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Production of Pigments and α-Tocopherol by Cell Cultures in Safflower (*Carthamus tinctorius* L.)

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Abstract

Safflower is an important medicinal plant that has been used in China, Korea and Japan for thousands of years. The red and yellow pigments obtained from the petals of safflower can invigorate blood, release stagnation and promote menstruation. In addition, these pigments are used safely in processed foods and soft drinks as naturally harmless color additives. On the other hand, the seed of safflower contains 30-40% oil with higher level of mono- and poly-unsaturated fatty acid profiles and elevated levels of α-tocopherol. In this paper, we describe advances in the production of pigments and α-tocopherol by cell culture in safflower.

Introduction

The dried flowers of safflower have been used in China, Korea and Japan for thousands of years. Its active compounds are the red and yellow pigments. The functions of these pigments are to invigorate blood, to release stagnation and to promote menstruation. It is used to stimulate blood flow and relieve pain by removing stagnant blood for the treatment of amenorrhea, chest and abdominal pain, painful swelling due to blood stasis, hepatomegaly and splenomegaly; it is also used to relieve pain from traumatic wounds (Liu and Xiao, 1993). Moreover, interest in food colorants has been directed to the use of natural pigments because of the restrictions on using synthetic pigments. Yellow and red pigments obtained from the petals of safflower are safely applicable to processed foods and soft drinks as naturally harmless color additives (Meselhy et al., 1993a; Watanable, 1997). Over 30 constituents have been extracted from the flower petals of safflower, and the most important group is the pigments, such as apigenin, carthamin, carthamone, safflower yellow A and safflower yellow B (Akito et al., 1998; Fukushima et al., 1997; Tsunajiam and Homma, 1997). It is a characteristic all of the pigments to have a common quinochalcone-C-glycoside structure. Carthamin and safflower yellow B are the most important pigments of the petals for drug, food and cosmetics purposes (Shingo et al., 1996). In addition, Wakabayashi et al. (1997) purified polysaccharide fractions that have immunomodulating activities from dried petals of safflower.

On the other hand, Safflower is one of the age old minor oil seed crops containing 30-40% seed oil with higher level of mono-and-poly-unsaturated fatty acid profiles and elevated levels of α-tocopherol (Cox et al., 1995). Over 25 compounds have been isolated from the seed of safflower (Lacey et al., 1998), such as α-tocopherol, *N*-[2-[5-(-D-glucosyloxy)-1*H*-indol-3-yl)ethyl]-p-coumaramide, *N*-[2-[5-(β-D-glucosyloxy)-1*H*-indol-3-yl)ethyl]ferulamide (Akito et al., 1998; Zhang et al., 1997). The yield of petals and seeds of safflower is limited in the field culture per year. Plant cell culture is an alternative means for producing useful chemicals. Safflower cells grow very quickly at artificial culture conditions

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(Hanagata et al., 1993). Thus, it is hopeful to produce pigments and oil compounds by cell cultures of safflower in commercial culture system. Up to now, the compounds produced by cell cultures in safflower are focusing on pigments and α-tocopherol. In this paper, we will describe advances in the production of pigments and α-tocopherol by cell cultures in Safflower.

Pigment Production

1. *MS-White medium system (Hanagata et al., 1992; 1993; 1994a; 1994b; 1994c; Hirose and Homma, 1996; Kawamoto and Matsuba, 1996)*

1.1. Callus induction

Cotyledons of safflower were inoculated on MS (Murashige-Skoog) agar medium containing 10^{-5} M NAA (Naphthyleneacetic acid) + 10^{-6} M kinetin. The calli were obtained after 4 weeks, and calli were subcultured on the same medium every 3 week.

1.2. Pigment production on agar medium

The Calli transferred to the White + 10^{-6} M NAA + 10^{-6} M kinetin or White + 10^{-6} M NAA + 10^{-5} M kinetin could produce pigment, but the pigment produced by calli on the White + 10^{-6} M NAA + 10^{-5} M kinetin was two-fold of the pigment produced by calli on the White + 10^{-6} M NAA + 10^{-6} M kinetin. However, these red calli became brown after 2 weeks and did not grow. Therefore, it was difficult to maintain the red calli. Hirose and Homma (1996) established experiments to produce flavonoids using the calli induced from the apical cells of sallower seedlings. The calli induced in the dark did not yield flavonoids, while five flavonoids were induced under the light. One of isolates from the calli grown under the light was quercetin.

