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Photosynthetic Independence of Light-induced Anthocyanin Formation in *Zea* Seedlings[1]

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ABSTRACT

Results are reported which support the view that the photosynthetic photosystems are not involved in the high irradiance response (HIR) phenomenon of light-dependent anthocyanin biosynthesis in dark-grown *Zea mays* L. seedlings. A negative correlation between change in greening rates and change in light-dependent anthocyanin accumulation rates with age was demonstrated. Lack of chlorophyll synthesis in a strain of maize possessing a temperature-sensitive lesion for chlorophyll synthesis could not be correlated with light-induced anthocyanin accumulation. Furthermore, seedlings totally lacking photosynthetic capabilities, either due to a genetic lesion or to excision of all photosynthetic tissue, had an enhanced rate of photoinduced anthocyanin formation. This evidence indicates that the HIR results in the initiation of processes that are in competition with chloroplast development for substrate in normal, intact seedlings.

Many investigators have attempted to connect PS[3] I and/or PS II with the HIR of higher plants. A majority of the investigations have been directed toward learning if there is photosynthetic involvement in the HIR of light-induced anthocyanin biosynthesis. Evidence has been gathered to support both the photosynthetic involvement and noninvolvement hypotheses.

Much of the argument for noninvolvement of photosynthesis in the HIR comes from greening and photosynthesis inhibitor studies. The greening inhibitors chloramphenicol (19, 31) and streptomycin (19, 24) enhance anthocyanin biosynthesis in developing seedlings. The PS II inhibitor DCMU (1, 23) and the cyclic photophosphorylation inhibitor NH_4^+ (15) are reported to have no effect on photoinduction of anthocyanin synthesis. Other evidence for the photosynthetic independence of anthocyanin synthesis has come from experiments in which normal anthocyanin (4) and betacyanin (20) synthesis occurred in the absence of CO_2.

Evidence in support of the involvement of photosynthesis in anthocyanin photoinduction has been offered repeatedly. Chlorophyll synthesis inhibiting concentrations of streptomycin (25) and levulinic acid (9, 30) have been shown to inhibit photoinduced flavonoid pigment formation. DCMU has been demonstrated to reduce photoinduced levels of anthocyanin (8, 11, 13, 21) and activities of PAL (7, 33), one of the key enzymes in the anthocyanin pathway. Cyclic photophosphorylation inhibitors such as NH_4^+, dinitrophenol, and antimycin A have also been demonstrated to inhibit anthocyanin photoinduction (25, 29, 30). The PS I inhibitor SAL has been shown to inhibit light-induced betacyanin formation (11). Both SAL (10) and NH_4^+ (23) also inhibit pigment formation under nonphotosynthetic conditions. Further support for photosynthetic participation in light-dependent anthocyanin biosynthesis is based on correlation of their action spectra (5, 12, 14) and an apparent dependency of PAL activity and flavonoid production on CO_2 supply (8).

As pointed out by Mancinelli *et al.* (24), while most of the evidence indicates there is no photosynthetic involvement in photoinduction of flavonoid pigments in most dark-grown seedlings, there does seem to be good evidence of such involvement in mature, fully greened tissue. There is a paucity of evidence supporting either argument which has been generated without involving metabolic inhibitors. Many of the inhibitors used are not highly specific and have much broader biological activities than those for which they were chosen in the experiments discussed above.

We report data in this paper which support the view that there is no photosynthetic involvement in photoinduction of anthocyanin formation in dark-grown maize seedlings. This evidence has been produced entirely without the use of photosynthetic or greening inhibitors.

