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THE INFLUENCE OF EXOGENOUS SUGAR FEEDING ON THE ACCUMULATION OF ANTHOCYANINS AND RUTIN IN BUCKWHEAT SEEDLING HYPOCOTYLS

Sugar feeding is generally known to be stimulatory for anthocyanin synthesis, and the view that various kinds of sugars act as promoters of anthocyanin accumulation is rather unanimously accepted by the majority of plant physiologists. One of the fundamental experimental evidences for this point of view was obtained already in the last century by E. Overton (1899), who showed in an extensive study that the floating of different plants or their organs in sugar solutions brings about a reddening of their cell cap. Since that time numerous sugar feeding experiments with various plants have been carried out, and a great number of qualitative as well as of quantitative data on this question have been collected, most of them supporting the general idea that sugars are stimulatory for anthocyanin formation. The voluminous literature on the relations between sugar feeding and anthocyanin biosynthesis up to about 1958 is comprehensively summarized in the reviews by F. Blank (1947, 1958; see also Thimann, Edmondson, 1949; Bogorad, 1958; Kandeler, 1960).

A number of more recent investigations lend further support to the observations made earlier. Sugar feeding was shown to enhance anthocyanin synthesis in isolated seedling parts and intact seedlings of different plant species (Alston, 1958; Arnold, Alston, 1961; Troyer, 1964a, b; Stafford, 1965; Malaviya, Laloraya, 1966; Havelange et al., 1967; Grill, 1967; Vince, 1968). A stimulation of pigment formation in sugar-containing media was also achieved in different tissue cultures (Szweykowska, 1959; Szweykowska et al., 1959; Straus, 1959), in detached petals of *Impatiens balsamina* (Klein, Hagen, 1961), in leaf disks (Eberhardt, Haupt, 1959; Creasy et al., 1965; Creasy, Swain, 1966) and apple skin pieces (Faust, 1965; Smock, 1966) floated in sugar solutions, and in whole apple fruits dipped or submerged for a certain time into solutions of different sugars (Smock, 1966). Only rare studies can be referred to in which the pigment-synthesizing plant material did not respond to sugar feeding positively (Beguin, 1964), whereas no report is known up to now in which an inhibitory effect of sugars on anthocyanin formation would have been observed. At the same time there are a number of data indicating that, besides anthocyanins, sugar feeding can enhance the biosynthesis of other flavonoids as well (Nick, 1953; Nöll, 1955; Creasy, Swain, 1966) and also stimulate the formation of some related polyphenolic compounds, such as chlorogenic acid (Ruckenbrod, 1955; Zucker, Levy, 1959) and lignin-like substances (Stafford, 1965).

Thus, there appears to be no doubt that sugar feeding can be considered as a factor permanently bringing about a stimulation of the formation of anthocyanins, if not other phenolic compounds, in plant tissues.

Surprisingly, in the feeding experiments with intact buckwheat seedlings carried out in this laboratory, the expected stimulatory effect of sugar (sucrose, in that case) could not be observed, but, instead, sucrose called forth a distinct suppression of anthocyanin formation (Margna, Otter, 1968a). This was the more unexpected as in Troyer's investigations conducted with excised buckwheat hypocotyls, sucrose feeding was found to favour anthocyanin formation (Troyer, 1964a, b). In our experiments a sucrose-stimulated increase in the anthocyanin formation of buckwheat hypocotyls was revealed only in seedlings with their protein synthesis blocked by a simultaneous treatment with 2.4-D (Margna, Otter, 1968b). Apparently, the mechanism of the sugar effect is not so simple as it may seem from the great majority of feeding data, but there must be some unknown relationships between the final effect of sugars and the metabolic activities of the organism studied.

In order to understand the nature of that dependence, the effect of feeding various sugars to intact, decotyledonized or derooted buckwheat seedlings and to excised buckwheat hypocotyls was studied in detail. In addition to examining sugar-induced changes in the formation of anthocyanins, we included measurements of rutin, known to be the only flavonoid compound besides anthocyanins and leucoanthocyanidins present in buckwheat hypocotyls (Troyer, 1955; Tohver et al., 1967).

