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EFFECT OF LIGHT ON BETALAIN AND CINNAMIC ACID BIOSYNTHESIS IN *AMARANTHUS CAUDATUS*

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INTRODUCTION

THE RATE of biosynthesis of amaranthin, the betalain pigment produced by *Amaranthus* species,[1,2] has been shown by several workers[3-5] to be controlled in seedlings by phytochrome, with the probable involvement also of a high energy light reaction.[6,7] In many other plants, such systems have been shown to control the biosynthesis of cinnamic acids and related flavonoid compounds,[8,9] and it is generally assumed that the major photoreceptor involved is again phytochrome.[8] In this latter case, the main effect appears to be to vary the activity of phenylalanine ammonia lyase (PAL), the enzyme responsible for the deamination of L-phenylalanine to cinnamic acid.[9]

Amaranthus species have long been known to contain, besides amaranthin and related pigments, both caffeic and ferulic acid derivatives,[10] and since the betalains and the cinnamic acids share, as a common precursor, L-phenylalanine,[1,8] it seemed of interest to investigate the effect of light on the biosynthesis of both classes of secondary product in seedlings of *A. caudatus*. In addition, we have examined the effect of light in this plant on the induction of PAL and the formation of hydroxybenzoic acids.

RESULTS AND DISCUSSION

In all experiments we have used 65 hr dark grown seedlings of *A. caudatus*. This period is a compromise based on the results of other workers.[3,7] Seedlings were then illuminated for 4 hr with either white or red light before being transferred back to darkness, and samples were taken at various periods for assay of amaranthin[3] (incidentally the levels of amaranthin per seedling quoted by Piatelli *et al.*[3] appear to be too high by a factor of 100: see

[1] MABRY, T. J. and DREIDING, A. S. (1968) in *Recent Advances in Phytochemistry*, Vol. 1, Appleton–Century–Crofts.
[2] KOHLER, K. H. (1973) *Pharmazie* **28**, 18.
[3] PIATELLI, M., GUIDICI DE NICOLA, M. and CASTROGIOVANNI, V. (1969) *Phytochemistry* **8**, 731.
[4] KOHLER, K. H. (1972) *Phytochemistry* **11**, 133.
[5] RAST, D., SKRIVANOVA, R. and WOHLPART, A. (1972) *Ber. Schweiz. Bot. Ges.* **82**, 213.
[6] GUIDICI DE NICOLA, M., PIATELLI, M. and AMICO, V. (1973) *Phytochemistry* **12**, 2163.
[7] FRENCH, C. J., PECKET, R. C. and SMITH, H. (1974) *Phytochemistry* **13**, to be published
[8] SMITH, H. (1973) in *Biosynthesis and its Control in Plants* (B. V. MILBORROW, ed.), p. 303, Academic Press, London.
[9] CAMM, E. L. and TOWERS, G. H. N. (1973) *Phytochemistry* **12**, 961.
[10] BATE-SMITH, E. C. (1962) *J. Linn. Soc. (Bot.)* **58**, 95.

French *et al.*[7] and our own results, Fig. 1), PAL activity[11] and the concentration of caffeic, ferulic, *p*-hydroxybenzoic, vanillic and gentisic acid derivatives,[12] which were separated by two-dimensional TLC.

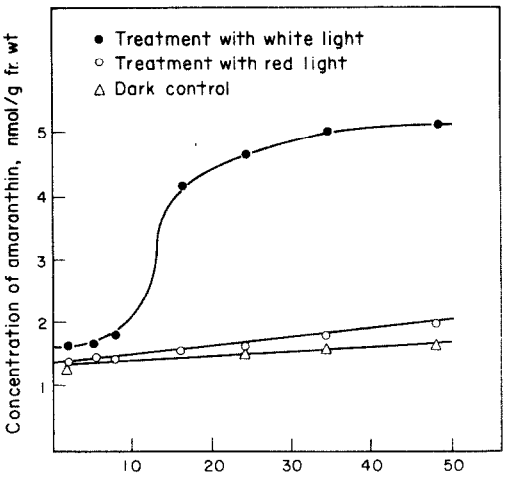

FIG. 1. THE INCREASE IN AMARANTHIN IN DARK-GROWN *Amaranthus caudatus* SEEDLINGS AFTER TREATMENT WITH LIGHT.
See Experimental for details.

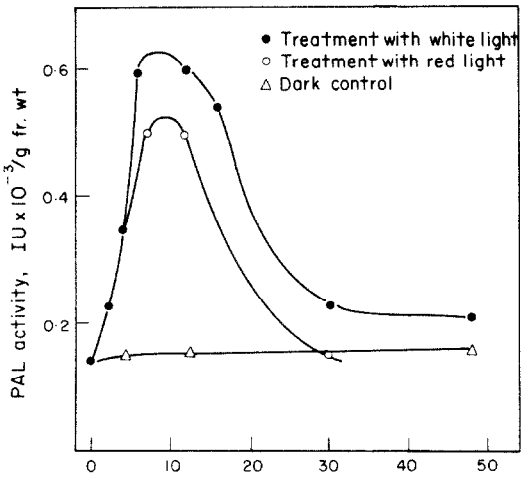

FIG. 2. THE INCREASE IN PAL IN DARK-GROWN *Amaranthus caudatus* SEEDLINGS AFTER TREATMENT WITH LIGHT.
See Experimental for details.