MATERIALS AND METHODS

Effects of Exicision of Photosynthetic Organs of *Zea* Seedlings on Photoinduction of Anthocyanin Biosynthesis. Seed of *Zea mays* L. cv Pioneer 3369A were sown in 1 mM $CaSO_4$ solution in Petri dish bottoms and grown at 25 C in complete darkness from the beginning of inhibition to 3.5 or 4.5 days. Then a cut was made through the coleoptilar node, excising all photosynthetic tissue. The decapitated seedlings were subsequently exposed to a continuous white light source (1.2 to 1.5 $\times$ 10^4 ergs/cm^2·sec) produced by General Electric cool fluorescent lamps supplemented with General Electric 40-w tungsten lamps. Successive anthocyanin extractions were made following exposure to light by grinding fresh tissue in measured volumes of cold methyl alcohol with 1% HCl, by centrifugation of the mixture at 1700*g* for 10 min, and by measuring the absorbance of the supernatant at 525 nm. All values presented are in terms of absorbance at 525 nm of the sample in 10 ml of solvent.

Differences in Light Induction of Anthocyanin Biosynthesis in Albino and Wild Type *Zea* Seedlings. Seedlings grown from seeds obtained from Turtox Biological Supply under stock No. 70V500 were used in these experiments. The seeds obtained produced green and albino seedlings in a 3 to 1 ratio. In these seedlings, homozygosity for a recessive albino allele results in a severe albino state with regard to Chl. Seedlings were grown in 1 mM $CaSO_4$ at 25 C in complete darkness from imbibition to 4.5 days of age. Then the seedlings were exposed to the white light source described above. After 48 hr of illumination, antho-

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[3] Abbreviations: PS: photosystem; HIR: high irradiance response; SAL: salicylaldoxime; PAL: phenylalanine ammonia-lyase.

cyanin and Chl extractions were made. Anthocyanin was extracted as described above and Chl extractions and quantifications were made according to Arnon's (2) method.

Time Courses of Chl and Anthocyanin Accumulation. In one group of experiments *Z. mays* L. cv Pioneer 3369A seedlings were germinated and grown as described above in complete darkness for 3.5, 4, 4.5, or 5 days. Then the seedlings were exposed to continuous white light as described above and anthocyanin and Chl extractions and quantifications made at various times after light exposure as described above. In other experiments *Z. mays* L. cv Pioneer 309B seedlings were grown for 4 days in complete darkness at 25 C and then placed in continuous white light at either 16 or 25 C. After 12, 24 and 36 hr of illumination, seedlings were sampled, and anthocyanin and Chl determinations were made.

RESULTS AND DISCUSSION

Effects of Excision of Photosynthetic Organs on Light-dependent Anthocyanin Biosynthesis. The time courses of anthocyanin accumulation in intact seedlings and those without photosynthetic tissue is shown in Figures 1 and 2 for roots and mesocotyls, respectively. There is a more rapid accumulation of anthocyanin in the mesocotyls of decapitated seedlings than those of intact seedlings. This effect is more pronounced in the mesocotyls of seedlings exposed to light after 4.5 days of dark growth than after 3.5 days (Fig. 2). In the roots (Fig. 1), the differences are not great. The seedlings lacking leaf tissue showed the greatest accumulation of anthocyanin at all times sampled. Anthocyanin accumulation was greatly reduced below treatments beginning at 3.5 days in both treatments beginning at 4.5 days.

The increased level of photoinduced anthocyanin production in seedlings lacking photosynthetic tissue may be due to elimination of competition for substrate between the greening process and anthocyanin synthesis. Greater enhancement of anthocyanin production in the mesocotyl than the root following coleoptile and leaf excision supports a substrate competition hypothesis since, as part of the shoot, the mesocotyl probably competes more directly for substrate with developing leaves than do the roots.

It follows from the evidence demonstrating PAL induction to be a wound response in several systems (7, 16, 17, 28, 32) that excision of the photosynthetic tissue itself could have stimulated PAL production which in turn could result in enhanced anthocyanin biosynthesis. However, this hypothesis does not appear valid since no anthocyanin was produced in seedlings kept in the dark after excision of coleoptiles and leaves.

