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EFFECT OF DIFFERENT SUCROSE AND NITROGEN LEVELS IN THE MEDIUM ON CHLOROPHYLL AND ANTHOCYANIN CONTENT IN *Clematis pitcheri* SHOOTS CULTURED *IN VITRO* AT DIFFERENT TEMPERATURES

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A B S T R A C T

The aim of the work presented was to determine the chlorophyll and anthocyanin accumulation in *Clematis pitcheri* shoots cultured *in vitro* at different temperatures on the medium with various sucrose and nitrogen level. Two concentrations of sucrose: 10 $g \cdot l^{-1}$ and 30 $g \cdot l^{-1}$, and two levels of nitrogen compounds: 100% and 50% of standard MS strength, were used. Shoots were cultured at 15°C, 20°C and 25°C. It was found that plantlets of *C. pitcheri* grown at 20°C contained the highest and at 15°C the lowest content of chlorophyll. The sucrose concentration in the medium had no or only a slight effect. Lower level of nitrogen compounds (50% N) stimulated accumulation of chlorophyll in shoots as compared to a normal strength (100% N), with the highest differences at 15°C. High sucrose (30 $g \cdot l^{-1}$) and nitrogen (100% N) concentrations and low temperature (15°C) significantly promoted anthocyanins accumulation. Reduction of nitrogen compounds level in the medium to 50% and lowering sucrose concentration to 10 $g \cdot l^{-1}$ leads to decrease of anthocyanins accumulation in the shoots cultured at 15°C and 20°C. In the case of explants cultured at 25°C, the sucrose and nitrogen concentration in the medium had no or only a slight effect on accumulation of anthocyanins in the shoots.

Key words: *Clematis pitcheri*, *in vitro*, nitrogen compounds, sucrose, anthocyanins, chlorophyll, temperature

INTRODUCTION

Clematis plants are propagated mainly by stem cuttings. However, similarly as in many other species and cultivars, there is a problem with poor rooting (Erwin et al., 1997). Studies on *Clematis* micropropagation were conducted previously (e.g. Lees et al., 1991b; Kreen et al., 2002) but they are not numerous. Mandegaran and Sieber (2000) described the possibility of somatic embryogenesis use for *C. integrifolia* x *C. viticella* and Luttman et al. (1994) for *C. tangutica* micropropagation. Dąbski and Parzymies (2006) investigated the influence of cytokinins on the proliferation of *C. integrifolia.* Nevertheless, due to the problems with apical dominance and/or with phenotype stability in plants propagated *via* callus, *in vitro* methods need additional studies.

In plant propagation *via* tissue culture the micropropagation efficiency and the quality of microcuttings are important. Because of these reasons, the composition of media and conditions of culture are very important and often specific for species and cultivars. For example, different forms and concentrations of nitrogen in the culture media may influence cell division, differentiation, growth and development, as well as the endogenous level of metabolites, proteins, organic acids, plant hormones and chlorophyll content, nitrate reductase and Rubisco activity, electron transport rate, photosynthetic rate and anthocyanin production (Mordhorst and Lorz, 1993; Preece, 1995; Gniazdowska-Skoczek, 1998; Guidi et al., 1998; Jain et al., 1999; Sotiropoulos et al., 2005; Ogura-Tsujita and Okubo, 2006). Several authors have suggested that some carbohydrate sources and concentrations in the culture medium may reduce the photosynthetic ability of plantlets cultured *in vitro* (Lees et al., 1991b; Fuentes et al., 2005).

The aim of the work presented was to determine the effect of sucrose and nitrogen concentrations in the medium on the chlorophyll and anthocyanins accumulation in *Clematis pitcheri* shoots cultured *in vitro* at different temperatures.

MATERIAL AND METHODS

The apical shoot explants (2-3 nodes) derived from *in vitro* grown *Clematis pitcheri* plants were placed on Murashige and Skoog's (1962) medium (MS) supplemented with 0.2 $mg \cdot l^{-1}$ m-Topolin, 10 $g \cdot l^{-1}$ and 30 $g \cdot l^{-1}$ sucrose and two levels of N-sources – KNO_3 and NH_4NO_3 (100% and 50% of standard MS strength). For that, 1.90 g KNO_3 and 1.65 g NH_4NO_3 (100% N) or 0.950 g KNO_3 and 0.825 g NH_4NO_3 (50% N) were used for 1 litre of MS medium. In each treatment 6 jars x 7 explants were used. After 8 weeks of growth at the temperatures of 15°C, 20°C or 25°C, plantlets from 1 jar were taken for chlorophyll analysis. The chlorophyll content was measured and calculated by the method of Bruinsma (1963). The fresh samples were extracted with acetone and the chlorophyll concentration in the extract was determined spectrophotometrically at