Experimental

The experiments were carried out on buckwheat (*Fagopyrum esculentum* Moench) seedlings grown under artificial illumination from white fluorescent tubes. Two growth regimes were used: A) the germinating seeds were held for 48 h in complete darkness; after that, until harvesting, the seedlings were transferred into a light chamber for further development under long-day conditions involving 3 light-dark cycles (16 h illumination + 8 h darkness); B) the developing seedlings were held in darkness for 72 h; then they were exposed to light for 10 h and after that were returned to darkness for an additional 14 h. In the experiments with decotyledonized or derooted seedlings the second growth regime was used only, the cotyledons and roots were removed before the light treatment. The seedlings were grown in glass dishes (25—30 seedlings per dish) on two layers of filter paper moistened with appropriate solutions. The light intensity used in all experiments was 27,300 ergs·cm^{-2}·sec^{-1}. The temperature was held constant at 25±1 °C throughout the whole period of seedling growth.

In the experiments three sugars were involved: sucrose, glucose and fructose. The fourth substance tested was mannitol, a sugar alcohol reported by some authors to be ineffective in promoting anthocyanin synthesis (Eberhardt, 1954). Three modes of sugar treatment were employed: a) sugars as a component of germination and growth medium — the seedlings were grown on filter paper moistened with a 1 or 0.5 per cent solution of the corresponding sugar (the control seedlings were grown in distilled water); b) sugars added to the growth medium of seedlings at a later stage of their development — all seedlings were grown in distilled water (growth regime B), but before the start of the illumination program to each of the dishes an adequate amount of a 2 per cent solution of the corresponding sugar was added (to control seedlings the same amount of distilled water was added, respectively); in the experiments with derooted seedlings the plants were placed with their cut ends into corresponding solutions, using special dishes covered with perphorated plastic plates; c) sugar solutions as floating media for excised hypocotyls — 72 h-old seedlings grown on distilled water in darkness were harvested, their cotyledons and roots were removed, and the excised hypocotyls floated for 60 minutes,

unless otherwise stated, in 1 per cent sugar solutions (control hypocotyls were floated in distilled water); after that, the hypocotyls were placed on filter paper moistened with the same solutions used for floating and then transferred into the light chamber to follow the illumination program analogous to the growth regime B.

Anthocyanins were assayed as described previously (Margna, Otter, 1968b). Rutin was measured by a procedure of repeated one-dimensional paper chromatography combined with a subsequent measurement of the optical density of the eluate of rutin spot spectrophotometrically at 360 nm (Margna, Margna, 1969). The content of flavonoid substances found in hypocotyls was expressed in optical density units (anthocyanins) or in micrograms (rutin) per seedling.

In radioactive-carbon experiments, a sample of uniformly ^{14}C-labelled glucose was used, the radioactive substance being fed to seedlings as a component of the initial growth medium, or of the solution added to the growth medium before the light treatment of seedlings. The radioactivity of the dried plant material was measured in a Vacutronic VA-Z-310 Geiger counter.

All experiments were carried out in 3 to 5 replications in space per treatment and were also replicated in time on 5 to 7 occasions. The results were subjected to evaluation by the statistical techniques of Student's significance test.

Results

The content of anthocyanins and rutin in the hypocotyls of intact buckwheat seedlings grown on sugar media. The results (Tab. 1) clearly show that when seedlings are grown on solutions of different sugars, the

Table 1

The content of anthocyanins and rutin in the hypocotyls of intact buckwheat seedlings grown on 1 per cent sugar solutions

The numbers here and in Tables 2—5 represent average values of at least 15 separate replications; the anthocyanin content is expressed in arbitrary units by the scale of optical density (O. D.×1000). Significant changes are marked with asterisks: $^{**}P \leqslant 0.01$; $^{*}0.01 < P \leqslant 0.05$.

