7. Langdon, O.G. and W.H. McKee, Jr. 1981. Can fertilization of loblolly pine on wet sites reduce the need for drainage? U.S. For. Serv. SE For. Expt. Stn. Genet. Tech. Rep. no. SE-34. p. 212–218.
8. Loucks, W.L. and R.A. Keen. 1973. Submersion tolerance of selected seedling trees. J. For. 71:496–497.
9. Loustalot, A.J. 1945. Influence of soil moisture conditions on apparent photosynthesis and transpiration of pecan leaves. J. Agr. Res. 71 (12):519–533.
10. Remy, P. and B. Bidade. 1962. Root asphyxia and collar rot in pome trees. The influence of the rootstock. Proc. Congr. Pomol. 92:17–28.
11. Sena Gomes, A.R. and T.T. Kozlowski. 1980. Growth responses and adaptations of *Fraxinus pennsylvanica* seedlings to flooding. Plant Physiol. 66:267–271.
12. Sena Gomes, A.R. and T.T. Kozlowski. 1986. The effects of flooding on water relations and growth of *Theobroma cacao* var. *catongo* seedlings. J. Hort. Sci. 61:265–276.
13. Shapiro, R.E., G.S. Taylor, and G.W. Volk. 1956. Soil oxygen contents and ion uptake by corn. Soil Sci. Amer. Proc. 20:193–197.
14. Thimann, K.V. and Y.H. Edmondson. 1949. The biogenesis of the anthocyanins. I. General nutritional conditions leading to anthocyanin formation. Arch. Biochem. 22:33–53.

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Development of Anthocyanin Pigments in Muscadine Grapes

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Additional index words. *Vitis rotundifolia*

Abstract. **The development of individual anthocyanins (Acy) in black muscadine grapes (*Vitis rotundifolia* Michx) during maturity was followed. Most of the grapes investigated contained the nonacylated 3,5-diglucoside of delphindinin (Dp), cyanidin (Cy), petundin (Pt), Peonidin (Pn), and malvidin (Mv) when pigment extracts were separated on a reverse phase HPLC C_{18} column. Dp was the most abundant anthocyanin (Acy) in most cultivars at maturity. The development of individual Acy, which is indicative of the nature of enzyme activity, occurred at different rates for each of the cultivars. A very high flavonoid 3′ hydroxylase activity was indicated by the fact that, in most instances, Dp content was higher than those of the other Acy at the onset of pigmentation, including Cy, which is the precursor of the other pigments. The total Acy content at maturity ranged from 12 to 338 mg/100 g of berries.**

The nature and content of grape anthocyanins (Acy) can serve as important chemical markers and improve understanding of the biochemical reactions leading to ripening of grapes. Ebel and Halbrook (3) and Markakis (7) noted that a chalcone is the first intermediate of the whole classes of flavonoids. Flavonone then is produced from chalcone isomerization by a chalcone isomerase, and this undergoes different enzyme catalyzed reactions leading to flavones, flavonols, isoflavones, or Acy. Although very little is known about the last transformation (3, 5), it appears to be quite evident that chemical modifications, such as hydroxylation, methylation, glycosylation, and esterification of the glucosides, occur. Roggero et al. (10) suggested a reaction pathway of Acy that involves the synthesis of cyanidin (Cy) from a flavonone after the isomerization of chalcone. Cy then can be converted to either delphindin (Dp) or peonidin (Pn) as a result of enzymatic activity. Flavonoid-3-hydroxylase (FH) catalyzes the conversion of Cy to Dp, while the formation of Pn is catalyzed by O-methyltransferase (MT). Dp then can be converted to malvidin (Mv) through Mt activity. Their results suggest that the hydroxylation of Pn to form Pt does not take place, and they noted that the percentage of Dp at the early stages of ripening is an excellent sign of enzyme activity in cultivars.

