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THE HORMONAL CONTROL OF BETACYANIN SYNTHESIS IN *AMARANTHUS CAUDATUS*

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Key Word Index—*Amaranthus caudatus*; Amaranthacae; betacyanins; GA_3; kinetin; ^{14}C-tyrosine and DOPA: tyrosine and DOPA oxidase.

Abstract—Gibberellic acid (GA_3) inhibits amaranthin synthesis whereas the growth retardant, phosphon D, enhances pigment levels in *A. caudatus* seedlings exposed to light. No effect was observed on chlorophyll and carotenoid synthesis. Radioactive tyrosine and DOPA were incorporated into amaranthin. The specific activity of amaranthin synthesised in the presence of ^{14}C-tyrosine or ^{14}C-DOPA in seedlings treated with GA_3 is higher than water controls. The specific activity of pigment from phosphon D treated tissue is relatively low. GA_3 treated tissue has lower active tyrosine and DOPA pools compared to phosphon treated seedlings. Tyrosine and DOPA-oxidase activity increases in GA_3 treated and H_2O control seedlings exposed to light. Kinetin stimulates the synthesis of amaranthin in dark-grown seedlings and this is not overcome by simultaneous GA_3 application. Dark-grown seedlings treated with different kinetin concentrations and incubated in ^{14}C-tyrosine synthesise radioactive amaranthin of similar specific activity. Kinetin treatment of dark-grown seedlings brings about an increased tyrosine and DOPA-oxidase activity. The results indicate that GA_3 controls the production and/or availability of tyrosine whereas kinetin can mimic light treatment and controls the utilisation of tyrosine probably by bringing about the synthesis or activation of tyrosine and DOPA-oxidase protein.

INTRODUCTION

Amaranthus caudatus L., in common with most members of the Centrospermae, contains a betacyanin pigment amaranthin which replaces the anthocyanins of other Angiosperm orders [1]. *Amaranthus* seedlings produce amaranthin in response to light [2,3] or, in the dark, in the presence of kinetin [3–4] and its production appears to be dependent on active protein synthesis [6]. The control of pigment synthesis is complex since gibberellins may be involved, exogenous GA_3, for example, inhibiting substantially the production of amaranthin [7]. This inhibitory response provides a rapid sensitive bioassay for GA_3 and GA_7 [8]. Low GA levels in *Amaranthus* seedlings induced by treatment with growth retardants (CCC, AMO 1618 and phosphon D) show a marked increase in pigment content [9]. The experiments reported here have been designed to ascertain the role of GA_3 and kinin control in betacyanin synthesis.

RESULTS

Gibberellic acid (GA_3) at 2.4×10^{-6} M inhibits amaranthin production by as much as 50% in seedlings grown in the dark for 2 days and the given a 24 hr light treatment. The growth retardant, phosphon D, on the other hand, promotes pigment levels by up to 30%. Phosphon D and GA_3 in combination produces similar levels of pigment as found in GA_3 treated seedlings (Table 1). Phosphon D and GA_3 have no effect on the photosynthetic pigments, chlorophyll and carotenoid, at the concentrations used in these experiments (Table

Table 1. The effect of GA_3 and phosphon D on pigment production in *A. caudatus* seedlings

Treatment	Amaranthin (nmol/g seeds)	Chlorophyll (μg/g seeds)	Carotenoid (μg/g seeds)
H_2O control	250	160	155
GA_3 (2.4×10^{-6} M)	135	150	140
Phosphon D (5×10^{-6} M)	370	145	140
GA_3 + Phosphon D	140	155	160

Seeds were germinated in 4 ml test solution for 2 days in the dark, transferred to the light for 24 hr and the levels of pigment in the seedlings measured.

Table 2. The effect of GA_3 and aromatic pigment precursors on amaranthin production in seedlings of *A. caudatus*

Treatment	Amaranthin (as % water controls)
GA_3 (2.4×10^{-6} M)	60
p-OH-Phenylpyruvate (5.5×10^{-3} M)	111
p-OH-Phenylpyruvate + GA_3	102
Tyrosine (5.5×10^{-3} M)	134
Tyrosine + GA_3	121
DOPA (5.5×10^{-3} M)	141
DOPA + GA_3	145

Seeds were germinated in 4 ml test solution for 2 days, given a 24 hr light treatment, after which amaranthin levels were determined.

