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ACCUMULATION OF BETANIN IN DISKS OF *BETA VULGARIS* LEAVES

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Abstract—Leaf disks of red beet plants (*Beta vulgaris*) accumulate betanin when cultured on agar media. Betanin accumulation is completely inhibited by cycloheximide. Pigment production is stimulated by light and sucrose. Kinetin and sucrose are ineffective in promoting pigment synthesis in the dark. It is suggested that the initial response triggering pigment production occurs at the gene level. The amino acids dihydroxyphenylalanine and tyrosine have no effect on pigment production.

INTRODUCTION

BETALAINS[1] and anthocyanins are vacuolar plant pigments found in roots, stems, leaves and flowers. Anthocyanins are distributed widely, while betalains occur only in plant families in the order Centrospermae.[2,3] The betalains comprise two pigment classes, a red–violet type called betacyanin and a yellow type called betaxanthin. The prominent red–violet pigment of the red beet (*Beta vulgaris*) is a betacyanin called betanin. We wish to report the finding that leaf disks of red beet plants (*Beta vulgaris*) are capable of accumulating betanin when maintained in the proper environment and to describe some of the physiological factors governing betanin formation.

RESULTS AND DISCUSSION

When disks of red beet leaves, lacking betanin, were cultured in the light on either of four media (A; A-S, 3%; A-N; A-N-S, 3% see Experimental) they accumulated betanin and in some cases, became completely red within 6 days. Betanin accumulated first at the edges of the disks near the injured cells and then spread over the entire disk. At no time was there leakage of pigment from the disks into the supporting medium. The pigment content increased up to ninety hours after excision and then levelled off or declined. It is of interest to note that pigment synthesis in *Beta vulgaris* seedlings follows a very similar time course.[4]

That the observed accumulation of betanin in the vicinity of the injured cells is in all likelihood a wounding response is shown by the fact that increasing the degree of injury by pricking small holes into the leaf disks, resulted in an increase in pigment production. Each site of injury acted as a center for pigment accumulation. Anthocyanins and betalains, as

[1] MABRY T. J. and DREIDING, A. S. (1968) in *Recent Advances in Phytochemistry* (MABRY, T. J., ed.), p. 145, Appleton–Century–Crofts, New York.
[2] MABRY, T. J., TAYLOR, A. and TURNER, B. L. (1963) *Phytochemistry* **2**, 61.
[3] MILLER, H. E., ROSLER, H., WOHLPART, A., WYLER, H., WILCOX, M. E., FROHOFER, H., MABRY, T. J. and DREIDING, A. S. (1968) *Helv. Chim. Acta* **51**, 1470.
[4] RAST, D., SKRIVANOVA, R. and WOHLPART, A., unpublished results.

well as other secondary plant products[5] and enzymes,[5,6] are known to accumulate at wound sites of plants that normally synthesize them. The view that this is a defense mechanism has been strengthened in the case of the anthocyanins by the findings of Ulrychova and Brcka[7] and in that of the betalains by those of Sosnova,[8] who showed that these pigments had an inhibitory effect on viral reproduction. Betalain accumulation at wound sites may thus serve as a defense mechanism protecting the plant against virus infection.

The precise site of pigment synthesis in the excised disks is not known. The pigment might be synthesized either at the wound site itself or at a location distal to it. In the latter case, the pigment would then be transported to and accumulate in the injured cells. The basis for the initiation of this response reaction is also unknown. The initial reaction may involve the activation of the genes coding for the enzymes responsible for betanin synthesis. Perhaps an oxidative process occurs, either at the level of a 'leuco' betalain or at that of a precursor. It is also possible that enzymes which had been compartmentalized in specific cellular organelles were released as a result of the injury and were then able to interact with the appropriate substrates to form betanin. The observation that cycloheximide, in concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M, prevented betanin synthesis in excised leaf disks, supports the idea that the initial reaction occurs at the gene level.

Although betanin is found mostly in the root of the red beet plant, its site of synthesis within the plant body is unknown. Betanin could be formed either in the leaves and transported to the root or in the root itself or in both the root and the leaves. Since the leaves do not contain any red pigment, except in major leaf veins, the beet itself is the presumed site of synthesis. Our results clearly indicate that leaves are capable of betanin synthesis; hence the distinct possibility exists that the beet root serves only as a storage organ and that the leaves are involved in betanin synthesis.