In *Amaranthus* seedlings, Colomos (6) found that excision of the cotyledon before exposure to continuous white light enhances betacyanin accumulation. In this case, the evidence indicates that the excision effect is hormonal. Although we have no evidence to refute this possibility in our excision experiments, the results with albino seedlings strongly support the substrate competition explanation.

Differences in Light Induction of Anthocyanin Biosynthesis in Albino and Wild *Zea* Seedlings. Table I shows enhanced anthocyanin biosynthesis in the mesocotyls of albino seedlings over wild type seedlings after 48 hr of illumination. This supports our hypothesis that elimination of the greening process results in enhanced anthocyanin synthesis. Mancinelli *et al.* (24) have speculated that streptomycin enhanced anthocyanin biosynthesis in dark-grown seedlings exposed to light could be due to an enlarged precursor pool resulting from elimination of chloroplast development. If elimination of chloroplast differentiation and chloroplast pigment production results in enhanced anthocyanin biosynthesis, there is no reason to postulate even a partial dependence of anthocyanin photoinduction on photosyn-

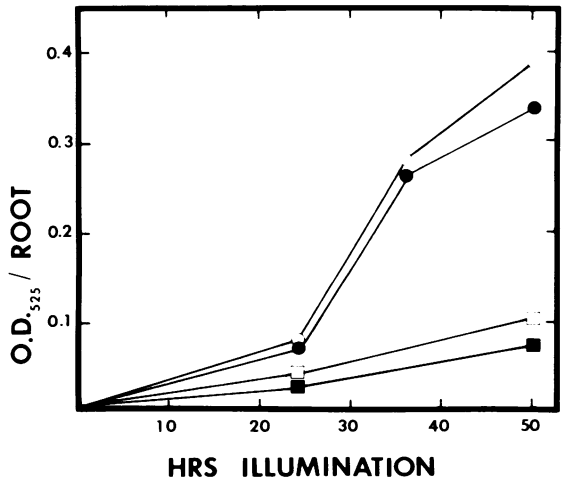

FIG. 1. Anthocyanin accumulation in the roots of intact maize seedlings (●, ■) *versus* the roots of seedlings with all tissue above the coleoptilar node excised (○, □). Seedlings were exposed to continuous white light after 3.5 (○, ●) or 4.5 days (□, ■) of dark development.

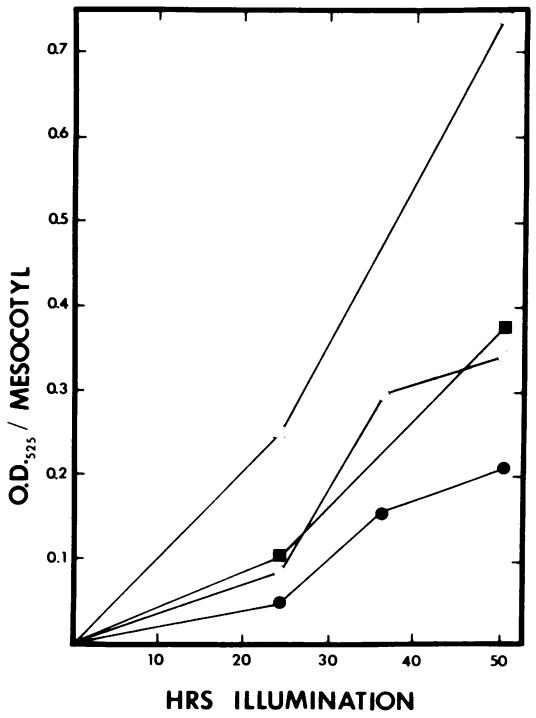

FIG. 2. Anthocyanin accumulation in the mesocotyls of intact maize seedlings (●, ■) *versus* the mesocotyls of seedlings with all tissue above the coleoptilar node excised (○, □). Seedlings were exposed to continuous white light after 3.5 (○, ●) or 4.5 (□, ■) days of dark development.

Table I. *Anthocyanin and Chl Content in Albino and Wild Type Maize Seedlings after Exposure to 48 Hr White Light*

Values are the averages of two experiments.

