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CARBOHYDRATE NUTRITION AND ANTHOCYANIN ACCUMULATION IN LIGHT GROWN AND ETIOLATED SHOOT CULTURES OF CAROB (*CERATONIA SILIQUA* L.)

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Abstract – Production of anthocyanins was studied in shoot cultures of carob at high (45.9 μmol $s^{-1}m^{-2}$) and low (9.2 μmol $s^{-1}m^{-2}$) irradiance levels and in darkness in relation to carbohydrate nutrition. Anthocyanin production was stimulated by light, but it also occurred in etiolated shoot cultures which developed in darkness. Anthocyanins were present in both leaves and shoot tips. The major factor affecting anthocyanin production was carbohydrate nutrition, with sucrose as a choice superior to fructose and glucose. The carbohydrate effect was clearly osmotic in nature, since anthocyanin production increased even at supraoptimal concentrations detrimental to the growth of shoot cultures. This conclusion was further confirmed in experiments in which sucrose was partly replaced with the sugar alcohols sorbitol and mannitol.

Key words: Carbohydrate nutrition, anthocyanin accumulation, light-grown and etiolated shoot cultures, *Ceratonia siliqua*

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INTRODUCTION

Carob is an evergreen xeromorphic Mediterranean species well adapted to conditions of high insolation and prolonged periods of drought. Carob shoot cultures can be obtained and maintained *in vitro* in two distinct morpho/anatomical forms, depending on the provided light regime. In light shoot cultures develop large pinnate leaves with prominent petioles. In cross-section, light-grown shoots develop massive circumferential rings of sclerenchyma and xylem consisting of cells with heavily lignified walls, the same as in plants growing free in nature. In conditions of continuous darkness, cultures undergo drastic structural and histological changes leading to the formation of etiolated shoot cultures (Vinterhalter and Vinterhalter, 1999). Etiolated shoots are long and slender, and their leaves are either totally absent or significantly reduced in size (Vinterhalter and Vinterhalter, 2001). Etiolated shoots are hypolignified, xylem is greatly reduced, and the sclerenchyma ring is replaced with a ring of parenchymatic cells (Vinterhalter and Vinterhalter, 2003). Although chlorophyll and other photosynthetic pigments are absent, anthocyanin production and accumulation in etiolated carob shoot cultures proceeds in the darkness (Vinterhalter, 1998).

Both anthocyanin function and biosynthesis and gene organization, expression, and regulation have been widely investigated (Dooner et al., 1991; Mol et al., 1996). A protective role for anthocyanins present in vegetative tissues has been assumed for a long time. However, it has recently been reconsidered by many authors, including Sherwin and Farrant (1998), Feild et al. (2001), Steyn et al. (2002), Close and Beadle (2003) and Hughes et al. (2005). Today a general opinion is steadily forming which holds that anthocyanins in vegetative tissues function as a light screen protecting photosynthetic tissues from excess light damage prior to their full functional establishment and later, when they become senescent.

Among factors which trigger or affect anthocyanin synthesis, light is exceptional. According to Mancinelli (1985), biosynthesis of anthocyanins in plant tissues either requires light or is enhanced by it. Our approach to the problem of anthocyanin synthesis was to use etiolated shoot cultures as a model system. They en-

able us to shut down light as the major factor and then to investigate the effect of carbohydrate nutrition alone as a second significant anthocyanin-inducing factor. When carbohydrate nutrition affects some process, a specific dilemma always appears – is this effect nutritional or osmotic in nature? The answer is often sought through the use of sugar alcohols (sorbitol, mannitol), which raise osmotic strength of the medium, but do not to affect the carbohydrate metabolism of cells. In this paper, therefore, we consider the effect of carbohydrate nutrition on anthocyanin production in etiolated and light grown carob shoot cultures.

MATERIAL AND METHODS

Shoot cultures of *Ceratonia siliqua* L. were isolated and cultured as previously described (Vinterhalter and Vinterhalter, 1992). Treatments consisted of culturing 15 *mm* long shoot explants cultured for 5 weeks on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 *mg* L^{-1} BA and 0.1 *mg* L^{-1} IBA. There were 5-6 shoots per 100 mL wide neck Erlenmeyer flask and six flasks per treatment, giving a total of 30-36 shoots per treatment. Treatments were replicated 3-7 times. Sucrose concentrations ranging from 29.2 to 438.9 mM were investigated at two irradiance levels, viz., 45.9 μmol s^{-1} m^{-2} (considered here as high) and 9.2 μmol s^{-1} m^{-2} (considered as low), and in darkness. Light was provided by cool white fluorescent lamps at a ratio of 16 h of light to 8 h of darkness and the temperature 25 ± 2^0 C. In some treatments, media also contained sorbitol and mannitol in addition to 29.2 or 58.4 mM sucrose. Glucose and fructose in concentrations of 58.4 mM or 146 mM were added individually or combined (in a 1: 1 ratio) in both light and darkness.

