Possibilities for Application of Photoautotrophy in Micropropagation of Dzhanka 4 (*Prunus cerasifera* Ehrt.) Rootstock

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Abstract

In vitro cultured plantlets have heterotrophic development, relying on the use of exogenous sugars as a carbon source for growth. During the acclimatization stage, plantlets are constrained to grow photoautotrophically, i.e. to synthesize carbohydrates through their own photosynthesis. Many researchers suggested that the photosynthetic competence of plants, when transferred to soil, might be an important factor in determining transplant survival.

The aim of the present investigation was to study photosynthetic ability and possibilities for application of photoautotrophy during micropropagation of *Dzhanka 4* (*Prunus cerasifera* Ehrt) rootstock.

Observations were made on net photosynthesis of plantlets at three stages of micropropagation (multiplication, elongation and rooting), cultured under conventional conditions and conditions favouring photosynthesis (light, gas-permeable closure and decreased sucrose concentration). In addition, the same photosynthetic characteristics were analyzed in photoautotrophically-rooted (in peat) plantlets.

The improved environmental conditions led to an increase of quantum yield and Rubisco activity. In result, the net photosynthetic rate increased by 63% (in multiplication) to 91% (in rooting).

The results from this research and the recent experiments showed that improved environmental conditions enhanced photosynthesis and enabled the application of photoautotrophy in micropropagation of *Prunus cerasifera* Ehrt.

Abbreviations: BAP-6-benzylaminopurine; IBA-indolyl-3-butyric acid; MS medium-Murashige and Skoog medium; Rd- dark respiration; Pn- net photosynthetic rate; DW-dry weight; Γ-compensation point; Rubisko-ribulose 1,5-bisphosphate carboxylase; PAR-photosynthetically active radiation.

INTRODUCTION

The use of clonal vegetatively-propagated rootstocks has an indisputable advantage over those produced by seed. The virus status of rootstocks, used for plum cultivar grafting, is of paramount importance, especially when "sharka" (plum pox virus) disease is concerned.

The controversial question of "Whether this disease is transmitted also by the pollen of seed-propagated plants?" instigated our idea to develop an effective in vitro technology for "Dzhanka 4" - a good compatible rootstock used in our country. The in vitro clone of Dzhanka 4", produced in this way, would enable the conducting of a real control by re-testing the nurseries established by it, in connection with the production of certified plum planting material.

A promising way to improve the conventional in vitro technology is the application of photoautotrophy at some stages of the cultivation period. The results obtained by us with clonal apple rootstocks (Nacheva and Ivanova, 1998; Nacheva, 2000) warranted our decision to study the response of Dzhanka 4 under these conditions.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

Micropropagated shoots of plum rootstock Dzhanka 4 (*Prunus cerasifera Ehrt.*) were subcultured conventionally for 3 weeks on MS nutrient medium (Murashige and Skoog, 1962) supplemented with vitamins (m-Inositol 100.0 mg l^{-1}, Thiamine.HCl 0.4 mg.l^{-1}). Growth regulators (0.56 mg l^{-1} BAP, 0.001 mg l^{-1} IBA), saccharose 30 g.l^{-1}, agar (Fluka) 5.8 g.l^{-1} were used. The elongation medium contained no growth regulators and had reduced saccharose concentration (20 g.l^{-1}). The plantlets were rooted in a nutrient medium with 25% MS macroelements, 100% MS microelements, m-Inositol 100 mg l^{-1}, Thiamine. HCl 0.4 mg l^{-1}, saccharose 15 g l^{-1} and IBA 0.3 mg l^{-1}. pH was adjusted to 5.6 and the medium was autoclaved at 121°C and 118 kPa for 20 min. In jars (600 ml volume and 78.5 cm^2 area) 30 explants were inoculated onto a 100 ml medium. The jars were tightly closed with glass caps and polyethylene folio. Cultures were kept in a growth chamber at 22± 2°C and a photoperiod of 16 h light (30 µmol m^{-2} s^{-1} PAR, fluorescent lamps OSRAM 40W) and 8 h dark.

The general description of the environmental conditions favouring CO_2-fixation and photomixotrophic (or photoautotrophic) growth of plantlets from three stages of micropropagation is given in Table 1. At the multiplication stage (MW) a polyester wool was placed under the glass cap, allowing a gas-exchange rate of 4,6 volumes per hour (Nacheva and Ivanova, 1998).

In the elongation stage (EW) the growth regulator-free medium with reduced to 10 g.l^{-1} saccharose, increased light intensity (48-50 µmol m^{-2} s^{-1} PAR) and the same gas-permeable closure of vessels was used. Then planlets were rooted in peat (R) at 50 µmol m^{-2} s^{-1} PAR, i.e. in photoautotrophic conditions.