1.3. Red pigment formation in suspension culture

A two-stage culture system was used for the suspension culture. At the first culture stage, a sample of 1.2 g fw of cultured cells were subcultured in a 300 ml Erlenmeyer flask containing 100 mL of MS liquid medium + 10^{-5} M NAA + 10^{-6} M kinetin every 10 day. The cells were transferred into the White liquid medium containing 10^{-6} M NAA+ 10^{-5} M kinetin for the second stage of culture. However, the red pigment was not formed in the suspension culture in spite of being formed in the agar medium of the same composition. The inhibition of red pigment formation was found to be mainly caused by the presence of magnesium in the medium, and it was found that a modified White medium (Micro-elements + Vitamins + $Ca(NO_3)_2 \cdot 4H_2O$ + NaH_2PO_4) was the most suitable for red pigment formation. This medium was called the red pigment production medium (RPP-I). When magnesium sulfate was added to the RPP-1 medium above at 100 mg/L, red pigment formation was completely inhibited. If only the RPP-1 medium was used, the formation of red pigment noticeably declined following the 15th subculture and no formation of red pigment was recognized in the 25th subculture. Therefore, 10^{-6} M NAA + 10^{-5} M kinetin were added to the RPP-1 medium, and using this medium (RPP-II), cultured cells had formed the red pigment until at the 100th successive subculture. $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and $KHPO_4$ in the MS medium of first culture stage were found to affect red pigment formation in the second culture stage. When the concentrations of these nutrients were increased to 2-, 3- and 8-fold, respectively in the first culture stage, the level of red pigment formation in the second culture stage increased by about 5-times.

1.4. Behavior of cell aggregates of cultured cells and its correlation with red pigment formation

About 80% of the cell aggregates in the growth medium were >1.00 mm in size. The growth rate of the large cell aggregates was more rapid than that of the small cell aggregates. Most cell aggregates of >0. 50 mm in size became larger or smaller than their original size during the culture. A high level of red pigment formation was observed when the cell aggregates obtained by pre-culture using cell aggregates of <1.00 mm in size were cultured in the production medium.

1.5. Elicitors

Twenty four polysaccharides were tested for activity as elicitors on pigment formation. Among them, thirteen stimulated pigment formation over 1.5 times the control and seven stimulated pigment formation over 2 times the control. When Xanthan gum or Fucoidan were used as an elicitor, the amount of red pigment formed was about 3 times the control. The amount of pigment produced was linearly proportional to the amounts added of Xanthan gum or Fucoidan up to 10 mg per 30 mL production medium. Above 10 mg, no significant increase in pigment production levels occurred. The maximum amount of pigment produced was approx. 4 mg/L, which was approx. six times the control. The autoclaved blue-green algae *Anabaena cylindrica, A. variabilis* and *Nostoc linckia* stimulated pigment formation more than 8 times the control level. However, no elicitor effect could be observed using green algae such as *Chlorella sorokiniana, Scenedesmus accuminatus, Chlorococcum sp.* and *Oocystis sp.*. The cell wall of *N. linckia* gave a larger effect than the crude extract. The

amount of pigment produced was linearly proportional to the amount of *N. linckia* cell wall added up to 4 mg per 30 mL production medium. The maximum amount of pigment produced was approx. 10 times the control. In addition, an extracellular polysaccharide from the culture of an unknown fungus strain SE-801 elicited pigment formation approx. 13 times that of the control. This extracellular polysaccharide consisted of two fractions, a 0-20% EtOH precipitating fraction and a 20-40% EtOH precipitating fraction. Both fractions possessed similar elicitor effects. The 0-20% EtOH precipitating fraction could be decomposed by cellulase and glucoamylase.

1.6. Analysis of red pigment formed by cultured cells

1.6.1. Preparation of red pigment formed by cultured cells

10 g of cellulose powder was added to 600 mL of safflower cultured suspension and stirred to absorb the red pigment. After 1 hr, cells and cellulose were harvested by filtration and washed with distilled water 3 times. Then red pigment was extracted from the cells and cellulose in 200 mL of acetone-methanol (80:20, v/v) twice. The extract was evaporated at less than 30℃ using a rotary evaporator. Red pigment obtained by evaporation was dissolved with 0.3 mL of 60% methanol and chromatographed on a column (1.5×18 cm) of Toyo Pearl HW-10F eluted with 60% methanol at a flow rate of 0.75 mL/min. Three pigments were obtained. One was red and the other two were purple. The red fraction was passed through the column again and evaporated below 30℃.

