Original Research Article
Induction of Anthocyanin Production in Established Callus Cultures of Roselle (*Hibiscus sabdariffa L.*) Using Yeast Extract

.

ABSTRACT

Aims: Hibiscus sabdariffa var. sabdariffa (Malvaceae), a shrub locally known as roselle has been valued for its vibrant red colored calyces that are used as food colorant and health drink. Its anthocyanin content has been known to have health promoting effects like antioxidant activity, antimicrobial and anti-cancer, among others. This study was done to establish callus cultures of roselle which are capable of producing anthocyanins.
Study design: An experimental study was done to look into the effects of different factors such as explant source and growth hormone concentration on the induction of roselle callus cultures. The effect of different concentrations of yeast extract as elicitor of anthocyanin production was also tested.
Place and duration of study: The study was conducted at the Biotechnology Laboratory for Natural Products, Institute of Biological Sciences, University of the Philippines Los Baños from June 2011 to May 2014.
Methodology: Callus induction was done using aseptically grown seedlings of the Thailand accession at MS medium with different combinations of growth hormones (T1: 0.5ppm 2,4-D and 1ppm kinetin; T2: 1ppm 2,4-D and 1ppm kinetin; T3: 1ppm 2,4-D and 2ppm kinetin). Established cultures were subjected to anthocyanins elicitation using yeast extract (Y1: 8g/L, Y2: 4g/L, Y3: 1g/L and Yc: no extract) as a biotic elicitor.
Results: Callus formation and ephemeral anthocyanin production were observed 2 weeks after inoculation. Addition of yeast extract increased the growth rate up to 10-fold (4g/L) but difference among treatments was not statistically significant. Callus cultures produced anthocyanins 2 weeks after transferred back to a growth medium without yeast extract.
Conclusion: Anthocyanin production was unstable and temporary but the calli proved competent for anthocyanin production. Yellow calli were also observed after exposure to yeast extract, TLC profile showed presence of chlorogenic acids which are possible precursors for anthocyanin production.

Keywords: Hibiscus sabdariffa, anthocyanin production, yeast extract, callus culture

1. INTRODUCTION

Hibiscus sabdariffa, also known as Roselle, is an annual crop which is valued for its red colored calyces that are used for making jam, jelly, and soft drinks (Da-Costa-Rocha et al., 2014; Ibrahim and Hussein, 2006). The characteristic red color of their calyces is due to the concentration of anthocyanins which can reach 1.5g/kg of dry matter (Cisse et al., 2009). Heightened interest in the health benefits of anthocyanins opens a new window of opportunities for the use of roselle extracts in a variety of applications. Roselle has high potential in the world market with its vibrant red color and health promoting attributes.

Product yield of anthocyanins from roselle has limitations such as: variability and seasonal availability of raw materials; fresh material losses due to insect infestations or to natural calamities; and pigment degradation upon extraction and storage. To counter limitations, establishment of plant cell and tissue cultures for pigment production and employing the tools of molecular biology in enhancing product yield can be done (Zhang and Furusaki, 1999).

Plant cell and tissue culture is a method wherein plant cells or unorganized tissues are grown under controlled conditions. This method has been an ideal system to study various aspects of anthocyanin formation. The process of induction and regulation, compartmentalization of biosynthesis and storage can be studied (Walton et al., 1999). A number of researches have been directed to increase productivity of plant cell cultures to be able to meet market demand for anthocyanins. Some of which focused on strain improvement, growth medium optimization, and selection of culture conditions (Sie et al., 2010). Specialized procedures such as elicitation and permeabilization are also employed (Zhang and Furusaki, 1999).

Anthocyanin synthesis and accumulation like other secondary metabolites are regulated in space and time in most plants. The specific localizations of production are related to the role of secondary metabolites for defense and survival (Wink, 1999). The plant must detect a stimulus about an attack to trigger the expression of genes for secondary metabolism. These corresponding signals are known as elicitors which can either be biotic or abiotic. Biotic elicitors are organic substances that may come from attacking microorganisms, or cell wall of spores and hyphae of pathogenic or nonpathogenic fungi. Abiotic elicitors are chemical or physical factors that put the plant under stress like heavy metals, compounds that interact with deoxyribonucleic acid (DNA), or ultraviolet (UV) rays (Reichling, 1999).

