Arch. Biol. Sci., Belgrade, 59 (3), 217-222, 2007. DOI:10.2298/ABS0703217V

EFFECT OF NITROGEN SALTS ON THE GROWTH OF *CERATONIA SILIQUA* L. SHOOT CULTURES

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Abstract – Effects of reduced nitrogen salt nutriton on the growth, lenticel hypertrophy and anthocyanin accumulation of carob (*Ceratonia siliqua* L.) shoot cultures were investigated in conditions of light and darkness. Growth of shoot cultures was not significantly affected until nitrogen salts were reduced to less than ¼ of full-strength MS (M u r a s h i g e and S k o o g, 1962) values. Cultures in darkness were less affected and their main shoots even increased in length. Appearance of hypertrophied lenticels in light decreased, while in darkness they were absent in all treatments. Reduced nitrogen salt nutrition strongly affected anthocyanin accumulation of shoots and leaves, which greatly increased in both light and darkness.

Key words: In vitro culture, mineral nutrition, lenticel hypertrophy, anthocyanins

UDC 581.133.1 : 634.46 : 57.08

INTRODUCTION

Nitrogen is an essential element in modern mineral salt formulations and is present in the form of both nitrate (NO_3^-) and ammonium (NH_4^+) ions. Murashige and Skoog medium (M u r a s h i g e and S k o o g, 1962) contains two nitrogen salts, ammonium nitrate (~20mM) and potassium nitrate (~20 mM), giving a total of ~60 mM for both nitrogen forms. This is considered to be a fairly high nitrogen concentration, supraoptimal for many woody species. For instance, the well-known "woody plant medium" formulated by L l o y d and Mc C o w n (1981) contains roughly four times less nitrogen than in MS medium. The first synthetic mineral media like Knop's and Hoagland's contained nitrogen only in the form of nitrate. The importance of ammonium as a mineral media constituent was demonstrated for the first time by G a m b o r g (1968). Gamborg's B5 medium contains only 1 mM ammonium in comparison to 20 times higher concentration in MS medium. However, plants containing ammonia as the sole source of nitrogen do not grow well and are subject to media acidification (K i r k b y, 1968).

In general, the lack of nitrogen results in decreased and malformed development of plants. T r e w a v a s (1983) compiled numerous observations which enabled him to propose a regulatory role for nitrogen nutrition. Similarly, P r e e c e (1995) compiled observations showing that inorganic nutrition can partly replace plant growth regulators.

The importance of nitrogen nutrition for the development of carob trees was pointed out by C o r e l l i a and M a r t i n s L o u c a o (1993). C r u z et al. (1993 a, b, c, d, e, f) studied the mineral uptake and transport of nitrate and ammonium ions in carob seedlings. Their major finding was that nitrogen in the form of ammonium ions was a much better choice than nitrate for overall plant development. In plants supplied with only one nitrogen form, ammonium uptake was much higher than nitrate uptake. Also, the presence of ammonium stimulated uptake of nitrate, while the presence of nitrate ion inhibited the uptake of ammonium ions. Under conditions of *in vitro* culture, decreased nitrogen nutrition was shown to affect leaf size in carob (V i n t e r h a l t e r et al. 2001).

MATERIALS AND METHODS

Carob shoot cultures were obtained and cultured according to previously published procedures (V i n t e r h a l t e r and V i n t e r h a l t e r, 1992). Cultures were maintained on MS medium supplemented with 2 % su-

crose, 0.64 % agar, BA 0.5 *mg* L^{-1}, and IBA 0.1 *mg* L^{-1}. Culture vessels were 100 mL wide-neck Erlenmeyer flasks stopped with cotton wool plugs permitting good aeration of cultures. Each flask had five shoot explants 18 – 20 *mm* long. Each treatment consisted of 5 – 6 flasks with a total of 30 – 36 shoot explants per treatment. Treatments were replicated 3 – 5 times. Subculture duration was 35 days. The light regime was 16 h of light to 8 h of darkness at a temperature of 25 ± 2^0C, with irradiance of 45.5 μmol s^{-1} m^{-2} from cool white fluorescent lamps followed by total darkness. Treatments contained MS inorganics with nitrogen salt concentrations adjusted to 1, 1/2, 1/3, 1/10 and 1/20 of the full-strength MS inorganic salt formulations which are here designated as MS N1/1, MS N1/2, MS N1/4, MS N1/10 and MS N1/20, respectively.