1.6.2. Preparation of carthamin standard

Dry petals of safflower were washed with water more than 20 times to remove yellow pigments. Red pigment was extracted in a 0.5% K_2O_3 solution for 3 hr with stirring. The pH of this extract was adjusted to 5.0 with 10% citrate, then cellulose powder was added to this solution. The cellulose was gently stirred for 1 hr to absorb red pigment, then cellulose was washed with distilled water three times to remove the residual yellow pigments. Red pigment was extracted from cellulose in acetone-methanol (80:20, v/v) for 3 hr and the extract was concentrated with a rotary evaporator at less than 30℃. Concentrated extract was put on a column (1.5×18 cm) of Toyopearl HW-40F (Tosoh Corporation, Tokyo) eluted with 60% methanol at a flow rate of 0.75 mL/min. The red fraction obtained by column chromatography was evaporated below 30℃.

1.6.3. Conditions of HPLC

For the preparative separation, a reversed-phase column, ODS-80T_M (300 mm×21.5 mm i.d., Tosoh) was used, and isocratic elution was carried out with methanol-water (60:40, v/v) at a flow rate of 0.5 mL/min. For analytical separation, ODS-80T_M (250 mm×4.6 mm i.d., Tosoh) was used, and a linear gradient elution from methanol-water (40:60, v/v) to methanol-water (80:20, v/v) in 15 min was performed at a flow rate of 1 mL/min. The detection was carried out at 515 nm.

1.6.4. Measurement of red pigment formed by cultured cells

Medium and cells were separated by centrifugation at 3,000 rpm for 5 min. To measure the level of the red pigment released into the medium, 3 mL of 60% acetone was added to 2 mL of the medium. Harvest cells were dried at 30℃ for 1 day, and washed with distilled water after homogenization. In order to extract the red pigment, 5 mL of 60% acetone was added to the homogenized cells and stirred for 5 hr, then filterated to remove the cells. The absorbance at 515 nm of the 60% acetone solution was measured.

1.7. Bioreactor culture

Hanagata and Karube (1994c) investigated reactors configuration for production of red pigment in a two-stage culture. Four reactors were used in the experiment. The first was a 300 mL Erlenmeyer flask agitated on a rotary shaker with an agitation diameter of 25 mm. The second was a stirred tank reactor (STR) (diameter = 16 cm, height = 20 cm) with six-flat-blade-turbine type impellers and sparger with eight holes. The third was a bubble column (diameter = 10 cm, height = 60 cm) with a gas sparger. The fourth was a seesaw (up and down) type reactor (diameter = 10 cm, length = 60 cm) with a gas sparger (seven holes) on the base and baffle plate was not provided. Air was supplied at a flow rate of 0.5 vvm for 5 min with an interval of 1 hr, oxygen therefore transferred to the medium from the liquid surface due to vibration of the reactor. The cylindrical reactor was vibrated by a motor in a vertical plane. The rocking frequency was controlled by the rotational speed of the motor. An amplitude of vibration was controlled by a light sensor attached to the reactor and the motor. The fularum of vibration was the center of reactor. The initial volumetric transfer coefficient (k_La) was calculated from the rate of recovery of the dissolved oxygen concentration in MS medium measured with an oxygen electrode from non-oxygen conditions obtained by the addition of sodium sulfate (Kato et al., 1975). For the first stage, growth levels of cells in the Erlenmeyer

flask on and the seesaw type reactor were higher than those in the STR and bubble column. Cells grown at a high k_L condition possessed high pigment forming capacity in the second culture stage. High pigment production levels were achieved by the periodic removal of pigment from the reactor. Production medium in the second culture stage could be reused by removal of the pigment formed.

In addition, Takeda et al. (1994) investigated the metabolic responses of safflower cells to hydrodynamic stress in a 550 cm^3 stirred tank reactor. At 0.5-2.3 m^2/S^3 of energy dissipation rate, the decreases in respiration rate and ATP content were larger than the decrease in the membrane integrity. Intercellular NAD(P)H was decreased and cytosolic calcium content was increased by agitation. EGTAL (ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid), verapamil and W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphtbalenesulfonamide) suppressed the decrease in NAD(P)H. These results suggest that the cytosolic calcium, as a second messenger, mediates the signal of the hydrodynamic stress into the metabolism that induces the decrease in respiration rate. Furthermore, a decrease in the filamentous action content due to agitation was observed.

