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THE INFLUENCE OF SUCROSE FEEDING ON ANTHOCYANIN FORMATION IN INTACT BUCKWHEAT SEEDLINGS AS A POSSIBLE FUNCTION OF PRIMARY CHANGES IN PROTEIN METABOLISM

Sucrose feeding is generally known to be promotive for anthocyanin biosynthesis. For example, a stimulation of pigment formation in sucrose-containing media was achieved in different tissue cultures (Slabecka-Szweykowska, 1952; Straus, 1959), in fronds of some species of duckweed (*Spirodela* spp.) (Thimann, Edmondson, 1949; Thimann et al., 1951), in leaf disks floated in sucrose solutions (Eberhardt, 1954; Creasy et al., 1965), and also in hypocotyls of different plant seedlings grown under experimental conditions of sucrose feeding (Eberhardt, 1954; Eddy, Mapson, 1951; Arnold, Alston, 1961). Recently such an increase in anthocyanin accumulation has been shown by Troyer in excised segments of buckwheat-seedlings hypocotyls (Troyer, 1964). It is generally supposed that the stimulatory effect of sucrose feeding is due to an increase in the amount of metabolites available in cells as structural or energetic material for anthocyanin synthesis. Though, in principle, this generalization may hardly be incorrect, the metabolic sequences following the initial sucrose absorption by cells up to its final effect on anthocyanin formation are far from being elucidated. The fact that the stimulation of pigment synthesis on sucrose-containing media is only rarely found to be correlated with higher content of soluble sugars in corresponding tissues (Straus, 1959; Creasy et al., 1965) strongly speaks in favour of the possibility that the whole process must be more complex and presumably proceeds in an indirect way. This view is substantially strengthened by the observations made in our recent feeding experiments in which the combined sucrose-ammonium nitrate nutritional media were used (Маргна, Оттер, 1968).

The data obtained in the present investigations lend further support to these ideas. We were able to show that in intact buckwheat seedlings the exogenous sucrose feeding is not at all connected with a stimulation of anthocyanin formation in hypocotyls but, on the contrary, leads to a considerable loss of pigment synthesizing capacity of the latter. Evidence was obtained that such an unexpected effect of sucrose is possibly a function of primary changes in the protein metabolism of the whole seedling.

Experimental

The experiments were carried out with 5-day-old buckwheat (*Fagopyrum esculentum* Moench) seedlings of the local Estonian variety 'Jõgeva'. The seedlings were grown in glass dishes at 22—25 °C on two layers of filter paper moistened with an adequate amount of distilled water (control seedlings) or with the same amounts of appropriate nutritional solutions (seedlings under treatment). The following nutritional media were used: 0.01*M* and 0.03*M* sucrose solutions in one set of experiments, and combined solutions of sucrose and 2.4-dichlorophenoxyacetic acid (2.4-D) in another. In the latter case the concentrations of solutions, with respect to sucrose, were equalled to those in the first set of experiments, whereas with respect to 2.4-D the 10^{-6}M or 10^{-7}M concentrations were chosen. The filter paper used as the growth basis for germinating seedlings was freed from contaminants by repeated boiling with distilled water during a total of 15 hours at least, before being employed in experiments.

During the first 48 h the germinating seeds were held in complete darkness; after that, until harvesting, they were placed into a light chamber for further development under long-day conditions (cycles: 16 h illumination+8 h darkness; illumination from fluorescent tubes, light intensity 31,500 erg · cm^{-2} · sec^{-1}).

For anthocyanin assay, the harvested hypocotyls were ground in a porcelain mortar with 10 ml of cool 1 per cent methanolic hydrochloric acid (methanol:water, 1:4, v/v); the crude extracts, together with hypocotyl tissue fragments, were quantitatively transferred into stoppered test-tubes, and left to stand for 1 hour at room temperature. After that the extracts were filtered through cotton wool, then centrifuged, and the resulting clear extracts were subjected to photocolorimetric assay for determining their optical density. The measurements were made on a Soviet FEK-56M photocolorimeter (green filter with absorption maximum at 540 nm, 10 mm cuvettes). The anthocyanin content of hypocotyls was expressed in arbitrary units (by the scale of optical density) per seedling or per 1 g of fresh weight.

