Stress Alterations in Growth Parameters, Pigment Content and Photosynthetic Functions of *in vitro* Cultured Plants

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Effects of different concentrations of glucose, sucrose and the natural growth regulator, triacontanol were studied under the unfavourable stress conditions of micropropagation of two woody plants, *Sorbus rotundifolia* L. and *Prunus* × *davidiopersica* 'Piroska'. After 4–6 weeks of cultures, the number and length of shoots, the photosynthetic activity as well as the chlorophyll, carotenoid and anthocyanin contents were investigated. As shown by the growth parameters, the optimal carbohydrate concentration was between 1.5–2.5%, whereas in higher concentrations, a definite inhibition could be observed. A similar response was found in changes of the anthocyanin content in *Prunus* × *davidiopersica* 'Piroska', but this effect was less pronounced with the photosynthetic pigments in both species. The Fv/Fm ratio representing the quantum yield of photosynthesis was low due to the inhibitory effect of stress and altered significantly by changing the carbohydrate concentrations. In all cases, the addition of 2–4 μg triacontanol/l further enhanced the stimulating effect of the optimal carbohydrate concentrations, which indicated the specific importance of the appropriate hormone balance under such stress conditions.

Introduction

The artificial environment of micropropagation (including the mechanical injury, changes of the nutrition and hormone availability, light intensity and quality, temperature, etc.) causes dramatic stress for developing plantlets. Amongst various factors, the concentration of carbohydrates and growth regulators in the culture medium play an important role in affecting the development of plantlets. For minimizing the stress effects induced by the artificial environment, optimization of the carbon source and hormone availability is specifically required.

Even sucrose or glucose, the most frequently used carbon sources have a retarding effect on growth in unfavorable concentrations. In tissue culture media for woody plants sucrose is usually used in a concentration of 3% (George, 1993), a rate that was originally recommended by Murashige and Skoog (1962) for callus culture of *Nicotiana.* In other studies the effect of various monosaccharides on the proliferation of woody plants was investigated and the "magic" 3% or very similar concentrations of carbohydrate source were applied as well (Belaizi and Boxus, 1995; Druart, 1995; Karhu, 1995). The effect of carbohydrates above or below the generally used concentration was only studied by Karhu and Ulvinen (1995) and Jámbor-Benczúr *et al.* (1996).

In micropropagation of woody plants, artificial growth regulators (benzyladenine, 2-isopentenyladenine) are generally used in the multiplication phase (Einset, 1991). Zeatin, a natural cytokinin would be the most favorable for plants, but it is seldom used as it is not economical. Therefore, it is of great importance to find natural growth regulators, which can be used to make mass propagation economical. Triacontanol, a long-chain primary alcohol which is a natural component of waxes was found to stimulate plant growth *in vivo* (Steffens and Worley, 1980; Ries, 1985; Srivastava and Sharma, 1991). Very recent work demonstrated that addition of triacontanol to the *in vitro* culture medium of *Melissa officinalis* resulted in high proliferation and significantly increased the

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chlorophyll content of the plantlets (Tantos *et al.*, 1999). Since the induction of proliferation in woody plants is difficult in most cases and the plantlets are usually pale due to the low pigment content induced by the stress condition of *in vitro* culture, triacontanol seemed to be a useful tool to overcome the above mentioned problems.

The aim of this work was therefore to optimize the concentrations of both carbon sources and triacontanol as a natural growth regulator in the micropropagation of two woody plants by monitoring alterations in their growth parameters, in their pigment content and in the photosynthetic activity characterized by fluorescence induction kinetics. Our data have clearly demonstrated that the usually applied micropropagation conditions are far from the optimum required for minimizing the harmful effect of the artificial environment.

