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THE EFFECT OF CARBON SOURCE IN CULTURE MEDIUM ON MICROPROPAGATION OF COMMON NINEBARK (*Physocarpus opulifolius* (L.) Maxim.) 'Diable D'or'

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Abstract Efficient plant micropropagation depends upon a number of factors one of which is the type and concentration of exogenously supplied carbon sources in the medium. This study tested several different sugars as carbon source on the efficiency of shoot proliferation and *in vitro* rooting of common ninebark (*Physocarpus opulifolius* (L.) Maxim.). Fructose, glucose, maltose and sucrose were tested at concentration ranging from 0–50 g dm^{-3}. The dry matter content, reducing sugars and sucrose in shoots were related to sugar concentrations in the medium and so was the rate of adventitious root formation. Sucrose did not stimulate shoot proliferation and glucose was completely ineffective in rooting induction. The highest number of shoots was produced on the fructose-containing medium. The concentration of 30 g dm^{-3} appeared to be optimal; the rate of proliferation at 30 and 40 g dm^{-3} were in fact similar, but the former produced a more favorable shoot length. The number of adventitious roots produced per shoot increased with increasing fructose concentration up to 30 g dm^{-3}. Fructose can be therefore recommended as the best C-source for the *in vitro* shoot proliferation and rooting in common ninebark.

Key words: carbohydrate, fructose, glucose, maltose, sucrose, *in vitro*

INTRODUCTION

Shrubs are very important ornamental plants, widely used in reforestation programs, soil retention systems and as essential components of the natural landscape, as well as of public and private recreation areas. Growing demand for ornamental nursery products compels the growers to intensify production but conventional methods are often little effective for propagation of woody plant species.

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Common ninebark is a member of the Rosaceae family; its cultivars are generally hardy. They grow well in full sunlight or under semi shade and tolerate a wide range of soil types. Its leaves are insensitive to air pollution, therefore this species is used for phytoremediation processes. It is used as the primary ornamental shrub in landscaping or for watershed protection. However, the species of ninebark is mostly propagated in generative way, but cultivars with colored leaves by cuttings. Nevertheless, nurseryman often has problems in conventional propagation of ninebark. The rooting rate is different among the cultivars (50–85%) [Pacholczak and Szydło 2008]. In order to induce the rooting of the cuttings it is necessary to apply „rooting induction powders" which are supplemented with auxins. As a consequence of the increased general proecological tendency in EU countries and increased restriction or complete withdrawn from use chemical compouds such as synthetic auxins [Pacholczak et al. 2010], *in vitro* micropropagation is an effective alternative to standard cuttings.

Growth and *in vitro* shoot multiplication are affected by many factors [Haque et al. 2003], one of which is the concentration and type of exogenous carbon sources added to medium as the energy source, and to maintain the osmotic potential [De Neto and Otoni 2003]. Root initiation and growth are high energy requiring processes that can only occur at the expense of available metabolic substrates, which are mainly carbohydrates [Thorpe 1982, De Klerk and Calamar 2002]. In plant cultured *in vitro*, a continuous supply of carbohydrates from the medium is necessary, since the photosynthetic activity is reduced under low light intensity, limited gas exchange and high relative humidity [Kozai 1991]. In general, sucrose is the carbohydrate of choice for the *in vitro* plant culture, probably because it is the most common carbohydrate present in the phloem sap of many plants [Thorpe 1982, Lemos and Baker 1998, Fuentes et al. 2000]. However, invertases released by explants into the medium split sucrose into glucose and fructose [George 1993, De Klerk and Calamar 2002]. As a consequence, the explants are usually exposed to a mixture of sucrose, glucose and fructose [Moing et al. 1992, Stoop and Pharr 1993, Sinclair and Byrne 2003]. According to Mosaleeyanon et al. [2004], the effects of the type and concentration of different carbohydrates on the *in vitro* growth and development of plant cultures are still relevant questions in micropropagation research, especially among woody species. This study attempted to quantify the effects of four sugars as carbon sources, and their concentrations, on the *in vitro* shoot proliferation and rooting of the ninebark.