The amount of total and nonprotein nitrogen in both cotyledons and hypocotyls was determined in two different portions of plant material by a modified Kjeldahl micromethod involving distillation of ammonia into a 2 per cent solution of boric acid and titration of the final solutions of borate complexes in the presence of a mixed indicator (Sher, 1955). The amount of protein nitrogen was calculated from the difference between the mean values of these two sets of measurements. The data of nitrogen assay were expressed in micrograms per seedling or per 100 mg of fresh weight.

All the experiments were run in at least five replications.

Results and discussion

Typical results obtained in one of the experimental series are collected in Table 1.

The results clearly indicate that when seedlings were grown on sucrose media, the absolute amount of anthocyanin synthesized in their hypocotyls during 5 days of development was considerably smaller than that in water-grown seedlings, the decrease in the intensity of pigment formation being the more, the higher the concentration of nutritional solution. True, when the data were expressed on the fresh weight basis, an opposite situation was noticed. In the present case this fact, however, cannot be indicative of a real stimulation of pigment biosynthesis, but merely shows a secondary change in pigment concentration in hypocotyls due to diminished growth of seedlings on sucrose media: an approximately 27 per cent inhibition of hypocotyl length as well as of their fresh weight on 0.03*M* sucrose solution and a corresponding 11 per cent inhibition of hypocotyl growth on 0.01*M* sucrose solution observed in our experiments (Table 2) were sufficient to mask completely the decrease in pigment formation actually taking place in hypocotyls under these conditions.

Characteristic changes were established in nitrogen metabolism. The growing of seedlings on sucrose media resulted in a more intense decrease

Table 1

Content of anthocyanins and nitrogen fractions in 5-day-old buckwheat seedlings grown in sucrose media

	Absolute data per seedling			Data expressed on fresh weight basis*		
	Water control	Sucrose		Water control	Sucrose	
		0.01*M*	0.03*M*		0.01*M*	0.03*M*
Hypocotyls:						
Anthocyanins (scale units)	**19.4**	**17.4**	**16.6**	816	830	975
Total nitrogen (μg)	75	76	79	323	327	412
Protein nitrogen (μg)	**28**	**30**	**34**	136	137	186
Nonprotein nitrogen (μg)	**47**	**46**	**45**	187	190	226
Ratio protein nitrogen/non-protein nitrogen	**0.60**	**0.65**	**0.76**	0.73	0.72	0.82
Cotyledons:						
Total nitrogen (μg)	220	214	198	1022	1009	1092
Protein nitrogen (μg)	172	169	150	803	793	845
Nonprotein nitrogen (μg)	48	45	48	219	216	247
Ratio protein nitrogen/non-protein nitrogen	3.58	3.76	3.12	3.67	3.57	3.42

* Anthocyanins — per 1 g, nitrogen — per 100 mg of fresh weight.

of the absolute content as well as in a decrease of the relative portion of the protein fraction of nitrogen in cotyledons, whereas in hypocotyls a more intense reverse tendency occurred as compared with the intensity of these processes in water-grown seedlings. Simultaneously with that, a more rapid decrease of total nitrogen in cotyledons and an increase of the latter in hypocotyls were revealed. This indicates that under the conditions of sucrose feeding the whole complex of biochemical reactions related to protein metabolism of seedlings is stimulated, including the enzymic breakdown of reserve proteins of cotyledons, translocation of amino acids released in that decomposition from cotyledons to other parts of young seedlings, and the formation of new proteins in the cells of hypocotyls and other organs of the developing seedlings.

Table 2

Hypocotyl growth of 5-day-old buckwheat seedlings grown in sucrose media
(average data of 5 separate experiments)

Sucrose concentration	Hypocotyl length (mm)	Hypocotyl fresh weight (mg)
0 (water control)	27	24
0.01*M*	26	21
0.03*M*	20	17

Similar results were also obtained in other experimental series with buckwheat seedlings carried out under analogous conditions of sugar feeding.

