

Original Research Article

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

ABSTRACT

Introduction: *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.

Methodology: Callus induction was done best using aseptically grown seedlings of the Thailand accession at MS medium with 1ppm 2,4-D and 2ppm kinetin. Callus formation and ephemeral anthocyanin production were observed 2 weeks after inoculation. Anthocyanins elicitation was done using yeast extract as a biotic elicitor.

Results: Addition of yeast extract increased the growth rate up to 10-fold (4g/L). When transferred to a growth medium without yeast, the callus cultures produced anthocyanins 2 weeks after subculturing.

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 (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). The 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. With its vibrant red color and health promoting attributes, roselle is considered as one plant source with high potential in the world market. Therefore, there is a need to address the limitations in using the anthocyanins of roselle to be able to meet the consumers' demand.

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 these limitations, one solution could be through the establishment of plant cell and tissue cultures for pigment production and employing the tools of molecular biology in enhancing product yield (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, in particular, the process of induction and regulation, compartmentalization of biosynthesis and storage (Walton et al., 1999). A number of researches have been directed to increase productivity of plant cell cultures to be able to meet the market demand for anthocyanins. Some of which focused on strain improvement, growth medium optimization, and selection of culture conditions. Specialized procedures such as elicitation and permeabilization are also employed (Zhang and Furusaki, 1999).

Synthesis and accumulation of secondary metabolites, such as anthocyanins, 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). Hence, to trigger the expression of genes for secondary metabolism, the plant must detect a stimulus about an attack. These corresponding signals are known as elicitors which can either be biotic or abiotic. Biotic elicitors are organic substances that may come from the attacking microorganism, cell wall of spores and hyphae of pathogenic or nonpathogenic fungi. On the other hand, 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. Wang and colleagues (2004) studied *Perilla frutescens* 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 previously 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 elicit anthocyanin production in established cultures of roselle using varying concentrations of yeast extract.

2. MATERIALS AND METHODS

Callus Culture

Explant were derived from the cotyledonary leaf and stem of 10-day old aseptic 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°C 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°C 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, MgCl₂ and CaCl₂ 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

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

3. RESULTS AND DISCUSSION

Callus Culture

Two weeks after inoculation, callus formation was observed. The leaf explants were less responsive with the highest percentage of callus formation 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.

Table 1. Physical, chemical and biological properties of experimental soil (0-20 cm)

Explant used	% of Callus Formation			% Anthocyanin Production		
	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

For some cultures, anthocyanin production was observed. The occurrence was highest in T3 cultures at 60%. Cultures started from stem explants exhibited only 30% of anthocyanin production in T2 (MS medium with 1ppm of 2,4-D and 1ppm kinetin).

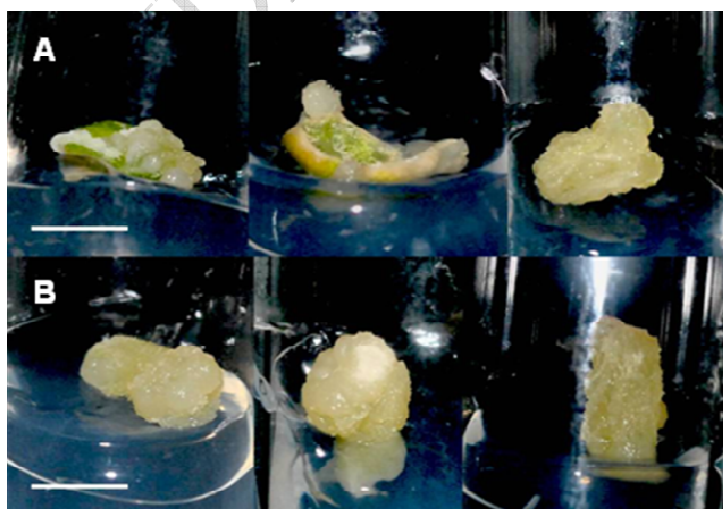


Fig. 1. Callus cultures from leaf (A) and stem (B) explants and induced in MS medium with 2ppm kinetin and 1ppm 2,4-D. (Scale bar = 1cm)