In an attempt to elucidate the nutritional and environmental conditions most favorable for pigment production, the leaf disks were cultivated in the light on a variety of media. It was found that the rate of pigment synthesis on a water-agar medium was about the same as on a medium containing inorganic ions and such organic constituents as thiamine, nicotinic acid, pyridoxine, and myo-inositol. None of these organic constituents thus seems to be a limiting factor in betanin synthesis in leaf disks. The addition of sucrose to either of these media, however, resulted in a substantial increase in the rate of betanin production. Pigment accumulation in excised leaf disks maintained in the light is thus to some degree dependent on an exogenous carbohydrate source. A complete nutritional medium plus sucrose resulted in maximum pigment production.

To establish whether the availability of sucrose was the sole factor controlling the rate of pigment production in the light, disks were cultivated on a water-agar medium containing different amounts of sucrose (1%, 5%, 10%). It is apparent from Table 1 (column A), that pigment synthesis was not markedly affected by increasing amounts of sucrose. A certain minimal level of sucrose is thus necessary for pigment production and any amounts above the minimum level have relatively little additional effect.

In order to determine whether or not the light history of the plant prior to disk excision influences betanin production, beet plants were grown for one week either in continuous light of high intensity (15 000 lx) or in the greenhouse under normal conditions. Leaf disks

[5] BASTIN, M. (1968) *Can J. Botany* **46,** 1339.
[6] GREEN, T. R. and RYAN, C A. (1972) *Science* **175,** 776.
[7] ULRYCHOVA, M. and BRCKA, J. (1967) *Phytopathol. Z.* **58,** 87.
[8] SOSNOVA, V. (1970) *Biologia Plantarum (Praha)* **12,** 424.

from these plants were then cultured on parallel media. The disks obtained from plants grown under continuous light accumulated considerably more pigment than disks excised from leaves of plants grown under normal light conditions (Table 2). This increase is especially noteworthy in the case of the media containing sucrose. These observations can be explained either on the basis of increased amounts of carbohydrates and/or energy-rich compounds or on the photo-stimulation of pigment synthesis in the plants receiving high light intensities. De Nicola *et al.*[9] have suggested that light and kinetin act on amarathin synthesis at two distinct levels, activation of genes and availability of energy-rich compounds. It is quite plausible that light plays a similar role in betanin production and that the observed photo-stimulation of pigment accumulation is realized via gene activation.

TABLE 1. EFFECT OF SUCROSE ON BETANIN ACCUMULATION IN LEAF DISKS MAINTAINED ON WATER-AGAR MEDIA

	Cultivation in either continuous light (A) or dark (B) (hr)					
	24		48		72	
	A	B	A	B	A	B
Sucrose (%)	Betanin in mol $\times 10^{-8}$/5 disks					
0	1·0	0·8	5·0	0·8	7·1	1·1
1	2·1	1·5	7·1	2·0	13·8	2·3
5	1·8	1·1	6·8	1·8	15·4	2·5
10	2·3	1·2	8·5	2·1	16·2	1·9

The effect of light and darkness on pigment production is illustrated in Table 1. Leaf disks were excised from plants grown in the greenhouse in normal light conditions and cultivated either on an agar-nutrient-sucrose and water-agar medium or on a water-agar medium containing varying concentrations of sucrose, in continuous light (15 000 lx) and total darkness. In both experiments, the disks maintained in the light on sucrose-containing media produced considerably more pigment than those grown in the dark.

TABLE 2. DIFFERENCES IN PIGMENT ACCUMULATION IN LEAF DISKS

	Cultivation in continuous light of disks obtained from plants grown in the greenhouse (A) or in continuous light (B) (hr)					
	36		72		84	
	A	B	A	B	A	B
Media	Betanin in mol $\times 10^{-8}$/5 disks					
Water-Agar (A)	2·0	3·1	3·0	7·1	8·2	10·3
Agar-Nutrient (A-N)	1·8	3·8	5·1	7·6	5·3	11·8
Agar-Sucrose (A-S, 3%)	2·5	5·2	9·1	10·2	14·3	27·3
Agar-Nutrient-Sucrose (A-N-S, 3%)	3·3	9·1	10·5	33·2	18·1	50·4

[9] DE NICOLA, M. G., PIATTELLI, M., CASTROGIOVANNI, V. and AMICO, V. (1972) *Phytochemistry* **11**, 1011.