Fungal elicitors are often used to enhance secondary metabolite production (Loc et al., 2014). Wang and colleagues (2004) studied *Perilla frutescence* cell cultures and their susceptibility to yeast elicitors. Results showed an increase in anthocyanin production of up to 10% dry weight. According to Molnar and colleagues (2011), yeast extract was used for growth medium as a source of amino acids and vitamins. They said that the provision of yeast extract was often found to be essential for tissue growth. Yeast extract was said to have unusual properties based on its amino acid content. George and colleagues (2008) presented that yeast extract can stimulate chalcone synthase activity leading to the formation of naringenin in *Glycyrrhiza echinata.*

This study was done to induce anthocyanin production in established cultures of roselle using varying concentrations of yeast extract.

2. MATERIALS AND METHODS

Callus Culture

Explants were derived from the cotyledonary leaf and stem of 10-day old aseptically grown seedlings. These were inoculated in solid Murashige and Skoog (1962) medium with 3% sucrose. Three treatments were prepared for the induction of callus, varying on the growth hormone levels.

T1: 0.5ppm 2,4-D (2,4-dichlorophenoxyacetic acid): 1ppm kinetin
T2: 1ppm 2,4-D:1ppm kinetin
T3: 1ppm 2,4-D:2ppm kinetin

The choice of growth hormone and their levels were as described by Sie et al. (2010). The cultures were observed for significant morphological changes. An explant with unorganized cell clusters growing at least 1 mm in size was considered “callusing.”

The induced callus cultures were maintained on MS medium with 1 ppm 2,4-dichlorophenoxyacetic acid and 2 ppm kinetin, 3% sucrose, and solidified with 0.2% agar (Gelrite®). Cultures were maintained in a culture room at 25±2℃ under 12/12 h photoperiod at 2,692.4 lux of fluorescent light. Subculturing of callus cultures was done every month on solid medium of same composition, unless otherwise stated.

Growth rate of the callus cultures was determined based on the dry weight of the callus. Dry weight was the constant weight achieved by the callus upon drying at 40℃ in a convection oven (Cassel®). Measurement was done on 3 replicates every week for a period of 1 month.

In the 16th month after initiation, callus cultures were transferred to a production medium to induce anthocyanin synthesis. As prescribed by Mizukami (1993), a modified LS medium with optimized concentrations of nitrogen source, phosphate, $MgCl_2$ and $CaCl_2$ were used as production medium. Twenty bottles were prepared and sampling was done for 1 month. Three samples per treatment were randomly picked, weighed, and observed for anthocyanin production.

Addition of Yeast Extract

From the 16 months old established callus cultures, 20 culture bottles were prepared for each treatment. Three concentrations of yeast extracts were used: 8g/L, 4g/L, 1g/L, and the control, 0g/L. Every week for one month, 3 samples per treatment were randomly picked, weighed, and observed for anthocyanin production.

Thin Layer Chromatography for Flavonoids

One gram powdered dried callus was extracted with 10 mL of methanol for 5 min on a water bath heated at 60℃ then filtered. Twenty µL aliquots were loaded on TLC plates (Silica gel 60 F_{254}, Merck®) using capillary tubes. The plates were developed using the developing solvent system: 100 mL ethyl acetate: 11 mL formic acid: 11 mL glacial acetic acid: 26 mL water. The plates were air-dried after development. The chromatograms were then viewed first under UV_{254} for quenching and under UV_{365} for fluorescence (Wagner and Bladt, 1996). Natural Products reagent (NP/PEG; Appendix A) was used as visualizing reagent (VR) to intensify the color of the bands under UV_{365}. Commercially available rutin tablet (Solgar®) was used as reference material instead of a pure rutin compound due to the unavailability of this standard.

