Effect of growth regulators on betalain profile in callus culture of *Celosia* sp.

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Abstract. Red and yellow inflorescence of *Celosia* indicated the content of betalain pigment, betacyanin and betaxanthin. In vitro system through callus culture is one of the strategies for producing plant bioactive compounds including betalain. Therefore, this study aims to analyse the content and profile of betalain pigments in *Celosia* callus in vitro induced by a combination of auxin and cytokinin. Callus was induced from in vitro cotyledonary and hypocotyl sprouts at 1 week after germination. Callus induction medium was MS + BAP + 2,4-D or NAA. Subsequently, the profile and content of betalain pigments in each type of pigmented callus and hypocotyl tissue and leaves in vitro were analysed by HPLC method. Repeated subcultures every two weeks resulted three types of pigmented callus: red, yellow and greenish white. HPLC analysis of in vitro hypocotyl, leaf and callus tissue derived from red and yellow *Celosia* inflorescence detected six types of compounds, namely 1) amaranthin, 2) isoamaranthin, 3) betalamic acid, 4) miraxanthin V, 5) 3-methoxytyramine betaxanthine and 6) (S)-tryptophan betaxanthine. Callus from red *Celosia* contains a small amount of betaxanthin which was higher in yellow *Celosia*. BAP:2,4-D combination produced higher total content of betalain pigments.

1. Introduction

Betalain is a nitrogenous water soluble pigment which consists of red-purple betacyanins and yellow-orange betaxanthins [1]. Betalain accumulates in vacuoles of hypodermic tissue [2] and epidermis [3] mainly because of their hydrophilicity [4,5]. In addition to inflorescence, inflorescences and fruits, the betalain is also found in the root, stem, and leaf tissue [6].

Betalains have significant taxonomic and evolutionary values [7] because it is mutually exclusive. It means that betalain replaces the presence of anthocyanin so that in the same plant betalain is never found together with anthocyanin [8,9]. In higher plants betalain is only found in the order Caryophyllales (except the family Caryophyllaceae and Molluginaceae). Consequently, the availability of betalain naturally is far less than anthocyanin. Beside from being a safe food colorant betalain is also a pigment that contributes to health because it has many biological activities [10] such as antioxidant properties [11], anti-inflammatory [12] and anticarcinogenic [13]. Many betalain benefits in the health sector increase the interest of the community to use it. Until now red beet extract is the only source of commercial betalain. Therefore, exploration of alternative plant species as a potential source of betalain is need to be done. *Celosia*, a member of the Amaranthaceae family has been suggested as an alternative candidate for betalain source [14].
Effort for increasing the production of secondary metabolites have been undertaken using plant tissue culture techniques (PTC). The use of PTC techniques serve many advantageous because independent of climatic and geographical conditions will provide a sustainable, economical and viable secondary metabolite production [15,16]. Modification of the growing environment can accelerate the accumulation of fresh biomass and increase the concentration of secondary metabolite compounds. Betalain engineering that has been carried out in a number of plant species through in vitro techniques opens new possibilities in the production of betalain biotechnology and the development of new ornamental varieties [1].

Callus culture and cell suspension culture composed of cells that actively proliferated. Propagation of callus cells and suspension through continuous callus induction is a challenge to manipulate the production of secondary compounds including pigments. Callus that is stable in producing betalain pigment is determined by many factors including the growth regulator in the culture medium. Plant growth regulators, used to control tissue development and to stimulate the increase of plant biomass and PDMC contents; Combination of BAP +2,4-D or 2,4-D alone is able to induce the synthesis of betalain pigment in callus culture of Chenopodium quinoa Willd [17], Alternanthera brasiliana [18] and Bougainvillea spp [19]. The profile of secondary metabolites in the in vitro system can vary when compared to the original plant [20]. Therefore, the optimal and continuous supply of callus for manipulation of betalain pigment production needs to be done. This study aims to analyze the effect of a combination of growth regulators on the profile and quantity of betalain pigment content in Celosia callus in vitro.