665, 652 and 645 nm. Anthocyanins content was determined using the modified Mancinelli et al. (1988) method. Plantlets collected from 5 jars were weighted and lyophilized. Dried samples were extracted at 4°C in 1% HCl in methanol (v/v) for 24 h in darkness. The extract was centrifuged for 10 min at 9000 rpm. The total content of anthocyanins was measured spectrophotometrically as the difference between the absorbance at 530 nm (peak of absorption of anthocyanin) and 657 nm. The formula A_{530}-0.25A_{657} was used to compensate for the absorption of chlorophyll degradation products at 530 nm. Results were expressed as milligrams of cyanidin-3-glycoside equivalent per gram of lyophilized sample.

All analysis were performed in 4 replicates. The data were subjected to an analysis of variance and Duncan's multiple range test was used for means separation at $p = 0.05$.

RESULTS AND DISCUSSION

In the previous reports it was showed that sucrose at concentrations 10 and 30 $g \cdot l^{-1}$ and nitrogen compounds at concentrations equal to 100% and 50% of standard MS strength strongly influenced the growth and development of *Clematis pitcheri* shoots cultured *in vitro*. The effect was also temperature-dependent. The highest number of axillary shoots was obtained on the explants growing at 25°C in the presence of lower level of nitrogen compounds (50% N) and sucrose at 10 $g \cdot l^{-1}$. The highest elongation of shoots was found on the medium with 10 $g \cdot l^{-1}$ sucrose, 100% N and at 20°C and 25°C. High concentration of sucrose inhibited the formation and elongation of axillary shoots. The shoots cultured at 20°C and 25°C had higher fresh mass and accumulated more carbohydrates per unit of dry matter than the explants cultured at 15°C (Gabryszewska et al., 2008; Kawa-Miszczak et al., 2008).

In the present study it was found that plantlets of *C. pitcheri* grown at 20°C contained the highest and at 15°C the lowest content of chlorophyll (Tab. 1). The sucrose concentration in the medium had no or only a slight effect. Lower level of nitrogen compounds in the medium (50% N) stimulated accumulation of chlorophyll in the shoots as compared to a normal strength (100% N), with the highest differences at 15°C (Tab. 1). On the other hand, shoots grown at 15°C produced more anthocyanins (Fig. 1). High sucrose (30 $g \cdot l^{-1}$) and nitrogen (100% N) concentrations and low temperature (15°C) significantly promoted anthocyanins accumulation. Reduction of nitrogen compounds level in the medium to 50% and lowering of sucrose concentration to 10 $g \cdot l^{-1}$ lead to some decrease of anthocyanins accumulation in the case of shoots cultured at 15°C and 20°C. However, in the case of shoots cultured at 25°C the sucrose and nitrogen concentration in the medium had no or only a slight effect on anthocyanins accumulation (Fig. 1).

Anthocyanins are pigments that are widely distributed in plant species. In studies on the regulation

Table 1. Chlorophyll content in *Clematis pitcheri* shoots depending on sucrose and nitrogen compounds concentration in MS medium

Sucrose [g·l^{-1}]	Nitrogen compounds [%]	Temperature [°C]			Mean
		15	20	25	
chlorophyll a [µg·100 mg^{-1} f.w.]					
10	100	65.6	84.0	78.2	75.9 a*
	50	85.7	86.5	81.2	84.5 b
30	100	65.8	81.7	72.8	73.4 a
	50	85.6	89.6	86.2	87.2 b
Mean		75.7 a**	85.5 b	79.6 a	
chlorophyll b [µg·100 mg^{-1} f.w.]					
10	100	22.3	43.5	33.4	33.1 ab
	50	29.8	48.3	33.8	37.3 b
30	100	22.4	36.5	29.2	29.4 a
	50	29.4	46.1	38.2	37.9 b
Mean		26.0 a**	43.6 c	33.7 b	
chlorophyll a + b [µg·100 mg^{-1} f.w.]					
10	100	85.6	132.1	113.0	110.3 a
	50	113.2	141.2	115.6	123.3 b
30	100	85.8	118.6	104.1	102.8 a
	50	112.1	140.5	125.0	125.9 b
Mean		99.2 a**	133.1 c	114.4 b	