MATERIALS AND METHODS

Plant material and culture conditions. Shoot tip explants of common ninebark (*Physocarpus opulifolius* (L.) Maxim.) cv. 'Diable D'or' were collected from eight-week old cultures maintained through monthly subculturing on the MS medium [Murashige and Skoog 1962], supplemented with 1.0 mg dm^{-3} BA, 0.1 mg l^{-1} NAA, containing 30 g dm^{-3} sucrose and solidified with 8 g dm^{-3} agar. In all experiments the culture medium was adjusted to pH = 5.8 before autoclaving at 121°C and 0.1 MPa for 20 min. The cultures were maintained under a photoperiod of 16 h light and 8 h darkness and at 24 ± 2°C. The photosynthetic photon flux density (PPFD) was 33 µmol m^{-2} s^{-1}.

There were 30 microcuttings in each treatment in 3 replication.

Shoot proliferation. For tests of shoot proliferation, similarly-sizes shoot tips (10 mm) were transferred to the mentioned media with one of the four sugars tested: fructose, glucose, maltose and sucrose at concentrations of 0, 10, 20, 30, 40 and 50 g dm^{-3}. After 8 weeks in culture the following data were collected: total number of shoots per proliferating explant, shoot length (cm), dry weight (mg) of shoots and sucrose or reducing sugars contents in microshoots.

For dry matter and sugar contents, the plant materials was finely chopped, thoroughly mixed, and the 0.5–1 g samples taken. Triplicate extracts were prepared for each analysis and three measurements were done for each extract producing nine readings for each data point.

For the dry matter content, 1 g samples were dried at 105°C to constant weight [Strzelecka et al. 1982]. Reducing sugar contents were measured by the colorimetric method of Somogyi in the Nelson's modification [Nelson 1944]. Sucrose was determined by the above method as a difference in reducing sugars after and before hydrolysis with 0.1 M HCl and expressed in mg glucose per gram of dry weight.

Rooting of microcuttings. For rooting studies, microshoots (2–3 cm long) were selected, cut midway in the internodal regions, and rooted on the MS medium supplemented with IBA at 1.0 mg dm^{-3}. Different carbon sources such as: fructose, glucose, maltose and sucrose were added individually to the rooting medium at concentrations as in proliferation stage.

The percentage of rooted microcuttings, the degree of rooting and the length of shoots was measured after 8 weeks from the start of the rooting experiment. The degree of rooting was evaluated on a 5-point scale describing the root ball development (tab. 1).

Table 1. Evaluation scale of the *in vitro* root development

No.	Characteristic of the degree of rooting	Score
1	Without visible roots	1
2	A few short roots	2
3	Roots with numerous branched roots, no root ball	3
4	Medium sized root system forming a root ball	4
5	Well developed, branched root system forming a root ball	5

Experimental design and statistics. Experiments were conducted in a completely randomized design. Data obtained was subjected to analysis of variance using StatGaphics 2.0. Multiple comparisons among means were done using Duncan T-test at $p = 0.05$.

RESULTS

Shoot proliferation. The type and concentration of the carbon source in the culture medium had significant effects on the number of shoots per explant. Fructose was the most effective of the four sugars tested. The highest number of shoots per proliferated explant (18.5) was obtained on medium suplemented in 40 g dm^{-3} fructose, but the longest shoots (1.2 cm) were observed by

30 g dm^{-3} fructose (fig. 1 a). Cultures grown on the glucose- or sucrose-supplemented media showed very poor response, with low numbers of shoots per proliferated explant (fig. 1 b, d). Increased concentration of maltose to 30–50 g dm^{-3} had an inhibitory effect on the number of shoots per proliferated explant and on the shoot length in compared to 20 g dm^{-3} (fig. 1 c). A similar response was also observed at the highest sucrose concentration (50 g dm^{-3}; fig. 1 d). These results indicate that different carbohydrates, and their concentrations, significantly affected the success rate of culture. Fructose at 30 g dm^{-3} produced the best shoot elongation, likewise sucrose at 10–20 g dm^{-3}. Limited growth and shoot length were observed on medium with glucose and maltose (fig. 1).