Data Analysis

Experimental results are expressed as mean ± SD. All measurements were replicated three times. The data were analyzed using analysis of variance (P =.05).

3. RESULTS AND DISCUSSION

Callus Culture

Two weeks after inoculation, callus formation was observed. The leaf explants were less responsive than the stem explants. The highest percentage of callus formation was observed in MS medium with 1 ppm 2,4-D and 2ppm kinetin (T3). All inoculated stem explants in the three treatments were able to show callus formation. Figure 1 shows the callus formed from leaf and stem explants. In both cases, white friable calli were produced.

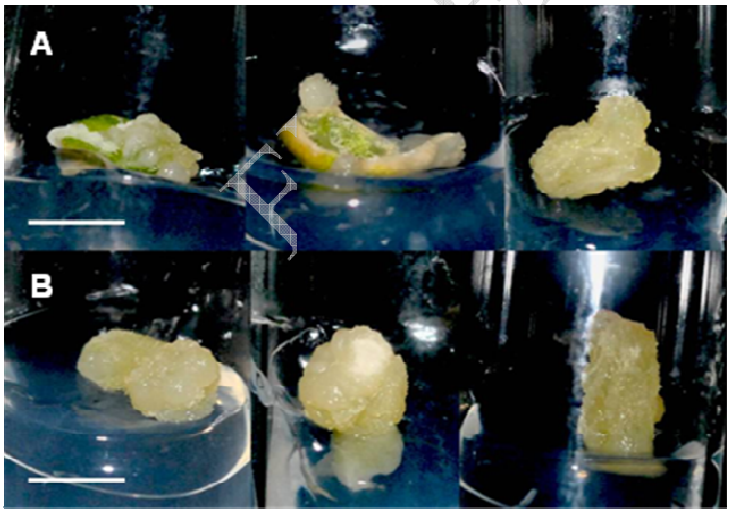

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Fig. 1. Callus cultures from leaf (A) and stem (B) explants and induced in MS medium with 1ppm 2,4-D and 2ppm kinetin. (Scale bar = 1cm)

Table 1 shows the percentage of callus formation and the associated anthocyanin production of different explant sources under the three treatments. For some cultures, anthocyanin production was observed. The occurrence was highest in T3 cultures from the leaf explant at 60%. Cultures induced from stem explants exhibited only 30% of anthocyanin production in T2 (MS medium with 1ppm of 2,4-D and 1ppm kinetin).

Table 1. The percentage callus formation and anthocyanin production in *H. sabdariffa* using the leaf and stem as explants and inoculated in MS medium with varying levels of 2,4-D and kinetin.

	% of Callus Formation			% Anthocyanin Production		
Explant used	T1	T2	T3	T1	T2	T3
Leaf	80	80	**90**	0	20	**60**
Stem	100	100	**100**	10	**30**	9

**T1= 0.5ppm 2,4-D:1ppm kinetin; T2= 1ppm 2,4-D:1ppm kinetin; T3= 1ppm 2,4-D:2ppm kinetin*

In Figure 2, callus cultures with anthocyanin production are presented. Pigment production was observed at the surface of the callus cultures at two weeks after inoculation of leaf and stem explants.

