Some Factors Affecting Anthocyanin Synthesis in Cress Seedlings

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Mapson & Cruickshank (1947) and Mapson, Cruickshank & Chen (1949), in their work on the effects of various sugars and salts upon the accumulation of ascorbic acid in cress seedlings, had noticed that the pinkness of their metaphosphoric acid extracts varied with the various treatments and that, in general, those seedlings containing most ascorbic acid also contained most anthocyanin. The present work describes the quantitative effects on anthocyanin production of feeding seedlings with various sugars and of certain other experimental treatments.

Few attempts have been made in the past to determine whether there is a quantitative relationship between the production of anthocyanin and the quantities of metabolites artificially supplied. Much work has been done, however, on the qualitative aspects of sugar feeding, especially in regard to the effects of different sugars. For reviews of the earlier work reference should be made to Wheldale (1916) and Blank (1947). More recently, Edmondson & Thimann (1947) and Thimann & Edmondson (1949) have shown that in *Spirodela oligorrhiza* the anthocyanin content is increased by feeding with sucrose and fructose, sucrose being the more effective. Glucose was found to have little or no effect.

The question of the necessity or otherwise of light for anthocyanin formation has never been resolved, although Linsbauer (1901), Kuilman (1930) and Karstens (1939) claimed to have shown that a photochemical reaction is necessary for the formation of anthocyanin in *Fagopyrum esculentum*. Edmondson & Thimann (1947) and Thimann & Edmondson (1949) found that pigment production in *Spirodela oligorrhiza* was increased by high light intensity (150–800 foot candles).

METHODS

Conditions of growth. To avoid the effects of variation in temperature and photosynthetic activity, all the experiments were carried out at 20° and, except when the effect of light itself was being investigated, in the dark. The seedlings were grown in white porcelain pots with metal lids, the details of the germination conditions being as follows. The floor of each pot was covered with a filter paper (Whatman no. 1) and 250 seeds (0.7 g.) were added. For the initial

watering, 9.0 ml. of glass-distilled water or of a solution of the sugar, containing in each case HgCl_2 (50 p.p.m.) to suppress bacterial and fungal growth, were given. This was sufficient for the first 2 days and, subsequently, each pot received 3.0 ml./day for the duration of the experiment.

Method of anthocyanin estimation. The pigment was identified by paper chromatography as chrysanthemin (cyanidin-3-monoside). Daily estimations were made on 200 seedlings from each pot from the 3rd to the 6th days of the experiment and the results are expressed as mg. chrysanthemin per 200 seedlings to avoid complications arising from the effects of the various treatments on the rate of growth. The anthocyanin was extracted by a modification of the method of Karstens (1939). This involved extracting with boiling acid ethanol and transference to distilled water after addition to the filtered extract of 1.6 vol. of a mixture of ether, amyl alcohol and benzene (1:1:2 by vol.).

The resulting solution was made up to 40 ml. with 50% ethanol and the pigment measured colorimetrically using a Spekker photoelectric absorptiometer. All the estimations were done in duplicate, the results plotted being the average of the values obtained. Duplicates differed from each other by less than 20%, the biggest discrepancies being obtained when the more extreme treatments, e.g. feeding with 4% glucose, were used.

Sugar estimations. Total reducing sugars were estimated by the method of Somogyi (1937), as modified by Hanes and described by Hockenhull & Herbert (1945). The reducing values are given in glucose equivalents and have been graphed as mg. per 200 seedlings. Pentoses and other non-fermentable substances were determined by estimating the reducing power after the fermentable sugars had been removed by incubation with yeast. The only hexose sugars present in the material had been shown to be glucose and fructose (Jermyn & Isherwood, 1949), and these were calculated as being equivalent to the total reducing sugars minus the non-fermentable fraction.