2. G-KP medium system (Wakayama et al., 1994; 1995)

2.1. Callus induction and cell line selection

Calli were induced from the leaves using the KG medium (modified MS medium) (Table 1). The medium was KG + 10^{-6} M NAA + 10^{-6} M BA (6-Benzylanimopurine) + gellan gum 0.2%. After induction of callus, the callus clumps were reduced to small aggregates by passage through nylon mesh (300 μm). After two weeks incubation, several cell clumps with a high proliferation rate were selected as host cells, on which a filter paper moistened with the MS medium was placed, and then the small clumps were set. Random combination of host cells (H 5, 8, 11) with a high proliferation rate and the aggregates (R 8, 15, 16, 20) with low growth rate was provided. The filter paper sandwiched by host cells and an aggregate was found to be pinkish. The aggregates were transferred into 50 mL of the liquid KG medium to establish a suspension culture. The growth of the two cell lines, R8 and R16, was improved in the suspension culture and the high proliferation rate was successively maintained for at least 5 months. The suspension cultures were employed to select the most promising cell lines with a high productivity of pigment. At last, the KB 7 cell line was selected.

2.2. Two-stage culture system for pigment production

For the cell growth, KB 7 cell line was pre-cultured in the KG medium, and for the pigment production, cells were transferred into the KP medium (Table 1). The pigment productivity reached the maximum at the 4th day after the pre-cultured cells were transferred into the KP medium.

Table 1. Pigment production medium (KR) in Safflower cell culture.

Material[a]	Conc.(mg / L)
NH_4NO_3	825
KNO_3	950
KH_2PO_4	85
$CaCl_2 \cdot 2H_2O$[b]	0 (220)
$MgSO_4 \cdot 7H_2O$[b]	0 (185)
$MnSO_4 \cdot 4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$NaMoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
FeSO4 · $7H_2O$	27.8
Na_2EDTA	37.3
myo-Inositol	100
Thiaminehydrochloride	0.1
Pyridoxinehydrochloride	0.5
Nicotinicacid	0.5
Glycine	2.0
Sucrose	30000
α-Naphthaleneaceticacid	0.186
N-6-Benzyladenine	0.225
D-Phenylalanine[b]	165.19(0)
Cellulosepowder[b]	40000 (0)

[a] 0.2% gellan gum was used in the solid KG medium
[b] Contents were changed to ( ) in the KG medium

2.3. Effects of various factors on pigment production

Addition of cellulose powder (3 g / 75 mL of KP medium) was found to be essential to the pigment production. Several plausible flavonoid precursors such as phenylalanine, tyrosine, tryptophan, cinnamic acid and sinapic acid were also added to the modified KG medium. The results showed that addition of D-phenylalanine (1 mM) and D-tyrosine (1 mM) markedly increased pigment productivity about 14.3 and 3.5 times, respectively. However, the corresponding L-isomers, as well as other plausible precursors, were not effective. The effect of metal ion deficiency on the pigment formation were examined. Elimination of Ca^{2+} and Mg^{2+} from the medium was found to be promising. The KP medium was finally established based on these results (Table 1).

2.4. Analysis and purification of the red pigment

For pigment analysis, the medium was filtered with sintered glass and the resulting pinkish powder was dried at 4℃ in the dark. One gram of the powder was soaked in 5 mL of pyridine and left to stand for 5 min. Absorbance of the supernatant was recorded at 520 nm with a Shimadzu UV-265.

For pigment purification, the filtered cake (950 g) was dried at 4℃ in the dark and soaked in 3.21 of acetone / methanol (1:1, v/v) at room temperature for 2 h, and the extract filtered through sintered glass. The filtrate was condensed in vacuo, and then passed through a Toyopearl HW-40 column (2.5×50 cm) using acetone / methanol (1:1, v/v) as an eluant. The red colored fractions were combined and concentrated to give a syrup, which was chromatographed on Sephadex LH-20 (1.6×60 cm) using methanol as an eluant. The resulting fractions were combined and concentrated to give a red residue. Recrystallization was carried out at room temperature using methanol. After purification, red crystals were obtained. Its UV/VIS spectrum as well as the HPLC behavior was clearly different from that of carthamin found in the mother plant and those of other typical plant pigments, suggesting that it was a novel compound. This pigment was named Kinobeon A.