As found by other workers, with white light (20 W/cm^2) there is an approximate 5 hr delay in pigment production in *A. caudatus* seedlings (Fig. 1), but this is not paralleled by PAL activity (Fig. 2) which shows an almost immediate increase. With red light ($\sim$1 W/cm, λ_{max} 610 nm), PAL activity is induced at approximately the same rate as in white light but not to the same overall extent (Fig. 2); however, under these conditions very little amaranthin is formed (Fig. 1). These results are similar to those of Köhler[4] who showed that red irradiation alone was ineffective in promoting amaranthin synthesis.

The changes in PAL activity after exposure to white or red light parallel those found previously.[9,11,13] It is interesting, however, that the biosynthesis of caffeic acid, like that of amaranthin, shows a lag of 3–5 hr (Fig. 3) but it should be noted that the other hydroxy aromatic acids show quite different behaviours (Fig. 3). Gentisic and *p*-hydroxybenzoic acid are hardly affected by light, whereas vanillic acid shows an immediate increase. Ferulic acid, on the other hand, shows an extended lag period (*ca* 24 hr) before increasing to 5-fold its original concentration. It should be noted that caffeic acid appears to be the major cinnamic acid in mature *A. caudatus* leaves and that no quercetin derivatives or other flavonoids are found in the seedlings.

The results shown in Figs. 1–3 show that light affects the biosynthesis of both types of secondary compounds from L-phenylalanine in dark grown *A. caudatus* in the same way. On the one hand, it stimulates an increase in the concentration of amaranthin and, on the other, it causes an increase in the PAL activity and in the amount of caffeic and, later,

[11] ZUCKER, M. (1965) *Plant Physiol.* **40**, 779.
[12] COOPER-DRIVER, G. A., CORNER-ZAMODITS, J. J. and SWAIN, T. (1973) *Z. Naturforsch.* **27b**, 943.
[13] ENGELSMA, G. (1967) *Planta* **77**, 49.

ferulic acid formed. It may be noted that the overall increase in these two hydroxycinnamic acids (no cinnamic or *p*-coumaric acid appears to accumulate to any detectable extent) is about 400 nmol over 48 hr (Fig. 3). Assuming that the two acids arise from L-phenylalanine, it can be seen that there is sufficient PAL present in the dark grown *A. caudatus* seedlings before illumination (Fig. 2) to account for this increase (0·15 mIU ≡ 9 nM hr or 430 nm/48 hr) (see Ref. 14). It seems possible, therefore, that the overall increase in total PAL activity (Fig. 2) may represent only an amplification in that enzyme responsible for lignin biosynthesis.[15]

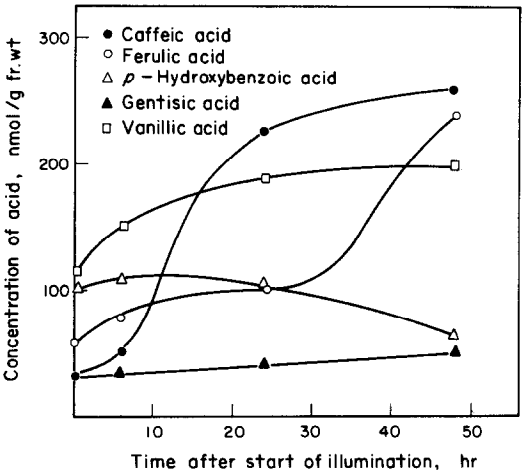

FIG. 3. THE EFFECT OF LIGHT ON THE INCREASE OF HYDROXY-BENZOIC AND -CINNAMIC ACIDS IN DARK-GROWN *Amaranthus caudatus* SEEDLINGS.

The fact that the two competing routes of biosynthesis from L-phenylalanine (to the betalains[1] and the cinnamic acids[8]) are both stimulated by light indicates the strong probability that there is a degree of compartmentalization. It may be noted, however, that the increase in amaranthin in molar terms is only about 1% of that of the total cinnamic acids. Nevertheless these results suggest that the other varied fates of L-phenylalanine (to protein, cinnamic acids, flavonoids, alkaloids, cyanogenic glycosides, glucosinalates, etc.) may similarly be all under separate control and this points to the need for a more sophisticated outlook on the interlocking pathways of biosynthesis than is usually expressed.