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1). The reduction in amaranthin level by GA_3 treatment could be overcome by the addition of *p*-hydroxyphenylpyruvate, tyrosine or DOPA (Table 2).

To investigate further the role of GA_3 in the control of amaranthin synthesis, the incorporation of known ^{14}C-labelled aromatic precursors [10–12] was measured. Before meaningful isotope experiments could be carried out it was necessary to investigate the kinetics of pigment production in the light and to develop relatively rapid techniques for the purification of amaranthin for radioactive assay. A lag phase of about 4 hr light is present after which exponential pigment synthesis begins and carries on for 18 hr light. After 18 hr the rate of synthesis declines. Table 3 gives the total amount of pigment synthesised and the rate of synthesis in seedlings treated with either GA_3 or phosphon D. Maximal rates of synthesis were found at 16 to 20 hr light in all treatments. Because of the close linearity in pigment synthesis between the 16 and 20 hr light period, radioactive incubation experiments were carried out on seedlings given a 16 hr light treatment, transferred to isotope and kept for a further 4 hr (and for comparisons, 8 hr) in the light. After the desired light period the tissue was thoroughly washed, frozen in liquid nitrogen, and amaranthin extracted for radioassay. Amaranthin purification was achieved relatively rapidly by precipitation of protein, a preliminary separation from labelled substrate amino acid (tyrosine or DOPA) on Sephadex-G10, (Sephadex-G10 was found superior to G-25 and gave a good cut between pigment and amino acid [13]).

Two-day old dark-grown seedlings treated with either GA_3 (2.4×10^{-6} M) or phosphon D (5×10^{-6} M) were given 16 hr light and then incubated in ^{14}C-tyrosine or ^{14}C-DOPA for a further 4 or 8 hr in the light. Typical results of such experiments are given in Tables 4 and 5. Very little difference in uptake of isotope occurred in the treatments at any incubation time. Treatment with GA_3, whilst reducing pigment levels by as much as 50% in all experiments, gave amaranthin of up to 3× the specific activity of the control in ^{14}C-tyrosine and ^{14}C-DOPA fed seedlings. On the other hand the spec. act. of pigment in phosphon D treated seedlings was always lower than that in the water controls.

Results expressed as pmol amino acid incorporated/nmol pigment synthesised makes the differences in specific activities between treatments even more clear (Tables 4 and 5). In nearly all cases more radioactivity from ^{14}C-DOPA compared to ^{14}C-tyrosine was found in amaranthin synthesised in all comparative experiments. From the data given in Tables 4 and 5, it is possible to give an estimate of the active pool size of endogenous tyrosine and DOPA accessible to amaranthin synthesis. The calculation is based on the amount of amaranthin produced and the dilution of the radioactive amino acid in the pigment. It has been assumed that *one* molecule of amaranthin requires *two* molecules of tyrosine or DOPA. The relative pool size of tyrosine and DOPA in tissue treated with GA_3 or phosphon D is shown in Table 6. It must be emphasised that the data are only of relative pool size and with this type of experiment are really only comparable within the same isotope incubation times. With this in mind, however, it should be noted that phosphon D treatment increases the relative active pool size of tyrosine and DOPA whereas GA_3 treatment lowers it. Because of the problem found in getting ^{14}C-tyrosine or ^{14}C-DOPA to enter tissue rapidly enough and in sufficient quantity it has proved difficult to carry out pulse labelling experiments and measurement of 'exact' pool size with any accuracy.

Table 3. The rate of amaranthin synthesis during specific time intervals in the light

Treatment	Time interval in the light			
	0–16 hr	16–18 hr	18–20 hr	20–24 hr
H_2O	1.4	2.8	2.4	1.9
GA_3 (2.4×10^{-6} M)	0.8	1.3	0.9	0.6
Phosphon D (5×10^{-6} M)	2.1	4.3	3.9	2.4

Rate expressed as nmol amaranthin synthesised hr/g fr. wt. Seeds were germinated in 4 ml test solution for 2 days in the dark and transferred to the light. Seedlings were harvested at time intervals up to 24 hr light and amaranthin content determined.

