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ANTHOCYANIN PRODUCTION IN STRAWBERRY LEAF DISKS

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Abstract—Leaf disks of *Fragaria vesca* L. var. "Alpine" floated on sucrose solutions produced anthocyanin at a constant reproducible rate for several days. Light was necessary to initiate anthocyanin production and stimulated its continued synthesis which was maximal at 25–30°, and with illuminated disks, at a sucrose concentration of 0·1 molal. Much higher sucrose concentrations were required for maximum production in the dark. Dinitrophenol was an effective inhibitor of anthocyanin production at $7{\cdot}5 \times 10^{-5}$ M in both light and darkness; NaF was inhibitory at 0·02 M, but only in darkness. Adenosine triphosphate stimulated production in the light only when sucrose was not supplied, but in the dark only when combined with sucrose. It was not effective if supplied at the beginning of the induction stage.

INTRODUCTION

THE physiology of anthocyanin formation has received considerable study,[1,2] especially the role of light.[1–6] Two distinct phases in anthocyanin formation are now evident from the photochemical studies.[3] The mediation of carbohydrate metabolism in the production of anthocyanins is also well-substantiated[7] and the feeding of sugars to plants frequently results in stimulation of anthocyanin formation.[7–11] The influence of temperature in controlling the production of anthocyanin is uncertain, as some workers stress the stimulation by low temperatures[11–14] while others find the maximum production at normal temperatures.[5,15]

We measured the effects of several variables known to influence anthocyanin formation on disks of mature strawberry leaves. Strawberry leaves were chosen because they produce leaves continually throughout the year under greenhouse conditions, contain little initial flavonoids but are able to synthesize such compounds rapidly when given suitable conditions. In addition to anthocyanin (predominantly cyanidin-3-monoglucoside) the leaves also

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produce (+)-catechin, leucoanthocyanins and several flavonols.[16] The number of flavonoid types produced by this tissue makes it a desirable one for studies of the comparison of the physiology of the formation of these compounds.

RESULTS AND DISCUSSION

Anthocyanin production proceeds after an initial illumination period called the induction stage.[3,6] Under our conditions this stage was about 15 hr after which anthocyanin production was linear for several days.

For examining the effect of temperature, leaf disks on 0·05 molal sucrose were exposed to light in controlled temperature rooms for 24 hr to complete the induction stage. Samples

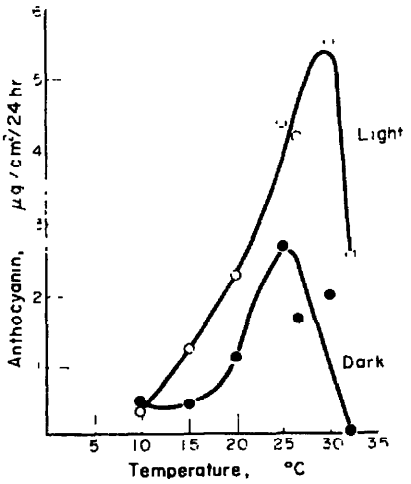

FIG. 1. EFFECT OF TEMPERATURE ON THE ANTHOCYANIN PRODUCTION OF LEAF DISKS BETWEEN 24 AND 48 HR FROM PREPARATION.

The disks were floated on 0·05 molal sucrose, and all were in the light for the first 24 hr.

were then removed for determination of anthocyanin content and half the disks at each temperature then were placed in the dark. The increase in anthocyanin was determined over the next 24 hr in both light and in darkness. As shown in Fig. 1, the maximum rate of anthocyanin production occurred at 30° in the light and at 25° in the dark. After the induction stage, the greatest stimulation by light (difference between the light and dark production) occurred at the higher temperatures. The photochemical reaction stimulating production must therefore involve a metabolite whose rate of production is limiting at the lower temperatures.

The influence of temperature on the length of the induction stage was not directly studied in this experiment, but by plotting the individual values for the anthocyanin content at 24 and 48 hr and then extrapolating to estimate the time production started, we concluded that temperature had no influence on the induction stage.

