The effect of nitrogen feeding on anthocyanin synthesis in isolated red cabbage embryos

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INTRODUCTION

The influence of mineral resp. nitrogen nutrition on anthocyanin synthesis in plants has been observed by several authors (Czartkowski 1914, Reinhold and Kochs 1935, Gassner and Straib 1937, Szweykowska 1952 and 1959) but the problem has not been sufficiently investigated till now.

Paech (1956) stated an increase of protein and soluble nitrogen and a decrease of carotenoids in etiolated wheat seedlings fed with supplementary nitrogen. He concluded this to be an evidence of a competition for carbohydrates between the nitrogen metabolism and secondary syntheses.

Frey-Wyssling and Blank (1943) estimated anthocyanin, sugar and nitrogen in red cabbage seedlings during the starvation metabolism but they did not find any relation between nitrogen and anthocyanin contents. In our previous paper on cotyledon-less embryos of red cabbage an inhibitory effect of nitrogen on anthocyanin formation was stated depending upon the sugar level in the medium (Szweykowska et al. 1959). Nitrogen had no effect on the amount of pigment when sugar was absent in the medium but it affected pigment formation distinctly when there was an optimal sugar concentration.

The purpose of this paper is to gain some information concerning the relations between sugar, nitrogen and anthocyanin pigment in red cabbage seedlings. The „in vitro” culture method has proved suitable for this work because of making possible the use of young cotyledon-less embryos and the control of supplying them with sugar and nitrogen.

MATERIAL AND METHODS

Red cabbage seeds, var. Koda, obtained from E. Freege, Cracow, were used in these experiments.

The methods of isolating and culturing embryos have been described in our previous paper (Szweykowska et al. 1959). After a 7 day incubation in the dark at 25°C the seedlings were removed from the tubes and the colourless roots cut out. The hypocotyls were weighed and then killed by a 10 min. heating in a water-

bath at 80°C and dried at 40°C to constant weight. The material was then ground and analyzed for anthocyanin, sugars (reducing and soluble) and nitrogen (total and soluble). All data refer to an average of 70—90 embryos, the standard error of the mean being 1,5—2,00%. The majority of experiments, especially of the more important ones (e.g. the influence of sugar and nitrogen feeding) were repeated.

Determination of anthocyanin. The pigment was extracted with a methanolic 10% HCl solution during 24 hrs. and the red extract centrifuged. The pigment was then determined in a Pulfrich photometer using S 53 filter (transmittance max. 5.330 Å). The standard curve was obtained using rubrobrassicin chloride.

Determination of sugars. About 20 mg of dry material was extracted with water in a boiling water-bath for 15 min. A mixture of CdSO₄ and NaOH was then added and the extract heated at 100° for 3 min. to precipitate the proteins. The extract was then filtered and filled up to 50 ml. To a 10 ml sample 1 ml of 1N HCl was added and heated 7 min. at 100°C to hydrolyze the soluble polysaccharides. After cooling and neutralizing with NaOH the sugars were determined by the modified Hagedorn-Jensen method. An excess of kalium ferricyanide in an alcalic phosphate buffer solution (2,52 g K₂HPO₄ + 7,65 K₃PO₄ in 1 l) was added and the tubes heated in the boiling water-bath for 15 min. Reduced ferrocyanide was precipitated with zinc sulphate and the excess of ferricyanide determined iodometrically with n/200 sodium thiosulphate. The sum of soluble sugars was expressed in mg glucose.

The reducing sugars of the extract (without hydrolysis) were estimated simultaneously. Their amount was in all cases proportional to the sum of soluble sugars (soluble polysaccharides + monosaccharides) and therefore only the results referring to soluble sugars will be given below.

The standard error of the estimations was 3,60%.

Determination of nitrogen. The nitrogen was estimated by the micro-Kjeldahl method. The ammonia was trapped into 5 ml of 20% boric acid and then titrated with n/28 hydrochloric acid using as indicator a mixture of methyl red and brom cresol green.