Treatment	Anthocyanins		Rutin	
	units/seedling	% change	µg/seedling	% change
Growth regime A:				
H_2O	17.8	—	17.8	—
Sucrose, 1%	14.9	—16.3**	19.6	+10.1*
Glucose, 1%	15.0	—15.7**	—	—
Fructose, 1%	17.3	— 2.8	—	—
Mannitol, 1%	15.9	—10.7**	—	—
Growth regime B:				
H_2O	9.14		12.9	
Sucrose, 1%	5.46	—40.3**	14.4	+11.6*
Glucose, 1%	6.36	—30.4**	13.8	+ 7.0
Fructose, 1%	6.75	—26.1**	14.5	+12.4*
Mannitol, 1%	7.07	—22.7**	14.0	+ 8.5

content of anthocyanins formed in their hypocotyls is considerably decreased. No marked differences could be revealed between the magnitudes of this inhibitory effect of sugars in 1 or 0.5 per cent (results not presented) solutions, but the suppression of pigment formation was more pronounced when the second growth regime (B) was employed. However, irrespective of which of the two regimes was used, the inhibition caused by sugar feeding remained highly significant in almost all cases, the same being the result in mannitol tests as well.

Entirely opposite was the effect of sugar feeding on the formation of rutin. In regard to this compound, all of the sugars tested, including mannitol, acted as promoters of its synthesis in most of the cases, the stimulation caused by sugars being sufficiently large to reach a statistically significant level (Tab. 1).

The effect of the removal of cotyledons. In order to clear up whether the sugar-induced inhibition of anthocyanin formation is related to the processes starting in the cotyledons after the onset of the illumination or is already determined by the earlier changes in the metabolism of the whole seedlings taking place during the preceding 72-hour period of the seedlings' growth on sugar solutions in darkness, the effect of the removal of cotyledons was studied. The results of the corresponding experiments indicate that sugar action was not modified by that treatment. As before, all three sugars as well as mannitol gave rise to a considerable decrease of pigment formation in hypocotyls (Tab. 2).

Table 2

Sugar-induced changes in the anthocyanin content in hypocotyls of buckwheat seedlings with cotyledons removed before light treatment

The seedlings were grown according to the growth regime B.

Treatment	Anthocyanins	
	units/seedling	% change
H_2O	6.31	—
Sucrose, 1%	3.99	—36.8**
Glucose, 1%	3.86	—38.8**
Fructose, 1%	4.97	—21.2**
Mannitol, 1%	4.37	—30.7**

The influence of sugars when introduced to seedlings before the onset of illumination program. This set of experiments was carried out on three seedling categories: on intact seedlings, on seedlings with their roots removed, and on decotyledonized seedlings. So far as rutin formation is concerned, no differences in their responses to sugar feeding worth to be taken into account were revealed between the three types of experimental material. In almost all cases (with the exception of mannitol), a rather strong stimulation of rutin accumulation in hypocotyls was observed (Tab. 3).

Table 3

Rutin content in the hypocotyls of buckwheat seedlings fed with sugars by introducing sugar solutions into the growth medium before light treatment

To each of the dishes with seedlings, 5 ml of a 2 per cent solution of the respective sugar was added to yield an approximately 1 per cent concentration of sugars in the resulting growth medium. Derooted seedlings were directly placed with their cut ends into 1 per cent solutions of corresponding sugars.

Treatment	Intact seedlings		Decotyledonized seedlings		Derooted seedlings	
	µg/seedling	% change	µg/seedling	% change	µg/seedling	% change
H_2O	19.5	—	17.0	—	19.4	—
Sucrose	25.0	+28.4**	21.2	+24.8**	23.0	+18.4**
Glucose	28.7	+47.2**	21.6	+27.1**	20.3	+ 4.7
Fructose	25.9	+32.8**	21.8	+28.0**	23.5	+21.2**
Mannitol	—	—	—	—	19.1	— 1.6

Somewhat different were the results in anthocyanin series. A significant inhibition of pigment formation, analogous to that found in the former experimental series, occurred only when the sugars were introduced

to derooted seedlings (Tab. 4), whereas the experiments with intact or decotyledonized seedlings failed to show stable results. Due to this uncertainty, a more detailed study of the question was undertaken.