*Means in columns and **in rows marked with the same letters are not significantly different according to Duncan's test at p = 0.05, separately for chlorophyll a, b and a + b

of secondary metabolism in cell and plant tissue culture, it has been observed that the cultures turned red due to the formation of anthocyanins upon the influence of some environmental conditions – temperature, phosphate, mineral nitrogen, phenylalanine and sucrose, with light as an essential stimulus (Piovan and Filippini, 2007). Increased sucrose concentration and low temperature were found to significantly enhance anthocyanin production by cell suspension cultures of many plants. Sugar types also affect anthocyanin accumulation. In the culture of *Prunus persica,* sucrose was more effective as carbon source than glucose, fructose or starch under a given nitrogen level (Cordts et al., 1987). In strawberry cell cultures, anthocyanin accumulation and cell growth were enhanced by glucose, sucrose and fructose (Mori and Sakurai, 1994). Also, increasing sucrose concentration was found to

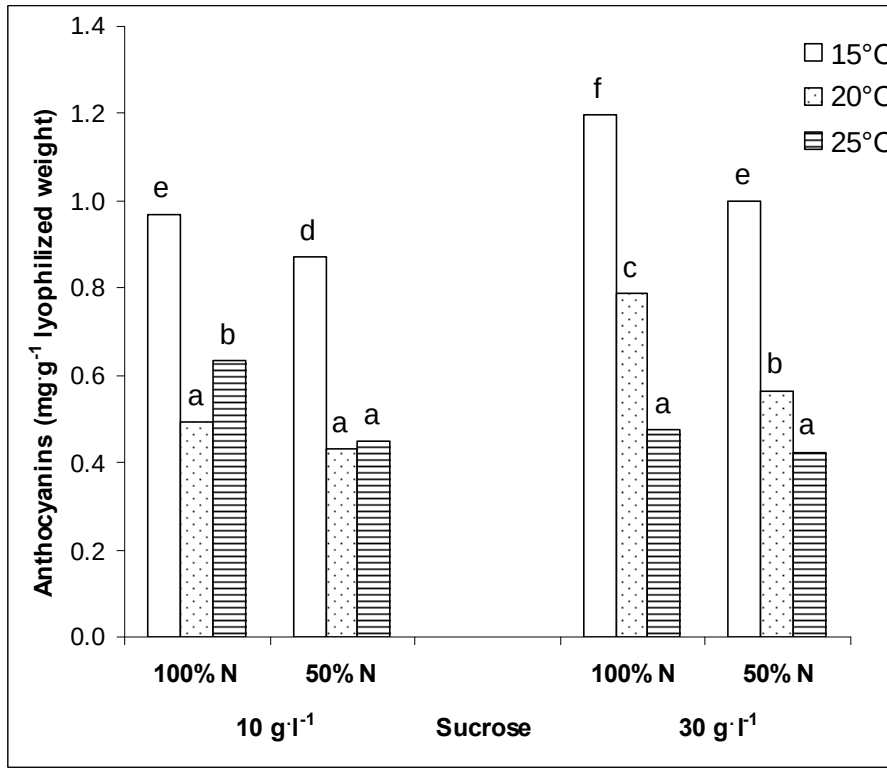

Figure 1. Anthocyanins content (expressed as cyanidin-3-glucoside equivalents) in *Clematis pitcheri* shoots depending on sucrose and nitrogen compounds concentration in MS medium; means marked with the same letter are not different according to Duncan's test at p = 0.05

enhance significantly anthocyanin yield in cell suspension cultures of *Catharanthus roseus* (Knobloch et al., 1982), *Aralia cordata* (Sakamoto et al., 1993), *Vaccinium pahalae* (Smith et al., 1997), *Vitis vinifera* (Decendit and Merillon, 1996), *Fragaria ananassa* (Mori and Sakurai, 1994) and *Daucus carota* (Rajendran et al., 1992). The low temperature stimulated anthocyanin production but reduced cell growth of *Perilla frutescens* (Zhong and Yoshida, 1993) and strawberry (Zhang et al., 1997). On the other hand, nitrate limitation has been shown to promote the accumulation of anthocyanins in cell cultures of *Catharanthus roseus* (Knobloch et al., 1982), *Euphorbia millii* (Yamamoto et al., 1989), *Fragaria ananassa* (Mori and Sakurai, 1994), *Aralia cordata* (Sakamoto et al., 1993) and *Daucus carota* (Rajendran et al., 1992). A medium with a relatively low nitrogen and high sucrose was most effective in stimulating anthocyanin production in peach leaves cultured *in vitro*

(Cordts et al., 1987). Growth of *Arabidopsis* seedlings on low nitrogen (0.1 mM) results in a significant reduction of chlorophyll and anthocyanin content (Martin et al., 2002). The addition of 100 mM sucrose to the nitrogen-depleted media results in a further reduction of chlorophyll content and an overall increase in anthocyanins. Repression of photosynthetic genes by carbohydrates can only be observed under low nitrogen conditions. Low nitrogen (0.1 mM) in the absence of exogenous carbohydrates results in a significant decrease in chlorophyll a/b-binding protein (Martin et al., 2002).

