# Physiological response of *in vitro* cultured *Prunus cerasifera* 'Nigra' to the type and concentration of the carbon source

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**Abstract**

In the present study, the effect of three carbohydrates – sucrose, glucose and fructose, applied as a carbon source in *in vitro* culture of *Prunus cerasifera* „Nigra" was assessed. The three tested carbohydrates were compared in mass concentrations of 20; 30 and 40 g/l. Mass concentrations were preferred because of the substantial difference in molar mass between glucose and fructose monosaccharides and the disaccharide sucrose, which allows sucrose to provide twice as many carbon atoms and energy in the growth medium. Plants grown on a medium containing fructose accumulated the maximum of dry weight of 53.98 mg; the highest average height of 15.83 mm and a significantly lower percentage of plants suffering from hyperhydration and symptoms of premature aging. For plants grown in sucrose-enriched medium, the highest fresh weight was 467.04 mg, the highest number of shoots was 6.89, but dry weight was the lowest – 44.28 mg; the smallest average height was 8.79 mm and the number of plants suffering from hyperhydration and premature aging – 86% was the highest. Plants cultivated for an extended period on a fructose-containing medium were established to have improved physiological performance in the first 2-3 subcultures. After 4 subcultures the percentage of failed micro plants dropped from 82% in plants grown in sucrose-enriched medium to 52% on medium enriched with 30 g/l fructose. An effect on the rooting process was also proven. The micro plants grown on a fructose-enriched medium expressed 77% higher survival rate and their rooting percentage exceeded with 125% that of the micro plants propagated on a sucrose-enriched medium.

**Keywords:** micropropagation; in vitro; carbohydrates; *Prunus cerasifera*

## INTRODUCTION

*Prunus cerasifera* belongs to Prunus genus and Euprunus subgenus, Rosaceae family. It is an ancient orchard culture important to modern fruit growing, mostly as rootstock for plum, apricot, peach and almond. It is a valuable ornamental plant, which most popular ornamental form is *Prunus cerasifera* 'Nigra'. In horticulture, the typical and ornamental forms of the plant are used as large bushes, small trees or for the formation of hedges and fences.

The requirements of *Prunus cerasifera* for the carbon source in *in vitro* culture have been poorly studied. Cheong & Chanhoon (2015) compared the effect of glucose, fructose sucrose in equimolar concentration on 8 species of Prunus (belonging to different subsets). They found that *P. cerasifera* micro plants were better grown on nutrient media containing glucose and fructose, while in a medium enriched with sucrose, a smaller number of shoots and smaller height were observed. In other studies for the cultivation of *Prunus cerasifera* genotypes *in vitro* sucrose was administered as a carbon source at concentration of 3% (Garland & Stoltz, 1981; Aier & Sharma, 1990; Ambrozic-Turk et al., 1990; Morini et al., 1992; Nacheva et al., 2002; Nowak et al., 2007; Liu et al., 2008).

Several authors point out the importance of the type of carbon source and the concentration in which it is applied in the medium (Kamenická, 1998; De

Neto et al., 2003; George et al., 2008; Gabryszewska, 2011; Gabryszewska & Sochacki, 2013). For example, in the micropropagation of some algae species there is a better development on glucose-enriched medium (Barghchi, 1988). Some species of orchids (Arditti, 1979), soybean (Wright, 1986), Castanea (Chauvin & Salesses, 1987) have shown significantly better development on media containing fructose as carbon source. In the case with *Magnolia* x *Solangiana*, fructose is found to be a more suitable carbon source than glucose and sucrose, both in the multiplication and in the rooting stages (Kamenická, 1998).

The mechanism of action of the carbon source on micro plants is very complicated and probably has at least several pathways of action that are relatively independent (De Neto et al., 2003). Carbohydrates are most commonly referred to as one of the main components in the media controlling the osmotic potential. Monosaccharides (glucose and fructose) have almost twice the molar mass of sucrose, suggesting that if used in equal weight concentrations (usually 3% by mass) will induce almost double the osmotic potential.

Several authors, however, report that in the autoclave process sucrose undergoes hydrolysis to monosaccharides to varying degrees, leading to an unintended increase in the osmotic potential of the medium (Bretzloff, 1954; George, 1993; George et al., 2008). The chemical composition of the medium can also be changed by caramelization and the formation of sugar acids. These processes are more typical for the monosaccharides (De Neto et al., 2003). In autoclaving of sucrose-containing media, the pH is generally low, whereas in autoclaving of monosaccharide-containing media, such as glucose and fructose, acidification might be significantly larger (Owen et al., 1991).