Total and soluble (non-protein) nitrogen were separately determined. The protein nitrogen was calculated from the difference of total and non-protein nitrogen. The soluble nitrogen was extracted with water in a boiling water-bath for 10 min. After the filtration the extract was examined for nitrogen in the way described above. For the reason of feeding the embryos with nitrates in some experimental series it seemed to be advisable to use a procedure of nitrate reduction before digestion. However, these efforts failed (the methods of titanous chloride, of reduced iron and of salicylic acid were applied). Simultaneously it was stated that when using the above described method of nitrogen determination the nitrates are not at all reduced and caused no deviations in parallel estimations. For this reason the nitrate estimations were omitted.

The standard error of the estimations was 3,50%.

THE INFLUENCE OF SUGAR ON GROWTH AND THE CHOICE OF REFERENCE UNITS

The effect of glucose and sucrose on fresh and dry weight and on the protein nitrogen content of the seedlings was examined. The influence of sugars on the fresh weight of hypocotyls is shown in fig. 1 and 2. The fresh weight decreases distinctly

Fig. 1. Effect of glucose feeding on the fresh and dry weight of hypocotyls

Fig. 2. Effect of sucrose feeding on the fresh and dry weight and on the protein nitrogen content of hypocotyls

in higher sugar concentrations, the hypocotyls become short and the roots longer (fig. 3). These changes of the fresh weight are not accompanied by similar changes of the dry weight of hypocotyls. The dry weight decreases in higher sugar concentrations only slightly (figs. 1 and 2). Simultaneously the water content of seedlings

Fig. 3. Cotyledon-less seedlings of red cabbage grown on sucrose media: left — on 40%, right — on 120% sucrose

decreases strongly (fig. 4). This fact is connected undoubtedly with the high osmotic value of the medium which renders more difficult the water supply. The better feeding effect of sucrose than that of glucose is to some extent caused also by the osmotic values of the media — the osmotic values of sucrose solutions are nearly half those caused by the same percent w/vol. concentrations of glucose.

Together with the worse water supply sugars accumulate in seedlings cultured in higher sugar concentrations as shown in fig. 6. The two facts, i.e. the variation

Fig. 4. Effect of sugars on the dry weight % of hypocotyls

of the fresh weight caused by different water contents and the variation of the dry weight caused by different sugar accumulations — make the fresh as well as the dry weight hardly useful as reference values for estimating anthocyanin, sugar and nitrogen. For this reason protein nitrogen has been chosen as reference value for analytical results. Protein nitrogen of embryos growing without cotyledons refers almost exclusively to plasmatic proteins and is most suitable for expressing the living and metabolizing part of the seedlings. The curve of protein nitrogen content (fig. 2) is in general similar to the curves of fresh and dry weight, but in higher sugar concentrations it evidently corrects the apparent growth alterations resulting from differences in the water and sugar content of the seedlings.

THE EFFECT OF SUGAR ON ANTHOCYANIN SYNTHESIS AND NITROGEN METABOLISM

Anthocyanin pigment of seedlings increases by increasing the sugar concentration in the medium. At the same time the soluble sugar content of the hypocotyls

increases (figs. 5—6). On the contrary, soluble nitrogen shows no relation to the anthocyanin content but is inversely related to protein synthesis. It increases in hunger metabolism when there is no sugar in the medium and protein synthesis is weak and also in high supraoptimal sugar concentrations when the protein synthesis is again inhibited. It reaches a minimum in optimal growth conditions, i.e. when

Figs. 5—6. Effect of sucrose on the pigment and sugar contents of hypocotyls

the sugar concentration in the medium is an optimal one (fig. 2 and 7).

The relations in reference to protein nitrogen are shown in figs. 5 and 7. They are very similar when referred to the fresh weight (figs. 6—7) as well as to the dry weight of the seedlings.

THE EFFECT OF NITROGEN FEEDING ON GROWTH

In a preliminary experiment an optimal sugar concentration of the medium ($40/0$) has been employed and four nitrogen sources were tested: nitrates ($\text{KNO}_3 + \text{Ca}(\text{NO}_3)_2$), urea, glycine and asparagine. Three concentrations (regarding N_2O_5 content of the substances used) were employed: 0.0, 3.7 and 7.4 mM. The 3.7 mM

Fig. 7. Effect of sucrose on the soluble nitrogen content of hypocotyls

Fig. 8. Effect of nitrates and asparagine on the fresh and dry weights of hypocotyls

Fig. 9. Effect of nitrates and asparagine on the protein nitrogen content of hypocotyls

conc. corresponds to that used in the basal medium (1/2 Knop solution). Urea and glycine strongly inhibit the growth of embryos having above all a harmful effect on root development. On the other hand, nitrates and asparagine promote the growth providing thus an evidence of being used in embryo metabolism. Five concentrations of nitrates and asparagine were tested and it was stated that a 5.5 mM concentration is optimal for fresh and dry matter production and 3.7 mM — for protein nitrogen formation (figs. 8—9).