Gas Exchange Measurements - Light and CO_2-Concentration Curves

The gas exchange rate of plantlets was analyzed by an open system (LCA -4, ADC, UK), based on infrared gas analysis of CO_2-concentration. The light and C_2O_2 curves were determined at 4 light intensities (approx. 0, 40, 120 and 250 µmol m^{-} s^{-} PAR) and 4 CO_2-concentrations (approx. 100, 350, 700 and 1500 vpm). The measurements were made at the end of each stage, without removing the plants from the medium, as well as after their reaching an almost steady-state photosynthesis at relevant light and CO_2 conditions. Before measuring the dark respiration rate (Rd), plants were dark adapted for at least 30 min.

The relationship between net photosynthetic rate and CO_2-concentration or PAR was fitted using the following photosynthetic response curve:

$$y = a + b\, e^{(-x/c)} \qquad (1)$$

where: y = Pn (apparent photosynthesis); *n*molCO_2 $(gDW)^{-1}$ s^{-1}

a = maximum photosynthesis Pn at saturated light and CO_2;

b = parameter, defining the difference between dark respiration and max Pn (Rd=a +b);

c =constant;

x_i = respective CO_2-concentration (µmol$CO_2$$mol^{-1}$, vpm) at Pn=f($CO_2$) or PAR (µmol m^{-2} s^{-1}) at Pn=f(PAR).

CO_2-compensation concentration (Γc, vpm) at saturated PAR, and light compensation point (Γ *l*) at saturated CO_2 were estimated using the following equation:

$$\Gamma = -\mathbf{c} \,.\, \ln(-a/b), \text{ vpm} \qquad (2)$$

The slope of the linear part of the curve, relating Pn to CO_2 concentration (dy/dx) at a saturating photon flux rate, indicates Rubisco activity (in non-destructive analysis). Under the conditions of CO_2-concentration saturation, the initial slope of the light curve (dy/dx) reflects the efficiency of light reactions in terms of regenerating the carboxylation substrate RuBP and is interpreted as quantum yield (Farquhar and von Caemmerer, 1980).

$$dy/dx = |a + b| / \Gamma \qquad (3)$$

RESULTS

For the plantlets from the multiplication stage (Fig. 1), the light saturation of photosynthesis (at CO_2-saturation concentration 1500 vpm) occurred under light intensity of about 200 μmol m^{-2} s^{-1}, the maximum Pn for plants with gas-permeable closure being 57,5 *n*molCO_2 g^{-1}(DW) s^{-1} which was 45% higher than that of plants cultured in tightly closed jars. A significant difference was observed also in the light compensation point, whose value for the conventionally closed plants was over 65 μmol m^{-2} s^{-1}, while that for the variant with a better gas exchange was significantly lower - 23 μmol m^{-2} s^{-1} PAR (Fig. 1, Table 2). This showed that even with a CO_2- saturation concentration and conventional light of culturing, the plantlets of the conventional variant could not develop a positive C-balance. A light intensity of about 250 μmol m^{-2} s^{-1} was saturating also for the plants at the elongation and rooting stages. The rooted plants showed a significantly higher maximum photosynthesis and quantum yield than that of the other variants. The maximum net photosynthesis (Pn) for the photoautotrophic rooting on peat substrate, was two-time higher than that established by the conventional technology of rooting on agar medium (Table 2).

The CO_2 dependence of photosynthesis was determined under saturating light intensity (250 μmol m^{-2} s^{-1}). For the plantlets at the multiplication stage, the photosynthetic saturation occurred at CO_2 > 900 vpm (Fig. 2). The maximim Pn in the saturation zone of plants cultured with a gas-permeable closure was 62% higher than that of plants grown in tightly closed jars (Table 3). This trend was also observed when comparing the variants from the other two stages. The plants rooted under photoautotrophic conditions were characterized by significantly higher maximum photosynthetic intensity than those from the other variants.

It is known that under the conditions of highly intensive PAR and low CO_2 concentration, all active sites of the Rubisco enzyme are occupied by RuBP (ribulose 1,5-bisphosphate) and the Pn kinetics is determined only by the rate of CO_2 fixation. The initial slope of the concentration curve - dy/dx - is defined as the fixed CO_2 amount at CO_2 concentration = 1vpm and expresses the carboxylase activity in non-destructive (*in vivo*) analyses (Farquhar and von Caemmerer, 1980). Both at the multiplication- and the elongation stage, the carboxylase activity, expressed by the initial slope of curves, increased significantly when the gas-exchange of vessels was improved (Table 3). The difference was still more significant in the rooted plants - about 6 times higher in the photoautotrophic variant. These results confirmed the suggestions of many authors, as well as our conclusions for the apple rootstock MM106, that the exogenous sugars in the nutrient medium are one of the main factors related to the low Rubisco activity in in vitro cultivated plants.