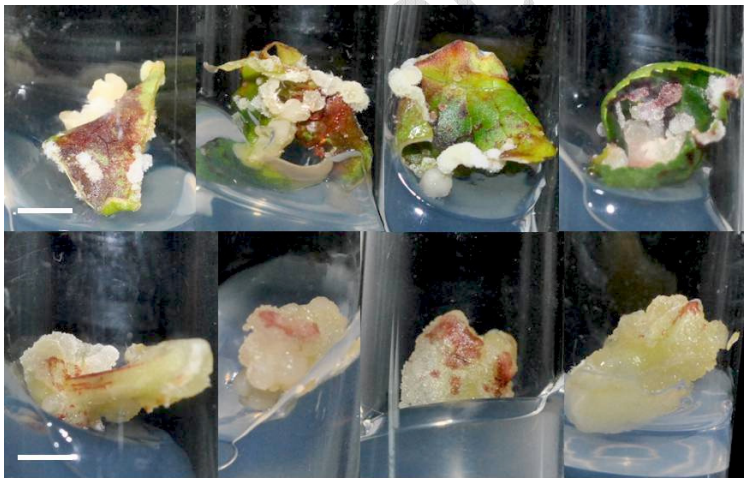

Fig. 2. Anthocyanin production in the leaf (A) and stem (B) explants two weeks after inoculation in MS medium with 2ppm kinetin and 1 ppm 2,4-D. (Scale bar = 1cm)

Based on the percentages of callus induction and anthocyanin production, T3 with 1ppm 2,4-D and 2 ppm kinetin was considered as the optimal combination of growth hormones for maintenance of roselle callus cultures. In addition, the use of leaf as explant source has proved to be a more favorable choice over stem for the establishment of roselle callus culture for pigment elicitation. A possible explanation for this is the leaf being functionally imposed to produce anthocyanins upon exposure to stress. A stress response is induced upon recognition of the stress at the cellular level. In this case, an abiotic stress like wound-

ing upon explant preparation induced the biosynthesis of secondary metabolites (Akula and Ravishankar, 2011). Regulation of gene expression for anthocyanin production is easily up-regulated in the leaf (Gould, 2004; Chalker-Scott, 1999).

Anthocyanin production was a temporary state. A month after the passage of induced calli with pigmented cells, cell growth increased and the anthocyanin production stopped.

Culture Elicitation for Anthocyanin Production

Addition of Yeast Extract

Elicitation of anthocyanin production was attempted by adding yeast extract to the culture medium. No pigment production was observed at the end of the one month observation period. Figure 3 shows the growth curve observed for the callus cultures under the different treatments. It can be observed in all treatments that at week 2 to 3 after inoculation was the log phase of growth. There was up to 10-fold increase in the culture growth based on dry weight increase as observed in treatment Y2 (4g/L). Statistical analysis resulted to no significant differences among the treatments.

Increase in growth may be due to the optimal effect of yeast extract on tissue growth as it serves as additional amino acid source (Molnar et. al., 2011). Increase in dry weight was also observed in cultures of *Solanum hainanense* exposed to yeast extract at 4g/L (Loc et al., 2014). For the Y1 (8g/L) treatment, although there was an increase in growth, most of the calli underwent necrosis.

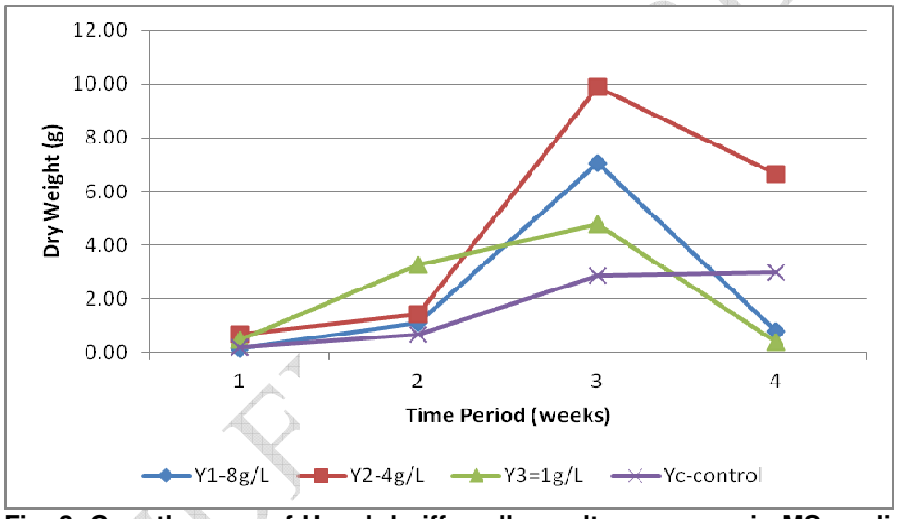

Fig. 3. Growth curve of H. sabdariffa callus cultures grown in MS medium with 1ppm 2,4-D and 2ppm kinetin with different yeast extract concentrations (Y1= 8g/L; Y2= 4g/L; Y3=1g/L; Yc=no yeast extract).