Thus, the results obtained here differ from those of the earlier investigators in that the pigment content of the buckwheat hypocotyls was not increased, but even decreased by feeding sucrose. Since exactly the same circumstances were fixed already in our previous investigations with seedlings grown in combined sucrose and nitrogen-containing media (Оттер, Маргна, 1967; Маргна, Оттер, 1968), there is no doubt that the decrease in the anthocyanin forming capacity of hypocotyls is characteristic and quite a regular response to exogenous sucrose feeding, at least when buckwheat seedlings if not other plant species are concerned. It seems to be very likely that the sucrose-induced inhibition of anthocyanin formation and the simultaneous stimulation of the protein metabolism of the whole seedling observed are closely connected with each other. Moreover, there is a good reason to believe that protein metabolism plays a leading role in the regulation of these interrelations. The regulatory mechanism of this relationship is most probably based on the competition between these two processes in energetical terms. It may be suggested that the increase in the total energetic potential of seedlings resulting from sucrose feeding is realized through its primary effect on the complex of reactions related to protein biosynthesis, which are known to be the most important energy-applying processes of young growing seedlings. Apparently, this results not only in quantitative, but also in some qualitative changes in the distribution of energy between different energy-applying processes of seedlings, so that in spite of the increase in the total energetic potential of seedlings by feeding sucrose, the intensity of some of the energy-limited processes of the latter can even be reduced. Hence, the decrease in pigment biosynthesis in hypocotyls can probably be considered to be a consequence of such a redistribution of the total energy of seedlings via the regulatory action of protein metabolism.

To check this hypothesis, another set of feeding experiments was conducted, in which the seedlings were allowed to grow in sucrose media combined with 2.4-dichlorophenoxyacetic acid (2.4-D), a compound known to be an inhibitor of protein biosynthesis (Воробьев, Ча Жу-би, 1960; Деева, Машгаков, 1965; Хотянович, Веденеева, 1965). In these series control seedlings were grown in 2.4-D solutions without an addition of sucrose.

Under these nutritional conditions unfavourable for biosynthetic incorporation of amino acid residues into protein molecules, the utilization of the energetic resources of the whole seedling for the need of protein synthesis would probably be considerably reduced and the regulatory effect of the latter would practically lose its previous importance. Consequently, the extra energy arising from sucrose feeding would presumably provide enhanced possibilities for secondary biosyntheses now more or less completely delivered from the competitive control of protein biosynthesis. One might expect therefore that under these conditions sucrose feeding gives rise to a certain intensification of the anthocyanin formation in hypocotyls or at least does not lead to a weakening of this process as it was found in experiments by feeding sucrose only.

This assumption was perfectly confirmed by experimental data.

As can be seen from the Table 3 presenting the results of one of the experimental series with sucrose-2.4-D combined media, the characteristics of nitrogen metabolism and pigment-forming processes of hypocotyls of buckwheat seedlings grown in mixed media are quite different and in part exactly opposite to those observed in seedlings fed with sucrose only. In the present case the absolute content of protein nitrogen in hypocotyls remained practically unchanged, although with increasing sucrose concentration in medium the intensity of decomposition of reserve proteins in cotyledons and the translocation of nitrogen from cotyledons to hypocotyls were continuously increased. In accordance with that, the amount of nonprotein nitrogen showed a permanent tendency to increase in hypocotyls, which

Table 3

Content of anthocyanins and nitrogen fractions in 5-day-old buckwheat seedlings grown in combined sucrose-2.4-D media

(absolute data per seedling)

	Only 2.4-D 10^{-7}M	2.4-D 10^{-7}M + sucrose 0.01M	2.4-D 10^{-7}M + sucrose 0.03M
Hypocotyls:			
Anthocyanins (scale units)	17.6	17.9	19.2
Total nitrogen (μg)	72	73	77
Protein nitrogen (μg)	28	29	29
Nonprotein nitrogen (μg)	44	44	48
Ratio protein nitrogen/nonprotein nitrogen	0.64	0.66	0.60
Cotyledons:			
Total nitrogen (μg)	211	210	191
Protein nitrogen (μg)	169	168	148
Nonprotein nitrogen (μg)	42	42	43
Ratio protein nitrogen /nonprotein nitrogen	4.02	4.00	3.44

was accompanied by a decrease of the ratio "protein nitrogen/nonprotein nitrogen" clearly indicating that protein biosynthesis in hypocotyls was blocked. At the same time the anthocyanin forming processes lasted with previous intensity, and with higher doses of sucrose in nutritional medium a marked increase of pigment formation in hypocotyls was actually observed. Similar were the results of other experimental series with sucrose-2.4-D media, irrespective of the 10^{-6}M or 10^{-7}M solutions of 2.4-D being employed.