In our experiments, results related to anthocyanins accumulation in *Clematis pitcheri* shoots grown *in vitro* are partially different. Low temperature and high sucrose concentration enhanced anthocyanin production, but nitrate limitation lead to decrease of anthocyanins accumulation in the shoots (Fig. 1).

Increased carbohydrate levels results in inhibition of photosynthesis and a decrease in chlorophyll content (Smeekens, 2000). In plant micropropagation, acclimatization to greenhouse conditions was more successful for plants which exhibited higher photosynthetic activity *in vitro* prior to transfer. Compared to the mature plant of *Clematis*, the *in vitro* system exhibits a considerable degree of pigment disorganization (Lees et al., 1991a). Lees et al. (1991b) studied the photosynthesis in *Clematis* x *patens* 'The President' during growth *in vitro* and subsequent *in vivo* acclimatization. The authors concluded that sucrose in the micropropagation medium inhibits photosynthesis *in vitro* and the use of glucose reduces the level of inhibition, and that photosynthetic competence affects subsequent survival in the nursery. It should be mentioned that Lees et al. (1991b) used only sucrose (30 $g \cdot l^{-1}$) for multiplication medium while rooting medium contained various sucrose (20 $g \cdot l^{-1}$ and 40 $g \cdot l^{-1}$) and glucose (10 $g \cdot l^{-1}$ and 20 $g \cdot l^{-1}$) concentrations. Nitrogen concentration, its forms and their proportion may also influence cell and plant growth and development *in vitro* and chlorophyll content (Guidi et al., 1998; Jain et al., 1999). Sotiropoulos et al. (2005) found that chlorophyll content in apple rootstock cultured *in vitro* was significantly increased in explants treated with KNO_3 + NH_4NO_3 in comparison to other treatments (KNO_3, NH_4NO_3, $NH_4H_2PO_4$, L-alanine).

In our experiments, we did not observe any effects of sucrose concentration in multiplication medium (10 $g \cdot l^{-1}$ and 30 $g \cdot l^{-1}$) on chlorophyll content in shoots, but chlorophyll accumulation was enhanced after lowering of nitrogen compounds level to 50% of standard MS medium strength (Tab. 1).

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WPŁYW TEMPERATURY ORAZ ZRÓŻNICOWANEGO POZIOMU SACHAROZY I AZOTU W POŻYWCE NA ZAWARTOŚĆ CHLOROFILU I ANTOCYJANÓW W PĘDACH *Clematis pitcheri* ROSNĄCYCH *IN VITRO*

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S T R E S Z C Z E N I E

Celem pracy było oznaczenie zawartości chlorofilu i antocyjanów w pędach *Clematis pitcheri* rosnących *in vitro* w zależności od temperatury oraz poziomu sacharozy i azotu w pożywce. Zastosowano dwa stężenia sacharozy – 10 $g \cdot l^{-1}$ i 30 $g \cdot l^{-1}$ oraz dwa poziomy związków azotowych – 100% i 50% standardowego składu pożywki MS. Kultury prowadzono w 15°C, 20°C i 25°C. Stwierdzono, że zawartość chlorofilu była najwyższa w pędach rosnących w 20°C, a najniższa w 15°C. Stężenie sacharozy w pożywce miało niewielki wpływ na zawartość chlorofilu w pędach. Natomiast obniżenie poziomu związków azotowych (50% N) stymulowało akumulację chlorofilu w pędach w porównaniu z normalnym poziomem soli azotowych (100% N), a największe różnice były w pędach rosnących w 15°C. Wyższe stężenie sacharozy (30 $g \cdot l^{-1}$) i azotu (100% N) w pożywce oraz niska temperatura (15°C) zwiększały akumulację antocyjanów. Zmniejszenie poziomu związków azotowych do 50% standardowego składu pożywki MS oraz niskie stężenie sacharozy (10 $g \cdot l^{-1}$) prowadziło do obniżenia zawartości antocyjanów w pędach rosnących w 15°C i 20°C. W przypadku eksplantatów rosnących w 25°C, stężenie sacharozy i azotu w pożywce nie wpływało lub miało niewielki wpływ na akumulację antocyjanów w pędach.

Słowa kluczowe: *Clematis pitcheri*, *in vitro*, związki azotowe, sacharoza, antocyjany, chlorofil, temperatura