After the experiment, callus cultures in treatment Y2 were subcultured back to MS medium with 1ppm 2,4-D and 2ppm kinetin. Two weeks after subculture, small sections of red pigmented callus appeared in two out of eight cultures (Figure 4). These anthocyanin-producing cells were unstable because in the next month after another subculturing, the pigments disappeared. Thus, the elicitation of anthocyanin production in the callus cultures was temporary and unstable. This may be due to the fact that anthocyanins were produced in response to stress induced by exposure to yeast extract but its return to maintenance medium removed the effect of stress.

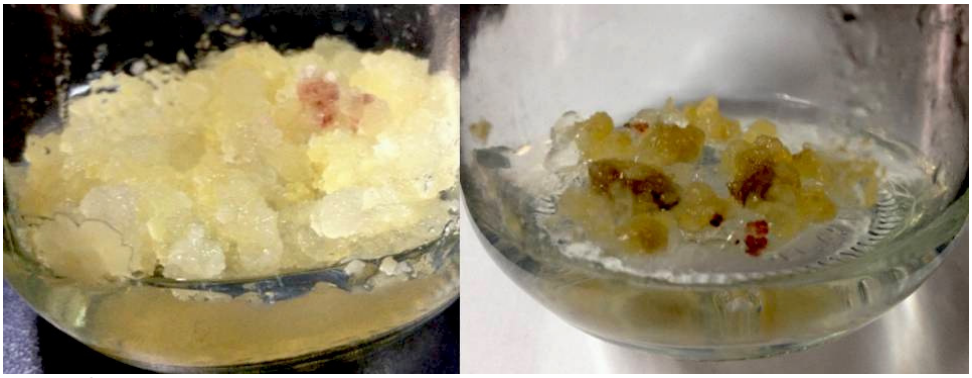

Fig. 4.Callus cultures showing anthocyanin production after being subcultured from MS medium with yeast extract to MS medium without yeast extract.

Callus cultures that were subjected to yeast extract containing medium turned yellow after several passages. Thin layer chromatography was done to identify the possible flavonoid that caused the pigmentation. Figure 5 shows a blue fluorescing band at the Hsyc lane which was identified as isochlorogenic acid (Wagner and Bladt, 1996). There was no anthocyanin production but the precursors needed for biosynthesis were present (Tanaka et al., 2008).