It is also known that carbohydrates have certain phyto-regulating functions on one hand and on the other hand, they might influence the absorption of nutrient salts and the activity of exogenously applied growth regulators (George et al., 2007). All this makes necessary to experimentally identify the most appropriate type and concentration of the carbon source for each plant species cultivated in vitro.

Various results regarding the rooting process have been reported in the available literature on micro-propagation of *Prunus cerasifera*. ½ Murashige and Skoog, 1962 (MS) is considered the most suitable medium for rooting (Garland & Stoltz, 1981; Liu et al., 2008), enriched with 30 $g.l^{-1}$ sucrose and Indole Butyric Acid (IBA) at concentration of 0.4 mg/l (Liu et al., 2008) or 0.2 $mg.l^{-1}$ (Garland & Stoltz, 1981). In plum, it achieves a maximum rooting of 54% by the addition of 1 mg/l IBA to Aier and Sharma rooting medium (1990).

The purpose of this study is to determine which of the three carbohydrates tested is more suitable as a carbon source for *in vitro* culture of *Prunus cerasifera* 'Nigra' and to establish an appropriate concentration in which it can be applied to the medium. Another task of the study is to establish the relationship between the used carbon source in the multiplication phase and the ability of micro plants to form roots.

## MATERIAL AND METHODS

*In vitro* culture of *Prunus cerasifera* 'Nigra' was created by introducing tops of growing shoots in the second half of April 2011. The explants were surface sterilized for 3 minutes by immersion in a 0.1% solution of mercuric chloride ($HgCl_2$). Murashige and Skoog medium (1962) with no growth regulators (G.R.) supplemented with 30 g/l sucrose and 7 $g.l^{-1}$ agar. In preliminary experiments the concentration of 0.5 $mg.l^{-1}$ 6-Benzylaminopurine (BAP) was found as the most suitable, and used it in the further experiments.

Propagated on MS media with vitamins enriched with 0.5 mg/l BAP, 30 $g/l^{-1}$ sucrose and 7 $g/l^{-1}$ agar agar, the grown plants were transferred for two subcultures on MS media enriched with 20, 30 and 40 $g/l^{-1}$ sucrose; 20, 30 and 40 $g/l^{-1}$ glucose and 20, 30 and 40 $g/l^{-1}$ fructose. In each variant, 30 micro plants (3 jars with 10 micro plants) were placed in the first subculture, and in the second subculture – 50. The results were recorded on day 60 of the second subculture. Fifty micro plants, grown in medium enriched with 30 $g.l^{-1}$ fructose were placed on the same medium for the 3rd and 4th subculture. At the end of each subculture (60th days of transfer of the micro plants) the rate of failure due to hyperhydration, accelerated aging or other physiological phenomenon was observed.

To determine the impact of the carbon source used in the multiplication phase, 50 micro plants

were grown for 3 subculturs on MS medium enriched with 0.5 mg.l$^{-1}$ BAP, gelled with 7 g/l$^{-1}$ agar and enriched with 30 g/l$^{-1}$ sucrose, 30 g/l$^{-1}$ glucose or 30 g/l$^{-1}$ fructose respectively. For rooting, MS medium with vitamins enriched with 0.2 mg.l$^{-1}$ indole butyric acid (IBA), 30 g/l$^{-1}$ sucrose and 7 g/l$^{-1}$ agar was used. On the 60th day the percentage of alive plants and the rooting percentage were recorded.

Micro plants were cultured in glass jars of 80 mm height, 95 mm in diameter and 400 ml volume in which 50 ml of the culture medium was poured. The jars were closed with metal caps. The micro plants were cultured in a cultivation room at temperature of 24 ± 2°C and artificial white light with an intensity of 30 μmol m$^{-2}$ s$^{-1}$ and a photoperiod of 16 hours day and 8 hours night. Philips 40W luminescent lamps (supplied by Philips - Bulgaria) were used for illumination.

The real multiplication rate was evaluated by the number of healthy new micro plants obtained from 1 explant. All micro plants exhibiting negative physiological features were excluded when calculating this factor.

The obtained data were subjected to a variance analysis (ANOVA) and a Duncan's multiple-range test ($p<0.05$).