Materials and Methods

Plant materials and growth conditions

In vitro cultures of a roadside tree, huckberry (*Sorbus rotundifolia* L. cv. 'Bükk Szépe'), and an ornamental peach tree with red leaves (*Prunus* × *davidiopersica* 'Piroska') were investigated for two years. The basal medium for *S. rotundifolia* 'Bükk Szépe' was MS medium (Murashige and Skoog, 1962) supplemented with hormones according to Molnár *et al.* (1994). *Prunus* × *davidiopersica* 'Piroska' was cultured on S-basal medium supplemented with 0.75 mg BA-ribose/l, 0.1 mg IBA/l and 0.1 mg GA_3/l (Jámbor-Benczúr and Márta-Riffer, 1990). Sucrose or glucose was added at 0.5 to 3% concentrations into the culture media of *S. rotundifolia* or *Prunus* × *davidiopersica* 'Piroska'. The combined effect of carbohydrates and triacontanol was studied on *Prunus* × *davidiopersica* 'Piroska' by supplementing the sugar-containing media with 2, 4 or 8 µg triacontanol/l. The *in vitro* cultures of both plants were illuminated with white light (Philips TLD 30W/827, F30W/33 and Tungsram 30W normal) at the intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by using 16/8 hours light/dark cycles with the day/night temperature of 22/16 °C, respectively.

Growth analysis

The number and length of the newly initiated shootlets of both *S. rotundifolia* and *Prunus* × *davidiopersica* 'Piroska' were counted on 8 explants of each treatment in 4 replications.

Determination of pigment contents

Chlorophyll (*a*+*b*) content of each plant was estimated in 80% acetone extracts as described by Arnon (1949). The concentration of carotenoids was determined according to Horváth *et al.* (1972). The anthocyanin content was measured in shoots of *Prunus* × *davidiopersica* 'Piroska' according to the method of Kho *et al.* (1975).

Photosynthesis measurements

Chlorophyll fluorescence induction kinetics were measured on huckberry plants by using a portable fluorimeter (Plant Efficiency Analyser, Hansatech). After 20 minutes of dark adaptation samples were excited with the actinic light 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of intensity at 650 nm. Fluorescence was detected for 60 s and the usually used parameter, the Fv/Fm ratio was calculated (Bolhár-Nordenkampf and Öquist, 1993). Six leaves freshly collected from a *Sorbus rotundifolia* L. cv. 'Bükk Szépe' tree in the Garden of the University were used as control samples.

Student's t-test was used for statistical evaluation of experimental data. The number of data (n), standard deviation and the level of significance are presented in the figures.

Results and Discussion

The various concentrations of different carbohydrates added to the culture medium strongly influenced the proliferation ability of *S. rotundifolia* shoots (Fig. 1). For either sucrose or glucose, the lowest and highest sugar concentrations yielded fewer shoots. Each concentration of glucose resulted in better sprouting compared to similar levels of sucrose. The highest shoot numbers were obtained at 1.5% glucose and 2% sucrose concentrations. As compared to the lowest sugar concentration longer shoots developed on the media containing 1.5 to 2.5% of both carbohydrates and even at 3% of glucose. Similar results were obtained with *Prunus* × *davidopersica* 'Piroska' where the optimal concentration of both carbohydrates was 2% (Fig. 2). In earlier work dealing with the micropropagation of different *Rosaceae*

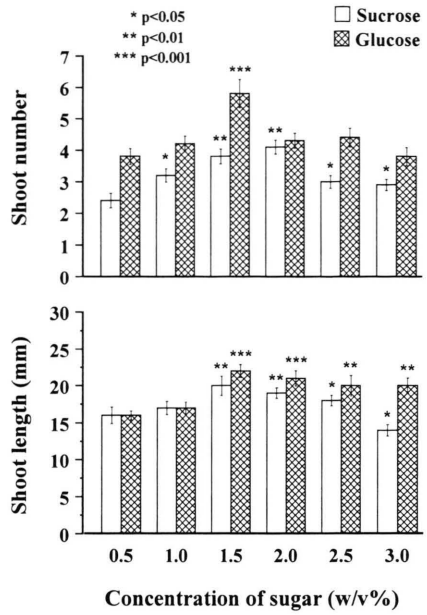

Fig. 1. Influence of increasing carbohydrate concentration of the culture media on the shoot number and length of micropropagated *Sorbus rotundifolia* (n = 32, stars indicate the level of significance at different sugar concentrations at 0.05, 0.01 and 0.001 probability).