Table 4. The incorporation of ^{14}C-DOPA into amaranthin in seedlings of *A. caudatus* exposed to light

Treatment and incubation time*	% uptake amino acid	Amaranthin synthesised†	Radioactivity in amaranthin (cpm)	Spec. act. pigment (cpm/nmol)	pmol DOPA incorporated nmol pigment
H_2O, 4 hr	9	10.1	1880	186	1.61
GA_3, 4 hr	10	5.2	2100	404	3.50
Phosphon D, 4 hr	8	17.3	2420	140	1.21
H_2O, 8 hr	84	19.6	9330	476	4.13
GA_3, 8 hr	85	8.4	7180	855	7.38
Phosphon D, 8 hr	86	27.8	7230	260	2.25

* Refers to the time in isotope after the 16 hr light period.

† Refers to pigment synthesised during the period of incubation in isotope in the light (nmol pigment/g fr. wt tissue).

Two-day-old-seedlings grown in either GA_3 (2.4×10^{-6} M) or Phosphon D (5×10^{-6} M) were placed in the light for 16 hr and then incubated in ^{14}C-DOPA (1 μCi, DL-3, 4-DOPA-2-^{14}C/g fr. wt seedlings; 19.2 nmol DOPA) for either 4 or 8 hr in the light. Amaranthin was then extracted, purified and assayed for radioactivity.

Table 5. The incorporation of ^{14}C-tyrosine into amaranthin in seedlings of *A. caudatus* exposed to light

Treatment and incubation time*	% uptake amino acid	Amaranthin synthesised†	Radioactivity in amaranthin (cpm)	Spec. act. pigment (cpm/nmol)	pmol DOPA incorporated/nmol pigment
H_2O 4 hr	53	10.4	725	69.7	0.062
GA_3, 4 hr	56	4.4	760	172.7	0.153
Phosphon D, 4 hr	48	16.4	825	50.3	0.045
H_2O, 8 hr	94	18.0	2220	123.3	0.110
GA_3, 8 hr	93	6.6	2830	428.7	0.380
Phosphon D, 8 hr	93	26.0	2550	98.1	0.088

* Refers to the time in isotope after the 16 hr light period.

† Refers to pigment synthesised during the period of incubation in isotope in the light (nmol pigment/g fr. wt tissue).

Two-day-old-seedlings grown in either GA_3 (2.4) $\times 10^{-6}$ M) or phosphon D (5×10^{-6} M) were placed in the light for 16 hr and then incubated in ^{14}C-tyrosine (1 μCi, L-tyrosine-^{14}C (U)/g fr. wt seedlings; 2 nmol) for either 4 or 8 hr in the light. Amaranthin was then extracted, purified and assayed for radioactivity.

Table 6. The relative pool sizes* of endogenous tyrosine and DOPA in seedlings of *A. caudatus* treated with either GA_3 or phosphon D

Treatment	Incubation time (hr)†	Pool tyrosine‡	Pool DOPA‡
H_2O	4	32.2	1.24
GA_3 (2.6×10^{-6} M)	4	13.0	0.57
Phosphon D (5×10^{-6} M)	4	44.6	1.65
H_2O	8	18.2	0.48
GA_3 (2.6×10^{-6} M)	8	5.2	0.27
Phosphon D (5×10^{-6} M)	8	22.9	0.89

* Calculated from data in Tables 5 and 6.

† Refers to the time (hr) in isotope after the 16 hr light period.

‡ Relative active pool size calculated as nmol amino acid/g fr. wt tissue.

Table 7. The effect of kinetin and aromatic amino acids on amaranthin production in dark grown seedlings of *A. caudatus*

Treatment	Amaranthin content*
GA_3 (2.4×10^{-6} M)	95
Kinetin (4.6×10^{-5} M)	150
Tyrosine (5.5×10^{-3} M)	120
DOPA (5.5×10^{-3} M)	148
p-OH-Phenylpyruvate (5.5×10^{-3} M)	111
Kinetin + tyrosine	280
Kinetin + DOPA	352
Kinetin + *p*-OH-Phenylpyruvate	207
Kinetin + GA_3	198
Kinetin + *p*-OH-Phenylpyruvate + GA_3	190
Kinetin + tyrosine + GA_3	264
Kinetin + DOPA + GA_3	362

* Amaranthin content expressed as a % of the water control. Seeds were germinated in 4 ml test solution for 3 days in the dark after which the level of pigment was measured.