## RESULTS AND DISCUSSION

After establishing a successful *in vitro* culture of *Prunus cerasifera* 'Nigra' in May 2011, it turned out that most micro plants hyperhydrate or aged quickly and died. The percentage of degraded plants on the 60th day of the subculture was very high (80-90%), and this was a major difficulty for further research on optimization of the protocol for micropropagation. Signs of hyperhydration and premature aging were observed also after the 30th day of the subculture. The shortening of the subculture period allowed successful multiplication, but the quality of the shoots at this stage was poor because they had not yet reached the optimum size.

Two monosaccharides were tested to solve the problem – glucose and fructose, having the same molar masses (180.16 g/mol) and osmotic potential; and sucrose, which is a disaccharide with a nearly double molar mass (342.3 g/mol), and inducing a similar osmotic potential as glucose and fructose in equimolar solutions. The results obtained showed that from the three studied carbon sources, fructose is the most suitable for the *in vitro* culture of *Prunus cerasifera* 'Nigra' (Table 1). Micro plants cultivated on a medium containing fructose had significantly better physiological performance than those grown on medium containing glucose.

Since these two monosaccharides have the same molar mass and the same osmotic potential, we assumed that the improved development of *in vitro* culture in the presence of fructose is not related only to the increased osmotic pressure of the medium. The fact that no significant differences were observed between micro-cultures grown on medium containing different concentrations of glucose and fructose also confirmed that the results obtained could not be explained solely by the osmotic potential of the medium. The only differences are evident in micro-cultures grown on medium enriched with different concentrations of sucrose (Table 1).

A trend was recorded of slow improvement of the physiological status of *in vitro* culture over three subcultures on a nutrient medium containing fructose. While in the second subculture 58% of micro plants showed symptoms of hyperhydration or accelerated aging, in the third subculture this percentage was 54 and in the fourth – 52%. The difference between the second and fourth subculture was 10.3% and statistically significant. An explanation of the reduction of the negative physiological phenomena in the micro-cultures can be sought in the possible direct effect of fructose and its derivatives on biochemical and regrowth processes in micro plants.

The real multiplication rate of micro plants varied considerably (Fig. 1). The highest multiplication factor was found in micro cultures grown on medium enriched with 30 and 40 g.l$^{-1}$ fructose, respectively, 2.13 and 2.05, and the lowest - in plants cultivated on 20 g.l$^{-1}$ sucrose 0.96.

The best results were obtained during the rooting of the propagated micro plants in a fructose enriched medium (Table 2). The worst results were obtained in micro-cultures grown in sucrose-enriched medium, where less than half of the micro plants survived by the 60th day, and only one third rooted. For micro plants propagated in a medium containing glucose, the results are inferior to those in a medium containing fructose. The percentage of rooted micro plants from the percentage of surviving to 60th day micro plants is an indicator which helped us to

address the question are the micro plants multiplied in different media capable of rooting. With respect to this indicator, micro plants multiplied in fructose and glucose enriched medium showed practically the same results, whereas those in sucrose-enriched medium significantly declined.

The results obtained in the process of rooting can be explained primarily by the physiological state of the shoots intended for rooting and by their quality. Increased height and lower hydration of the stem was observed for the shoots grown on fructose and glucose, most of the shoots had stiffness of the base or the entire lower half of the stem. All this implies an objectively better rooting in micro plants grown in a medium containing fructose as carbon source. We assume that the carbon source used in the multiplication process affects the levels of endogenous cytokines. This assumption is based on the significantly higher number of shoots seen in micro-cultures grown on medium enriched with sucrose compared to those grown in fructose-enriched medium (Table 1). The level of endogenous growth regulators can also be responsible for the unsatisfactory rooting of micro plants propagated in sucrose-enriched medium.

**Table 1.** Influence of the type and concentration of the carbon source on the growth and physiological state of the micro plants of *Prunus cerasifera* 'Nigra'