One further point must be noted. It has often been assumed that benzoic acids in higher plants arise from their cinnamic acid counterparts by β-oxidation.[16] It would appear from the results shown in Fig. 3 that this is very unlikely. Thus, vanillic acid increases as soon as the *A. caudatus* seedlings are exposed to light whilst the corresponding cinnamic acid, ferulic acid, as mentioned earlier, shows a distinct lag period. Furthermore, there is no parallel between the amounts of these two acids which are synthesised. Nor is there any recognisable association between the other benzoic and cinnamic acids. Unlike *p*-hydroxybenzoic acid, no *p*-coumaric acid accumulates nor is there any cinnamic acid equivalent of gentisic acid. In fact, the only event which is obvious from Fig. 3 is that there is probably a sequential formation of ferulic acid from caffeic acid.

[14] SWAIN, T. and WILLIAMS, C. A. (1970) *Phytochemistry* **9**, 2115.
[15] AHMED, S. I. and SWAIN, T. (1970) *Phytochemistry* **9**, 2287.
[16] ZENK, M. (1971) IN *Pharmacognosy and Phytochemistry* (WAGNER, H. and HÖRHAMMER, L. eds.), p. 314, Springer, Berlin.

It seems obvious to us that much more work needs to be done on "competing" biosynthetic pathways before we can understand the overall biochemistry of secondary metabolites in plants. The concentration of effort on six or seven species, while obviously necessary initially, needs now to be replaced, in our opinion, by a broader comparative approach.

EXPERIMENTAL

Plant material. *Amaranthus caudatus* seedlings (washed for 3 min in 1% NaOCl) were germinated in darkness at 28°, in 10 cm Petri dishes on moist filter paper, and grown in the dark for 65 hr before treatment. All experiments were carried out in triplicate.

Light sources. For white light treatments, the seedlings were illuminated by a bank of 40W Warmwhite Cryselco fluorescent tubes (20 W/cm^2). Red light was from the same source with No. 6 and No. 5A Cinemoid filters (1 W/cm^2).

Extraction and assay of amaranthin. Seedlings (1 g) were macerated in acetate buffer, pH 4·5, (3 ml) at 5°. The homogenate was centrifuged (10000 **g** for 20 min) and the absorbance of the supernatant was measured at 540 nm. Amaranthin was determined using ϵ, $5{\cdot}66 \times 10^3$.[7] High voltage electrophoresis on TLC silica gel in 0·1 M formic acid, pH 2·4 (1000V for 15 min.) was used to determine the number of betalains in the *Amaranthus* seedling extracts.[17] A control sample of amaranthin was extracted from mature *A. caudatus* flowers by freezing and thawing and its homogeneity determined electrophoretically.

Extraction and assay for PAL. Seedlings were ground with an equal weight of sand at 5° in 0·05 M Tris buffer, pH 8·0 (5 ml/g) and the homogenate centrifuged at 15000 *g* for 30 min. The supernatant was concentrated using Diaflo membranes at 2000 *g* for 2–3 min. PAL activity was determined spectrophotometrically:[18] reaction mixtures (3·0 ml) contained 30 μmol L-phenylalanine in 0·05 M Tris buffer, pH 8·8 and 0·5 ml enzyme extract. They were incubated at 37°, and the increase in absorbance at 290 nm recorded. The reaction was linear over periods of at least 60 min (1 μM/min gives an absorbance increase of 0·63 per hr).

Identification and quantitative assay of phenolic acids. Seedlings were hydrolysed at 100° for 20 min with 2M HCl and the soln extracted 3 × Et_2O. The Et_2O was extracted with 0·1 M $NaHCO_3$, and the latter, after acidification, was re-extracted with Et_2O. The Et_2O was taken to dryness and the residue dissolved in 0·5 ml 80% MeOH. The spectral characteristics were recorded under acidic and neutral conditions, and in the presence of $AlCl_3$ and molybdate.[12] An aliquot was chromatographed on Whatman No. 1 paper in isoPrOH–*n*-BuOH–*t*-BuOH–conc. NH_3–H_2O (4:2:2:1:2) followed by C_6H_6–HOAc–H_2O (125:72:3), and on cellulose TLC run in the C_6H_6–HOAc–H_2O solvent and then in 1M NaOH–HCOOH–H_2O (150:8:42). The phenolic acids were visualized with diazotized *p*-nitroaniline (0·5% *p*-NO_2, 1 ml, 5% $NaNO_2$, 0·1 ml, 2M Na_2CO_3, 5 ml, and H_2O, 5 ml). Comparison of R_f vs and colour reactions with those of authentic specimens were used for identification.[12] Quantitative estimation was effected by elution from 2-D cellulose TLC plates with 80% MeOH. Absorbances at λ_{max} for each acid were determined, and the amounts present estimated from calibration curves prepared for standard acids.

[17] BOGLE, A. L., SWAIN, T., THOMAS, R. D. and KOHN, E. D. (1971) *Taxon* **20**, 473.
[18] HAVIR, E. A. and HANSON, K. R. (1968) *Biochemistry* 7, 1896.