comparing the effect of various carbohydrates, the sugar concentration was generally 3% (Druart, 1995; Karhu, 1995). Our results have clearly demonstrated that the sugar concentration used earlier for the micropropagation of *Sorbus sp.* is far from the optimum and perhaps can rather be considered as a stress agent. This conclusion agrees with the results of Molnár *et al.* (1994) and Jámbor-Benczúr *et al.* (1995) where 2% sugar was found to be the most suitable concentration for micropropagation of *S. rotundifolia* and *Prunus* × *davidopersica* 'Piroska', and higher concentrations of both sugars harmful by affecting the plantlets.

The effect of triacontanol combined with various carbohydrate concentrations is also shown in Fig. 2. At 0.5% of carbohydrates, all triacontanol concentrations applied resulted in higher yields in shoot proliferation. The highest shoot number was obtained with 2 μg/l triacontanol at 2% sugar and further elevation of the sugar or triacontanol concentrations inhibited proliferation. This agrees with an earlier finding where 2 μg triacontanol/l was found to be optimal for the micropropagation of *Melissa officinalis* (Tantos *et al.*, 1999).

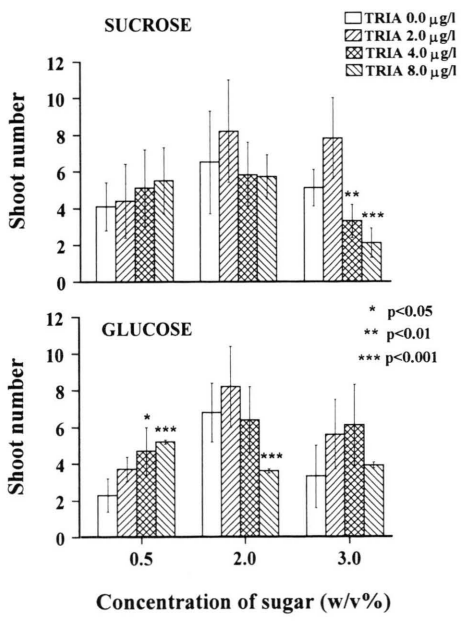

Fig. 2. Comparison of the triacontanol effect at different concentrations on the shoot number of *Prunus* × *davidiopersica* 'Piroska' (n = 32, stars indicate the level of significance at different triacontanol concentrations at 0.05, 0.01 and 0.001 probability).

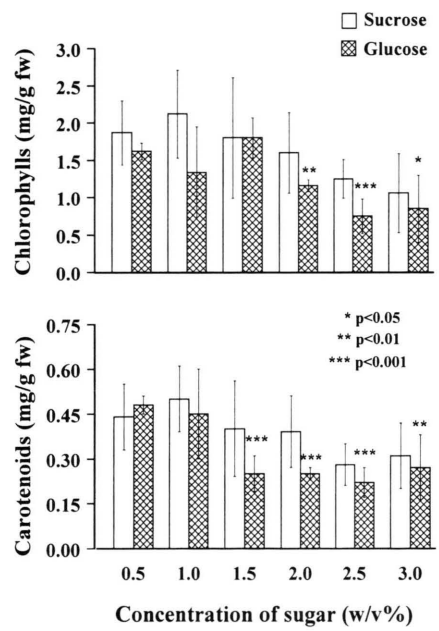

Fig. 3. Influence of different sucrose and glucose concentrations of the culture media on the photosynthetic pigment content of micropropagated *Sorbus rotundifolia* shootlets (n = 8, stars indicate the level of significance at different sugar concentrations at 0.05, 0.01 and 0.001 probability).