	Albino	Wild
A_{525}/g fresh wt	2.35	1.76
Total Chl(mg)/g fresh wt	0.0009	0.6626
A_{525}/mesocotyl	0.423	0.311
Total Chl(mg)/seedling	0.0001	0.9754

thesis in seedlings in which this enhancement has been demonstrated.

One of Mohr's (26) principal arguments against photosynthetic involvement in the HIR is the lack of correlation between the time courses of HIR phenomena and those for attainment of photosynthetic capabilities under continuous far red light. His conclusions have been based on work with species in which there is abundant anthocyanin photoinduction in far red light. In maize seedlings, we have found very little anthocyanin produced under continuous far red light; however, one may suppose that if there is a dependence on photosynthetic processes for anthocyanin production in maize seedlings, there should be a direct correlation between changes in the time courses of greening and changes in those for anthocyanin biosynthesis with alteration of temperature or age at time of exposure to light.

Chlorophyll Accumulation *versus* Anthocyanin Accumulation. Chlorophyll and anthocyanin accumulation by seedlings exposed to continuous white light after different periods of dark growth are shown in Figures 3 through 7. It can be seen that Chl accumulation was enhanced by age at the time of light exposure up to 5 days (Fig. 3). This correlates negatively with the changes in anthocyanin accumulation with age at exposure shown in Figures 4 through 7. In both roots and mesocotyls, there was a decreased rate of accumulation of anthocyanin/g fresh weight (Figs. 4 and 5) with increasing age at the time of light exposure. This relationship also holds for anthocyanin accumulation per root (Fig. 6) and per mesocotyl after 24 hr of illumination (Fig. 7). Rapid mesocotyl elongation in the dark is a confounding factor in comparing anthocyanin accumulation per mesocotyl in treatments exposed to light after different periods of dark growth.

The observed decrease in anthocyanin accumulation rate with exposure age could be due to a decline in potential for photoinduction of anthocyanin biosynthesis with age which is unrelated to greening. Light-dependent anthocyanin synthesis has been demonstrated to be a function of seedling age at the time of exposure to light (3, 18, 22). Our results and those of others could possibly be due to the enhanced ability, with age, of the greening process to compete with anthocyanin biosynthesis for substrate.

Apparently, the greening process competes more successfully with anthocyanin synthesis after longer periods of dark seedling growth. This hypothesis is supported by the excision experiment results which show that removal of the leaves after a longer period of dark development results in greater differences in anthocyanin accumulation between the mesocotyls of decapitated and intact seedlings.

To further establish lack of interdependence of greening and anthocyanin biosynthesis a maize mutant (cv. Pioneer 309B)

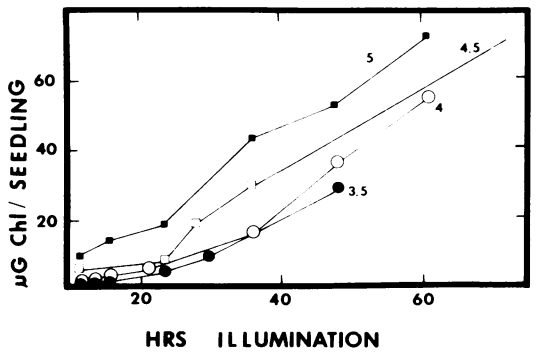

FIG. 3. Total Chl accumulation in maize seedlings after 3.5, 4, 4.5, and 5 days in complete darkness before exposure to continuous white light.