Anthocyanin content of shoots and leaves was measured using absorbance at l 533 *nm*. Frozen tissue (1 *g*) was homogenized in 10 mL of cold 1% HCl in methanol. Measurement was performed on a Shimadzu UV-160 spectrophotometer using 2 mL of supernatant obtained after centrifuging for 15 min at 3300 rpm. Since anthocyanin composition of carob is yet unknown pigment content was calculated as cyanidin-3-glucoside with MW = 442 and molar absorptivity of 26900 and expressed as *mg* g^{-1} of fresh tissue. Absorbance measurements were performed separately for every replication within a treatment.

RESULTS

In carob shoot cultures, anthocyanins are present in both light grown and etiolated cultures. In light-grown shoot cultures, intensity of the red color was directly correlated with the concentration of sucrose added to the culture medium. At the higher irradiance level (45.9 μmol s^{-1} m^{-2}), cultures were dark-green on 1-2 % sucrose, pink

Table 1. Effect of sucrose on accumulation of anthocyanins in shoots and leaves at high (45.9 μmol s^{-1} m^{-2}) and low (9.2 μmol s^{-1} m^{-2}) irradiance and in darkness. Within each column means followed by the same letter were not significantly different according to the LSD multiple range test at p = 0.05 nm* - not measured.

Sucrose % (mM)		Anthocyanins, *mg* g^{-1} fresh weight ± SE				
		45.9 μmol s^{-1} m^{-2}		9.2 μmol s^{-1} m^{-2}		Darkness
		Shoots	Leaves	Shoots	Leaves	
1	(29.2)	0.12 ± 0.02 a	nm*	0.09 ± 0.01ab	nm	0.02 ± 0.007 ab
2	(58.4)	0.17 ± 0.01 a	0.22 ± 0.04 a	0.10 ± 0.01 a	0.08 ± 0.01 a	0.02 ± 0.002 a
3	(87.6)	0.19 ± 0.02 a	0.25 ± 0.01 ab	0.09 ± 0.01 a	0.16 ± 0.02a b	0.02 ± 0.003 a
5	(146.0)	0.31 ± 0.03 b	0.33 ± 0.04 abc	0.13 ± 0.02 ab	0.22 ± 0.03 b	0.06 ± 0.01 b
8	(233.6)	0.54 ± 0.04 c	0.36 ± 0.04 abc	0.19 ± 0.03 b	0.24 ± 0.02 b	0.09 ± 0.02 c
10	(292.0)	0.71 ± 0.05 d	0.39 ± 0.01 bc	0.28 ± 0.05 c	0.35 ± 0.03 c	0.13 ± 0.01 d
15	(438.0)	0.80 ± 0.09 d	0.47 ± 0.01 c	0.04 ± 0.03 d	0.25 ± 0.02 bc	0.25 ± 0.01 e

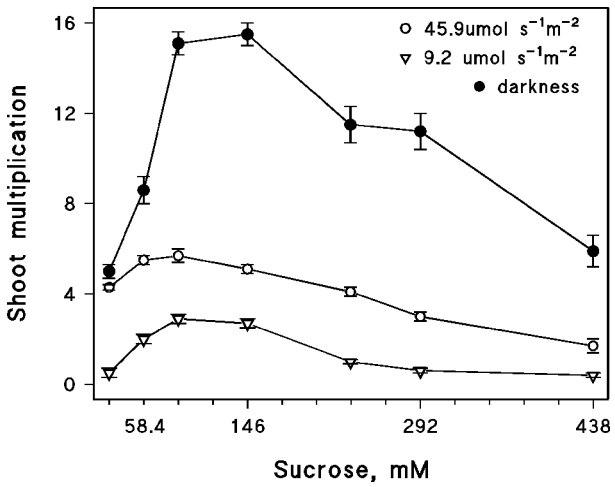

Fig. 1. Effect of sucrose concentration on the shoot multiplication at high (45.9 μmol s^{-1} m^{-2}) and low (9.2 μmol s^{-1} m^{-2}) irradiance and in darkness.