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

RESULTS

Growth parameters

The effect of reduced nitrogen nutrition on standard growth parameters of carob shoot cultures is presented in Table 1 for light and in Table 2 for treatments in darkness. In light, reduction of nitrogen nutrition decreased all growth parameters. Such decrease was gradual and in MS N1/4 reached ~20 % for all growth parameters. On the medium with the lowest nitrogen content (MS N1/20), length of the main shoot decreased only ~30%, while the decrease of other parameters was prominent, 4.3-fold for the length of axillary shoots, 3-fold for the multiplication index and 2.1-fold for the number of active nodes producing axillary buds.

In dark treatments the multiplication index on MS N1/4 also decreased by ~20%, as in light. The number of active nodes producing axillary shoots and the length of axillary shoots were the same as on MS N1/1 (full-strength MS). However, length of the main shoot increased by ~25 %. When the nitrogen content in darkness was further reduced to MS N1/20, the multiplication index decreased by 45%, length of axillary buds decreased by 69%, the number of active nodes producing axillary buds remained the same, and length of the main shoot increased by 50%. It is interesting to note that on MS N1/1 and MS N1/2, the main shoots of explants did not elongate at all. On these media, the only growth was by the greatly elongated axillary shoots. Decrease of nitrogen nutrition apparently stimulated elongation of the main shoot and depressed that of axillary shoots.

It is apparent that reduced nitrogen nutrition in general decreases growth parameters of carob shoot cultures. However, this decrease for shoot elongation was less than what we expected.

Anthocyanin accumulation

The effect of reduced nitrogen nutrition on anthocyanin accumulation is presented in Table 3. In shoots, anthocyanin accumulation was strongly stimulated in both light and darkness, reaching the highest values on MS N1/20. Anthocyanin production in dark-grown etiolated shoots, although much lower than in light was more prominent due to the absence of masking chlorophyll pigments.

In leaves, the highest anthocyanin content in light was registered on MS N1/4 and then declined with reduced nitrogen nutrition. In darkness, shoots are etiolated and leaves fail to develop.

The effect of various types of nitrogen salts on anthocyanin accumulation is presented in Table 4. The data presented here indicate that on MS N1/10, there were no significant differences between various types of nitrogen salts and the NO_3^-/NH_4^+ ratio. It follows that nitrogen nutrition affects anthocyanin production via the total concentration of nitrogen and not through the ionic form and accompanying anions and cations.

Lenticel hypertrophy

Lenticel hypertrophy was studied in shoots cultured in light (Fig. 1), since in darkness hypertrophied lenticels were absent regardless of media composition. Reduced nitrogen nutrition decreased the appearance of hypertrophied lenticels on the three basal internodes. On MS N1/4 and media with lower nitrogen content, hypertrophied lenticels did not develop on the third internode and their

Table 1. Effect of decreased nitrogen nutrition on standard growth parameters of carob shoot cultures in light. Within each column means followed by the same letter were not significantly different according to the LSD multiple range test at $p \leq 0.05$.

Nitrogenous salts of MS (conc.)	Multiplication index ± SE	Length of main shoot, mm± SE	No. of nodes ± SE	No. of active lateral nodes ± SE	Length of axillary buds ±SE
1/1	5.0 ± 0.2c	30.4 ± 1.0c	6.1 ± 0.2 c	3.4 ± 0.1c	12.0 ± 0.7d
1/2	4.7 ± 0.2c	28.7 ± 0.9c	5.8 ± 0.2 c	3.3 ± 0.1c	9.2 ± 0.6c
1/4	4.7 ± 0.2c	24.3 ± 0.9b	5.2 ± 0.2 b	3.1 ± 0.1b	5.7 ± 0.3b
1/10	3.6 ± 0.1b	22.1 ± 0.6ab	4.7 ± 0.2 ab	2.8 ± 0.1b	3.2 ± 0.2a
1/20	1.7 ± 0.2a	19.9 ± 0.6a	4.4 ± 0.1 a	1.6 ± 0.2a	2.8 ± 0.2a

numbers on the first two internodes decrease. A decrease was apparent in the number of internodes with high lenticel hypertrophy (10 or more hypertrophied lenticels per internode).

DISCUSSION

Reduced nitrogen nutrition (nitrogen deficiency) in plants generally results in stunted growth. It affects biosynthesis of proteins and nucleic acids in cells of meristematic tissues. Nitrogen deficiency is often accompanied by leaf necrosis, formation of slender woody shoots, and increased production of anthocyanins.