Kinetin and amaranthin production in dark-grown seedlings

Kinetin, as reported previously, will enhance amaranthin levels in dark grown seedlings especially in the presence of exogenous tyrosine or DOPA. Typical results are given in Table 7. Kinetin in the presence of tyrosine, DOPA and even *p*-OH-phenylpyruvate give in most cases higher pigment levels than expected from the levels obtained with the additives given alone. Treatment with GA_3 in any combination with kinetin or with the aromatic amino acids has no inhibitory effect on amaranthin production. It should be noted, however, that the levels of amaranthin in dark-grown seedlings treated with kinetin was usually only 20% (70 nmol/g seeds) of that found in untreated seedlings grown for 2 days dark followed by 24 hr light treatment (300 nmol/g seeds).

Table 8. The effect of kinetin on ^{14}C-tyrosine incorporation into amaranthin in *A. caudatus* seedlings grown in the dark

Concentration kinetin ($\times 4.6 \times 10^7$)	Amaranthin (nmol/g seeds)	Spec. act. amaranthin (cpm/nmol amaranthin)
100	66	525
10	54	612
1	50	490

Seedlings were grown in the dark for 42 hr and then incubated with ^{14}C-tyrosine (1 μCi, L-tyrosine-^{14}C (U)/g fr. wt seedlings; 2nmol) for 6 hr after which kinetin was added and the seedlings left for a further 24 hr in the dark before pigment extraction and estimation.

To investigate in more detail the effects of kinetin on the dark-synthesis, *Amaranthus* seedlings were incubated with ^{14}C-tyrosine for 6 hr after 42 hr growth in the dark, kinetin was then added and the seedlings kept in the dark for a further 24 hr. Amaranthin was then extracted, purified and assayed for radioactivity. The results of such an experiment are given in Table 8. The specific activity (cpm/nmol amaranthin) was similar in all kinetin treatments even though the pigment levels show a progressive reduction, the lower the kinetin concentration.

Table 9. Total amino acid and tyrosine in seedlings of *A. caudatus* given various light and hormone treatments

Treatment	Total amino acid (mg/g fr. wt)	Tyrosine (pmol/g fr. wt)	Tyrosine (% total amino acid)
2 days dark*			
control	3.3	911	5
+ 4 hr light	3.5	773	4
+ 8 hr light	3.6	795	4
+ 16 hr light	3.5	773	4
+ 20 hr light	3.3	732	4
+ 24 hr light	3.5	773	4
+ GA_3 + 24 hr light†	3.5	773	4
+ phosphon D + 24 hr light	3.2	534	3
3 days dark‡	3.6	761	4
+ kinetin	3.2	884	5

* 2-day dark-grown seedlings were transferred to the light and analysed at regular intervals for total amino acid and tyrosine content.

† Seedlings grown in GA_3 (2.4×10^{-6}M) or phosphon D (5×10^{-6}M) and transferred to the light for 24 hr before amino acid analysis.

‡ Seedlings grown in the dark for 3 days in the presence of kinetin (4.6×10^{-5}M).

Total amino acid and tyrosine levels

The effect of the various treatments which effect amaranthin levels, on the total amino acid and tyrosine content of seedlings has been investigated. Amino acid preparations from *Amaranthus* tissue were analysed on an amino acid analyser and the results for tyrosine (DOPA could not be detected) are given in Table 9. In seedlings synthesising amaranthin in the light, the total amino acid recovered varied from 3.3 to 3.6 mg/g fr. wt the percentage tyrosine in all samples being similar. Results for GA_3 and phosphon D treatments after exposure to light and for seedlings grown completely in the dark in the presence of kinetin were also similar. On a pmol/g fr. wt basis tyrosine showed little variation in any of the treatments, indicating that this probably represents a pool of largely inactive tyrosine probably present in the cell vacuole.