2. Material and Methods

2.1 Preparation of explant sources
The seeds of two accessions of the Celosia plants which had red and yellow inflorescences collected from the Bantul region of Central Java were sterilized with commercial bleach and germinated in vitro. The medium contains only water that is compacted with agar without the addition of macro and micro nutrient components and growth regulators. Two weeks after germination of parts of hypocotyl and cotyledon sprouts have been used as explants for callus induction.

2.2 Induction and maintenance of callus
Two weeks old in vitro Celosia sprouts were used as sources of explants. A hypocotyl of about 0.5 cm is placed horizontally on a callus induction medium while cotyledons are placed with the abaxial side in contact with the media. The response of callus formation was observed in three types of callus induction medium, namely MS base medium with the addition of 1) BAP 2 mg / l + NAA 2 mg / l, 2) BAP 1 mg / L + 0.25 2,4-D, and 3) BAP of 1 mg / L + 0.5 2,4-D. Each type of explants was repeated 10 bottles and each culture bottle was filled with three explants for each type. Furthermore, all cultures were maintained at 24 ± 2 ºC with 60-65% relative humidity and continuous lighting at an intensity of about 600 lux provided by white fluorescent light. Morphological changes that occur in each type of explant were observed during the process of primary callus formation. Callus friability and color were also observed along with callus mass development.

Primary calluses that have reached a certain volume are selected based on their pigmentation and subcultured in fresh medium with the same growth regulating composition as callus induction. Callus which has shown stable pigmentation is then ready to do content analysis and betalain pigment profile.

2.3 Analysis of betalain profile by HPLC
All solutions, both standard solutions, sample solutions, and mobile phase (eluent) solutions are filtered with a PTFE membrane and then degassed. The standard solution and sample solution before being injected into HPLC are filtered again with cellulose nitrate membrane on the HPLC injector syringe. HPLC analysis was performed with Shimadzu with the SPD M20-A Photo Diode Array detector. The analysis was determined at 35 ºC in the 5 µm VP ODS Shim-pack column, 150 x 4.6
mm. The mobile phase with isocratic method uses formic acid (0.1% v/v) and acetonitrile (0.1% v/v) by comparison (70:30). Analyses were performed at wavelengths of 535-550 nm for betacyanin and 475 - 480 nm for betaxanthin with a flow rate of 0.4 ml / min for 10 minutes. The volume of extract solution being injected is 20 µl.

2.4 Data analysis
Qualitative data during callus development were analyzed descriptively. Descriptive analysis was also carried out on the profile and content of the results of the chromatographic analysis using the HPLC method.

3. Result and Discussion

3.1 Callus induction
The seed of both Celosia plant accession which was inoculated on a solid medium without the addition of plant growth regulator (PGR) began to germinate at one week of culture. Celosia sprouts with red inflorescences have red hypocotyls and reddish green cotyledons (Figure 1A). Whereas the yellow-inflorescence Celosia sprouts have green hypocotyls (Figure 1B).

![Figure 1. In vitro sprout of Celosia one-week old culture. A. Celosia with red inflorescence type, B. Celosia with yellow inflorescence type.](image)

The cotyledon and hypocotyl explants will initially swell or enlarge (Figure 2A-B) which indicated active division, thereby increasing the number of cells and tissue size. Active proliferation accompanied by the process of dedifferentiation was characterized by the start of callus tissue formation in the wounded area which extends to other areas of the explant tissues (Figure 2C).

![Figure 2. Callus induction 2 weeks after culture. A-B. Cotyledon explants, C. Hypocotyl explants](image)

The combination of BAP (1 mg / l) with 2,4-D (0.25 and 0.5 mg / l) is able to induce proliferation and de-differentiation of explant cells so produced a large callus mass (Table 1). A large callus mass
was also obtained in the medium with the addition of BAP: NAA with the same concentration, 2 mg / l. However, when BAP 2 mg / l was combined with NAA at a reduced concentration to 0.25 mg / l was unable to induce callus formation. The reduced ability of callus induction cannot be improved by adding 0.1 mg / l kinetin.