Pigment accumulation in *Beta vulgaris* seedlings is similarly affected in the dark.[10] It is apparent that sucrose is unable to completely replace the effect of light in the dark-cultivated disks, although it does stimulate accumulation to some degree. This observation, in conjunction with the fact that disks obtained from plants grown in continuous light produce considerably more pigment than disks excised from plants maintained under normal light conditions (Table 2), support the idea that light affects pigments synthesis by some other mechanism than simply supplying carbohydrates and/or energy-rich compounds.

Cytokinins are able to replace light as the causative agent in certain plant responses. It is interesting that the production of betacyanins in seedlings of certain *Amaranthus* species with an absolute light requirement for pigment synthesis, falls into this category.[9,11] In these cases, kinetin was able to overcome the light requirement and pigment production in dark-grown seedlings was equal to that in light-grown ones. Koehler[12] reported that kinetin does not have a stimulatory effect on pigment synthesis in dark-grown seedlings of *Beta vulgaris* var. *rapa*. Since light stimulates betanin biogenesis in leaf disks and kinetin is known to overcome the light requirement in amaranthin biosynthesis, the effect of kinetin on betanin biogenesis in leaf disks maintained in the dark was studied. Kinetin, in concentrations of 10^{-14}, 10^{-5} and 10^{-6} M was unable to replace the effect of light in dark-cultivated disks maintained either on a water-agar or an agar-nutrient-sucrose medium. Kinetin is thus ineffective in replacing light in betanin biogenesis in *Beta vulgaris* seedlings[12] as well as leaf disks.

The following dichotomy with respect to betacyanin synthesis thus exists. Plants either do or do not have an absolute light requirement for betacyanin synthesis.[4,11] Plants that have an absolute light requirement are unable to produce any pigment in the dark and kinetin is able to overcome the effect of light. Plants that do not have an absolute light requirement produce less pigment in the dark than in the light and kinetin is ineffective in promoting pigment synthesis in dark-grown plants. The basis for this differential effect of kinetin in amaranthin and betanin synthesis is not known. Koehler[13] has shown that amaranthin biosynthesis in *Amaranthus caudatus*, having an absolute light requirement for pigment synthesis, is controlled by phytochrome in the presence of kinetin and by kinetin alone and that their effect on amaranthin biosynthesis is realized via nucleic acid and protein synthesis.[9] In plants not having an absolute light requirement for betacyanin synthesis, the following possibilities thus exist; either the genes for betanin synthesis are already on or the internal concentration of factors such as kinetin or active phytochrome are high enough to cause gene activation in the dark. In this case, light further enhances pigment production by other means than supplying carbohydrates.

The amino acids tyrosine and dihydroxyphenylalanine (DOPA) are known precursors of the betalains.[3,14] Administration of tyrosine and DOPA increased the pigment content of *Amaranthus* seedlings.[15] In line with these observations, we expected an increase in the amount of pigment in leaf discs grown in the presence of these precursors. No such increase was observed, however. The amino acids DOPA and tyrosine, either administered in agar-nutrient-sucrose or water-agar had no effect on the quantity of betanin formed in the concentration ranges at which they were applied ($5{\cdot}5 \times 10^{-4}$ M, $2{\cdot}8 \times 10^{-4}$ M, $5{\cdot}5 \times$

[10] WOHLPART, A. and MABRY, T. J. (1968) *Plant Physiol.* **43**, 457.
[11] BAMBERGER, E. and MAYER, A. M. (1960) *Science* **131**, 1094.
[12] KOEHLER, K. H. (1970) *Biol. Rundschau* **8**, 50.
[13] KOEHLER, K. H. (1972) *Phytochemistry* **11**, 133.
[14] MINALE, L., PIATTELLI, M. and NICOLAUS, R A. (1965) *Phytochemistry* **4**, 593.
[15] GARAY, A. S and TOWERS, G. H. N. (1966) *Can J. Botany* **44**, 231.