As a result it was found that glucose is the only one of the three sugars tested which maintains its ability to suppress anthocyanin formation in buckwheat hypocotyls when introduced to intact or decotyledonized seedlings before the onset of the light treatment. In the majority of replicate experiments conducted in that series, the content of anthocyanins in glucose-treated hypocotyls did not reach the level of pigment content in water controls, and though the average decrease of anthocyanin formation remained much smaller (—5.3%) than it was when the seedlings were grown on glucose media, it was still large enough to reach a statistically significant level ($P<0.05$). In sucrose and fructose series neither inhibition nor stimulation of pigment formation could be firmly established.

Table 4

Anthocyanin content in the hypocotyls of derooted buckwheat seedlings placed with their cut ends into sugar solutions before light treatment

Treatment	Anthocyanins	
	units/seedling	% change
H_2O	14.6	—
Sucrose, 1%	13.0	—11.0*
Glucose, 1%	12.3	—15.9*
Fructose, 1%	13.0	—11.0*
Mannitol, 1%	11.5	—21.2**

The experiments with excised hypocotyls floated in sugar solutions. No more inhibition of anthocyanin formation could be observed in these experiments, and with regard to the formation of both rutin and anthocyanins, two of the three sugars tested — sucrose and fructose — showed a clear-cut stimulatory action (Tab. 5). Glucose was less effective and it

Table 5

The content of anthocyanins and rutin in excised buckwheat hypocotyls floated in sugar solutions

Treatment	Anthocyanins		Rutin	
	units/seedling	% change	µg/seedling	% change
Unfloated control	7.2	—	21.3	—
H_2O, 60 min	9.4	—	22.8	—
Sucrose, 1%, 60 min	10.2	+ 8.5*	25.6	+12.3*
Glucose, 1%, 60 min	9.5	+ 1.1	24.0	+ 5.3
Fructose, 1%, 60 min	10.5	+11.7**	29.5	+29.4**
Mannitol, 1%, 60 min	10.1	+ 7.4	23.3	+ 2.2
H_2O, 10 min	9.1	—	—	—
Glucose, 1%, 10 min	10.0	+ 9.9*	—	—

was only in experiments with a shorter duration of floating procedure that we could obtain a significant stimulation of pigment formation comparable with the effect of the two other sugars. When mannitol solutions were used as floating media of hypocotyls, a tendency towards a stimulatory action was obtained in anthocyanin series, only, whereas the intensity of rutin accumulation remained practically unchanged.

Radioactive carbon experiments. In sugar feeding experiments a possibility often remains that the changes observed in the biochemical features of a plant or its organs are not due to an actual entering of sugars into the plant tissues, followed by their direct utilization in the

metabolic processes of the plant, but are merely a secondary phenomenon caused by an increased osmotic pressure of nutritional medium due to the presence of sugars. That might be also the case in the present studies in particular in those experimental series in which the sugars were fed to intact seedlings through their root systems. To check that possibility, special experiments with seedlings fed with uniformly labelled glucose-^{14}C were carried out, and the radioactivity of the separate organs of the seedlings at the end of the experimental period was measured.

Table 6

Radioactivity of buckwheat seedlings grown on glucose-^{14}C solutions

Seedling part	Growth regime A			Growth regime B		
	imp/min/ seedling	%	µM of radioact. glucose/ seedling	imp/min/ seedling	%	µM of radioact. glucose/ seedling
Cotyledons	3764	26.3	0.145	4422	31.2	0.170
Hypocotyls	2438	17.0	0.094	2757	19.5	0.106
Roots	8132	56.7	0.313	6982	49.3	0.268
Total seedling	14334	100	0.552	14161	100	0.544

The results (Tab. 6) point to a sufficiently high level of radioactivity in the seedlings treated, speaking clearly in favour of the supposition that glucose (and probably all other sugars tested) is intensively absorbed by roots and actively transported from roots to the other parts of the seedling. Calculations showed that during the experimental period about 10 per cent of the radioactive glucose added to the growth medium was absorbed by the seedlings grown on these solutions, and about 1 per cent of the total dry matter of a seedling harvested at the end of this period was due to the radioactive sugar taken up by its roots. As expected, the major part of the label found in the seedlings was located in the roots, but the radioactivity of the hypocotyls and cotyledons was also considerable.