3. Biotransformation in the cultured cells of safflower

In cultured safflower cells, safflower yellow B is converted to carthamin and safflomin-A in MS medium free of ammonium nitrate and potassium diphosphate (Kawamoto and Matsuba, 1996; Onodera et al., 1995). A crude enzyme preparation from the flower cell extraction converted safflower yellow B to safflomin-A and an unstable intermediate which was easily converted to an unstable yellow precursor and subsequently to carthamin (Hirose and Homma, 1996). Phenylalanine labelled with ^{14}C was administered to the cultured cells and the intact flowers of safflower. The cultured cells took up the fed substrate, but they could not incorporate the label into carthamin, while incorporation of the radioactivity from phenylalanine into the red pigment occurred in the intact flowers. The activities of polyphenol-oxidizing enzymes were operative normally in the mother explant, whereas their activity patterns changed altogether in the cultured cells, where kurenamin, a new reddish pigment is produced actively (Saito et al., 1993a).

Production of α-tocopherol and oil fractions

1. Production of α-tocopherol

1.1. Callus culture

Calli were induced from root, hypocotyl, cotyledon and flower buds of safflower. All calli had the capability to synthesize α-tocopherol. Among these calli, the hypocotyl-callus was better than others in cell growth rate and α-tocopherol content. Culture conditions could intensively influence the growth rate and α-tocopherol production of the calli. Sucrose (30 g/L) was good for callus growth and glucose (30 g/L) was good for α-tocopherol accumulation. A high concentration (0.55%) of inositol could stimulate both the growth rate and the α-tocopherol synthesis of calli. The inoculum quantity for best callus growth was 0.035 to 0.067 g dry wt / flask (50 mL volume). In addition, α-tocopherol content was effectively increased by a culture callus in high CO_2 concentration. Studies showed that the callus growth rate, α-tocopherol content and yield were 1.88, 2.03 and 3.30 times, respectively, as high as the control by the administration of 0.45%-0.55% inositol, 10% coconut milk, 0.1-0.5% casamino acid, 30 g/L sucrose and 10 g/L glucose (Gan and Zheng, 1991; Zhou and Zheng, 1989a; 1989b).

1.2. Cell culture

A plating culture technique was used for screening high-producing α-tocopherol variants from the culture cells of safflower. The content of α-tocopherol in cultured cells was measured by HPLC. The cell variants were selected from more than 200 clones induced from single cell or small cell aggregates (2-8 cells), and it was found that these clones were significantly different in their α-tocopherol synthesis and cell growth. α-Tocopherol content varied from 0-138.9 μg / g DW, and cell growth rates varied from 0.20-0.53 g DW / l.d at the first generation of clones. There was not a significant correlation between α-tocopherol productivity and cell growth in these clones. A more stable high-producing original strain CT-289 was found, its α-tocopherol content was 15.83 μg / g DW. The growth rate of CT-289 was 0.424 g DW / l.d, and it was similar to the original strain (0.417 g DW / l.d) (Gan and Zheng, 1992a; Zhou and Zheng, 1989a; 1989b)

Kinetics of cell growth and α-tocopherol production in cell cultures of safflower were studied using a structured model. A compartmental model for plant cell culture was proposed. This model considered respiratory intermediates as the rate-limiting components in the growth of cells and the formation of secondary metabolites. Cell structural components and storage carbohydrates were also considered as constituting compartments. The storage carbohydrates were considered as a reservoir of sub-

strates for the respiratory intermediates. Growth regulation by intracellular phosphates was considered the major factor controlling interconversion between the respiratory intermediates and the storage carbohydrates. The growth of safflower cells and α-tocopherol production were described using this model. The calculated profile of cell growth and α-tocopherol formation agreed with the experimental values in both batch cultivations and semi-continuous cultivations. Furthermore, the conditioning effect (which is assumed to be responsible for lag phase) was included in this model with the assumption of respiratory intermediate release (Furuya et al., 1987; Toshiya et al., 1998).