| MS+ 0,5 mg/l$^{-1}$ BAP + | Fresh weight | Dry weight | Height | Number of shoots | % failed micro plants |
|---|---|---|---|---|---|
| 20 g/l$^{-1}$ sucrose | 467,04 $^{c}$ | 44,28 $^{a}$ | 8,79 $^{a}$ | 6,89 $^{cd}$ | 86 $^{d}$ |
| 30 g/l$^{-1}$ sucrose | 445,96 $^{c}$ | 42,86 $^{a}$ | 8,93 $^{a}$ | 6,57 $^{c}$ | 82 $^{c}$ |
| 40 g/l$^{-1}$ sucrose | 461,34 $^{c}$ | 46,14 $^{ab}$ | 8,49 $^{a}$ | 7,24 $^{d}$ | 88 $^{cd}$ |
| 20 g/l$^{-1}$ glucose | 320,63 $^{b}$ | 48,82 $^{b}$ | 12,11 $^{b}$ | 5,32 $^{b}$ | 72 $^{b}$ |
| 30 g/l$^{-1}$ glucose | 321,22 $^{b}$ | 50,27 $^{b}$ | 11, 64 $^{b}$ | 5,28 $^{b}$ | 74 $^{b}$ |
| 40 g/l$^{-1}$ glucose | 318,75 $^{b}$ | 49,61 $^{b}$ | 11, 93 $^{b}$ | 5,44 $^{b}$ | 70 $^{b}$ |
| 20 g/l$^{-1}$ fructose | 307,86 $^{ab}$ | 54,86 $^{bc}$ | 15,83 $^{c}$ | 4,51 $^{a}$ | 62 $^{a}$ |
| 30 g/l$^{-1}$ fructose | 294.17 $^{a}$ | 54,79 $^{bc}$ | 15,56 $^{c}$ | 4.44 $^{a}$ | 58 $^{a}$ |
| 40 g/l$^{-1}$ fructose | 298,11 $^{a}$ | 55,37 $^{c}$ | 15,28 $^{c}$ | 4,67 $^{a}$ | 60 $^{a}$ |

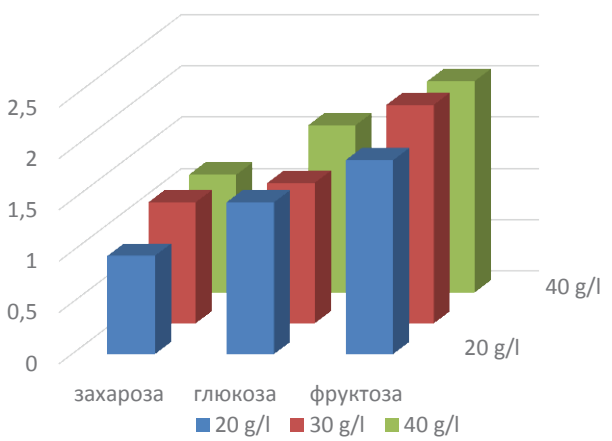


**Figure 1.** Real multiplication rate for micro plants of *Prunus cerasifera* 'Nigra' grown in media enriched with different carbon sources in different concentrations

**Table 2**. Influence of the type of carbon source in the multiplication stage on the survival and rooting of micro plants of the reddish gruff *Prunus cerasifera* 'Nigra'

| Medium | Surviving live plants on the 60th day (%) | Rooted plants on the 60th day (% of all) | Rooted plants on the 60th day (% of live) |
|---|---|---|---|
| MS + 30 sucrose | 44 $^{a}$ | 32 $^{a}$ | 72,73 $^{a}$ |
| MS + 30 glucose | 66 $^{b}$ | 60 $^{b}$ | 90,91 $^{b}$ |
| MS + 30 fructose | 78 $^{c}$ | 72 $^{c}$ | 92,03 $^{b}$ |

## CONCLUSIONS

1. The type of carbon source has more significant effect on the growth and physiological state of the micro plants than the concentration in which it is used.

2. The effect of monosaccharides glucose and fructose on the growth and physiological state of the micro plants was to a significant degree similar to the effect of sucrose, namely, a decrease in fresh weight, the average number of shoots and the percentage of failed micro plants and an increase in the average dry weight and the average height of the micro plants.

3. Fructose as a carbon source has been found to induce better physiological state and a higher multiplication rate than the other two carbohydrates tested.

## REFERENCES


**Aier, N. B. & Sharma, S. D.** (1990). Micropropagation in some plum cultivars. *Fruit Science Reports, 17*(2), 57-63.

**Ambrozic Turk, B., Smole, J., & Šiftar, A.** (1990). Micropropagation of a plum ecotype (Prunus domestica L.) as rootstock for apricots. *In Vitro Culture, XXIII IHC 300*, 111-114.

**Arditti, J.** (1979). Aspects of the physiology of orchids. *Advances in Botanical Research,* 7, 421- 655.

**Barghchi, M.** (1988). Micropropagation of Alnus cordata (Loisel.) Loisel. *Plant cell, tissue and organ culture*, *15*(3), 233-244.