Sucrose was estimated by determining the difference in reducing value after hydrolysis with invertase, and fructose was estimated by the method suggested by Roe (1934) and modified by Cole (1942) as follows. A sample was made up to 4 ml. with water and to it were added 6 ml. resorcinol reagent (0.15% in 96% (v/v) ethanol) and 6 ml. HCl-FeCl_3 reagent (7.5 mg. FeCl_3 /100 ml. conc. HCl). The mixture was heated for 30 min. at 75°, together with a blank and two standard fructose solutions treated similarly. The colour developing was estimated in a Spekker photoelectric absorptiometer previously calibrated with standard fructose solutions.

Glucose was taken as being the total fermentable value of the solution less the fructose equivalent.

RESULTS

Effect of sugar feeding on anthocyanin content

Seedlings were grown in the dark on 1% solutions of the following sugars: glucose, sucrose, fructose, invert sugar, sorbose, galactose and arabinose. Water controls were grown simultaneously. Fig. 1a shows the changes in the anthocyanin and sugar contents of the controls, and Figs. 1b-h those due to the various sugars. With all the sugars there was a stimulation of anthocyanin production above that in seedlings grown on water alone.

Effect of sugar feeding on sugar content

In cress seedlings grown on water only (Fig. 1a) the initial sucrose content of 20 mg./200 seedlings decreased, until by the 6th day it had a value of about 3 mg./200 seedlings. At the same time the total fermentable hexose content rose from zero to a peak at about 96 hr. The hexose is made up of glucose and fructose in roughly constant proportions. There was a slight rise in non-fermentable reducing substances from 13 to about 20 mg./200 seedlings.

Fig. 1. Changes in the anthocyanin and sugar contents of cress seedlings grown on water and sugars. ●—●, anthocyanin; x—x, hexose (glucose + fructose); ■—■, glucose; ○—○, fructose; ▲—▲, sucrose; □—□, non-fermentable sugars.

Fig. 2 shows the changes in anthocyanin and sugar contents found on feeding with 2 and 4% glucose respectively. There was a 150% stimulation with 2% glucose, and the curve obtained with 4% glucose suggests that the peak stimulation might be of the order of 450%. Fig. 3 shows the relationship between the anthocyanin content and the hexose (glucose + fructose) and total sugar contents. It will be seen that the anthocyanin is more closely related to the latter than to the former.

Feeding 1% solutions of glucose, sucrose, fructose or invert sugar (Fig. 1b-e) caused a slight lag in the disappearance of sucrose and an increase in the total hexose to a value about 23 mg./200 seedlings higher than that in the water-fed seedlings. This was made up of glucose and fructose in approximately constant proportions. Glucose, sucrose and fructose are apparently in equilibrium in the plant, and artificially supplied glucose, sucrose or fructose are interconverted to restore the internal balance.

Fig. 2. Changes in the anthocyanin and sugar contents of cress seedlings grown on 2 and 4% glucose.
●—●, anthocyanin; x—x, hexose; ▲—▲, sucrose.

Fig. 3. Relationship between anthocyanin and hexose (●) and anthocyanin and total sugar (x).
(a) straight plot; (b) logarithmic plot.

The additions of 1% sorbose and arabinose (Fig. 1*f, h*) appear to accelerate the sucrose breakdown and, especially with sorbose, to depress the production of hexoses, possibly by affecting the rate of fat mobilization. In both cases there was a considerable increase in the non-fermentable fraction, i.e. from about 13 to 50 mg./200 seedlings.

With 1% galactose (Fig. 1*g*) there was a smaller increase in the non-fermentable fraction and also an increase in the fermentable hexoses. Galactose, however, has always been reported to be non-fermentable by yeast, and this fact was checked experimentally. It may be that galactose is converted by the plant into a fermentable hexose or that it is less readily absorbed than other sugars (thus accounting for the lower non-fermentable fraction) and accelerates the mobilization of fat reserves (thus causing an increase in the hexose content).

therefore, germinated in the following solutions, and anthocyanin estimations and sugar analyses carried out to find if there was a similar effect on the pigment production: (i) water; (ii) 0.02M-ammonium sulphate; (iii) 0.02M-ammonium sulphate + 1% glucose; (iv) 0.02M-ammonium sulphate + 2% glucose.