Liao and Luh (6), and Ribereau-Gayon (9) reported that the color of red muscadine wine bears a direct relationship with the Acy composition. They also reported that these pigments are the nonacylated 3,5-diglucosides of Mv, Pn, Pt, Cy, and Dp. These findings also were supported by the research conducted by Ballinger et al. (1). Nesbitt et al. (8) demonstrated that good red wine color bears a direct relationship to the presence of a large amount of Mv, and, to a lesser degree, of Pt. It was suggested that increased methylation of individual Acy enhanced pigment stability. Although Dp was the most abundant form of all the Acy present, there appeared to be no correlation between the quantity of Dp present in the grapes and good wine quality. Ballinger et al. (2) in a subsequent study also confirmed these results. They reported that Mv is the most stable Acy in wines, followed in decreasing order by Pn, Pt, Cy, and Dp. Similar observations were made in muscadine juices and jellies by Flora (4), who showed that cultivars with relatively high Mv and total Acy contents yielded products with the highest quality and most suitable colors. The objective of this study was to separate and quantify the Acy pigments that are present in some black muscadine grape cultivars at different stages of maturity.

Grapes from the cultivars Chief, Jumbo, Albermarle, Cowart, Noble, Regale, and Pink Hunt were harvested at different stages of maturity from experimental vineyards located on the university campus. Each cultivar then was separated visually into three categories based on the degree of coloration (verasion, half color development, and full color, respectively). Each subgroup again was separated into three batches for analysis. The sugar content of the grapes was determined with an American Optional temperature-compensated hand refractometer.

Color of whole grapes was measured on a Perkin-Elmer Lambda 3B Spectrophotometer equipped with an external integrating sphere and a data station. Grapes were placed in the external sphere, and transmittance values for scans between 780 and 380 nm were determined at 1 nm intervals. Luminance "L' values were determined using the CIE standard source C on Perkin Elmer IFL3 and COLOR software. Total Acy content in the grapes was determined on freeze-dried, dewaxed grapes (30 g). Wax removal was done by soaking whole grapes in ether for 5 hr. Pigments then were extracted exhaustively in 1% HCl in methanol by successive extractions. Absorbance values of these solutions were read at 535 nm and Acy content was estimated using a molar extinction coefficient (E) 2.1×14^4 and a molecular weight of 683 (4).

For HPLC analysis, the dry powders obtained from freeze drying the grapes (30 g) were mixed with acidified methanol (20 ml) and left overnight in the refrigerator. The dissolved pigments were separated from solid materials by centrifugation at 5000 × g for 10 min. Separation of individual Acy in the supernatant was done with a Hitachi 655A-11 system after filteration through a 0.45 Teflon filter, using a 250 mm × 4 mm I.D. Hibar C_{18} column and an L-3000 multichannel UV-VIS photodetector. Acy were monitored at 520 nm. The solvent system used was H_3PO_4 (1%) in acetic acid-water (10:90). Samples (20 µl) were injected into the column and eluted at a flow rate of 1.0 ml/mm (11), and peaks were integrated with a Hitachi D-2000 Chromato-Integrator. Peaks were identified from their retention times using standards obtained from Alpine Chemical Co., England. Statistical analysis of data was carried out using the Student *t*-distribution.

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Table 1. "L" values, percent soluble solids and anthrocyanin development in muscadine grapes