**Table 1.** Effect of variations in auxin: cytokinin combination on MS medium on response of callus formation

| No | Auxin/Cytokinin (mg/l) | Response of callus formation* |
|----|------------------------|-------------------------------|
|    | 2,4-D | NAA | BAP | Kinetin |
| 1  | 0.5  | -   | 1   | -       | +++   |
| 2  | 0.25 | -   | 1   | -       | +++   |
| 3  | -    | 2   | 2   | -       | +++   |
| 4  | -    | 0.25| 2   | -       | -     |
| 5  | -    | 0.25| 2   | 0.1     | -     |

Note *: +++: many callus formed; -: few callus formed

Callus induced from both hypocotyl explants and cotyledon of the two accessions of the *Celosia* plant was able to synthesize red pigment (betacyanin) (Figure 3A) and yellow (betaxanthin) (Figure 3B) according to the inflorescence color of the *Celosia* accession plant.

**Figure 3.** Callus accession Celosia 2 weeks after subculture. A. Callus induced from explants with red inflorescences, B. Callus induced from explants with yellow inflorescences.

3.2 *Analysis of betalain pigment profile by HPLC method*

Chromatographic analysis of betalain profiles by HPLC in leaf and callus tissue in vitro originating from red and yellow inflorescence *Celosia* detected six types of compounds in the order of appearance, namely 1) amaranthin, 2) isoamaranthin, 3) betalamic acid, 4) miraxanthin V, 5) 3-methoxytyramine betaxanthine and 6) (S) -tryptophan betaxanthine (Table 2; Figure 4). Isoamaranthin and amaranthin were the main betacyanin in the red *Celosia* callus while miraxanthin V was detected as the most betaxanthin followed by 3-methoxytyramine betaxanthin in the yellow *Celosia* callus.
Table 2. Composition of betalain pigment identified by HPLC method in extracts of callus and Celosia leaf in vitro

| No peak | Betalain types                  | Betalain group | Retention time (min) |
|---------|---------------------------------|----------------|----------------------|
| 1       | amaranthin                      | betacyanin     | 16,205               |
| 2       | isoamaranthin                   | betacyanin     | 17,212               |
| 3       | betalamic acid                  | chromofor      | 20,418               |
| 4       | miraxanthin V                   | betacyanin     | 23,062               |
| 5       | 3-methoxytyramine betaxanthin   | betaxanthin    | 27,524               |
| 6       | (S)-tryptophan betaxanthin      | betasanthin    | 34,709               |

Figure 4. HPLC chromatogram of betalain pigment extracted from in vitro Celosia callus. A. Callus derived from red inflorescence Celosia, B. Callus derived from yellow inflorescence Celosia

Primary callus produced in the combination of PGR generally has two white-green-red / pink / violet colour combinations and white-green-red / pink / yellow / orange (Table 3). Callus produced in each type of PGR combination provided different responses synthesis of betalain pigments. This is indicated by variations in the content of betalain pigments in the callus formed (Figure 5).
Table 3. Callus colours produced on several mediums with variations of auxin:cytokinin combination

| No | Accession Code | Combination of PGR | Types of tissues       |
|----|----------------|--------------------|------------------------|
| 1  | Red Celosia ID 01 | B2N0,25K0,1        | red hypocotyl          |
| 2  | Red Celosia ID 02 | B2N2               | red callus             |
| 3  | Red Celosia ID 03 | B1 D0,5            | red callus             |
| 4  | Red Celosia ID 04 | B1 D0,25           | red callus             |
| 5  | Red Celosia ID 05 | B1 D0,25           | Greenish yellow callus |
| 6  | Yellow Celosia ID 06 | B2N0,25          | green leave            |
| 7  | Yellow Celosia ID 07 | B2N2            | yellow callus          |
| 8  | Yellow Celosia ID 08 | B1 D0,25          | yellow callus          |
| 9  | Yellow Celosia ID 09 | B1 D0,25          | yellow callus          |