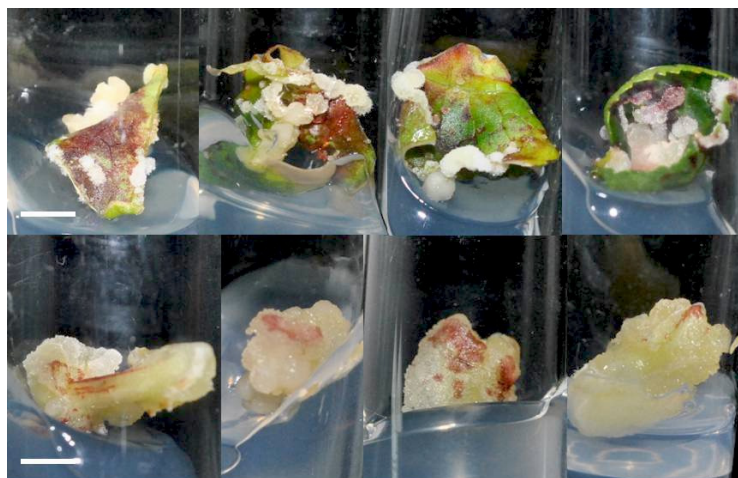


Fig. 2. Anthocyanin production in the leaf (A) and stem (B) explants during callus induction in MS medium with 2ppm kinetin and 1 ppm 2,4-D. (Scale bar = 1cm)

In Figure 2, callus cultures with anthocyanin production are presented. Pigment production was observed at the surface of the callus cultures at first passage two weeks after inoculation of leaf and stem explants. 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. Regulation of gene expression for anthocyanin production is easily up-regulated in the leaf (Gould, 2004).

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.

A change in nutrient medium was introduced, from growth medium to production medium, to explore the potential of applying two stage culture method for efficient anthocyanin production (Wink, 1999). Growth rate and pigment production were monitored in a period of one month. Figure 3 shows the growth rate of roselle callus cultures in growth medium and in production medium. Continuous growth of callus can be observed in both culture media throughout the four-week experiment but the increments of change in weight decreases through time. There was no visual indication of anthocyanin production.

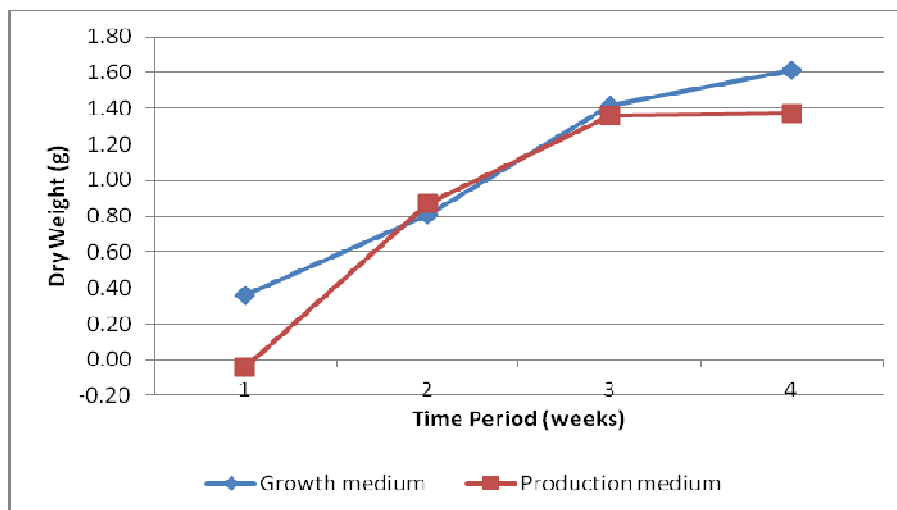


Fig. 3. Growth curve for *H. sabdariffa* callus cultures grown in MS medium with 1ppm 2,4-D and 2ppm kinetin (Growth medium) and in a modified LS medium with 0.22 ppm 2,4-D and 0.22 ppm kinetin (Production medium).

Culture Elicitation for Anthocyanin Production

Addition of Yeast Extract

Elicitation of anthocyanin production was attempted by adding yeast extract to the culture medium. At the end of the one month observation, still no pigment production was observed. Figure 4 shows the growth curve observed for the callus cultures under the different treatments. It can be observed that at week 2 to 3 after inoculation was the log phase of growth. In terms of the increase in dry weight, there was up to 10-fold increase in the culture growth as observed in treatment Y2 (4g/L). Increase in dry weight was also observed in cultures of *Solanum hainanense* exposed to yeast extract at 4g/L (Loc et al., 2014). 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). For the Y1 (8g/L) treatment, although there was an increase in growth, most of the calli underwent necrosis.

