Biosynthesis of anthocyanins in maize: presence and role of o-diphenolase and monophenolase enzymes and their isoenzyme polymorphism

During the course of chemico-genetical investigations on anthocyanin biosynthesis in maize, the cut ends of silks in many lines were found to turn brownish within a few minutes, developing a melanin type of pigment. Interest in polyphenol oxidases arises from their role in phenolic biosynthesis, oxidation of phenols and formation of dark colored pigment. By screening the genetic background of these lines it may be possible to ascertain the role of polyphenols. Polyphenols are also known to aid in pollen germination and tube growth *in vitro* and thus their presence in silks may have a more definite role in aiding pollen germination rather than in the synthesis of anthocyanins.

With this view in mind, silks from some 97 lines of different genetic background were studied for the activity of polyphenol oxidases and their isoenzyme polymorphism.

The enzyme was extracted by homogenizing 5 gm silks with 15 ml prechilled (-15 C) 0.05 M phosphate buffer (pH 6.6) and centrifuging at 20,000 xg for 20 min at 5 C. The supernatant extract was assayed immediately for polyphenol oxidase activity using catechol (10 mg/ml) and L-tyrosine (1.0 mg/ml) as substrates. The reaction mixture consisted of 2 ml catechol, 0.1 ml of enzyme preparation and 0.05 M phosphate buffer (pH 6.6) to bring the total volume to 5 ml. The mixture was incubated at 37 C for 3 min before addition of enzyme. Absorbancy was measured at 430 nm at intervals of 15 seconds. For measuring the monophenolase, 2 ml of L-tyrosine (1.0 mg/ml) solution and 0.5 ml of crude enzyme extract were used. After oxygenating the substrate for a few minutes, absorbancy was measured at 430 nm after 3 hr of incubation at 37 C. Controls consisted of everything else except substrates.

Isoenzyme studies were carried out by electrophoresis in 7.5% polyacrylamide, using tris-glycine buffer (pH 8.3). The gels were stained with L-tyrosine (1.0 mg/ml in 80% ethanol), destained and stored in 30% alcohol.

In about 10 lines no o-diphenolase activity and only negligible monophenolase activity were recorded. These lines showed no browning of cut ends of silks. In the rest of the lines definite polyphenol oxidase activity was observed, mainly the activity of o-diphenolase. The latter was also found to give 5-7 isoenzyme bands of similar nature. Unlike o-diphenolase, no multiple forms were observed for the monophenolase enzyme.

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Biosynthesis of anthocyanins in maize: in vitro tissue culture

Tissue culture of certain critical plant parts affected by each allelic form might prove useful in studies on gene action. It was found indispensable to standarize a suitable nutrient medium for culturing maize kernels *in vitro* from the early stages after anthesis, to trace the anthocyanin biosynthetic pathway based on enzyme activities and specificities. Moreover, it is possible to incorporate enzyme inhibitors or inducers in the culture medium in order to induce or inhibit the synthesis of anthocyanins.

Attempts were made to develop such a medium, and a basal nutrient medium has been standarized which gives profuse growth of maize kernels inoculated the 10th day after pollination. The medium consists of White's major elements and vitamin mixture, Nitsch's trace elements, 2% sucrose, 0.8% agar and 0.5% yeast extract (pH 6.1 - 7.0), and can be used to culture whole kernels, endosperm or embryo alone. Microscopic examination of transverse sections of kernels developed *in vitro* 20 days after inoculation revealed tissue differentiation--i.e., formation of a distinct aleurone layer.

By culturing the kernels at 10 days post-pollination, studies were carried out in vitro on the role of Spm and Spf in causing patched aleurone patterns (a-ml and R-rEm) and also on the 'Anthocyanin Decolorizing Enzyme Complex.' The results confirm our findings from studies in vivo.

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Biosynthesis of anthocyanins in maize: Presence and role of an 'Anthocyanin Decolorizing Enzyme Complex'

During in vivo and in vitro studies on the role of the regulatory systems Spm and Spf in causing spotted aleurone patterns (a-ml and R-rEm), the presence of an 'Anthocyanin Decolorizing Enzyme Complex' has been demonstrated in maize for the first time. The object was to ascertain whether anthocyanins in colorless patches are not synthesized at all or are decolorized in localized areas only after synthesis, leading to spotted patterns.

The crude enzyme complex was isolated from endosperm of R-rEm, R-sd2 and a-ml; pericarp of R-rEm, R-sd2 and a-ml; and aleurone (colorless portions) of R-sd2 and a-ml by homogenizing about 1 gm tissue in cold (-15 C) acetone. The homogenate was vacuum filtered in a Buchner funnel, and the residue was suspended in cold 0.05 M phosphate buffer (pH 6.6) and centrifuged at 2000xg for 15 min at 4 C. The supernatant was used immediately for assaying activity with substrates prepared from aleurone of R-rEm and pigmented portions of aleurone of a-ml and R-sd2. For preparation of substrates the tissues were extracted with cold 1% methanolic HCl, evaporated to dryness and dissolved in enough dilute HCl to get a solution with maximum 5% transmittance at 515 nm.

The assay mixture consisted of 4 ml of freshly prepared substrate, 2.5 ml of enzyme preparation in 0.05 M phosphate buffer (pH 6.6) and water to bring the total volume to 8 ml. The anthocyanin decolorizing capacity of the enzyme complex was measured spectrophotometrically by observing changes in transmittance at 515 nm after intervals of 45 min, 2 hr, 4½ hr and 24 hr.

The results given in Table 1 indicate that the enzyme complex from aleurone tissue of a-ml dilutes R-rEm colored aleurone substrate, enzyme complex from peri-

Table 1. Activity of 'Anthocyanin Decolorizing Enzyme Complex.'

Source of enzyme complex	Substrate*	% Transmittance				
		0 hr	45 min	2 hr	4½ hr	24 hr
R-rEm endosperm	A	58	60	63	69	49
	C	63	68	60	56	59
R-sd2 endosperm	C	66	50	44	46	38
a-ml endosperm	A	48	52	52	50	44
	B	65	64	69	61	62
	C	60	64	68	68	-
R-rEm pericarp	A	60	65	67	68	68
	C	52	56	56	56	51
R-sd2 pericarp	C	66	71	74	67	70
a-ml pericarp	A	48	48	56	46	42
	B	65	71	68	70	72
	C	62	49	48	52	-
R-sd2 aleurone (colorless portion)	B	80	82	-	-	87
	C	86	89	88	-	85
a-ml aleurone (colorless portion)	A	66	72	73	76	78

*Key for substrates: A = R-rEm aleurone; B = a-ml aleurone (pigmented portion); C = R-sd2 aleurone (pigmented portion)