Figure 2 shows the effect of sucrose concentration on the anthocyanin production of leaf disks. Leaf disks were placed in the light on the various sucrose concentrations at 20–22°

[16] L. L. CREASY, E. C. MAXIE and V. L. SINGLETON, *Proc Am. Soc. Hort. Sci.* (in press).

and the anthocyanin measured after 48 hr. Duplicate disks were put in the dark after 48 hr light and sampled after an additional 24 hr. These disks therefore all went through the induction stage in the light and the quantities plotted are those produced during the dark period only. An optimum sucrose concentration of 0·1 molal was observed in the light but no optimum response was found for the production in a dark period following a light period.

Since, after the induction phase, the effect of light at 20° appears limited by the production of some metabolite, the stimulation of anthocyanin production in light with increasing sucrose concentration is not unexpected. However, beyond 0·1 molal the stimulation decreases. This cannot be due to any osmotic factor since it does not happen in the dark. The effect might be explained by assuming inhibition of the light reaction by sucrose, or by the diversion of anthocyanin intermediates into a competing light reaction. The linear increase in anthocyanin production in darkness with increasing sucrose concentration is presumably due both

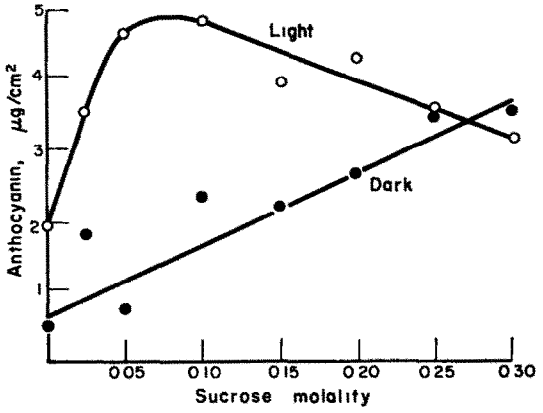

FIG. 2. EFFECT OF SUCROSE CONCENTRATION ON THE ANTHOCYANIN PRODUCTION BY LEAF DISKS. Production during light is the anthocyanin content at 48 hr from preparation. Dark production is the additional amount produced when the disks were kept in darkness for a further 24 hr.

to a greater supply of intermediates and to the increased potential for energy production.

The effect of 2,4-dinitrophenol (DNP) as an inhibitor of anthocyanin synthesis has been reported in various plant tissues[7,10,17] and suggests the mediation of ATP in the process. We tested inhibition separately on the induction and production stages. Dinitrophenol at $7{\cdot}5 \times 10^{-5}$ M in 0·05 molal sucrose had no effect on the duration of the induction stage, since treatment with DNP for 20 hr followed by removal from DNP, resulted in the same quantity of anthocyanin at 48 hr as in untreated disks (3·75 μg/cm^2 and 3·25 μg/cm^2 respectively). If DNP was supplied after the 20-hr induction stage or continuously for the 48 hr, inhibition occurred (2·45 and 2·40 μg/cm^2 respectively). Higher concentrations of DNP resulted in injury to the disks, as shown by reductions in chlorophyll content (absorbancy at 650 mμ) and by visible necrosis. The inhibition was the same in light or darkness.

Sodium fluoride at 0·02 M in 0·05 molal sucrose resulted in less anthocyanin production in 24 hr in light induced leaf disks moved to darkness (0·38 μg/cm^2) than in controls with only sucrose (1·18 μg/cm^2). In continuously illuminated samples the NaF treated disks produced

[17] L. J. STADLER, *Am. J. Botany* **29** (abstract), 17s (1942).

more anthocyanin (5·05 μg/cm^2) than comparable controls (3·78 μg/cm^2). The presence of NaF in the solutions did not noticeably alter the duration of the induction stage.

Treatment of leaf disks with the sodium salt of adenosine triphosphate (ATP) stimulated the formation of anthocyanin production in the light (Fig. 3). Stimulation of anthocyanin production was previously demonstrated in seedlings.[1] No stimulation in production during dark periods was found in the absence of added sucrose with strawberry leaves. All the disks received an initial 24 hr of light to complete the induction stage, were then transferred to the ATP solutions and given a further 24 hr in the light for the uptake and the anthocyanin increment was measured over the next 24-hr period in light and in darkness. In the presence of added sucrose, ATP stimulated production in darkness in previously illuminated leaf disks treated as the preceding ones. There were 0·98 μg cm^2 formed per 24 hr of darkness with 0 05 molal sucrose alone and 2·5 μg/cm^2 with 0·05 molal sucrose containing 0·01 M ATP.