Photosynthetic pigment analysis of huckberry plantlets grown at different sugar concentrations indicated that with the exception of the very low concentrations (0.5–1.0%), higher amounts of sucrose or glucose in the culture media led to significant less the chlorophyll or carotenoid content of the leaves (Fig. 3). At most sugar concentrations, the chlorophyll content of plantlets was higher on sucrose than on glucose, but the high sucrose concentrations were also inhibitory to proliferation. This result is in agreement with Donelly and Vidaver (1984) who found less pigment content and photosynthesis at higher concentration of sugar in red raspberry *in vitro* and *ex vitro* cultures. Our results prove that high concentrations of both sugars can be considered as stress factors, which is very probably related to their osmotic effect (Sato *et al.*, 1996).

The combined effect of sugars and triacontanol on the chlorophyll content of shootlets of *Prunus* × *davidopersica* 'Piroska' is shown in Fig. 4. The effect of sucrose and glucose was basically similar to the result obtained with *S. rotundifolia* (c.f. Fig. 3 and Fig. 4). The lower concentrations (2 to 4 μg/l) of triacontanol could overcome the inhibitory effect of the higher sucrose concentrations. The high concentrations of triacontanol were also found to be inhibitory similar to results previously described by Tantos *et al.* (1999). The inductive effect was less pronounced at 0.5% sucrose concentrations but at 2% sucrose 2 μg triacontanol/l produced significantly higher chlorophyll content. Applying triacontanol together with glucose, however, enhanced the chlorophyll content at each glucose concentration.

Photosynthetic efficiency is usually characterized by the quantum yield of photosynthesis represented by the ratio of the maximal (Fm) and variable (Fv) components of the fluorescence induction curve. Under normal conditions, the Fv/Fm ratio is around 0.832±0.004 and its decrease is a good indicator of the manifestation of stress (Bolhár-Nordenkampf and Öquist, 1993). Regarding the Fv/Fm ratio of *in vitro* cultured *S. rotundifolia* shoots, we found significant lower Fv/Fm values as compared to that of the control leaf (Fv/Fm = 0.838). No differences were found at different glucose concentrations but it was strongly varied with changing the sucrose concentration of the medium. The overall decrease of the Fv/Fm

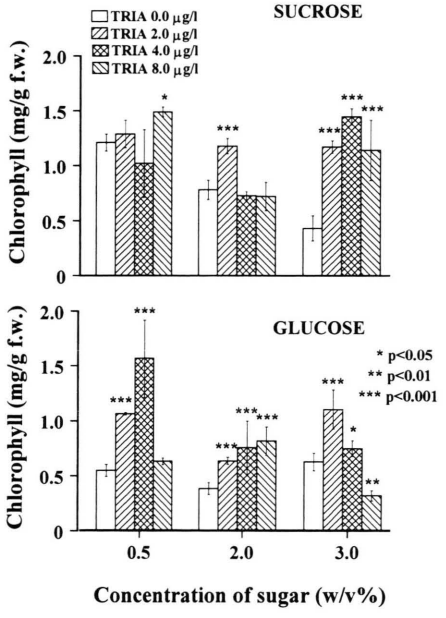

Fig. 4. Effect of various concentrations of triacontanol of the culture media on the accumulation of chlorophylls in micropropagated *Prunus* × *davidiopersica* 'Piroska' (n = 8, stars indicate the level of significance at different triacontanol concentrations at 0.05, 0.01 and 0.001 probability).