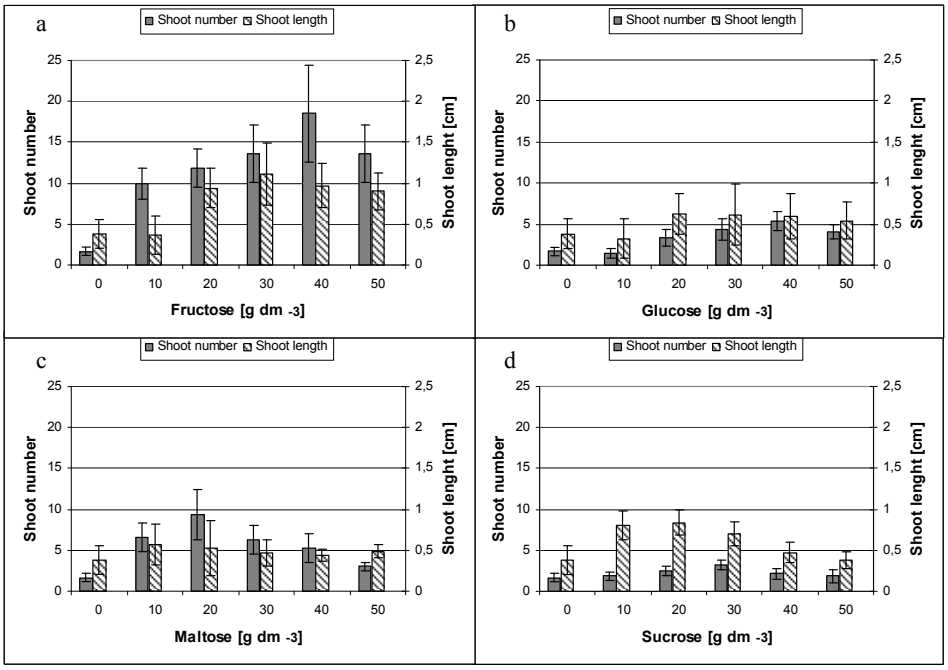

Fig. 1. Multiplication rate and shoot length after 8 weeks on MS medium with 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA supplemented with different concentrations of: a – fructose, b – glucose, c – maltose and d – sucrose

Dry weight of microshoots recorded after 8 weeks of culture increased together with the increasing C-source concentration in the medium, being slightly lower in plantlets cultured on fructose (fig. 2 a).

The reducing sugar contents and sucrose were related to the concentrations of fructose, glucose and sucrose in the medium. The highest concentration of reducing sugars and sucrose in the plant material was found in shoots cultured on media with 40–50 g dm^{-3} of each of the sugars. Cultures grown on media without a carbon source had low levels of reducing sugars and sucrose (fig. 2).