Polyphenol oxidase activity

From the results given it would appear as though GA_3 and kinetin are operative at two different sites in the control of amaranthin synthesis. Kinetin is responsible for controlling the utilisation of tyrosine and DOPA and GA_3 in controlling the production and/or availability of these amino acids. Mabry [1,12] has reviewed the evidence that betacyanins are synthesised from DOPA. DOPA is formed from tyrosine and is converted to DOPA-quinone which can be spontaneously converted to DOPA-chrome. Providing dehydrogenation activity is well in excess of hydroxylation then tyrosine oxidase can be measured by the formation of DOPA-chrome upon the addition of tyrosine to the enzyme preparation. With this assay we have attempted to measure oxidase activity in seedlings given various treatments to promote or retard pigment production. Because DOPA-chrome absorbs at 475 nm, an area in which amaranthin also absorbs, it was necessary to develop methods for the rapid separation of pigment from protein samples. This was readily achieved by passing the pigment/protein extract through Sephadex-G25 when protein was eluted immediately after the void volume followed by amaranthin [13].

Oxidase activity was assayed at regular intervals in seedlings grown for 2-days dark in GA_3 or phosphon D and exposed to light for 24 hr. The results are given in Fig. 1. and show that tyrosine oxidase, although at a much lower activity than DOPA-oxidase increases

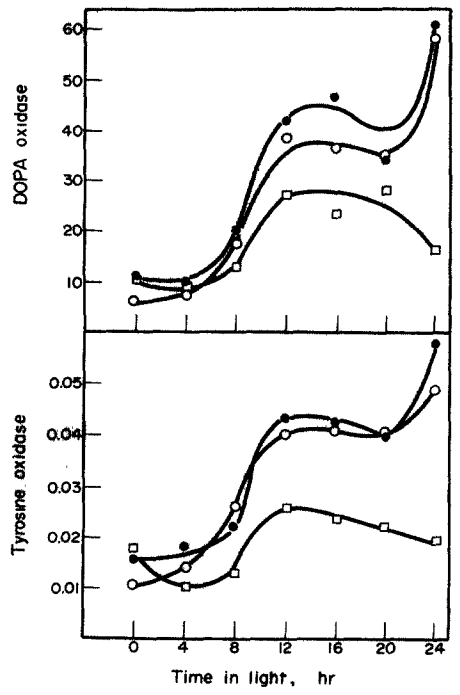

Fig. 1. The effect of GA_3 and phosphon D on tyrosine-, and DOPA oxidase in seedlings of A. caudatus. Two-day old seedlings were grown in GA_3 or Phosphon D and exposed to light for 24 hr. At regular intervals in the light seedlings were assayed for oxidase activity. Activity is expressed as nmol DOPA chrome formed mg^{-1} protein min^{-1}.
O——O GA_3 seedlings; □——□ phosphon D seedlings; ●——● water controls.

Table 10. The effect of kinetin on tyrosine and DOPA oxidase in dark-grown seedlings of *A. caudatus*

Treatment*	Tyrosine-oxidase†			DOPA-oxidase†		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
Kinetin (4.6×10^{-6} M)	0.016	0.025	0.022	16	19	21
H_2O	0.010	0.014	0.016	15	14	14

* 4.6×10^{-5} kinetin gave similar results

† Oxidase activity expressed as nmol DOPA-chrome formed mg protein/min.

Seedlings were grown for 2 days dark and then given kinetin after which oxidase activity was measured at regular intervals.

rapidly after 8 hr light treatment. DOPA-oxidase exhibits a similar development with light treatment. Enzyme preparations from seedlings treated with GA_3 and exposed to light had similar levels of tyrosine oxidase and DOPA-oxidase activity as the water controls. Phosphon D treated tissue on the other hand, had reduced levels of oxidase activity (Fig. 1). Hydroxylation of coumaric acid gave similar results as found for tyrosine and DOPA oxidase [13].

Oxidase activity was also measured in dark-grown tissue treated with kinetin. The results (Table 10) show that kinetin in fact stimulates both tyrosine and DOPA oxidase in dark-grown seedlings. Maximum oxidase activity was observed 12 hr after kinetin treatment and was nearly double that found in the water control.