10^{-5} M, $5{\cdot}5 \times 10^{-6}$ M). A similar observation has been made by Constabel and Nassif-Makki[16] in callus cultures of *Beta vulgaris* species. They suggest that the developmental stage of the tissue rather than the availability of exogenous precursors is the limiting factor in pigment formation. This interpretation can also be applied to the accumulation of betanin in leaf disks.

EXPERIMENTAL

Plant material. Red beets (*Beta vulgaris*, var. Detroit Dark Red and Burpee's Red Ball) were either purchased on the local market and transplanted into 30 cm pots or raised from seedlings in such pots. They were grown either in the greenhouse under normal light conditions or in plant growth chambers under continuous light (25°, 15 000 lx, cool white fluorescent lamps).

Preparation of leaf disks. Leaf disks were excised with a No. 3 cork borer. Care was taken not to include any major leaf veins in the disks. The disks were surface-sterilized in a 2% chlorox solution, rinsed in sterile H_2O and collected in sterile H_2O until about 600 disks had been accumulated. Disks were then picked at random and transferred to the appropriate media.

Cultivation of leaf disks. Ten disks were placed on each of three plastic Petri dishes (9 cm) containing 25 ml of the appropriate medium and maintained in plant growth chambers at 25° either in continuous light (15 000 lx, cool, white fluorescent lamps) or in total darkness for up to four days.

Basic media. Leaf disks were cultivated on a variety of media to study the effects of such factors as nutrition, light, hormones, selected amino acids and an inhibitor of protein synthesis, on betanin production. The following media were utilized: water-agar medium (A), 1% agar in H_2O; agar-sucrose medium (A-S, 3%), 1% agar and 3% sucrose; agar-nutrient medium (A-N), 1% agar and the medium of Witham *et al.*;[17] agar-nutrient-sucrose medium (A-N-S, 3%), the A-N medium plus 3% sucrose. The pH of all media was adjusted to 5·9 with dil. HCl prior to autoclaving at 121° for 15 min at 15 lb/in.^{2}

Cycloheximide medium. Cycloheximide dissolved in sterile water, was added to the warm autoclaved agar-nutrient-sucrose medium (A-N-S, 3%) to a final concentration of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol $l.^{-1}$.

Kinetin medium. Kinetin dissolved in sterile, dil. NaOH, was added to the autoclaved agar-nutrient-sucrose medium (A-N-S, 3%) and to the agar medium (A) to final concentrations of 10^{-4}, 10^{-5} and 10^{-6} mol $l.^{-1}$.

Amino acid media. The amino acids D,L-dihydroxyphenylalanine and D,L-tyrosine dissolved in sterile H_2O, were each added separately to the autoclaved agar-nutrient-sucrose medium (A-N-S, 3%) and to the agar medium (A) to final concentrations of $5{\cdot}5 \times 10^{-4}$, $2{\cdot}8 \times 10^{-4}$, $5{\cdot}5 \times 10^{-5}$, $2{\cdot}8 \times 10^{-5}$ and $5{\cdot}5 \times 10^{-6}$ mol $l.^{-1}$.

Isolation, identification, and quantitative determination of betanin. Five leaf discs were ground in a mortar with 3–5 ml of a celite-water slurry. The homogenate was spun down in a clinical centrifuge at full speed for 2–3 min. The supernatant was saved and the pellet was reextracted with 2–3 ml of dist. H_2O. The absorption of the combined supernatants was determined at 547 nm. The amount of pigment in mol was calculated on the basis of an ϵ value of 60 500.[18] The solution containing the pigment was concentrated in *vacuo* to about 2 ml and aliquots were then subjected to paper strip electrophoresis (0·05 M pyridine-formate buffer) and PC (1% formic acid). The migrational distance of the extracted pigment was compared to that of an authentic betanin sample. Each experimental parameter was tested in duplicate in two independent experiments.

[16] CONSTABEL, F. and NASSIF-MAKKI, H. (1971) *Ber. Dtsch. Bot. Ges.* **84,** 629.

[17] WITHAM, F. H., BLAYDES, D. F. and DEVLIN, R. M. (1971) *Experiments in Plant Physiology*, p. 194, Van Nostrand Reinhold, New York.

[18] WILCOX, M. E., WYLER, H., MABRY, T. J. and DREIDING, A. S. (1965) *Helv. Chim. Acta* **48,** 252.