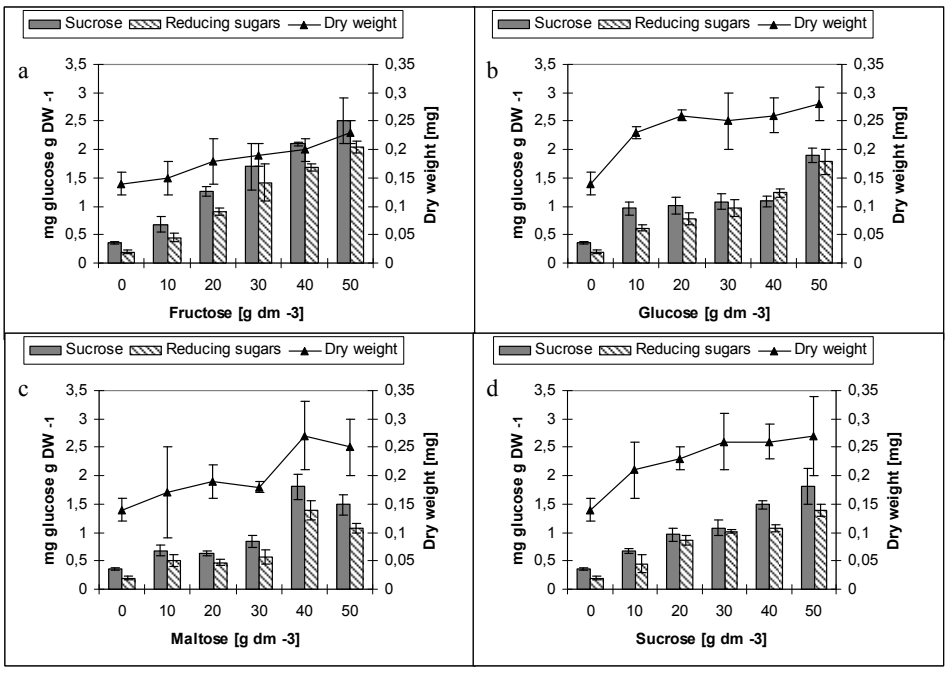

Fig. 2. Contents of reducing sugars, sucrose and dry weight of microshoots after 8 weeks on MS medium with 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA supplemented with different concentrations of: a – fructose, b – glucose, c – maltose and d – sucrose

Rooting of microcuttings. Rooting percentage depended on the type and concentration of the carbohydrate present in the medium (fig. 3). Maximum rooting percentage (75%) was observed in the medium containing fructose at 20–30 g dm^{-3} while at the same concentration of glucose rooting was only 0–15%. Similarly, fructose at 20–30 g dm^{-3} resulted in the highest degree of rooting (fig. 3 a and fig. 5 c, d). Likewise, the shoot length increased with a sugar concentration increasing up to 20 g dm^{-3}. Plantlets rooted in the medium supplemented with 40–50 g dm^{-3} glucose became more compact. Plantlets grown on fructose in concentration at 20–30 g dm^{-3} reached 1.6 cm (fig. 4 and fig. 5).