DISCUSSION

The specific activities of amaranthin synthesised in the light from ^{14}C-tyrosine or ^{14}C-DOPA, in seedlings treated with GA_3 or phosphon D indicate that GA_3 is bringing about the reduction in pigment levels by effecting the production and/or availability of tyrosine. This is also reflected in the calculated relative pool size of tyrosine and DOPA in seedlings treated with GA_3 or phosphon D. We have suggested in an earlier report [9], the possiblity that GA may be causing a drain on available tyrosine by increasing general protein synthesis. Certainly from the isotope data it is clear that gibberellin can control the production of amaranthin at some point prior to tyrosine synthesis. At the moment work is in progress examining the activity of enzymes of the shikimate pathway but as yet no evidence for GA_3/phosphon D affecting activity has been found.

The specific activity of amaranthin synthesised from ^{14}C-substrates in the dark in the presence of kinetin indicates that kinetin can influence pigment synthesis by affecting a point(s) after tyrosine/DOPA production. Kolhler [3] implicates kinetin in controlling, at the gene level, the production and/or activation of probably an oxidase system utilising DOPA. Copper chelators also appear to inhibit pigment synthesis [3]. Certainly here, light brought about an increase in the tyrosine/DOPA oxidase system as did kinetin treatment of dark grown seedlings. Kinetin then, can substitute for light to some extent in the synthesis of amaranthin in dark-grown seedlings. French *et al.* [14] suggests from precursor studies, that light may be responsible for effecting at least two steps, one between tyrosine and DOPA and another between DOPA and pigment. Nicola *et al.* [15] working with isolated *Amaranthus* cotyledons, conclude that light effects the synthesis of the dehydropyridine moiety of the pigment, but could produce no evidence for or against a controlling step between tyrosine and DOPA.

The overall control of amaranthin synthesis in seedlings of *Amaranthus caudatus* appears therefore to be quite complex with phytochrome as well as two different hormones involved (Fig. 2)

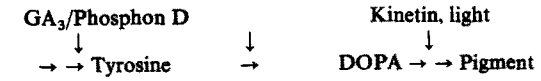

Fig. 2. Overall control of amaranthin synthesis.

Whether the gibberellin effect is important physiologically is not clear, however endogenous GA levels [9] as well as applied GA, effect pigment production.

EXPERIMENTAL

A. caudatus L. seeds were purchased from Thompson & Morgan Limited, Ipswich, UK. Phosphon D was a gift from Perifleur Limited, UK. Radioactive amino-acids were obtained from the Radiobiochemical Centre, UK.

Seedling growth and pigment extraction. 0.2 g seeds were spread on Whatman 1 filter discs in 9 cm petri dishes containing 4 ml H_2O or test solution. After 2 days dark-germination and growth at 25° the seedlings were either given a 24 hr light treatment (white fluorescent light giving 6500 lx at seedling level) or a further 24 hr dark period before pigment extraction. Pigment was extracted in a small vol. H_2O in a pestle and mortar. For every 4 ml H_2O used 1 ml 25% TCA was added. After centrifugation the supernatant was removed and its A at 537 nm measured. Amaranthin concs were calculated using a molar extinction coefficient of 5.66×10^{-4} [2]. Where necessary, chlorophyll and carotenoids could be extracted from the same tissue by 3 extractions in 80% Me_2CO followed by two in H_2O (5 ml solvent/g fr. wt tissue). Extracts were bulked and partitioned against Et_2O. The fat soluble pigments were recovered in the Et_2O fraction leaving the amaranthin in the Me_2CO/H_2O layer. Chlorophyll was determined in Et_2O using the equations of Comar and Zscheile [16]. Carotenoids were determined in the non-saponifiable Et_2O fraction and determined at 450 nm assuming an average E value of 2000 [17].