Cultivar	°Brix	L	Total Acy content (mg/100g fruit)	Peak area (%) Dp	Cy	Pt	Pn	Mv
Chief 1[x]	9.9	51.52	8	100	---[y]	---	---	---
Chief 2	11.2	45.14	50	46.5 a[z]	36.6 b	9.3 c	4.7 d	1.0 e
Chief 3	17.2	38.86	130	42.8 a	22.2 b	25.7 b	9.0 c	0.3 d
Jumbo 1	10.2	40.51	26	100	---	---	---	---
Jumbo 2	11.9	35.05	147	91.6 a	3.8 b	---	---	---
Jumbo 3	14.7	32.35	232	80.0 a	11.3 b	7.2 c	0.8 d	0.8 d
Albemarle 1	10.9	41.59	14	---	100.0	---	---	---
Albemarle 2	12.8	36.57	66	56.2 a	23.6 b	2.8 c	0.1 d	8.9 e
Albemarle 3	15.9	32.66	135	68.0 a	20.4 b	8.2 c	2.7 d	0.6 e
Cowart 1	10.1	57.89	11	20.5 a	47.6 b	11.9 c	20.0 a	---
Cowart 2	12.5	51.03	16	30.9 a	33.2 a	11.0 b	22.6 c	2.3 d
Cowart 3	16.0	47.51	42	25.9 a	44.7 b	4.9 c	13.4 d	0.9 e
Noble 1	9.6	47.37	19	88.5 a	11.5 b	---	---	---
Noble 2	11.0	37.61	139	34.0 a	11.6 b	16.7 c	14.6 bc	23.1 d
Noble 3	15.5	34.19	388	33.6 a	15.5 bc	19.8 c	18.2 c	13.1 b
Regale 1	8.4	47.91	16	66.2 a	33.8 b	---	---	---
Regale 2	11.4	40.71	139	74.6 a	11.1 b	12.7 b	---	---
Regale 3	14.0	35.30	367	59.7 a	3.0 b	28.1 c	0.2 d	9.0 e
Pink Hunt 1	12.1	50.29	5	100.0	---	---	---	---
Pink Hunt 2	13.0	47.79	9	64.5 a	35.5 b	---	---	---
Pink Hunt 3	15.2	41.96	12	19.6 a	29.2 b	22.5 c	28.7 c	---

[x]Category of color development: 1 = verasion; 2 = half color development; 3 = full color.
[y]Acy content was below detection limit for procedure used.
[z]Values in each row separated by t test. ($P = 0.05$).

"L" values decreased with maturity, indicating that the grapes became darker as they matured. The development of Acy occurred at different rates during ripening. In all the cultivars except Regale, pigment development did not occur until the sugar content was ≈10°Brix (Table 1).

Acy contents of mature grapes ranged from 12 in 'Pink Hunt' to 388 mg/100g of berry weight in 'Noble'. Most of the cultivars had all five nonacylated 3,5-diglucosides at optimum ripeness. Mv was not detected in 'Pink Hunt'. The Mv contents of 'Cowart', 'Jumbo', and 'Albemarle' were also lower than those of the other Acy. Pn was present in mature 'Regale' only in trace amounts. As observed by Nesbitt (8) and Ballinger (2), Dp was the most abundant Acy in many of the cultivars. 'Cowart' and 'Pink Hunt' showed bigger peaks for Cy than the other Acy. The relative individual Acy contents varied considerably among cultivars.

In most instances, Dp was the predominant Acy detected at the onset of pigmentation and the most dominant Acy when the grapes were fully ripe. Although 'Cowart' and 'Albemarle' had more Cy than the other Acy at the early stages of coloration, the relative contents of 'Cowart' decreased with ripening. With the exception of 'Noble', Mv development did not take place until Acy formation was well underway. In all cultivars, except 'Regale,' its formation appears to cease before Acy concentration peaked.

Changes in relative Cy contents during ripening should be indicative of the FH and MT activities in the grapes. Since all the other Acy are synthesized from Cy, the ideal behavior should be that of gradual decrease with maturity (3, 10). In many of these cultivars, however, Dp was the most prominent peak at the onset of color development. This observation suggests a relatively low MT activity at this stage and a very high FH activity, which causes the formation of Cy to be rate limiting. Cy then is converted rapidly to Dp as soon as it is formed. The only exception was 'Cowart', which had relatively high Pn content at the very early stage of pigment development, indicative of a significant MT catalyzed conversion of Cy. In 'Cowart' and 'Albemarle', the initial Cy contents suggest a relatively low enzyme activity at this stage of pigmentation. An increase in enzyme activity occurs as the grapes develop, with a higher FH activity in 'Albemarle', which resulted in the high Dp content at maturity. 'Pink Hunt' showed a gradual decrease in FH activity with ripening and an apparent increase in the activity of MT, which caused the Pn content to be higher than that of Dp. Pn and Mv, owing to their monophenol structures, are more stable than the other Acy. 'Noble' showed a relatively high Mv and Pn content, whereas 'Regale' had little or no Pn content. The Dp contents of 'Regale' during ripening also indicate low MT activity throughout the ripening process.