THE EFFECT OF NITROGEN FEEDING ON ANTHOCYANIN SYNTHESIS

Several combinations were tested using:

- I. Two nitrogen sources: nitrates and asparagine
- II. Three N_2O_5 concentrations: 0.0, 5.7 (optimum) and 9.2 mM
- III. Three sucrose concentrations: 0, 4 (optimum) and 100/0.

Fig. 10. Effect of asparagine on the pigment, sugar and soluble nitrogen contents of hypocotyls of seedlings kept on a sugarless medium

Fig. 11. Effect of asparagine on the pigment, sugar and soluble nitrogen contents of hypocotyls of seedlings kept on a medium with 40% sucrose

12. Effect of nitrates on the pigment, sugar and soluble nitrogen contents of hypocotyls of seedlings kept on a medium with 40% sucrose

If there is no sugar in the medium asparagine slightly promotes anthocyanin synthesis without increasing the soluble sugar content of the seedlings (fig. 10).

Using 4 and 100% sugar concentrations, the anthocyanin content was highest when there was no nitrogen present. Supplying embryos with nitrogen a strong decrease of pigment formation was stated beginning with lowest concentration used (which is optimal for protein formation). A further increase of nitrogen in the medium had nearly no effect on pigment formation (figs. 11 and 12).

The soluble nitrogen of embryos growing on asparagine accumulate according to the asparagine concentration in the medium (fig. 11) and that of embryos growing on nitrates remains almost constant (fig. 12). In the latter case the intensity of nitrate reduction, kept only on the level needed for protein synthesis, is probably the limiting factor (as it was pointed out, „soluble nitrogen” does not include nitrates). The relations in respect to media containing 100% sucrose are shown in tables 1—2.

Table 1
The effect of nitrates

N ₂ O ₅ mM	g/g protein nitrogen		
	pigment	sugar	soluble nitrogen
0.0	2.8	17.5	1.0
5.5	0.9	5.0	0.6
9.2	1.0	5.3	0.6

Table 2
The effect of asparagine

N ₂ P ₅ mM	g/g protein nitrogen		
	pigment	sugar	soluble nitrogen
0.0	2.1	13.8	0.7
5.5	1.5	4.8	3.2
9.2	1.6	5.6	3.9

The lack of a direct relation between nitrogen and anthocyanin pigment and a close relation between pigment and sugars is obvious. This statement is true in the case of affecting pigment synthesis by an increasing sugar content in the medium as well as in the case of affecting it by depriving the medium of nitrogen, because nitrogen affects an anthocyanin formation only if it affects simultaneously the soluble sugar level in the tissues. Fig. 13 shows the relation between the pigment and soluble sugar in hypocotyl tissues on various nutrient media and in hypocotyls and cotyledons of whole embryos growing on water-agar medium, without any nutrient substances added. The majority of points are placed along a straight line showing thus an intimate connection of these two factors. An exception constitute only some points referring to asparagine media. The deviations may be caused by the carbon chain of asparagine which is formed in the Krebs cycle and undoubtedly may also

enter into this cycle. It is known that at least a part of the rubrobrassicin of red cabbage is formed of products of respiratory sugar breakdown (Eberhardt 1954, Grisebach 1957 and 1958). In this way the carbon chain of asparagine may be used in anthocyanin synthesis without being previously converted to sugar. It is not included in the results of sugar analyses and therefore points resulting from plotting anthocyanin against sugar are placed differently for embryos kept on asparagine media.