on 3-5 % sucrose, and dark red on 8-15 % sucrose. At the lower irradiance (9.2 μmol s^{-1} m^{-2}), the differences in color were less prominent, while in darkness color ranged from white and pale-pink to saturated dark-pink. However, in the absence of chlorophyll the presence of anthocyanins in etiolated shoots was prominent. The anthocyanin content of shoots and leaves at various sucrose concentrations and light regimes is presented in Table 1. According to these data, anthocyanins accumulated in both shoots and leaves, and their accumulation at both irradiance levels and in darkness was clearly stimulated by increased sucrose concentration in the media. At equal sucrose concentrations, anthocyanin accumulation was always higher at the higher irradiance level. At 1% sucrose in both light and darkness, leaves were either absent or very small and anthocyanin determination could not be performed. It is interesting to note that anthocyanin content in shoots and leaves progressively increased with sucrose concentration even though sucrose concentrations above 146 mM were supraoptimal and actually detrimental to the growth of shoot cultures (Fig. 1). It follows that the stimulative effect of sucrose on anthocyanin production and accumulation is not only a nutritive but also an osmotic effect of high carbohydrate nutrition.

To investigate the effect of sucrose constituents (glucose and fructose) on anthocyanin production, sucrose was replaced with equimolar concentrations of fructose, glucose or both in a 1:1 ratio (Table 2). Two concentrations were investigated, viz., 58.4 mM (considered as low carbohydrate nutrition) and 146 mM (considered as moderately high carbohydrate nutrition).

In media supplemented with glucose, fructose, or glucose + fructose in a 1:1 ratio, anthocyanin production in leaves and shoots was higher on media containing more carbohydrates. However, none of these carbohy-

Table 2. Effect of fructose and glucose on accumulation of anthocyanins in shoots and leaves in light (high irradiance) and darkness. Within each column means followed by the same letter were not significantly different according to the LSD multiple range test at p = 0.05.

Carbohydrate type	mM	Multiplication index ± SE		Anthocyanins, *mg* g^{-1} fresh weight ± SE		
				Leaves	Shoot	
		light	darkness	light	light	darkness
sucrose	58.4	5.5 ± 0.3 b	8.3 ± 0.6 b	0.22 ± 0.03b	0.17 ± 0.01 b	0.02 ±0.002 a
fructose	58.4	3.3 ± 0.1 a	1.3 ± 0.1 a	0.15 ± 0.02 a	0.09 ± 0.01 a	0.04 ± 0.005 b
glucose	58.4	3.6 ± 0.1 a	1.5 ± 0.2 a	0.17 ± 0.02 ab	0.10 ± 0.01 a	0.04 ± 0.006 b
fruct+gluc	58.4	3.7 ± 0.2 a	1.2 ± 0.1 a	0.18 ± 0.02 ab	0.10 ± 0.01 a	0.04 ± 0.006 b
sucrose	146.0	5.1 ± 0.2	15.5 ± 0.5 b	0.33 ± 0.04 b	0.31 ± 0.3 b	0.06 ± 0.01 b
fructose	146.0	4.1 ± 0.2	4.2 ± 0.2 a	0.24 ± 0.02 ab	0.16 ± 0.03 a	0.03 ± 0.004 a
glucose	146.6	3.7 ± 0.2	3.7 ± 0.2 a	0.23 ± 0.04 ab	0.14 ± 0.02 a	0.04 ± 0.006 ab
fruct+gluc	146.6	3.4 ± 0.2	3.7 ± 0.2 a	0.22 ± 0.01 a	0.14 ± 0.02 a	0.04 ± 0.004 ab

Table 3. Effect of joint sorbitol + sucrose supplementation on accumulation of anthocyanins in shoots. Within each column means followed by the same letter were not significantly different according to the LSD multiple range test at p = 0.05.

Sucrose (mM)	Sorbitol (mM)	Multiplication index ± SE	Anthocyanins, $mg\ g^{-1}$ fresh weight
29.2	0	4.7 ± 0.5 d	0.12 ± 0.02 a
	29.2	3.8 ± 0.3 c	0.19 ± 0.02 ab
	58.4	2.1 ± 0.3 b	0.24 ± 0.4 ab
	116.8	2.4 ± 0.3 b	0.35 ± 0.06 bc
	204.4	1.8 ± 0.2 b	0.46 ± 0.05 c
	262.8	0.7 ± 0.2 a	0.51 ± 0.7 c
58.4	0	5.1 ± 0.3 e	0.17 ± 0.01 a
	29.2	4.3 ± 0.3 c	0.22 ± 0.02 ab
	87.6	4.9 ± 0.2 d	0.31 ± 0.04 b
	175.2	3.0 ± 0.2 b	0.51 ± 0.5 c
	233.6	2.1 ± 0.2 a	0.57 ± 0.5 c