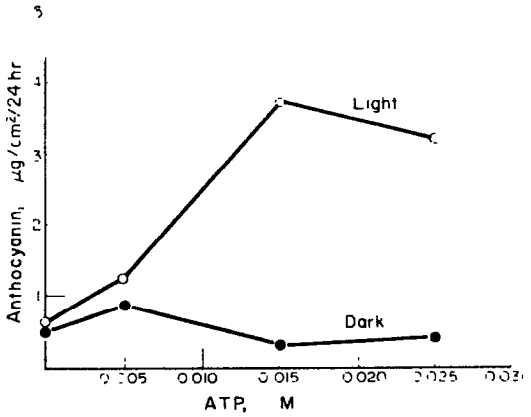

FIG. 3. EFFECT OF ATP CONCENTRATION ON THE ANTHOCYANIN PRODUCTION BY LEAF DISKS NOT TREATED WITH EXTERNAL SUCROSE.

The production plotted is for the period between 48 and 72 hr from preparation, after a preceding light treatment of 24 hr on distilled water and 24 hr on the respective ATP concentrations.

This suggests that during the production phase, anthocyanin synthesis can utilize external ATP in the light when precursors are supplied by photosynthetic processes and in darkness when the precursors can be supplied by the metabolism of added sugar.

The ATP effect is different if the compound is supplied during the induction stage, and anthocyanin formation is not stimulated even in the light. After 2½ days of light, disks contained 2·4 μg/cm^2 anthocyanin if maintained on phosphate buffer alone, and 2·3 μg/cm^2 if 0·01 M ATP was added, while disks started without ATP, left in the light for 15 hr to complete the induction stage, and then moved to 0·01 M ATP for the remainder of the 2½ days contained 4·1 μg/cm^2. The effect of ATP therefore was associated with some interference with the induction stage. In an experiment with 0·015 M ATP, production of anthocyanin was completely suppressed for a 2-day period compared to disks not started on ATP.

Anthocyanin was not the principal flavonoid produced by this tissue. In experiments with phenylalanine-^{14}C, the incorporation of radioactivity for a 44-hr light period into (+)-catechin and a specific leucoanthocyanin was 30 and 7 times as great respectively as into anthocyanin. Studies into the metabolism of these flavans are in progress.

It can be seen from the above results that anthocyanin production in strawberry leaves is influenced by some factors similar to those previously shown to affect production of these compounds in other plant materials. The influences are here reported for a mature, chlorophyllous, non-growing tissue which has the ability to form numerous flavonoids. The effects of temperature and of sucrose concentration on anthocyanin production in strawberry leaves are greater than has been reported with other material. Metabolic inhibitors such as DNP are not effective during the induction stage thereby substantiating that this stage is strictly photochemical. The anomalous effects of ATP solutions are difficult to interpret because the action of externally supplied ATP on intact cells is not clear.

EXPERIMENTAL

Plants of the red-fruited, runnerless, perpetually flowering type of *Fragaria vesca* L., cv. "Alpine", were grown from seed in pots in the greenhouse. Disks were cut from the lamina of the leaves with a cork borer (1·0 cm diameter). The disks were placed with the lower epidermis upward floating in solutions in petri dishes. The dishes were placed 9 cm from 20 W fluorescent lamps providing an intensity of about 350 ft-c. The same distance and light source were used for all illuminated samples.

Ten disks were extracted by steeping in 10 ml of a 1 % solution of HCl in absolute methanol at −18°. The extracts were stable for several days.

Anthocyanin was measured by the method of Swain and Hillis,[18] to eliminate absorption by the large amounts of nonanthocyanin pigments in the leaves. It was found that phaeophytin was not destroyed by the H_2O_2 used to "bleach" the anthocyanin in the 20-min waiting period, but only after several hours. The anthocyanin content of the leaf disks is expressed in micrograms per sq cm of leaf area ($\mu g/cm^2$). An approximation of 30,000 for the molar absorptivity of cyanidin-3-monoglucoside was based on similar values for other related anthocyanins.[19]

[18] T. Swain and W. E. Hillis, *J. Sci. Food Agri.* **10**, 63 (1959).
[19] J. B. Harborne. Personal communication.