The result for (i) is shown in Fig. 1*a* and for (ii), (iii) and (iv) in Fig. 4*a-c*. The anthocyanin curves for (i), (ii) and (iii) lie so close together that the difference between them is well within the experimental error. The curve for (iv) is similar to that obtained by feeding cress seedlings with 1% glucose in the absence of ammonium sulphate. Although feeding with 0.02M-ammonium sulphate reduced the internal sugar content to about one-third of its normal value, there was no appreciable reduction in the anthocyanin production. On the other hand, restoration of the content to its normal value by artificial

Fig. 4. Changes in the anthocyanin and sugar contents of cress seedlings grown on $(\text{NH}_4)_2\text{SO}_4$ with and without glucose.

●—●, anthocyanin; ×—×, hexose; ▲—▲, sucrose.

Feeding with either 2 or 4% glucose resulted in an initial synthesis of sucrose—about 5 mg./200 seedlings with 2% and 20 mg. with 4%. The increased synthesis with 4% glucose accounts for the fact that there is very little more free hexose to be found on feeding 4% glucose than on feeding 2%. The extra sucrose is, however, soon broken down and does not appear to bear a direct relationship to the amount of free hexose present. From the figures obtained it is impossible to deduce a threshold value for hexose above which sucrose is synthesized, although it may be that this varied according to the stage of germination of the seedlings.

Effect of ammonium sulphate

Mapson & Cruickshank (1947) and Mapson *et al.* (1949) showed that if cress seeds were germinated on 0.02M-ammonium sulphate the sugar contents of the seedlings were reduced with a concomitant decrease in the amount of ascorbic acid formed. Seeds were,

feeding resulted in no stimulation. Feeding ammonium sulphate with 2% glucose, however, pushed the internal sugar content towards that obtained by feeding 1% glucose without ammonium sulphate and there was a similar increase in the anthocyanin content.

Effect of light

Several workers in the past have claimed to have demonstrated that a photochemical reaction is an essential part of the anthocyanin-producing mechanism. For this reason, and because seedlings grown in the dark are developing under abnormal conditions, the effect of light upon the anthocyanin production of cress seedlings was investigated. The seeds were germinated in white porcelain pots as before, but with glass lids in place of the usual metal ones. For the light source, electric lamps of various wattages were used, either naked or masked, and the intensities were measured in foot-candles

(ft.c.), using an 'Avo' direct reading photoelectric light meter. The effects of 0.5, 1.5, 4 and 15 ft.c. and of 4 ft.c. for 48 hr., followed by darkness, were investigated.

the dark, and in which the amount of pigment can be increased threefold by an illumination of 15 ft.c. and more than doubled by 0.5 ft.c. That cress seedlings produce a certain amount of anthocyanin in the dark

Fig. 5. Effect of light on anthocyanin production by cress seedlings grown on water. ●—●, darkness; ○—○, 0.5 ft.c.; ▲—▲, 1.5 ft.c.; ■—■, 4 ft.c.; □—□, 15 ft.c.; ×—×, 4 ft.c. for 48 hr. and then darkness.

The results are shown in Fig. 5, and in Fig. 6 the chrysanthemin production is plotted against the amount of light supplied in ft.c.hr. Light of an intensity of 0.5 ft.c. resulted in a stimulation of about 130%, and as the intensity was raised to 15 ft.c. the stimulation rose to 200%. Transferring seedlings

Fig. 6. Relationship between anthocyanin production and amount of light supplied.

which had been grown in the light for 48 hr. into the dark showed that the effect of the light can manifest itself after the source of illumination has been removed. In the 24 hr. after removal to the dark these seedlings produced 0.88 mg. of anthocyanin/200 as compared with control seedlings which had been kept in the dark from the beginning of the experiment and which produced only 0.18 mg. In the same 24 hr., however, those seedlings which had been in the light all the time produced 1.48 mg.