In this study, we showed that reduced nitrogen nutrition manifests distinct morphogenetic effects in carob shoot cultures. Most prominent of these is stimulation of elongation of the main shoot in etiolated cultures. We previously showed (V i n t e r h a l t e r and V i n t e r h a l t e r, 2003) that both shoot elongation and multiplication are significantly stimulated in etiolated shoot cultures. This stimulation was directly dependent on carbohydrate (sucrose) nutrition. We have now demonstrated that apart from sucrose, reduction of nitrogen nutrition also participates in the elongation of etiolated carob shoots.

A second important finding reported here is that the nitrogen salts of MS medium are supraoptimal for carob shoot cultures. Both in light and darkness, cultures could be successfully maintained on media with two to four times lower content of nitrogen salts. We could not confirm that one of the nitrogen forms (ammonium or nitrate) is preferred by cultures or that they in any way affect standard growth parameters.

Stimulated anthocyanin production as a consequence of reduced nitrogen nutrition is well known from the literature (V i n t e r h a l t e r et al., 2007) and was

Table 2. Effect of decreased nitrogen nutrition on standard growth parameters of carob shoot cultures in darkness. Within each column, means followed by the same letter were not significantly different according to the LSD multiple range test at $p \leq 0.05$.

Nitrogen salts of MS (conc.)	Multiplication index ± SE	Length of main shoot, mm± SE	No. of active lateral nodes ± SE	Length of axillary buds ± SE
1/1	10.5 ± 0.4d	20.3 ± 1.2a	2.7 ± 0.1a	29.6 ± 1.2c
1/2	9.4 ± 0.3c	19.2 ± 1.3a	2.7 ± 0.1a	32.2 ± 1.2c
1/4	8.4 ± 0.3b	25.5 ± 2.5b	2.8 ± 0.1a	29.7 ± 1.7c
1/10	6.3 ± 0.3a	31.0 ± 2.2c	2.6 ± 0.1a	20.7 ± 1.6b
1/20	5.8 ± 0.3a	30.6 ± 2.5bc	2.8 ± 0.1a	9.2 ± 0.9a

Table 3. Effect of reduced nitrogen salts nutrition on accumulation of anthocyanins in shoots and leaves in light and darkness. Within each column, means followed by the same letter were not significantly different according to the LSD multiple range test at $p \leq 0.05$.

Nitrogen salts of MS (conc.)	Anthocyanins, *mg* g^{-1} fresh weight ± SE		
	Leaves	Shoots	
		light	darkness
1/1	0.23 ± 0.04 a	0.17 ± 0.01 a	0.02 ± 0.002 a
1/2	0.31 ± 0.02 a	0.24 ± 0.01 a	0.02 ± 0.004 a
1/4	0.43 ± 0.06 b	0.59 ± 0.09 bc	0.04 ± 0.008 a
1/10	0.32 ± 0.03 ab	0.52 ± 0.06 b	0.10 ± 0.02 b
1/20	0.20 ±0.01 ab	0.75 ± 0.01 c	0.25 ±0.02 c

therefore expected. Such findings were registered in many plant species and different model systems including cell suspension and callus cultures (Mizukami et al., 1993; Sakamoto et al., 1993, 1994) and shoot cultures (Cordts et al., 1987).

Finally, the observation that nitrogen nutrition affects lenticel hypertrophy is also new. Formation of hypertrophied lenticels in carob shoot cultures has been studied in detail as a rare physiological disorder (Vinterhalter et al., 1992; Vinterhalter and Vinterhalter, 1992) caused by a number factors, including aeration of culture vessels, cytokinin content of the medium, and light. We also demonstrated previously that increased sucrose nutrition efficiently prevents lenticel hypertrophy in carob shoots (Vinterhalter and Vinterhalter, 2003).

We have now established three different processes in carob shoot cultures which are jointly regulated by su-

Table 4. Effect of various nitrogen salts on accumulation of anthocyanins in light and darkness. Within each column, means followed by the same letter were not significantly different according to the LSD multiple range test at $p \leq 0.05$.

Nitrogen salts	Concentration mM	NO_3^-/NH_4^+ Ratio	Anthocyanin content, *mg* g^{-1} fresh weight ± SE	
			light	darkness
NH_4NO_3 KNO_3	20 20	40 : 20	0.17 ± 0.01 a	0.02 ± 0.002 a
NH_4NO_3 KNO_3	2 2	4 : 2	0.52 ± 0.06 cd	0.10± 0.02 e
KNO_3 $(NH_4)_2SO_4$	2 2	2 : 4	0.47 ± 0.11 bcd	0.04 ± 0.01 ab
$(NH_4)_2SO_4$	3	0 : 6	0.38 ± 0.06 b	0.09 ± 0.01 cde
NH_4Cl_2	6	0 : 6	0.39 ± 0.06 bc	0.07 ± 0.01 bcde
$(NH_4)_2HPO_4$	3	0 : 6	0.53 ± 0.06 bcd	0.06 ± 0.002 bcd
$NH_4H_2PO_4$	6	0 : 6	0.45 ± 0.08 bcd	0.08 ± 0.02 bcde
NH_4NO_3	3	3 : 3	0.62 ± 0.09 d	0.05 ± 0.004 abc
KNO_3	6	6 : 0	0.47 ± 0.08 bcd	0.09 ±0.02 de