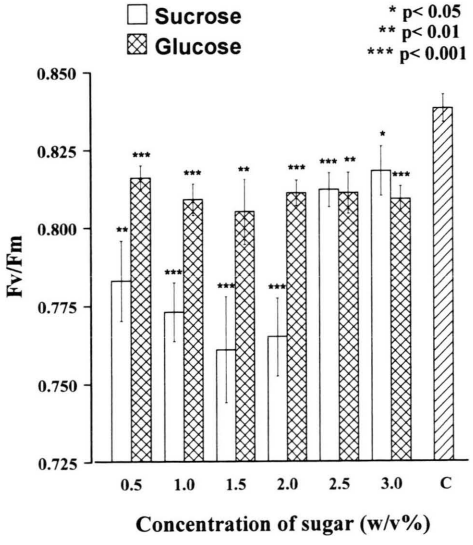

Fig. 5. The photosynthetic quantum yield of *S. rotundifolia* at various sugar concentrations as measured by chlorophyll fluorescence induction. (C – control tree leaves of the Garden, n = 12, stars indicate the level of significance at different sugar concentrations at 0.05, 0.01 and 0.001 probability).

ratio indicated the general stress effect of micropropagation (Fig. 5). Van Huylenbroeck and Debergh (1996) also experienced a 10% decrease in the Fv/Fm ratio and the photosynthetic pigment content at the beginning of sucrose treatment but after 14 days of the acclimation, the photosynthesis recovered and the pigment content increased. In contrast, Karhu and Ulvinen (1995) measured practically no decline in the Fv/Fm ratio in various sucrose media as compared to other carbohydrate treatments.

Prunus × *davidiopersica* 'Piroska' contains anthocyanin, the red pigment in its leaves. In general the increasing anthocyanin content in leaves is considered as an indicator of the appearance of stress because the activity of phenylalanine ammonia lyase increases under unfavorable conditions (Tholakalabavi *et al.*, 1997). Since anthocyanin is a natural constituent of *Prunus* × *davidiopersica* 'Piroska', the presence of red color does not mean a disadvantage for the plantlets. As shown in Fig. 6 the increasing concentrations of carbohydrates enhanced the anthocyanin accumulation in leaves which peaked at 2% of both sugars mainly in glucose. The stimulating effect of triacontanol is more pronounced in the presence of sucrose than glucose but the highest triacontanol concentration had an inhibitory effect similar to that observed with chlorophylls, shoot number and length. We assume, therefore, that the elevated anthocyanin content at the optimal concentration of carbohydrates and triacontanol represents the restoration of the normal anthocyanin synthesis, rather than a manifestation of unfavourable stress conditions.

This work demonstrates that for *Sorbus rotundifolia* and *Prunus* × *davidiopersica* 'Piroska' the inhibitory effect of stress induced by micropropagation can be reduced by optimizing the concentration of carbohydrate source and the conditions can further be improved by adding triacontanol to the culture.

Acknowledgement

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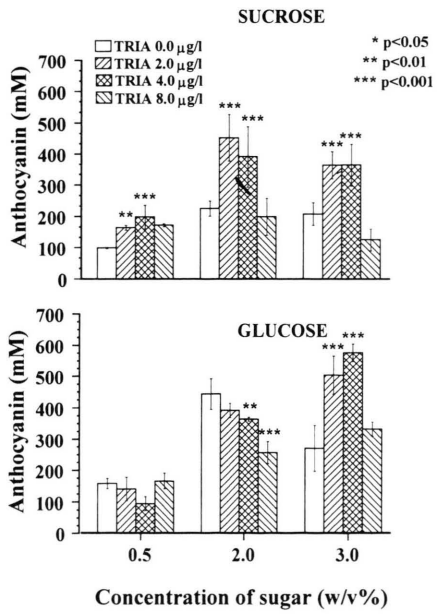

Fig. 6. Combined effect of different concentrations of sugars and triacontanol of the culture media on the anthocyanin accumulation in micropropagated *Prunus* × *davidiopersica* 'Piroska' (n = 8, stars indicate the level of significance at different triacontanol concentrations at 0.05, 0.01 and 0.001 probability).

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