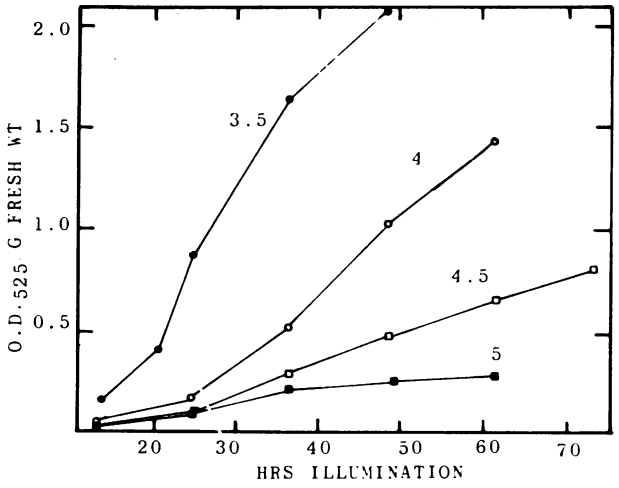

FIG. 4. Anthocyanin accumulation/g of fresh maize seedling root tissue after 3.5, 4, 4.5, and 5 days in complete darkness before exposure to continuous white light.

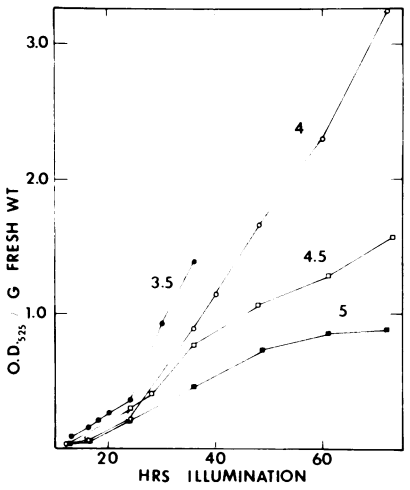

FIG. 5. Anthocyanin accumulation/g of fresh maize seedling mesocotyl tissue after 3.5, 4, 4.5, and 5 days in complete darkness before exposure to continuous white light.

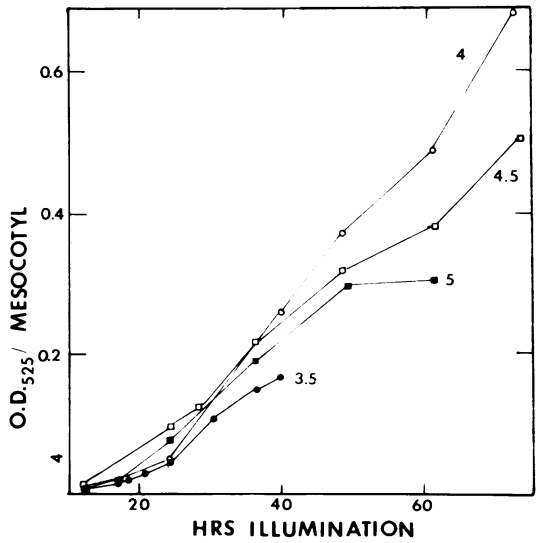

FIG. 6. Anthocyanin accumulation/maize seedling mesocotyl after 3.5, 4, 4.5, and 5 days in complete darkness before exposure to continuous white light.

shown by Naylor and McWilliams (27) to produce very little Chl at 16 C under high light intensities was employed. Chl accumulation (Fig. 8) and anthocyanin accumulation with time by roots and mesocotyls (Fig. 9) at 16 and 25 C in *Z. mays* L. cv Pioneer 309B show a reduction in anthocyanin biosynthesis at 16 C of about one-sixth to one-thirtieth the 25 C levels, while Chl levels were reduced to roughly one-hundredth the 25 C levels. Although our results indicate a temperature-sensitive lesion exists at some point in the biosynthesis of anthocyanin, the severity of the lesion is not as great as that for Chl accumulation.