Total Acy contents obtained for mature grapes are comparable to those reported by Ballinger (2) and Flora (4) for the same cultivars. However, The ratio of the individual Acy were not in agreement in some instances. Similar differences exist between the ratios reported by Flora (4) and Ballinger et al. (2) as well as those of Ballinger et al. (2) and Nesbitt et al. (8), for the same cultivars. These differences do not support the findings of Ribereau-Gayon (9), who showed that although Acy content of *Vitis vinifera* grapes may vary from year to year, the ratio of Acy contents of grapes remains relatively constant. The differences observed in the relative Acy contents for these *Vitis rotundifolia* grape cultivars might be due to differences in geographical locations and prevailing climatic conditions. Research is needed to determine the nature and characteristics of muscadine grape enzymes and the factors that influence their behavior during grape ripening.

Literature Cited

1. Ballinger, W.E., E.P. Maness, W.B. Nesbitt, and D.E. Carroll. 1973. Anthocyanins of black grapes of 10 clones of *Vitis rotundifolia* Michx. J. Food Sci. 38:909–910.
2. Ballinger, W.E., E.P. Maness, W.B. Nesbitt, D.J. Makus, and D.E. Carroll. 1974 A comparison of Anthocyanins and wine color quality in black grapes 39 clones of *Vitis rotundifolia* Michx. J. Amer. Soc. Hort. Soc. 99:338–341.
3. Ebel, J. and K. Halbrook. 1982. Biosynthes, p. 641–679. In: The flavonoids: Advances in research. J.B. Horborne and T.J. Marbry (eds.). Chapman and Hall, N.Y.
4. Flora, L.F. 1978. Influence of heat, cultivar and maturity on the anthocyanin-3,5-Diglucosides of muscadine grapes. J. Food Sci. 43:1819–1821.
5. Hrazdina, G. 1982. Anthocyanins, p. 135–188. In: The flavonoids: Advances in research. J.B. Harbrone and T.J. Mabry (eds.). Chapman and Hall, N.Y.
6. Liao, F.W.H. and B.S. Luh. 1970. Anthocyanin pigments in Tinto Cao grapes. J. Food Sci. 35:41–46.
7. Markakis, P. 1974. Anthocyanins and their stability in Foods. CRC Crit. Rev. Food Technol. 4:437–456.
8. Nesbitt. W.B., E.P. Maness, W.E. Ballinger, and D.E. Carroll. Relationship of Anthocyanins of black muscaidne grapes (*Vitis rotundifolia* Michx) to wine color. Amer. J. Enol. Vitic. 25:30–32 (1974).

9. Ribereau-Gayon, P. Les Composes phenolique du raisin et du vin. Ann. Physiol. Vig 6:211–242 (1964).
10. Roggero, J.P., S. Coen, and L.B. Ragonnet. 1986. High performance liquid chromatography survey on changes in approach to Anthocyanin metabolism Amer. J. Enol. Vitic. 37:77–83.
11. Williams, M., G. Hrzdina, M.M. Wilkinson, J.G. Sweeny, and H.A.J. Iacobucci. 1978 Chromatog. High-pressure liquid chromatographic separation of 3-glucosides, 3,5-diglusides, 3(6-0-p-coumaryl) glucosides and 3-(6-0-p-coumarylglucoside)-5-glucosides of anthyocyanins. J. Chromatog. 155:389–398.

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Carbon/Nitrogen Ratio and Greening and Protocorm Formation in Orchid Callus Tissues

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Abstract. **Callus tissues of orchid *Aranda* Tay Swee Eng (*Archnis* Lily Chong x *Vanda* Piha Moon) were cultured in Vacin and Went's medium that contained between 0% and 2% fructose. The chlorophyll content of callus tissues measured after 31 days of incubation ranged from 59 to 133 $\mu g \cdot g^{-1}$ tissue and was inversely proportional to the C/N ratio of the initial culture medium. The number of protocorm developed on callus tissue was instead inversely proportional to the residual C/N ratio of the 31-day-old culture medium and ranged from 13 to 74. The interrelationship of the C/N ratio of the medium, chlorophyll content, and protocorm formation in *Aranda* callus tissues is discussed.**