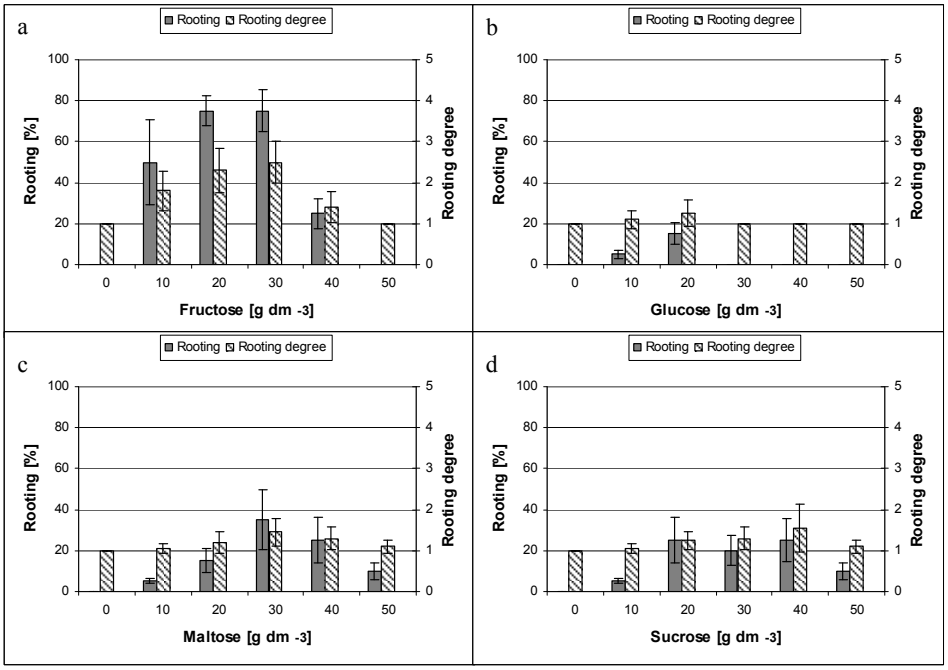

Fig. 3. Percentage of rooting of microshoots and root development after 8 week on MS medium with 1.0 mg dm^{-3} IBA supplemented with different concentrations of: a – fructose, b – glucose, c – maltose and d – sucrose

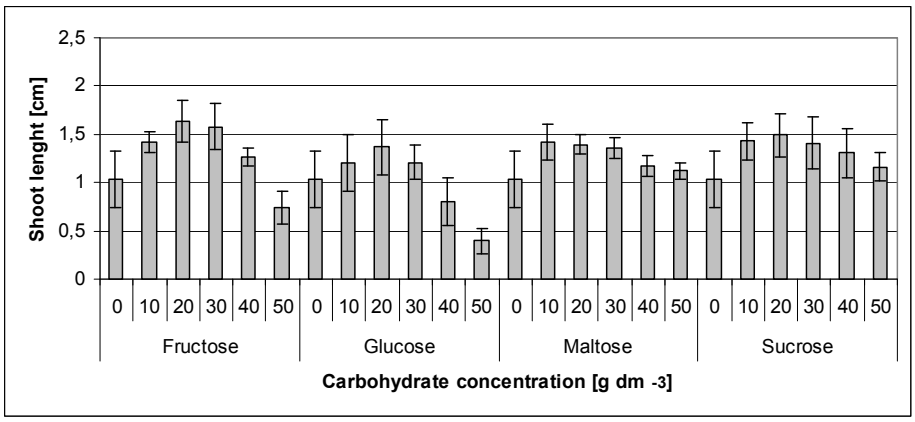

Fig. 4. Growth of microshoots after 8 weeks on MS medium with 1.0 mg dm^{-3} IBA supplemented with different carbon sources

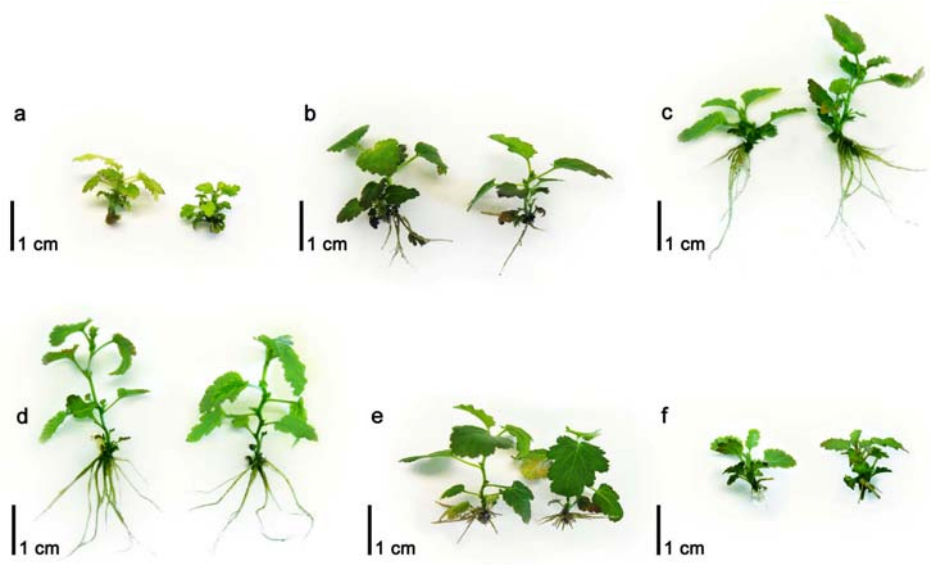

Fig. 5. Microshoots after 8 weeks on MS medium with 1.0 mg dm^{-3} IBA: a – without C-source and supplemented with different concentration of fructose: b – 10 g dm^{-3}, c – 20 g dm^{-3}, d – 30 g dm^{-3}, e – 40 g dm^{-3} and f – 50 g dm^{-3}