Our correlation studies of Chl biosynthetic rates with light-dependent anthocyanin accumulation indicate photosynthesis has little or no influence on photoinduced anthocyanin formation in dark-grown maize seedlings. The experiments involving albino seedlings and seedlings with their photosynthetic organs

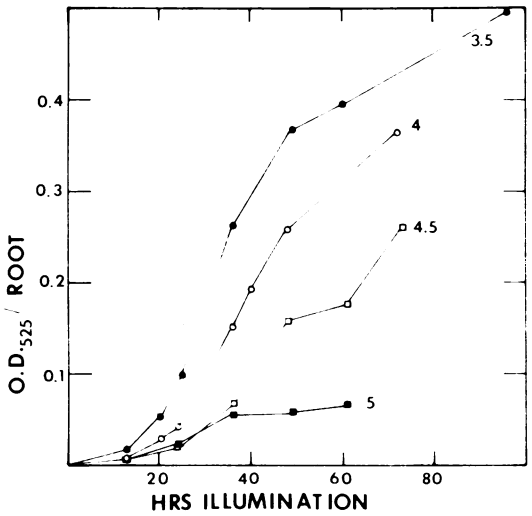

FIG. 7. Anthocyanin accumulation in maize seedling roots after 3.5, 4, 4.5, and 5 days of complete darkness before exposure to continuous white light.

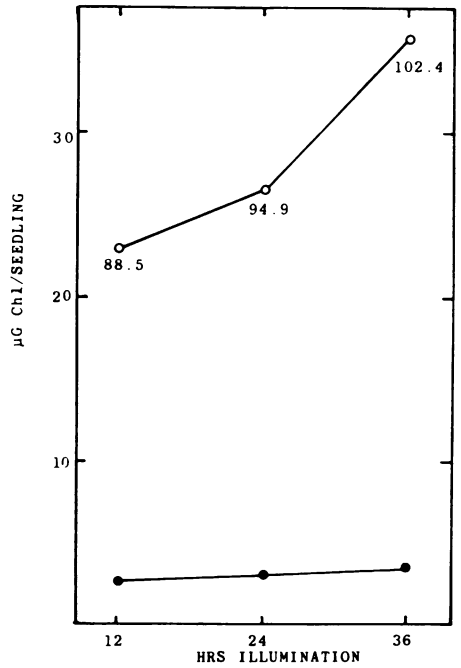

FIG. 8. Chlorophyll accumulation in seedlings of *Zea mays* L. cv. Pioneer 309B at 16 C (●) and 25 C (○). Numbers under open circles represent 25 C/16 C ratios.

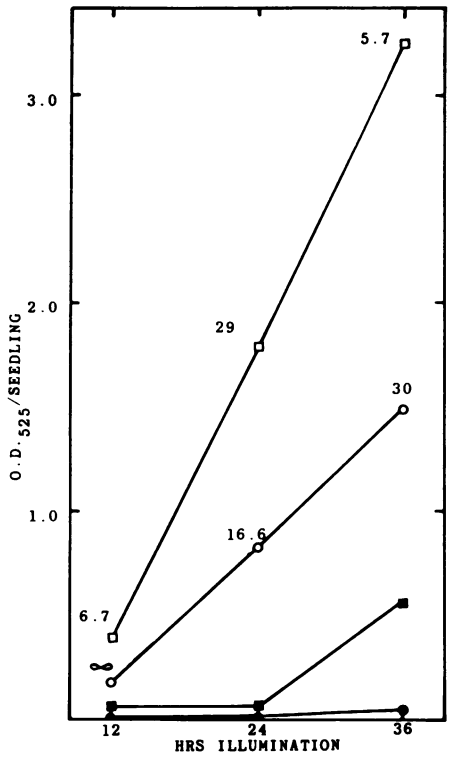

FIG. 9. Anthocyanin accumulation in seedlings of *Zea mays* L. cv. Pioneer 309B at 16 C (●, ■) and 25 C (○, □) in roots (□, ■) and mesocotyls (○, ●). Numbers above open figures represent 25 C/16 C ratios.

excised strongly indicate that the overall greening process competes with anthocyanin biosynthesis for substrate. The contention of Mancinelli *et al.* (24) that there is no good evidence for contribution of the photosynthetic process in HIR mediation of anthocyanin biosynthesis in developing seedlings is well supported by our findings with maize.

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