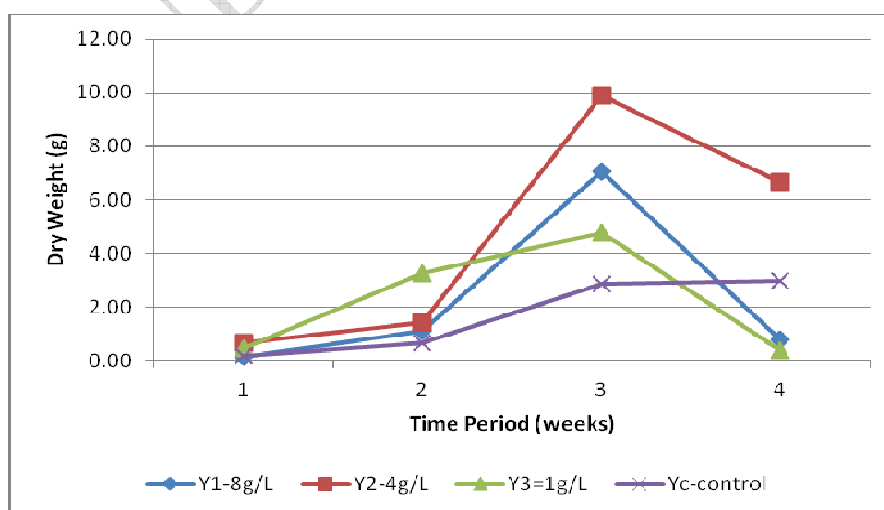


Fig. 4. Growth curve of *H. sabdariffa* callus cultures grown in MS medium with 1ppm 2,4-D and 2ppm kinetin with different yeast extract concentrations (Y1= 8g/L; Y2= 4g/L; Y3=1g/L; Yc=no yeast extract).

After the experiment, callus cultures in treatment Y2 were subcultured back to MS medium with 2ppm kinetin and 1ppm 2,4-D. Two weeks after subculture, small sections of red pigmented callus appeared in two out of 20 cultures (Figure 5). 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. 5. 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 6 shows a blue fluorescing band at the Hsync lane which was identified as isochlorogenic acid (Wagner and Bladt, 1996). There was no anthocyanin production but the precursors needed for biosynthesis were present.

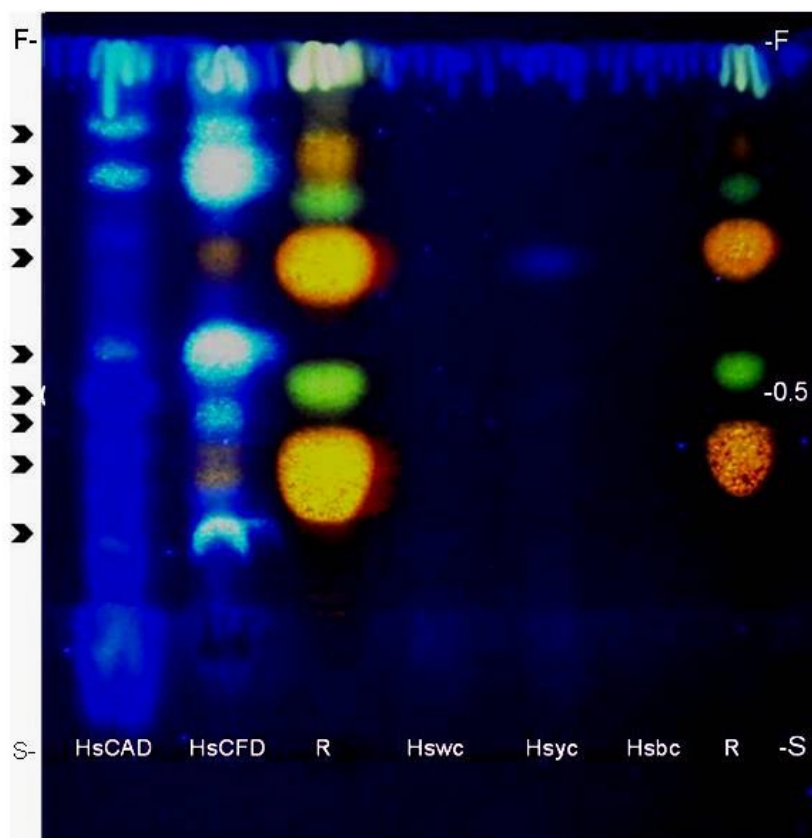


Fig. 6. Thin layer chromatogram showing the yellow colored callus (Hsyc) produced after elicitation with yeast extract that showed fluorescence at $R_f = 0.7$ (arrow) which corresponds to isochlorogenic acid.

Elicitation of anthocyanin production in roselle callus cultures using yeast extract was not easy. The concentration of extract, growth conditions, and follow-through after exposure to yeast extract should be optimized for a more efficient elicitation. The occurrence of anthocyanin production, even if it was ephemeral, indicated that the callus cultures were competent of expressing genes responsible for the biosynthesis.