1.3. Elicitors

Different kinds of oligosaccharides isolated from the cultured cells of Panax ginseng could increase the cell growth rate and α-tocopherol content of the safflower cell cultures. Among them, the effects of oligosaccharides VI, VII and VIII were very significant. The optimal effective concentrations of oligosaccharides VI, VII and VIII were higher in the callus culture than in the suspension culture. It was found that the α-tocopherol content was increased after the addition of oligosaccharides VI, VII and VIII for 1-3 days. The cell growth rate was increased by 18.11%, α-tocopherol content and yield were increased by 3.5- and 4.3-fold, respectively, when 2 mg/L of oligosaccharides VI and VII, and 1 mg/L of oligosaccharide VIII were added at the same time (Furuya et al., 1994; Gan et al., 1990; 1991; 1992a; 1992b). Another oligosaccharide isolated from the cell cultures of *Panax ginseng* was named ginseng-oligosaccharin M. Its optimum concentration on callus culture was 5 mg/L. When ginseng-oligosaccharin M was added to the suspended safflower cells, the duration of exponential growth was shortened and the cells could be harvested earlier. The cell growth rate during the exponential growth stage was increased approx. by 21%-23% and the α-tocopherol content was increased 1.4-1.8-fold(Gan et al., 1992c).

2. *Production of oil and fatty acid proportions*

Callus cultures of cotyledonary and leafy origin were analysed for relative oil content and the degree of unsaturation in fatty acid profiles of safflower. Four test formulations supplemented with various energy levels and growth promoting organic additives were made in combinations onto semi-gelled MS basal medium. A differential rate of oil accumulating potential in each tissue type was observed as the degree of unsaturation in the fatty acid profiles increased or decreased. Results indicated leafy tissues had a higher oil percentage with a greater degree of stability in desaturation predominantly of oleic, linoleic and linolenic acids in their storage lipid fractions, while cotyledonary tissues had a fast trend towards reversal of the fatty acids constituents. The presence of additional sucrose in the medium (4%, w/v) induced the formation of more unsaturated fatty acids in both tissue types. The cell behavior indicated that leafy cultures had a higher potential for unsaturation both in the control and in the treated cells (Singh et al., 1993).

Added precursors (choline chloride, serine, mevalonic acid and mesoinositol), organic supplements and extra energy source were found to be conducive for oil yield in the MS basal medium during the incubation phase. Medium containing a higher sucrose content (5%, w/v) enhanced the oil level up to two-fold while the inclusion of precursor bases of lipid biosynthesis yielded a comparatively low level of oil. Moreover, the addition of casein hydrolysate and coconut water increased the oil percentage further. A combination of all these components in the medium resulted in a synergistic impact on the biosynthesis and biotransformation of additives for *in vitro* oil synthesis and accumulation (Singh et al., 1991).

Discussion

Tissue and cell culuture is a economical and quick way for the production of active compounds in safflower. Tissue culture techniques have been used in multiplication of safflower (Ko and Ro, 1996). Baker and Dyer (1996) reported the optimal protocol for inducing root formation. Tejovathi et al. (1997) obtained multiple shoots from the cotyledonary leaves of 4, 5 and 6 day old seedlings of three Indian cultivars of safflower B hima (S-4), APRR-3 and Mangira. Mondal et al. (1995) induced somatic embryos directly on the adaxial surface of cotyledonary leaves. Safflower is easy to cultivate in the field, but the yield of flower and seeds is limited. Therefore, the production of active compounds by tissue and cell culture is more important than other use of tissue and cell culture in safflower.

Now, the pigment production by cell culture of safflower is at a standstill. We think one of the key reasons is that the researchers did not collaborate cell culture with studies of phytochemistry and pharmacology. As far as we know, the structure of pigments produced by cell cultures has not been defined clearly. Meanwhile, whether the pigments produced from cell cultures of safflower have active functions or not, it still needs pharmacological experiments to confirm. In addition, the previous study

only focused on production of red pigments from cultured cells, we think the future study should be carried out for getting both red and yellow pigments, because safflower yellow B has been demonstrated to have active functions (Liu and Xiao, 1993). Morever, many oil compounds isolated from safflower seeds have active efffects for human health (Zhang et al., 1997). So, more experiments need to be established for getting other oil fractions by cell cultures of safflower, not only for getting α-tocopherol.

Metabolism study is the base for increasing the production rate of pigment and oil contents in safflower cell cultures. Some experiments had been established to investigate the pigment metabolism in intact flower of safflower (Saito, 1994; 1995), and it was found that glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* could catalyse the conversion of precarthamin to carthamin (Saito, 1993a). Because some pigments produced by safflower cell cultures are different from the pigments produced from the intact plant (Saito et al., 1993b), the cultured cells may have different metabolism pathways from the intact plant cells. Up to now, almost no report about the pigment metabolism in the cultured cells of safflower. We think the future study of pigment synthesis and degradation in the cultured cells of safflower will be a helpful step toward the increase of pigment production.