In terms of these radioactivity data, the amount of glucose absorbed by the seedlings was approximately of the order of 0.5 micromoles of sugar per seedling, an amount that seems to be large enough to bring about marked changes in the seedling metabolism. It may be supposed, however, that the actual amount of glucose taken up by the seedlings and translocated from their roots to hypocotyls and cotyledons was still greater than that found in those organs by radioactive measurements. A part of the labelled glucose taken up by the seedlings was undoubtedly involved in the respiratory processes of the latter, resulting in certain losses of radioactivity due to the evolution of carbon dioxide into the surrounding atmosphere. There remains, therefore, no doubt that the effect of glucose and other sugars on flavonoid formation in buckwheat seedlings is a result of their active involvement in the metabolic processes of the seedling tissues.

Discussion

The results unequivocally show that in buckwheat seedlings the effect of feeding sugars depends upon the state of the seedling material at the moment of sugar treatment, and on the stage of their development at the beginning of the treatment. Sugar action was clearly inhibitory for pigment formation when the seeds were allowed to germinate on sugar solutions and the latter were used as growth medium during the whole period of

the subsequent development of seedlings. Similar was the response when the sugars were introduced to seedlings which for the first 72 h were grown on water in darkness and then, just before the onset of illumination and sugar addition, were derooted, but the inhibition of anthocyanin formation was less pronounced when the sugars were introduced, at the same point of seedling development, to intact or decotyledonized seedlings through the root system. Finally, the sugars acted as stimulators of anthocyanin accumulation when they were fed, by a floating procedure, to excised hypocotyls. Thus, in buckwheat seedlings, providing they are treated as intact organisms or that the integrity of their metabolic processes is maintained more or less undisturbed, the feeding of exogenous sugars is principally not stimulatory but inhibitory for anthocyanin formation. An increase of pigment accumulation observed in excised hpocotyls seems to be rather untypical for that kind of seedlings.

It is not easy to imagine what might be the reason of such an unusual effect. It seems most reasonable to suppose that the effect is somehow related to the type of metabolic patterns specifically characteristic of developing buckwheat seedlings, which, in turn, is presumably determined by the prevalent carbohydrate and protein nature of the reserve substances stored in buckwheat seeds. The results provide evidence that these patterns must involve a particular regulatory mechanism possessing the ability to direct the utilization of the extra amounts of energy and carbon units supplied by the sugars added. It is likely that the mechanism represents a part of a more general regulatory system normally operating in young seedlings. The maintenance of metabolic as well as of morphological integrity of seedlings seems to be one of the prerequisites for the functioning of that system, but the system also appears to remain active in seedlings with only roots or cotyledons removed. In isolated hypocotyls the regulating ability of the mechanism is apparently completely lost.

In attempts to explain the possible nature of the postulated regulatory mechanism, the following aspects seem to be of particular importance. Firstly, the mechanism must at least partly operate at the energetical level, and the sugars fed to seedlings are probably used mainly as a source of the extra energy for the synthetic processes of seedlings. If the sugars had been mainly used as a source of additional structural material for the building of carbon compounds, it could not be understood why they acted as stimulators for rutin biosynthesis, but not with regard to anthocyanin formation, though the molecules of both of the two types of flavonoid compounds are materially built up exactly in the same manner and from the same precursors. In addition, feeding sugars to buckwheat seedlings gives rise to a considerable intensification of their metabolic activities related to protein metabolism (Otter, Margna, 1967; Margna, Otter, 1968a), a complex of biochemical reactions which is known to require much energy for its completion, but, in seedling material, does obviously not need any additional carbon units of structural importance.