The plateau of concentration curves reflects the limiting of photosynthesis by the electron transport rate. The great differences in the maximum Pn and the quantum yield under saturating CO_2 concentration among the plants from the tightly closed jars, the permeable closures, and particularly the photoautotrophically rooted ones (Table 2), suggested that some structural and functional changes of the photosynthetic apparatus occurred just in this direction. This led to the important conclusion that the optimizing of the external factors in the process of in vitro propagation could increase not only the photosynthetic efficiency of plantlets, but could also contribute to their better adaptability to environmental conditions during the period of adaptation.

High rooting percentages were established for both variants tested (88% for the conventional control (RK) and 91% for the photoautotrophic variant) with no significant difference between them.

DISCUSSION

The net photosynthesis of plantlets from different plant species, removed from culture vessels and measured under optimal environmental conditions, was comparable to that of the respective seedlings (Pospisilova et al., 1996). The results obtained by us for the plum rootstock Dzhanka 4, as well as for the apple rootstock MM106 (Nacheva and

Ivanova, 1998; Nacheva, L. 2000), showed that at suitable parameters of environmental factors (mainly light intensity, CO_2 concentration and saccharose in the nutrient medium) the in vitro plants could develop a positive carbon balance, i.e. they could create their organism at the expense of their own photosynthesis.

During the successive stages of the micropropagation process, the photosynthetic apparatus of plants underwent some development leading to an increase in their capacity of CO_2 photofixation. From 34 $n$molCO$_2$ g^{-1}(DW) s^{-1} in the conventional multiplication (MK) plants, the maximum photosynthetic intensity (Pn max) increased to 40.9 $n$molCO$_2$ g^{-1}(DW) s^{-1} in the process of elongation (Ef) and reached 68.5 $n$molCO$_2$ g^{-1}(DW) s^{-1} in the rooting on agar medium (RK), (Table 3).

Similar conclusions have previously been reached for other micropropagated plants - rose (De Riek et al.,1991); statice, cymbidium, carnation, potato (Kozai, 1991a, b; Kozai et al.,1992), gardenia (Serret et al., 1997). Yet the development of photoautotrophy in plant micropropagation was probably restricted by the tightly closed vessels (leading to reduced CO_2 concentration inside the jars during the photoperiod). Our results provided evidence that the development of photoautotrophy in micropropagated plum seemed to be restricted by the tightly closed jars (resulting in lower levels of CO_2 inside the jars during the photoperiod) and in part by the presence of sugars in the medium (Figs 1 and 2, Tables 2 and 3).

The photosynthetic productivity of plantlets could be increased significantly by adequate control of the environmental factors. By the use of a gas-permeable closure (polyester wool), allowing a gas exchange rate of 4,6 volumes per vessel per hour, and by light intensity of about 50µmol m^{-2} s^{-1}, the potential of plantlets for photosynthetic CO_2 assimilation increased significantly. Similar results were obtained by us for the apple rootstock MM106 (Nacheva, 2000).

The positive effect of the increased ventilation of vessels through the use of a polyester wool and a higher light intensity, in combination with reduced saccharose concentration in the nutrient medium, outlined the possibilities for the gradual development of the photosynthetic apparatus in in vitro cultured plants and their transition to photoautotrophy as early as the stage of rooting. Deng and Donnelly (1993) reported that photoautotrophic plantlets performed better than photomixotrophic plantlets after ex vitro transplantation. Similar results were reported about rice regenerants (Seko and Nishimura, 1996). Namely, the preparation of the plantlets under photoautotrophic conditions may reduce the loss of plantlets during the preparation stage, and provide plantlet quality prior to transplanting.

Omitting sugar from the medium benefits the plantlets not only by promoting photoautotrophy in in vitro plants but also by reducing the loss of plantlets due to the biological contamination of the culture medium (Kozai, 1991a). The fact established by us that plum rooting might be successful in photoautotrophic conditions when agar medium is replaced by peat substrate is also of significant importance. Thus, some of the main photoautotrophic technique advantages are also combined, i.e.: working under non-sterile conditions, no necessity of controlling CO_2-diffusion in culture vessels, agar saving. At the same time, a significant stress reduction is achieved when rooted plantlets are transferred for adaptation as this is done together with the substrate. In this way, plants are partly acclimatized which increases their survival during the adaptation period. Using the method proposed here, we may eliminate in vitro rooting and decrease the ex vitro acclimatization period. It leads to reducing the overall costs for mass propagation of fruit plants.