**Bretzloff Jr, C. W.** (1954). The growth and fruiting of Sordaria fimicola. *American Journal of Botany*, 58-67.

**Chauvin, J. E., & Salesses, G.** (1987). Advances in chestnut micropropagation (Castanea sp.). In *International Symposium on Vegetative Propagation of Woody Species 227* (pp. 340-345).

**Cheong, E. J. & Chanhoon, A.** (2015). Effect of Carbohydrates on in vitro Shoot Growth of Various Prunus Species. *Korean Journal of Plant Research, 28*(3), 357-362.

**De Neto, V.B.P. & Otoni, W.C.** (2003). Carbon sources and their osmotic potential in plant tissue culture: does it matter? *Scientia Horticulturae*, *97*(3-4), 193-202.

**Emershad, R. L., & Ramming, D. W.** (1994). Effects of media on embryo enlargement, germination and plant development in early-ripening genotypes of Prunus grown in vitro. *Plant cell, tissue and organ culture*, *37*(1), 55-59.

**Gabryszewska, E.** (2011). Effect of various levels of sucrose, nitrogen salts and temperature on the growth and development of Syringa vulgaris L. shoots in vitro. *Journal of Fruit and Ornamental Plant Research*, *19*(2), 133-148.

**Gabryszewska, E.** (2014). Rozmnażanie in vitro Helleborus purpurascens Waldst. et Kit. *Wiadomości Botaniczne*, *58*(1-2).

**Gabryszewska, E. & Sochacki, D.** (2013). Effect of various levels of sucrose and nitrogen salts on the growth and development of lily bulblets *in vitro*. Acta Horticulturae, 1002, 139-145.

**Garland, P., & Stoltz, L. P.** (1981). Micropropagation of Pissardi plum. *Annals of Botany*, *48*(3), 387-389.

**George, E. F.** (1993). *Plant propagation by tissue culture. Part 1: The technology* (No. Ed. 2). Exegetics limited.

**George, E. F., Hall, M. A., & De Klerk, G. J.** (2007). Plant propagation by tissue culture 3rd Edition. *The Background. Exegetic Basingstone. UK*.

**Kamenická, A.** (1998). Influence of selected carbohydrates on rhizogenesis of shoots saucer magnolia in vitro. *Acta Physiologia Plantarum*, *20*, 425-429.

**Liu, C. Q., Chen, X. S., Wu, C. J., Zhang, H., Cui, X. P., Shi, J., & Guo, L.** (2008). Tissue culture of wild myrobalan plum (Prunus cerasifera) stem segment, leaf explant and its plantlet regeneration. *Journal of Fruit Science*, *1*, 49-53.

**Morini, S., Sciutti, R., & Fortuna, P.** (1992). In vitro growth response of Prunus cerasifera shoots as influenced by different light-dark cycles and sucrose concentrations. *Plant cell, tissue and organ culture*, *28*(3), 245-248.

**Murashige, T., & Skoog, F.** (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

**Nacheva, L., Milusheva, S., & Ivanova, K.** (2001, August). Elimination of plum pox virus (PPV) in plum (Prunus domestica L.) cvs Kyustendilska Sinya and Veljevka through in vitro techniques. In *VII International Symposium on Plum and Prune Genetics, Breeding and Pomology 577* (pp. 289-291).

**Nowak, B., Miczyński, K., & Hudy, L.** (2007). The effect of total inorganic nitrogen and the balance between its ionic forms on adventitious bud formation and callus growth of 'Węgierka Zwykła'plum (Prunus domestica L.). *Acta Physiologiae Plantarum*, *29*(5), 479-484.

**Owen, H. R., Wengerd, D., & Miller, A. R.** (1991). Culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. *Plant Cell Reports*, *10*(11), 583-586.

**Quoirin, M., & Lepoivre, P. H.** (1977, September). Improved media for in vitro culture of Prunus sp. In *Symposium on Tissue Culture for Horticultural Purposes 78* (pp. 437-442).

**Sokolov, R. S., Atanassova, B. Y., & Iakimova, E. T.** (2014). Physiological response of in vitro cultured Magnolia sp. to nutrient medium composition. *Journal of Horticultural research*, *22*(1), 49-61.

**Sokollov, R., Atanasova, B., & Yakimova, E.** (2014). Influence of Vitamins on Growth Performance of in vitro Cultured Magnolia sp. *Plant Science (Bulgaria).*

**Wright, M. S., Koehler, S. M., Hinchee, M. A., & Carnes, M. G.** (1986). Plant regeneration by organogenesis in Glycine max. *Plant cell reports*, *5*(2), 150-154.