Secondly, protein biosynthesis together with the related processes seems to act as a primary metabolic factor responsible for the control and regulation of the utilization of the chemical energy of sugars fed to seedlings. This is evidenced by the fact that intact seedlings with their protein synthesis blocked are practically deprived of the regulating ability (Margna, Otter, 1968b). The situation is in a sense comparable with that arising in excised hypocotyls after the removal of cotyledons. In that case, the hypocotyls are isolated from the protein reserves of seedlings located in cotyledons, and the regulatory interference of protein metabolism is therefore practically excluded.

Thirdly, an important role in this phenomenon may be played by competitive-like relations between the protein synthesis and processes leading to the formation of anthocyanins and other flavonoids, arising probably from the presence of only a restricted pool of metabolically active phenylalanine — a key-metabolite for flavonoid biosynthesis — in seedling cells. The occurrence of such a competition in buckwheat seedlings is clearly proved by a number of experimental facts reported earlier (Margna et al., 1969; Margna, 1971), including those which suggest, as already mentioned, that a sugar-induced decrease in anthocyanin-forming capacity of seedlings is accompanied by a marked intensification of the processes of protein metabolism.

It may well be that under favourable conditions of sugar feeding the phenylalanine pool present in seedlings is predominantly used to supply the processes responsible for protein biosynthesis which are of first-order importance in plant life. As a result, the amount of phenylalanine available for other syntheses must be reduced, giving rise to a decrease in the accumulation of anthocyanin pigments. Alternatively, an opposite effect of sugar feeding on rutin formation makes possible that the decrease in anthocyanin accumulation observed in sugar-fed seedlings might at least partly be due to certain shifts in the balance of substrate supply within the whole group of flavonoids itself, indicating that an additional competition for phenylalanine-precursor between different types of flavonoid compounds may exist in intact seedlings. Finally, a possibility still remains that a certain role in the sugar effects on the formation of flavonoids in the plant material may be also enacted by competition-like relations between the protein metabolism and secondary processes at an energetical level, as emphasized by us earlier (Margna, Otter, 1968a).

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SUHKRUTE MÕJU ANTOTSÜAANIDE JA RUTIINI MOODUSTUMISELE TATRAIDANDITE HÜPOKOTÜÜLIDES

Resümee

Näidatakse, et suhkrute sisseviimine nii intaktsetesse kui ka eraldatud juurte või idulehtedega tatraidanditesse nõrgendab antotsüaanide kogunemist nende hüpokotüülides, mitte aga ei stimuleeri seda protsessi nagu tavaliselt teistes objektides. Pigmendisünteesi mõningane stimulatsioon ilmnes ainult siis, kui suhkrutega mõjustati isoleeritud hüpokotüüle. Suhkrute toimes rutiini kogunemisele niisuguseid erinevusi ei esinenud: nende sisseviimisel suurenes rutiinisisaldus hüpokotüülides alati. Analüüsitakse põhjusi, mis võisid tingida selle ebatavalise suhkruefekti antotsüaanide moodustumisel.

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УДО МАРГНА, ТИЙУ ВАЙНЪЯРВ, ЭВИ МАРГНА

ВЛИЯНИЕ ЭКЗОГЕННОГО САХАРНОГО ПИТАНИЯ НА НАКОПЛЕНИЕ АНТОЦИАНОВ И РУТИНА В ГИПОКОТИЛЯХ ПРОРОСТКОВ ГРЕЧИХИ

Резюме

Показано, что введение сахаров в интактные проростки гречихи, а также в проростки с удаленными корнями или семядольными листочками приводит к ослаблению накопления антоцианов в их гипокотилях, а не к стимуляции этого процесса, как обычно наблюдается в других объектах. Некоторая стимуляция пигменто-образования обнаруживалась только при введении сахаров в изолированные гипокотили. В действии сахаров на накопление рутина аналогичных различий не было установлено, при введении сахаров его количество в гипокотилях всегда возрастало. Обсуждаются возможные причины установленного необычного эффекта сахаров на накопление антоцианов.

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