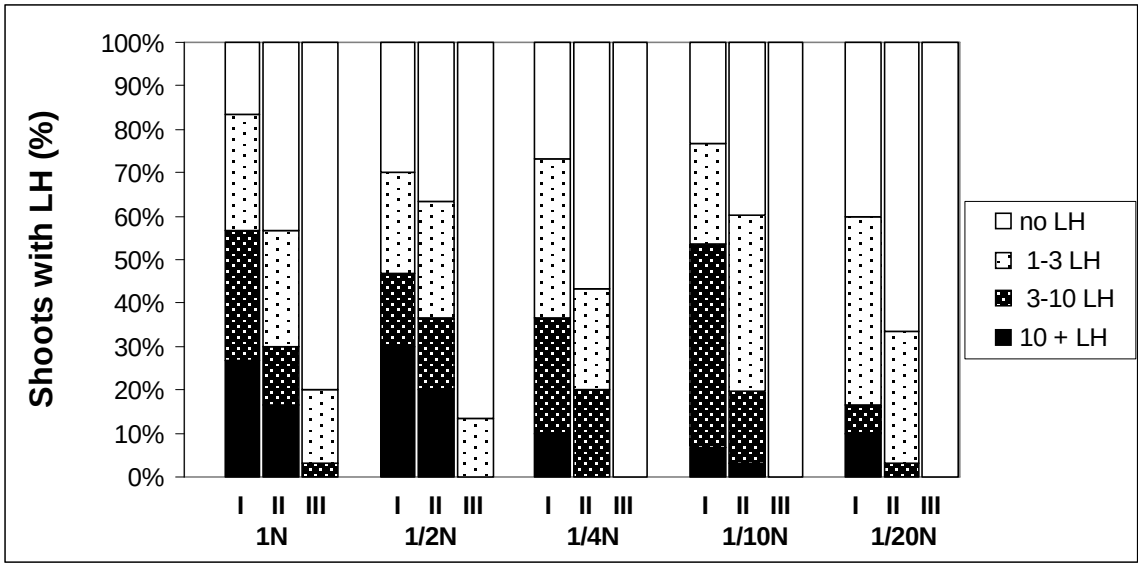

Fig. 1. Frequency of hypertrophied lenticel formation on the three basal internodes of shoots cultured on media with reduced nitrogen content**.**

crose and nitrogen nutrition. They are: elongation of the main shoot in etiolated cultures, anthocyanin accumulation, and prevention of lenticel hypertrophy. In all three instances, the relationship between sucrose and nitrogen nutrition is inverse, meaning that nitrogen salts will be inhibitory in processes found to be stimulated by increased sucrose nutrition.

Acknowledgment – This work was supported by the Serbian Ministry of Science and Environment Protection (Grant No. 143026B). The authors thank Prof. Dr. Bato Korać for help in quantitative determination of anthocyanins.

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УТИЦАЈ АЗОТНИХ СОЛИ НА РАСТЕЊЕ КУЛТУРА ИЗДАНАКА РОГАЧА (*CERATONIA SILIQUA* L.)

БРАНКА ВИНТЕРХАЛТЕР, СЛАВИЦА НИНКОВИЋ, СНЕЖАНА ЗДРАВКОВИЋ-КОРАЋ, АНГЕЛИНА СУБОТИЋ и Д. ВИНТЕРХАЛТЕР

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Код култура изданака рогача испитивани су ефекти смањења концентрације азотних соли у подлози MS минералног раствора на мултипликацију и издуживање изданака, хипертрофију лентицела и синтезу и акумулацију антоцијанина на светлости и у мраку. Смањење концентрације N у подлози битно мења параметре растења изданака (мултипликацију и издуживање) тек на ¼ (на светлости) тј. 1/10 (у мраку). Хипертрофиране лентицеле се не развијају на етиолираним изданцима гајеним у мраку док су код изданака гајеним на светлости бројне. Смањењем концентрације N у подлози број HL се смањује као и њихово акропетално простирање. Синтеза и акумулација антоцијанина у изданцима и листовима рогача била је директно зависна од концентрације N у подлози и на светлости и у мраку.