DISCUSSION

Several reports have proved that the carbon source affects the *in vitro* morphogenesis of different plant species [Fuentes et al. 2000]. Carbohydrates control morphogenesis by acting as the energy source and by altering the osmotic potential of the culture medium, which in turn alters such cell wall properties as extension, hardening, and composition, followed by subsequent modification in morphogenesis [Pritchard et al. 1991].

Carbohydrates are necessary in living cells as a source of energy and carbon skeletous for biosynthetic processes. The internal carbohydrate pool is suggested to have an important role in morphogenesis of several woody species [Kromer and Gamian 2000, Li and Leung 2000] and this can be influenced by the exogenous supply of carbon sources [De Neto and Otoni 2003]. It is well established that carbohydrate requirements depend upon the stage of culture, and may vary from species to species [Thompson and Thorpe 1987].

Proliferation of common ninebark cultures in this study was strongly affected by carbon source. The interactions between different types of carbohydrates used were significant for such parameters as shoot number, shoot height, and length. Medium supplemented with 30 g dm^{-3} fructose greatly enhanced shoot proliferation and elongation while sucrose did not stimulate shoot proliferation and glucose did not stimulate rooting of microcuttings. Poor response to sucrose in shoot proliferation might be due to

slow break up of sucrose into glucose and fructose. Invertase is required for an efficient conversion of sucrose into glucose and fructose [Pua and Chong 1984], and its levels may vary depending on the species and tissues used. More success with shoot proliferation on the medium containing fructose may be associated with a better utilization of fructose with the sufficient availability of enzymes that help in the hydrolysis of fructose. The enzymatic conversion of fructose into fructose-6-phosphate can make fructose readily available for the tissues and thus used as carbon structure for new growth [Dennis and Greyson 1987].

Shoot elongation is a critical step in the micropropagation system, closely related to the nutritional composition of the media [Chen et al. 2003]. An increase in shoot length on fructose may be attributed to its effective role in cell expansion, which is driven by turgor pressure, and fructose may be one of the major osmolytes used to generate turgor [Bianco and Rieger 2002]. Our results showed that fructose was more effective than other sources of carbon. Similarly, growth of shoots from non-dormant buds of mulberry is not promoted by sucrose, but can be promoted by fructose, maltose or glucose [Oka and Ohyama 1982].

Our study indicated that higher shoots dry weights were observed at the highest 40–50 g dm^{-3}) concentrations of all sugar used. This observation confirms data of Al-Khateeb [2008], who reported an increase of dry weight of *Phoenix dactylifera* L. shoots in response to a high sugar concentration.

Steinitz [1999] and Da Silva [2004] sugested that carbohydrates are perceived by cells as chemical signals, with very high concentrations *in vitro* acting as stress agents. Our study showed that fructose stimulated a higher level of rooting, a better rooting degree and higher lengths of rooted microshoots. Shoots cultured on medium without carbon source did not produce roots, indicating the importance of sugar in root formation. Thorpe [1982] presumed that root initiation and growth were high energy requiring processes that could only occur at the expense of available metabolic substrates, which were mainly carbohydrates. The results of this study provide evidence that as far as root formation is concerned, proliferated shoots of common ninebark can utilize fructose better than sucrose. In ninebark, perhaps the osmotic adjustment regulated by reducing carbohydrates also affects the initiation of root primordia [Pua and Chong 1984], and high concentration of carbohydrates in the medium have a negative impact. Eliasson and Brunes [1980] reported that such a negative impact could result from accumulation of rooting inhibitors, the reduction of promotors, and the transformation of added sugars into in soluble and storage forms [Haisig 1984]. Carbon source, each at 3% concentration (fructose, glucose, lactose, maltose and sucrose) was tested for root induction in *Withania somnifera* and sucrose gave the best result at 100% rooting while fructose gave 65% root induction [Sivanesan and Murugesan 2008]. This study shows a better percentage of rooted plantlets on fructose, and this appears to be the most favorable source of carbon for this species.