Amaranthin purification. The deproteinated pigment extracts were freeze dried and taken up in a small vol. 1% HOAc. Preliminary purification from amino acids was achieved on Sephadex G-10 swollen in 1% HOAc. The amaranthin fraction was collected in the first 2 ml of eluate following the void vol. The 2 ml fraction was freeze dried and taken up in a small vol. 1% HOAc. The pigment was subjected to 2D PC on Whatman 1 (1st direction, BuOH–HOAc–H_2O(12:3:5) 2nd direction, 1%

HOAc). Amaranthin was eluted in 1% HOAc, freeze dried, and where necessary further purified on columns of Dowex 50W × 2 (H^+), before counting.

^{14}C-tyrosine and DOPA feeding. In all feeding experiments 1 g fr. wt seedlings were placed in 10 ml bathing soln (0.01 M Tris/HCl, pH 7.2) containing the appropriate test compound and 1 μCi of either L-tyrosine-^{14}C (U) (2 nmol) or DL-3, 4-DOPA-2^{14}C (19.2 nmol). Incubation was for either 4 or 8 hr in the light or for the appropriate period in dark-grown kinetin treated seedlings. Radioactive amaranthin was counted by liquid scintillation in a Triton X/toluene based scintillant (666 ml toluene + 334 ml Triton-X 100 + 4 g PPO + 0.2 g POPOP) and corrected for quenching.

Amino acid analysis. Tissue samples were exhaustively extracted in hot 80% EtOH and an amino acid fraction obtained by passing through Zeocarb 225 (H^+) and eluting in 2N NH_4OH. Total amino acids were determined on an amino acid analyser.

O-Diphenol: oxygen oxidoreductase (EC 1.10.3.1). Enzyme preparations for the determination of tyrosine and DOPA oxidase activity were prepared from Me_2CO powders of the plant material. Powders were extracted in Pi buffer (0.1 M, pH 6) and protein separated from amaranthin on columns of Sephadex—G25. The enzyme was assayed by following A_{475} at 30° upon the addition of substrate (1 μmol L-tyrosine or DL-DOPA in a total reaction vol. of 1.1 ml) dopachrome concns were calculated using an E value of 3160 [18].

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REFERENCES

1. Mabry, T. J. (1966) in *Comparative Phytochemistry* (Swain, T. A. P. ed.) p. 231.
2. Piatelli, M., Guidici de Nicola, M. and Castrogiovanni, V. (1969) *Phytochemistry* **8**, 731.
3. Kohler, K. H. (1972) *Phytochemistry* **11**, 133.
4. Kohler, K. H. (1965) *Naturwissenschaften* **52**, 561.
5. Piatelli, M., Giudici de Nicola, M., and Castrogiovanni, V. (1971) *Phytochemistry* **10**, 281.
6. Piatelli, M., Giudici de Nicola, M., and Castrogiovanni, V. (1970) *Phytochemistry* **9**, 785.
7. Stobart, A. K., Pinfield, N. J. and Kinsman, L. T. (1970) *Planta* **94**, 152.
8. Kinsman, L. T., Pinfield, N. J. and Stobart, A. K. (1975) *Planta* **127**, 149.
9. Kinsman, L. T., Pinfield, N. J. and Stobart, A. K. (1975) *Planta* **127**. 207.
10. Garay, A. S. and Towers, G. H. N. (1966) *Can. J. Botany* **44**, 231.
11. Hörhammer, L., Wagner, H. and Fritzch, W. (1964) *Biochem. Z.* **339**, 398.
12. Mabry, T. J., Kimler, L. and Chang, C. (1972) *Structural and Functional Aspects of Phytochemistry* (Runeckles, E. and Tso, T. C. eds.) p. 105.
13. Kinsman, L. T. (1974) Ph.D. Thesis, University of Bristol.
14. French, C. J., Pecket, R. C. and Smith, H. (1973) *Phytochemistry* **12**, 2887.
15. Giudici de Nicola, M., Amico, V., Sciuto, S. and Piatelli, M. (1975) *Phytochemistry* **14**, 479.
16. Comar, C. L. and Zschiele, F. P. (1942) *Plant Physiol.* **17**, 191.
17. Goodwin, T. W. (1955) in *Modern Methods of Plant Analysis* (Peach, K. and Tracey, M. V. eds.) Vol. 3, p. 272. Springer-Verlag, Berlin.
18. Parish, R. W. (1972) *Z. Pflanzenphysiol.* **66**, 176.