Sugar in culture media is not only responsible for the nutritional effects on growth of plant tissues *in vitro,* but also has a host of regulatory effects upon plant development. Vanseveren-van (8, 9) found chloroplast development and organogenesis of *Cattleya* and *Cymbidium* tissues in culture to be enhanced at low sucrose concentrations. Others (4) noted that removal of sugar might be necessary for promoting greening of protocorms of *Phalaenopsis.* Also, proliferation and shoot production by callus tissues of *Aranda* and *Aranthera* were induced by the removal of sucrose from the medium (3). Similar views were also held by Nyman et al. (5), who reported that, in cultured shoots of taro (*Colocasia esculenta* var. Antiquorum), greening and compact growth were necessary prerequisites to organogenesis.

This paper examines the effect of fructose concentration on chlorophyll formation and organogenesis and the relationship between the two processes. The results indicate that although both chlorophyll formation and organogenesis (such as protocorm formation) are direct responses to the sugar concentration in culture medium, they may not be directly related events.

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The orchid hybrid *Aranda* Tay Swee Eng used in this study is commonly cultivated in Singapore for cut flowers. Callus tissues were initiated through the culture of apical meristems. Before the experiments, callus tissues were maintained in Vacin and Went's medium (7) which contained 2.50 $g \cdot liter^{-1}$ $NaNO_3$ and 10% (v/v) coconut water as a growth adjunct. The pH of the medium was adjusted to 5.1 before autoclaving at 15 psi for 20 min.

Between 0% and 2% fructose was added to the basal culture medium and appropriate amounts of mannitol were added to maintain the osmotic concentration of the culture medium equivalent to the presence of 2% fructose.

The starting materials were green calli derived from the same stock culture and contained 0.165 mg chlorophyll per gram of tissue. Green callus tissues were blotted dry using sterilized filter paper and weighed. Approximately 1 g of these tissues was inoculated into 100-ml conical flasks each containing 25 ml of culture medium. The flasks were then sealed with a double-layer of sterile aluminum foil and placed on an orbital shaker (Chittern Scientific S570) set at 100 rpm. The cultures were maintained in a culture room at 23° ± 2°C under fluorescent light for 16 $hr \cdot day^{-1}$. Light intensity on the surface of the culture flasks was 45 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

The initial and residual carbohydrate concentrations in the culture media were determined by the Anthrone test (6). Glucose was used as the standard. Total chlorophyll con-

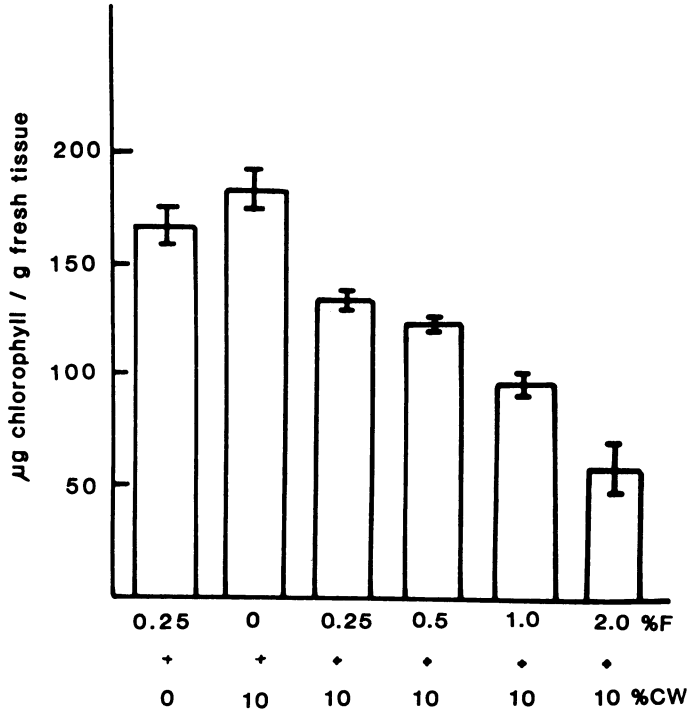

Fig. 1. Chlorophyll concentration of *Aranda* Tay Swee Eng callus tissues cultured in media with various concentrations of fructose for 31 days. F = fructose, CW = coconut water.