The results suggest the general conclusion that anthocyanin synthesis depends on the level of simple organic compounds (e.g. sugars) serving as building material as well as an energy source for pigment synthesis. Protein synthesis is a competing

Fig. 13. The dependence of pigment synthesis upon the sugar level in seedling tissues

process and the depressing effect of nitrogenous compounds is based on its promoting effect on protein formation. Under its influence occurs a decrease of organic material (e.g. sugars) being left at the disposal of secondary processes.

DISCUSSION

The problem of the role of nitrogen in anthocyanin synthesis has been undertaken by Frey-Wyssling and Blank in a paper dealing with anthocyanin physiology in seedlings of red cabbage (1943). The authors estimated protein, soluble and total nitrogen and did not find any constant ratio of the pigment to any of the nitrogen fractions. Similarly they found that there is no constant proportion between pigment and sugars and suggested a connection between them to be highly proble-

matic. If it really exists it is only so distant as for instance an artificial sugar supply — increased metabolism — increased anthocyanin production. However, the results of the present paper indicate that a relation of anthocyanin to sugar undoubtedly exists and that it is a close although indirect one (at least in part — which is proved by recent works on biochemical steps of anthocyanin synthesis). This is evidenced by the linear dependence of the anthocyanin content upon the sugar amount in the seedlings (fig. 13). This dependence is shown not only in the case of cotyledonless seedlings growing on various nutrient media, but also in the case of seedlings similar to those used by Frey-Wyssling and Blank, i.e. etiolated seedlings with cotyledons (hypocotyl and cotyledons being analyzed separately) growing on 10% water-agar medium. The line is of the type $y = ax + b$ thus showing that some amount of pigment (the term b of the equation) is formed independently of the actual sugar content in seedlings. This pigment amount may be formed directly from a precursor stored in embryos. This may be supported by one experiment of Frey-Wyssling and Blank who brought about the formation of violet pigment in subepidermal tissue of red cabbage embryos by a simple treatment of seeds or colourless embryos with 1.5–20% hydrogen peroxide. With reference to the total sugar amount the ratio „anthocyanin : sugar” ($y:x$ instead of $(y-b):x$) will not be constant but it will decrease together with increasing sugar concentration. This fact was perhaps one of the reasons of inconstant ratio „anthocyanin : sugar” found by Frey-Wyssling and Blank (1943).

The influence of nitrogenous compounds on pigment synthesis is undoubtedly connected also with sugar. When present in the medium nitrogen causes a strong decrease of sugar in the seedlings and vice versa — the sugar content increases in the absence of nitrogen. The amount of pigment formed varies together with the variation of the sugar level in the tissues, according to the linear diagram in fig. 13 which was discussed above.

Paech (1956) stated a similar relation of nitrogen to carotenoids in etiolated wheat seedlings growing on urea. The conditions of a high nitrogen supply resulted in increased protein synthesis and decreased carotenoid formation. Paech suggested these relations to be a manifestation of a sugar competition between the protein metabolism and carotenoid formation. The processes of secondary syntheses (e.g. carotenoid or anthocyanin formation) are generally characterized by a relatively weaker intensity than those belonging to the primary metabolism (e.g. protein synthesis). They can use only those products which had not been consumed in primary metabolism. In this way the secondary product formation must decrease in conditions favourable for protein synthesis. The present investigation on the influence of nitrogenous compounds on protein synthesis and anthocyanin formation and the results of sugar analyzes confirm Paech's conclusions and extend their general significance.

The author is indebted to Prof. Dr J. Czosnowski for his advice and assistance in preparing this paper.

SUMMARY

The influence of sugar and nitrogen feeding on anthocyanin synthesis in isolated red cabbage embryos has been investigated. Sugar promotes pigment synthesis and nitrogen acts in an opposite direction. The analyses of soluble sugars and of protein and soluble nitrogen have shown that nitrogen feeding results in an increase of protein synthesis and in a decrease of the soluble sugar content of the seedlings. On the other hand, the absence of nitrogen in the culture medium results in an increase of soluble sugars and of pigment synthesis. The author stated a linear dependence of the pigment amount upon the sugar content of the hypocotyls or cotyledons calculated on protein nitrogen unit. In this way a direct relation of anthocyanin synthesis to sugar metabolism is proved.

The results obtained are discussed together with those obtained by Frey-Wyssling and Blank (1943) and Paech (1956) and together with conclusions they reached.

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