On the other hand, many studies of lipid metabolism had been carried out in developing cotyledons of safflower (Stobart et al., 1997; Vogel and Browse, 1996). It was found that the activity of 1-acylglycerophosphocholine O-acyltransferase varied during maturation of safflower seeds (Ichihara et al., 1995). Knutzon et al. (1992) partially purified two oleoyl-acylcarrier protein (18:1-ACP) thioesterase from developing safflower seeds. Expression of the two safflower cDNA clones in *Escherichia coli* resulted in a 50- to 100-fold increase in the level of 18:1-ACP thioesterase activity. In 1994, Bhella and MacKenize presented the nucleotide sequence of a full-length cDNA encoding a GPTA isolated from a λgt22 cDNA library made from mRNA of developing safflower seeds. However, almost no report has approached the lipid metabolism in cultured cells of safflower. Increasing the production of α-tocopherol and other oil compounds by cell culture is critically dependent on our knowledge of understanding the regulatory mechanisms involved in steps of lipid assembly in cultured cells. Thus, metabolism studies in cultured cells appear to be specifically channeled into the increase of production of both pigments and oil compounds by cell cultures in safflower.

A culture of hairy roots, induced by infection with *Agrobacterium rhizogenes*, is an excellent starting material for the production of secondary metabolites by plant cells because of the stability of important characteristics, such as morphology, the capacity for growth, and the productivity of secondary metabolites (Mano, 1989). However, there are only a few reports concerning *Agrobacterium tumefaciens*-mediated transformation of safflower cultivar 'Centennial' (Ying et al., 1992). 'Centennial' explants inoculated with *Agrobacterium tumefaciens* containing NPT II and GUS genes produced kanamycin-resistant calli from which buds were regenerated. Orlikowska et al. (1995) obtained the transgenic shoots by the *Agrobacterium tumefaciens* strain EHA 105 / p35S GUS Int. But these research did not result in cells, calli, shoots or roots that had a high content of active compounds. Thus, further studies are needed to increase the content of the active compounds by different gene transformation methods.

To date, large-scale plant cell cuture processes have been developed almost exclusively using dispersed cell suspensions. This form of culture is preferred in commercial system for several reasons, including the applicability of many reactor design and scale-up procedures developed for microbial fermentations, and the availability of engineering technology for aseptic inoculation and downstream transfer (Verpoorte et al., 1998). Now the airlift bioreactors have been successfully used in cultures of yew tree cell, potato seedling, lily bulblet, and multiple adventitious root of ginseng (Son et al., 1999). As stated in the above review (Hanagata and Karube, 1994c; Takeda et al., 1994; 1998), only a few experiments of bioreactor culture have been established in safflower, and the bioreactors used for safflower cell culture are small-sized and their style are not suitable for plant cell culture. Therefore, bioreactor culture is thought to be the key step for the commercial production of pigments and oil compounds by cell cultures in safflower, and airlift bioreactors appear to be the optimal style.

Conclusion

1. The pigments obtained from the petals of safflower can be used as pharmaceuticals and food additives. Meanwhile, the seeds of safflower contains high level of oil compounds. Cell culture is an alternative and hopeful means to produce pigments and oil compounds in safflower.
2. The structure of pigments produced by cell cultures need to be defined, and pharmacological experiments to need to be carried out to investigate these pigments functions. In addition, The future cell culture should be established for getting both

red and yellow pigments, as well as other oil fractions besides α-tocopherol.

3. The cultured cells may have different metabolism pathways from the intact plant cells. Metabolism studies in cultured cells appear to be specifically channeled into the increase of production of both pigments and oil compounds by cell cultures in safflower.
4. A culture of hairy roots, induced by infection with *Agrobacterium rhizogenes*, has been confirmed to be an excellent starting material for the production of secondary metabolites in many medicinal plants. Thus, further studies are needed to increase the content of the active compounds by different gene transformation methods.
5. To date, large-scale plant cell cuture processes have been developed very quickly. The airlift bioreactors have many advantages for plant cell culture comparing with other bioreactors. Therefore, bioreactor culture is thought to be the key step for the commercial production of pigments and oil compounds by cell cultures in safflower, and airlift bioreactors appear to be the optimal style.

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