in normal LS medium. In the production medium, 12 mM NH_4Cl and 48 mM KNO_3 were used as nitrogen source, $MgSO_4$ was omitted and phosphate and $CaCl_2$ were reduced to one-fifth of those in normal LS medium. The rest of the medium ingredients were not changed in both the growth and production media.

As shown in **Table 1**, fresh weight and anthocyanin content of the roselle callus cultured in the growth medium were 142% and 7%, respectively of those cultured in LS medium, whereas fresh weight and anthocyanin content of roselle callus cultured in the production medium were 42% and 250%, respectively, of those in LS medium. Such an inverse relationship between growth and secondary mtabolite accumulation has been reported for many plant cell cultures[19]. The two-phase culture system is based on the concept that an efficient production of secondary metabolite will be attainable if cells grown in a medium devised for growth are then transferred to a medium optimized for secondary product formation. It has been successfully employed in *Lithospermum* suspension cultures[20] and in cell suspension cultures of *Salvia miltiorrhiza*[21].

The development of such a two-phase culture system is under investigation for large-scale production of anthocyanin pigment by roselle cell cultures.

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《和文要約》

ローゼル（*Hibiscus sabdariffa* L.）のカルス培養における アントシアニン生成に対する培地成分の効果

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(要約)　ローゼル（*Hibiscus sabdariffa* L.）カルス組織の生長とアントシアニン色素生成に対する培地多量成分の効果について検討した．取り上げた種々の要因の中で，炭素源と窒素源の種類と濃度ならびにリン酸塩の濃度が生長とアントシアニン形成に対して 顕著な影響を及ぼした．実験結果に基づいて改良した増殖用培地および色素生産用培地を用いることによって，ローゼルカルスの増殖用およびアントシニン含量は，Linsmaier-Skoog の培地を用いた場合と比較して，それぞれ 1.4 倍，2.5 倍に増加した．これら結果は，ローゼル培養細胞を用いるアントシアニン生産において 2 段階培養法を検討することの有用性を示唆している．