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Tables

Table 1. Experimental conditions of the different variants

Variants	Light (μmol m^{-2} s^{-1}) PAR	vessel closure	saccharose gl^{-1}	duration days
conventional system				
multiplikation (MK)	30	glass cap+polyethylene folio	30	21
elongation (EK)	30	glass cap+polyethylene folio	20	10
rooting (RK)	30	glass cap+polyethylene folio	15	13
improved system				
multiplikation (MW)	30	glass cap+polyester wool	30	21
elongation (EW)	48	glass cap+polyester wool	10	10
rooting in peat (R)	48		---	12

Table 2. Values for the functional photosynthetic parameters, calculated from the curves of net photosynthetic rate versus photon flux density (Pn=f (PAR)) at saturated CO_2-concentration (1500 vpm).

Variants	Max net photos. rate $n$molCO$_2$ (gDW)$^{-1}$ s^{-1}	Dark respiration rate $n$molCO$_2$ (gDW)$^{-1}$ s^{-1}	Light comp. point (Γl) µmolCO$_2$m^{-2} s^{-1}	Quantum yield dy/dx
MK	39.67	-32.21	65.8	0.489
MW	57.57	-20.85	23.3	0.894
EK	58.36	-8.94	27.2	0.328
EW	76.40	-0.78	1.1	0.706
RK	72.35	-2.79	27.6	0.101
R	139.87	-27.53	11.1	2.479

Table 3. Values for the functional photosynthetic parameters, calculated from the curves of net photosynthetic rate versus CO_2-concentration (Pn=f (CO_2)) at saturated photon flux density (250 µmol m^{-2} s^{-1}).

Variants	Max net photos. rate $n$molCO$_2$ (gDW)$^{-1}$ s^{-1}	Dark respiration rate $n$molCO$_2$ (gDW)$^{-1}$ s^{-1}	CO$_2$-comp. point (Γc) vpm	Carboxylase activity dy/dx
MK	34.03	-9.26	109.5	0.085
MW	55.57	-18.31	69.3	0.264
EK	40.89	-7.35	65.0	0.113
EW	71.99	-10.20	56.9	0.179
RK	68.43	-11.86	93.3	0.127
R	133.76	-108.87	150.8	0.721

Figures

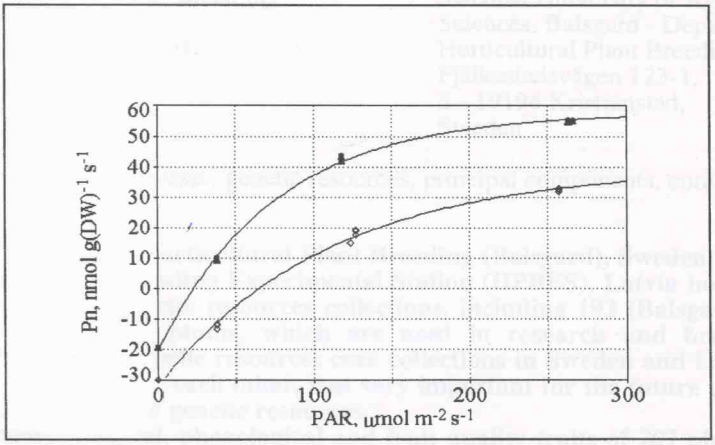

Fig. 1. The relationship between net photosynthetic rate (Pn) and PAR at saturated CO_2-concentration (1500 vpm) stage ***multiplication***, (◊-conventional system (MK); ■- improved system (MW)).

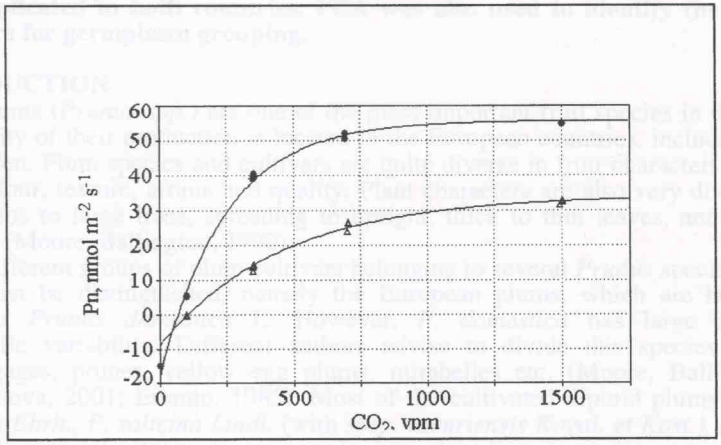

Fig. 2. The relationship between net photosynthetic rate (Pn) and CO_2-concentration at saturated PAR (250 μmol m^{-2} s^{-1}) stage ***multiplication***,(Δ-conventional system (MK); ■- improved system (MW)).