The results of the analysis showed that amaranthin / isoamaranthin and miraxanthin V were the dominant pigments in the red and yellow callus Celosia, respectively (Figure 5). However, the quantity of miraxanthin V pigment was less (228.45 - 462.72 ug / g FW) than amaranthin / isoamaranthin (256 - 779.32 ug / g FW). Similar to what was reported by [21] this study also showed that miraxanthin V was present in the callus Celosia in lower concentrations than betacyanin. Amanthanthin / isoamaranthine content in secondary callus maintained on BAA:NAA and BAA:2,4-D medium was higher than in hypocotyl tissue in BAP:NAA: Kin (Figure 5, *). On the other hand, the content of miraxanthine V in callus tissue maintained in BAA:NAA and BAA: 2,4-D was lower than in vitro leaves cultured in BAP:NAA medium. This showed that the same combination of PGR can had different effects on the ability of synthesis or accumulation of different types of pigment betalain. Decreasing 2,4-D concentration from 0.5 mg / L to 0.25 mg / L increased the quantity of amaranthin / isoamaranthin and miraxanthin V in callus although it still lower than leave content. These results are different from C. cristata callus culture which is able to accumulate betalain with a quantity close to the yield in plants in vivo [21]. The analysis of HPLC on greenish yellow callus produced from the red inflorescence Celosia accession explant detected amanthanthin / isoamaranthine pigment content similar to that produced by red callus on medium with a combination of BAP: NAA (Figure 5).

![Figure 5. Effect of combination of auxin:cytokinin on betalain content in callus culture of Celosia.](image)
Celosia species are characterized by inflorescences that have a wide range of colors ranging from yellow to red due to the presence of betalain pigments [14,22,23,24]. [14,23,25] show a common pattern of betacyanin in Celosia cristata L. species, consisting of amaranthin / isoamaranthine, betanin / isobetanin, Celosianin I / isoCelosianin I, and Celosianin II / isoCelosianin II [22] while betanin / isobetanin, Celosianin I / isoCelosianin I, and Celosianin II / isoCelosianin II [22] whereas in Celosia plumosa hort, only amaranthin and its diastereomer [14]. The absence of Celosianin/ isoCelosianin in callus in vitro in this study indicated that the accession of sample plants was C. plumosa.

Celosia inflorescence colors can vary from yellow to various red and purple colors due to betalain pigments [14]. Six pigments found in Celosia have been reported: amaranthin, isoamaranthin, betalamic acid, dopamine-bx, 3-methoxytryamine-bx and S-tryptophan-bx. However, the amount of this pigment varies according to the species and color of the inflorescence. In the cristata species, dopamine-bx dominates the yellow inflorescence; amaranthin / isoamaranthin and dopamine-bx are found in the same amount in orange inflorescences; whereas amaranthin / isoamaranthin is the main pigment in red inflorescences, this shows that in the biosynthesis of pigments there is a gradual change from dopa-cyclo-dopa to amaranthin and dopa-dopamine into betaxanthin [20]. Phytohormone also affects the synthesis of betalains [25]. Callus culture of C. cristata L. (Amaranthaceae) can accumulate betalains in amounts close to the amount produced by the plant [21]. The very red colored callus culture of C. cristata has been identified to contain betacyanin and betaxanthin. Amarantine as the main betacyanin in the callus, followed by celoscristatin, betanin, phyllocactin, and other minor betacyanin. Similar to the results of this study, dopamine-based betalain, miraxanthin V, was detected as the main betaxanthin in the callus but at a much lower concentration level than betacyanin.

4. Conclusion
The results of this study indicate that exogenous PGR added to culture medium can affect the ability of callus cells / cells to synthesize betalain pigments.

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