These facts unequivocally point to the postulated relationship between anthocyanin biosynthesis and protein metabolism in buckwheat seedlings and bring forth strong evidence in support of the hypothesis that the relationship is competitive in its nature, that it is based on competition for energy, and that protein biosynthesis acts as a primary metabolic factor responsible for the control and regulation of this relationship.

The suggestion proposed seems to be most satisfactory for explaining the peculiarities of the relationship between anthocyanin biosynthesis and nitrogen metabolism discovered in this study with sucrose feeding of intact buckwheat seedlings. Furthermore, the hypothesis is not inconsistent with Troyer's results mentioned above, although in his work, contrary to our data, sucrose feeding was found to favour anthocyanin formation in buckwheat hypocotyls (Troyer, 1964). In that case the experiments were conducted with hypocotyls detached from cotyledons with their nitrogen reserves and, therefore, the competition between pigment formation and protein metabolism for energy source as well as the regulatory interference of protein biosynthesis were practically excluded.

Further investigations on this question are in progress.

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SAHHAROOSI TOIME ANTOTSÜAANIDE BIOSÜNTEESILE TATRAIDANDEIS KUI VALGU AINEVAHETUSES TOIMUNUD PRIMAARSETE MUUTUSTE FUNKTSIOON

Resümee

Uuriti antotsüaanide biosünteesi ja lämmastikuühendite ainevahetuse iseärasusi viiepäevastes tatraidandites nende kasvatamisel erineva kontsentratsiooniga sahharoosilahustel kas ilma või koos 2,4-dikloorfenoksüäädikhappe (2,4-D) lisandiga.

Vastupidiselt enamikule suhkrute toimet käsitlenud uurimustes saadud tulemustele tehti kindlaks, et tatraidandite hüpokotüülides antotsüaanide absoluutne sisaldus sahharoosi mõjul mitte ei suurene, vaid märgatavalt väheneb. Samal ajal täheldati iseloomulikke muutusi lämmastikuühendite ainevahetuses: toitekeskkonna suhkrukontsentratsiooni tõustes väheneS valgufraktsiooni suhteline sisaldus idulehtedes, suurenes aga hüpokotüülides, millega samaaegselt kaasnes lämmastikuühendite üldhulga vähenemine idulehtedes ja suurenemine hüpokotüülides. Seega põhjustas sahharoos elavnemise kogu valgu ainevahetusega seotud reaktsioonide kompleksis, kaasa arvatud idulehtede varuvalkude lagunemine, vabanenud aminohapete ümberpaigutumine idulehtedest idandi teistesse osadesse ning uute valkude süntees areneva idandi organeis, sealhulgas hüpokotüülides.

Katsetes, kus tatraidandeid kasvatati 2,4-D lisandiga toitelahustes, avaldus sahharoosi toime teisiti. Valgu sünteesi pärssimise tõttu jäi valgufraktsiooni absoluuthulk hüpokotüülides praktiliselt muutumatuks, selle suhteline sisaldus hüpokotüülide lämmastikuühendite üldhulgas aga keskkonna sahharoosikontsentratsiooni tõustes pidevalt kahanes. Antotsüaanide biosünteesi intensiivsus sahharoosi toimel seevastu suurenes, erinedes sahharoosi mõjust antud protsessile neis tatraidandeis, mis olid kasvatatud ilma 2,4-D lisandita lahustel.