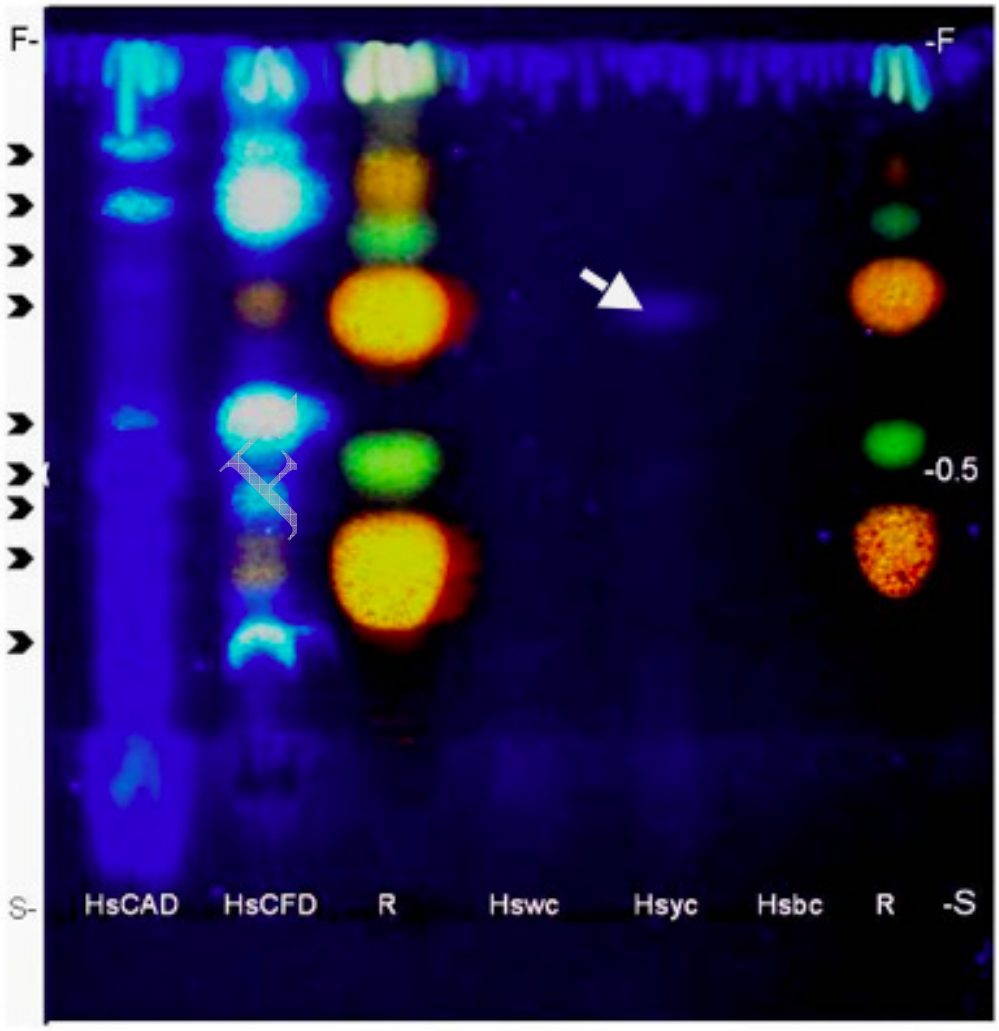

Fig. 5. Thin layer chromatogram showing the yellow colored callus (Hsyc) produced after elicitation with yeast extract that showed fluorescence at Rf= 0.7 (arrow)

which corresponds to isochlorogenic acid. [HsCOD (*H. sabdariffa* calyx oven dried), HsCFD (*H. sabdariffa* calyx freeze dried), R (rutin tablet as reference), Hswc (*H. sabdariffa* white callus)]

4. CONCLUSION

Tissue culture establishment was done to be able to manipulate growth conditions to achieve increased production of anthocyanin pigment. Growth hormone concentration was optimized to bulk up the biomass of the callus cultures to be used in anthocyanin elicitation. Based on the results, growth was optimal at MS medium with 1 ppm 2,4-D and 2ppm kinetin.

Elicitors such as yeast extract were employed to induce anthocyanin production. The basis for choosing elicitors is to achieve pigment production as a result of stress response upon exposure to harmful stimuli. The use of yeast extract as an elicitor proved to be difficult because yeast extract can affect the growth of the callus. Using the right amount of yeast for elicitation is necessary. Cultures exposed to yeast were responsive to the stress caused by its addition to the growth medium. Ephemeral anthocyanin production was observed upon changing growth medium composition. This indicates that the callus cultures were competent to express genes for pigment biosynthesis even if it was temporary. Yellow colored calli developed for cultures that were previously exposed to yeast extract. TLC showed they contained chlorogenic acids which are possible precursors for the biosynthetic pathway of anthocyanins.

Consent, Ethical: NA

5. ACKNOWLEDGEMENTS

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6. AUTHORS' CONTRIBUTIONS

Both authors initiated the concept of the research. The first author conducted the literature search, collected the plant materials, performed the laboratory work, formulated recommendations, and prepared the write-up for publication. The second author identified some issues, formulated recommendations, and reviewed the paper.

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