Carbohydrates can be also a signaling molecules and play similar role as growth regulators. Regulation of metabolic processes, growth and development with the participation of sugars followed by the expression or repression of genes determining the appropriate enzymes. The role of carbohydrates during cell division and cell differentiation is closely related to their effect on plant metabolism and development [Rolland et

al. 2006]. It can be assumed that the effect of carbohydrates in the process of multiplication and rooting of shoots of common ninebark is also related to its signaling role. Gabryszewska [2011] has shown inhibitory effect of high concentration of sucrose in growth of axillary shoots of *Syringa vulgaris*. In this study we can found that high concentration of carbohydrates also inhibited the growth of axillary shoots.

Responses of *in vitro* cultures to different kinds of carbohydrates in the medium are tested frequently and results appear species-specific. Although carbon sources are of prime importance for *in vitro* organogenesis, carbon metabolism *in vitro* is still not clearly understood [Kozai 1991].

CONCLUSIONS

1. Proliferation and rooting of ninebark microshoots depend on a suitable carbon source in a culture medium.
2. Content of reducing sugars and succrose in microshoots depend on type of C-source in proliferation medium.
3. Fructose is the best carbohydrate for micropropagation of ninebark.

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WPŁYW RODZAJU CUKRU W POŻYWCE NA MIKROROZMNAŻANIE PĘCHERZNICY KALINOLISTNEJ (*Physocarpus opulifolius* (L.) Maxim.) 'Diable D'or'

Streszczenie. Wydajność mikrorozmnażania roślin uzależniona jest od wielu czynników, m.in. od koncentracji i rodzaju węglowodanów dodawanych do pożywki. Celem pracy było określenie wpływu różnych źródeł węgla na poziom proliferacji (współczynnik rozmnażania) i stopień ukorzenienia mikrosadzonek pęcherznicy kalinolistnej (*Physocarpus opulifolius* (L.) Maxim.) w warunkach *in vitro*. Do pożywek dodawano fruktozę, glukozę, maltozę i sacharozę w stężeniach 0–50 g dm^{-3}. Zawartość suchej masy, cukrów redukujących i sacharozy w pędach związana była ze stężeniem węglowodanów w pożywce i wpływała na proces rizogenezy u mikrosadzonek. Sacharoza nie wpływała stymulująco na proliferację nowych pędów, natomiast glukoza okazała się być całkowicie nieefektywna w ukorzenianiu mikrosadzonek. Najwyższy współczynnik rozmnażania zaobserwowano na pożywce wzbogaconej we fruktozę. Pomimo że współczynnik rozmnażania na pożywce zawierającej 30 i 40 g dm^{-3} był zbliżony, to długość wytworzonych pędów była istotnie większa na pożywce zawierającej 30 g dm^{-3}, dlatego stężenie to uznano za optymalne. Najwyższy stopień ukorzenienia mikrosadzonek zaobserwowano na pożywce z dodatkiem 30 g dm^{-3} fruktozy. Fruktoza może być więc zalecana jako najlepsze źródło węgla do namnażania i ukorzeniania pęcherznicy kalinolistnej w warunkach *in vitro*.

Słowa kluczowe: fruktoza, glukoza, *in vitro*, maltoza, sacharoza, węglowodany

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