In the case of the cress, we have a system in which a limited amount of anthocyanin is formed even in

cannot be regarded as very strong evidence against the idea of a photochemical reaction playing a part in anthocyanin formation, since it can be argued that in this case a limited supply of the products of the photochemical reaction may be present in the ungerminated seed. In favour of this idea is the fact that when the seedlings are grown in the light for 48 hr. and then transferred to the dark, most of the total anthocyanin formed is produced after the seedlings have been removed from the light.

DISCUSSION

The results obtained agree with those of the earlier workers in that the anthocyanin content of the seedlings was increased by feeding with glucose, fructose, sucrose and invert sugar. They differ, however, in that the pigment content was also increased by feeding sorbose, galactose and arabinose. The reason for using these latter three sugars was to try to obtain evidence for or against the suggestion which has been put forward that the extra sugar is acting as a substrate for the chain of reactions leading to the production of the anthocyanin molecule, as implied by the hypothesis of Robinson (1935), or whether it upsets the balance of intermediary metabolism, anthocyanin production following as a secondary consequence. If it had been found that only glucose, fructose and sucrose promoted anthocyanin formation, this would have supported the former view. Since, however, such diverse sugars as galactose, sorbose and arabinose

also stimulate anthocyanin production, one must favour the view that the effect of sugar is indirect. Several previous writers (Mirande, 1907; Palladin, 1908; Combes, 1910; Noack, 1922; Blank, 1947) have favoured the latter view.

It is, however, possible that while the glucose, fructose and sucrose feeding was directly supplying a substrate, the feeding of the other sugars was having a different effect. An additional fact in favour of the view that sugar is acting indirectly is that when 4 % glucose is fed, an increase of 90 mg. of sugar results in an increase of only 2.9 mg. of anthocyanin.

It is necessary, therefore, to consider the possible effects on general metabolism, especially with regard to growth, of increasing the internal sugar content. Feeding up to 2 % sugar had no visible effect on either germination or the rate of growth but, in 4 % glucose, elongation of the hypocotyl was retarded, resulting in stunted seedlings. The expansion of the cotyledons appeared to be unaffected, however.

If we now consider the effect of decreasing the internal sugar content by ammonium sulphate feeding, it is necessary to find an explanation why this treatment has comparatively little effect on anthocyanin production. There are two possible lines of explanation. First, it may be that most of the anthocyanin produced in the absence of sugar feeding is formed from pre-existing glycosidic precursors whose conversion into anthocyanin is independent of sugar concentration. The second, and more probable, arises from the fact that the hyperbolic form of the relationship between anthocyanin production and sugar content determines that, at low sugar concentration, large differences are accompanied by only slight variations in anthocyanin content.

Low light intensities can stimulate anthocyanin formation independently of sugar feeding, showing

that more pigment can be produced even in the absence of an increased supply of nutrient. All the above considerations tend to support the view that the effect of sugar feeding is stimulatory rather than due to the piling up of substrate for the chain of synthetic reactions. Nevertheless, there is no conclusive proof that sugar is not the immediate substrate for these reactions.

SUMMARY

1. The normal anthocyanin accumulation of cress seedlings grown on water in the dark can be increased by feeding 1 % solutions of the following sugars: glucose, fructose, sucrose, invert sugar, galactose, sorbose and arabinose.

2. Whereas 1 % glucose causes a stimulation in the anthocyanin production of 50 % above normal, 2 % glucose causes a stimulation of 150 % and 4 % glucose a stimulation of 450 %. Anthocyanin production is related more closely to total sugar content than to the content of any one sugar.

3. Although feeding with 0.02M-ammonium sulphate causes a reduction in the internal sugar content to approximately one-third of its normal value, there is no concomitant reduction in anthocyanin content. The possible reason for this is discussed.

4. Low light intensities stimulate the anthocyanin production of cress seedlings grown on water, the curve relating the amount of pigment produced to the amount of light received having the form of a rectangular hyperbola. It has been shown that the effect of the light may manifest itself after the source of the illumination has been removed.

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