drate sources produced as much anthocyanin as sucrose. Only in shoots cultured in darkness was 58.4 mM sucrose less effective than equimolar glucose, fructose, and 1:1 glucose + fructose. However, 146 mM sucrose in darkness again stimulated anthocian accumulation more than its constitutive hexoses. Column multiplication was added in Table 2 mainly to demonstrate the high morphogenic effect which sucrose exerts on shoot multiplication in etiolated shoot cultures.

To distinguish whether increased anthocyanin production is connected with the nutritional or osmotic effects of carbohydrate nutrition, treatments were prepared in which sucrose was partly replaced with a sucrose alcohol, sorbitol or mannitol (Table 3). Results with mannitol (not presented) were similar to results obtained with sorbitol. The column multiplication index was added to demonstrate the adverse effect of sorbitol on the shoot multiplication, an important growth parameter of shoot cultures. The results indicate that increase of anthocyanin production is stimulated by increased carbohydrate nutrition even when it lowers the growth of cultures. This stimulative effect therefore seems to be mainly osmotic in nature.

DISCUSSION

Anthocyanin production in carob shoot cultures was highly influenced by carbohydrate nutrition and irradiance levels at which the cultures were grown. In darkness,anthocyanin production, although prominent due to the absence of other pigments was significantly lower than in light. In conditions of low irradiance (dim light) (9.2 μmol s^{-1} m^{-2}), anthocyanin production was lower than at high irradiance (45.9. μmol s^{-1} m^{-2}), but still much higher than in darkness.

According to M o l et al. (1996) light is the major regulatory signal in the biosynthesis of anthocyanins. Anthocyanin synthesis is directly regulated by phytochrome (M a n c i n e l l y, 1985) and many enzymes in the pathway of anthocyanins biosynthesis require activation by light. The ability of certain species to synthesize anthocyanins in darkness is not unusual. Thus, there are varieties of potato which can produce and accumulate anthocyanins in darkness (L e w i s et al., 1998). It is well known that in callus tissues sporadic mutations may appear in which cells attain the ability to synthetize anthocyanins in darkness (N a k a m u r a et al., 1999). In carob, anthocyanin production in darkness obviously is not

completely arrested and it is stimulated by increased carbohydrate nutrition.

The osmotic effect of carbohydrate nutrition on the anthocyanin production was demonstrated in two separate sets of experiments. First it was shown that anthocyanin production progressively increases with sucrose even though the concentrations applied are supraoptimal and suppress the growth of shoot cultures. Further experiments showed that sorbitol, which is not a nutritional media component, but rather an osmotic one, also significantly stimulates anthocyanin production. We thus confirmed the osmotic effect of carbohydrates on anthocyanin production in plant cell and suspension cultures (D o and C o r m i e r, 1990).

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УТИЦАЈ УГЉЕНИХ ХИДРАТА НА АКУМУЛАЦИЈУ АНТОЦИЈАНИНА У КУЛТУРИ ИЗДАНАКА РОГАЧА (*CERATONIA SILIQUA* L.) ГАЈЕНИХ НА СВЕТЛОСТИ И У МРАКУ

БРАНКА ВИНТЕРХАЛТЕР, СЛАВИЦА НИНКОВИЋ, БРАНКА КОЗОМАРА и Д. ВИНТЕРХАЛТЕР

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Продукција антоцијанина истраживана је у културама изданака рогача на високом (45.9 μmol s^{-1} m^{-2}) и ниском (9.2 μmol s^{-1} m^{-2}) интензитету светлости и у мраку под утицајем исхране угљеним хидратима. Продукција антоцијанина коју је стимулисала светлост одвијала се и у етиолираним културама изданака у мраку. Антоцијанини су били присутни у листовима и у врховима изданака. Главни фактор који је утицао на продукцију антоцијанија била је исхрана угљеним хидратима а сахароза је била супериорна у односу на глукозу и фруктозу. Ефекат угљених хидрата био је јасно осмотске природе јер се продукција антоцијанина повећавала и на супраоптималним концентрацијама које су биле штетне за растење култура изданака. Овај закључак је додатно потврђен у огледима у којима је сахароза делимично замењена шећерним алкохолима сорбитолом и манитолом.