Saadud tulemuste põhjal tuilakse järeldusele, et antotsüaanide biosüntees ja valgu

ainevahetus on tatraidandeis teineteisega tihedas seoses, kusjuures määrav tähtsus selle seose reguleerimisel normaalselt funktsioneerivates idandites arvatakse olevat nende protsesside vahelisel interferentsil (konkurentsil) energeetilise kindlustatuse tasemel. Otsustav osa selles kuulub nähtavasti valkude biosünteesiga seotud reaktsioonide kompleksile kui tähtsaimale energiatarbijale idandeis. Seejuures on tõenäoline, et mistahes muutus idandite üldises energiabilansis realiseerub tema primaarse toime kaudu valkude biosünteesile, mille lõpptulemuseks on nii kvantitatiivsed kui ka kvalitatiivsed muutused kogu summaarse energia jaotamises idandite üksikute energiat tarbivate protsesside vahel. Arvatakse, et sahharoosi sisseviimisele järgnev antotsüaanide biosünteesi nõrgenemine tatraidandite hüpokotüülides ongi taolise energia ümberjaotamise tulemuseks valgu ainevahetuse regulatoorse toime vahendusel, olgugi et idandite üldine energeetiline potentsiaal neis tingimustes tegelikult suureneb.

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ВЛИЯНИЕ ПИТАНИЯ САХАРОЗОЙ НА ОБРАЗОВАНИЕ АНТОЦИАНОВ В ИНТАКТНЫХ ПРОРОСТКАХ ГРЕЧИХИ КАК ВОЗМОЖНАЯ ФУНКЦИЯ ПЕРВИЧНЫХ ИЗМЕНЕНИЙ В БЕЛКОВОМ ОБМЕНЕ

Резюме

Изучали особенности биосинтеза антоцианов и азотного обмена у 5-дневных проростков гречихи, выращенных либо на растворах сахарозы разной концентрации, либо на среде с сахарозой в комбинации с 2,4-дихлорфеноксиуксусной кислотой (2,4-Д).

В противоположность результатам большинства предыдущих работ установлено, что у интактных проростков гречихи питание сахарозой не приводит к стимуляции формирования антоцианов, а, наоборот, вызывает значительное уменьшение абсолютного количества пигментов, синтезированных в гипокотилях в течение их 5-дневного развития. В то же время наблюдались характерные изменения в процессах азотного обмена: уменьшение относительной доли белкового азота в общей сумме азотсодержащих веществ в семядольных листочках и увеличение доли той же фракции в гипокотилях, некоторое снижение количества общего азота в семядолях и увеличение его в гипокотилях. Это показывает, что под воздействием сахарозного питания значительно оживляется весь комплекс биохимических реакций, связанных с белковым обменом, включая расщепление запасных белков семядолей, транслокацию освободившихся аминокислот из семядольных листочков по всем органам молодых проростков и образование новых белков в клетках развивающихся органов, в том числе в гипокотилях.

В экспериментах с выращиванием проростков в присутствии 2,4-Д влияние сахарозы обнаруживалось иначе. Из-за блокирования реакций биосинтеза белков вызванное сахарозой ускорение расщепления запасных белков семядольных листочков не сопровождалось увеличением количества белковой фракции в гипокотилях, как это отмечалось в экспериментах без 2,4-Д в среде, а, наоборот, приводило к заметному уменьшению относительной доли этой фракции среди азотсодержащих веществ гипокотилей. В то же время образование антоцианов в гипокотилях заметно ускорилось — противоположно тому, что было обнаружено в проростках при введении только сахарозы.

Делается вывод, что биосинтез антоцианов и процессы белкового обмена проростков гречихи тесно связаны между собой и что регуляция указанной взаимосвязи в нормально функционирующих проростках определяется механизмом, в основе которого лежит интерференция (конкуренция) на уровне энергетической обеспеченности. Первостепенное значение принадлежит здесь комплексу процессов биосинтеза белка как важнейшему потребителю доступной энергии и любое изменение в общем энергетическом балансе проростков реализуется, по-видимому, через его первичное влияние на биосинтез белка. Конечным результатом этого, по всей вероятности, являются не только количественные, но и качественные изменения в распределении суммарной энергии между всеми процессами проростков, потребляющими энергию. Предполагается, что ослабление формирования антоцианов в гипокотилях при выращивании проростков гречихи в сахарозных средах и является следствием такого перераспределения суммарной энергии проростков через регулярное действие белкового обмена, несмотря на то, что общий энергетический потенциал проростков в этих условиях увеличивается.

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