4. CONCLUSION

Tissue culture establishment was done to be able to manipulate growth conditions to achieve increased production of anthocyanin pigment. Callus was initiated using the aseptically germinated seedlings of the Thailand accession of roselle. 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. Two weeks after inoculation, callus formation was already observed and some already showed anthocyanin production. After a year of continuous passage, the cultures were transferred in a production medium that was optimized by Mizukami (1993) to induce anthocyanin production in the white, friable callus cultures. Growth rate of the callus cultures was not affected. However, no anthocyanin elicitation occurred in the production medium.

Elicitors such as yeast extract was also 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 as a biotic elicitor can affect the growth of the callus. Hence, using the right amount of yeast for elicitation is necessary. The callus cultures exposed to yeast were responsive to the stress induced by its addition to the growth medium. Ephemeral anthocyanin production was observed 2 weeks after subculturing to maintenance MS medium. This indicates that the callus cultures were competent to express anthocyanin production even if at certain times it loses the pigment. 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.

REFERENCES

- CISSE M., M. DORNIER, M. SAKHO, A. NDIAYE, M. REYNES, O. SOCK. 2009. Le Bissap (*Hibiscus sabdariffa* L.): Composition et Principales Utilizations. *Fruits*. 64(3): 179-193. [2]
- GEORGE E.F., M.A. HALL, G-J. DE KLERK. 2008. 3rd Ed. *Plant Propagation by Tissue Culture*. Springer. Netherlands. 115-173. [9]
- GOULD K.S. 2004. Nature's Swiss Army Knife: The Diverse Protective Roles of Anthocyanins in Leaves. *Journal of Biomedicine and Biotechnology*. 5(2004):314-320. [12]
- IBRAHIM M.M., R.M. HUSSEIN. 2006. Variability, Heritability, and Genetic Advance in Some Genotypes of Roselle (*Hibiscus sabdariffa* L.). *World Journal of Agricultural Sciences*. 2(3): 340-345. [1]
- LOC N. H., N.H.T. ANH, L.T.M. KHUYEN, T.N.T. AN. 2014. Effects of yeast extract and methyl jasmonate on the enhancement of solasodine biosynthesis in cell cultures of *Solanum hainanense* Hance. *J. BioSci. Biotech*. 3(1): 1-6. [13]
- MIZUKAMI H. 1993. *Hibiscus sabdariffa* L. (Roselle): In vitro Culture and the Production of Anthocyanins. *Medicina and Aromatic Plants V* ed. by Y.P.S.Bajaj. *Biotechnology in Agriculture and Forestry*. 24: 218-228. [11]
- MOLNAR Z., E. VIRAG, V. ORDOG. 2011. Natural substances in tissue culture media of higher plants. *Acta Biologica Szegediensis*. 55(1): 123-127. [8]
- MURASHIGE T., F. SKOOG. 1962. A revised medium for rapid growth and bioassay of tobacco tissue culture. *Physiol. Plantarum* 15: 473-497. [9]
- REICHLING J. 1999. Plant-microbe interactions and secondary metabolites with antiviral, antibacterial and antifungal properties. In WINK M. (Ed.) *Functions of Secondary Metabolites and their Exploitation in Biotechnology*. *Annual Plant Reviews* 3:187-273. [6]
- SIE R.S., G. CHARLES, H.F. SAKHANOKHO, Y. TOUEIX, Y. DJE, A. SANGARE, M. BRANCHARD. 2010. Protocols for Callus and Somatic Embryo Initiation for *Hibiscus sabdariffa* L. (Malvaceae): Influence of Explant Type, Sugar, and Plant Growth Regulators. *Australian Journal of Crop Science*. 4(2):98-106. [10]
- WAGNER H., S. BLADT. 1996. *Plant Drug Analysis*. 2nd ed. Springer-Verlag Berlin Heidelberg. Germany. Pp: 281-289. [14]
- WALTON, N.J., A.W. ALFERMAN, M.J.C. RHODES. 1999. Production of secondary metabolites in cell and differentiated organ cultures. Ed. M. Wink. *Functions of Secondary Metabolites and their Exploitation in Biotechnology*. *Annual Plant Reviews* 3:311-345. [4]
- WANG, J.W., Z.H. XIA, J.H. CHU, R.X. TAN. 2004. Simultaneous production of anthocyanin and triterpenoids in suspension cultures of *Perilla frutescens*. *Enz. Micro. Tech*. 34:651-656. [7]
- WINK, M. 1999. *Functions of Secondary Metabolites and their Exploitation in Biotechnology*. *Annual Plant Reviews* 3:1-14. [5]
- ZHANG W. and S. FURUSAKI. 1999. Production of Anthocyanins by Plant Cell Cultures. *Biotechnol. Bioprocess Eng*. 4:231-252. 02;32(4):489-98. [3]