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Effect of carbon dioxide enrichment during *in vitro* cultivation and acclimation to *ex vitro* conditions

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Abstract

Tobacco and carnation plantlets were grown *in vitro* on Murashige and Skoog's medium with 2 % saccharose. Carnation plantlets were also grown fully photoautotrophically on a medium without saccharose. The ambient CO_2 concentration was increased from 0.6 to 10 or 40 g m^{-3} during the last 3 weeks of *in vitro* cultivation or during the first 3 weeks of acclimation to *ex vitro* condition (plantlets transplanted to pots with sand and nutrient solution) or during both growth phases. CO_2 enrichment during *in vitro* cultivation markedly stimulated growth of tobacco plantlets, and also of carnation plantlets, both with and without saccharose. CO_2 enrichment during the acclimation period promoted plant growth more effectively in plantlets grown *in vitro* at a CO_2 concentration of 0.6 g m^{-3} than in plantlets grown in either growth phase at higher CO_2 concentrations.

Additional key words: *Dianthus caryophyllus*, *Nicotiana tabacum*, saccharose.

Introduction

The goal of micropropagation is to obtain a large number of genetically and physiologically uniform plantlets with high photosynthetic potential, able to survive the tranfer to *ex vitro* conditions. One way to reach this is photoautotrophic cultivation of plantlets on a medium without saccharides. These plantlets usually need CO_2 enrichment and higher than conventionally used irradiance (for reviews see, *e.g.*, Kozai 1991, Pospíšilová *et al.* 1992, 1997, Buddendorf-Joosten and Woltering 1994, Jeong *et al.* 1995, Kozai and Smith 1995). Such environmental control enables promotion of plantlet growth, higher survival percentage and a reduction in biological contamination. The CO_2 enrichment can be achieved by

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1) using a gas permeable film for vessel closure, *2*) increasing CO_2 concentration around the cultivation vessels or *3*) by direct supply of CO_2 into the vessels, *e.g.*, by forced ventilation. Individual growth parameters are affected by increased CO_2 concentration differently in individual plant species and ontogenetic stages (*e.g.*, Figueira *et al.* 1991, Fournioux and Bessis 1993), while interactions with other environmental factors especially irradiance and medium composition also have to be considered (Kozai and Iwanami 1988, Kozai *et al.* 1988a,b). The after-effect of increased CO_2 concentration accompanied by increased irradiance or decreased air humidity during *in vitro* cultivation of *Asparagus*, *Eucalyptus*, *Fragaria* and *Rubus* plantlets promotes their growth during acclimation to *ex vitro* conditions (Laforge *et al.* 1991, Deng and Donelly 1993, Kirdmanee *et al.* 1995). Less attention has been paid to CO_2 enrichment during the acclimation period of transplanted plants. Nevertheless, it could be also useful, especially in combination with supplemental lighting (Desjardins *et al.* 1987, Hayashi and Kozai 1987).

The aims of this paper were *1*) to determine the effects on plant growth rate of CO_2 enrichment during the last phase of *in vitro* cultivation, during acclimation to *ex vitro* conditions and during both stages; *2*) to compare the responses of two plant species, tobacco and carnation; and *3*) to determine the effect of the presence of saccharose in the cultivation medium on plantlet response to CO_2 enrichment.

Materials and methods

Plantlets of tobacco (*Nicotiana tabacum* L. cv. White Burley) were cultivated on the agar-solidified medium of Murashige and Skoog (1962) with 2 % of saccharose at 16/8 h photoperiod, irradiance (400 - 700 nm) of 100 μmol m^{-2} s^{-1}, and day/night temperature of 25/20 °C. Starting one week after culture initiation, plantlets were exposed to three different CO_2 concentrations: 0.6 g(CO_2) m^{-3} [0.1 M solutions of $NaHCO_3$ and Na_2CO_3 mixed in the ratio 77/23 (v/v)], 10 and 40 g(CO_2) m^{-3} [3 M solutions of $NaHCO_3$ and Na_2CO_3 mixed in the ratios 50/50 and 73/27 (v/v)] (for detail see Solárová *et al.* 1989). After 3 weeks the plantlets were transplanted into pots with coarse sand and IBP nutrient solution (Hewitt 1966) and grown under similar irradiance and temperature as during the *in vitro* cultivation. Plantlets grown *in vitro* under each CO_2 concentration were after transplantation divided into three groups which were grown under the three different CO_2 concentrations (0.6, 10 or 40 g m^{-3}) for a further 3 weeks. Thus the growth parameters (leaf number, leaf area, leaf, stem and root dry masses, *etc.*) were determined in 3 variants after 3 weeks of *in vitro* cultivation and in 9 variants after the whole treatment (3 weeks *in vitro* and 3 weeks *ex vitro*).

Similar experiments were done with carnation (*Dianthus caryophyllus* L. cv. Mascotte Rose) plantlets which were cultivated on agar-solidified MS medium without or with 2 % saccharose with the aim of comparing the effect of CO_2 enrichment under fully autotrophic and mixotrophic conditions. Photoperiod was also 16 h, irradiance 100 μmol m^{-2} s^{-1} and day/night temperature 25/20 °C. Starting one week after culture initiation, plantlets were exposed to atmospheric CO_2

concentration 0.6 g(CO_2) m^{-3} or increased to 40 g(CO_2) m^{-3} or decreased to almost 0 g(CO_2) m^{-3} (absorption of CO_2 by 10 % KOH solution). After 3 weeks carnation plantlets were transplanted in a similar way to tobacco plantlets, and grown under two the different CO_2 concentrations (0.6 or 40 g m^{-3}) for a further 3 weeks. Thus the growth parameters (leaf number, leaf area, leaf, stem and root dry masses, *etc.*) were followed in 6 variants after 3 weeks of *in vitro* cultivation and in 8 variants 3 weeks after transplantation.

Irradiance and air temperature and air humidity were measured with a *LI 185B* radiometer with a quantum sensor (*Li-Cor*, Lincoln, USA) and with a *JUMO Humitherm TDAc-70* (*M.K. Juchheim*, Fulda, Germany), respectively. Dry mass was determined in samples oven-dried at 90 °C to constant mass. The experiments were repeated 3 times with similar results.

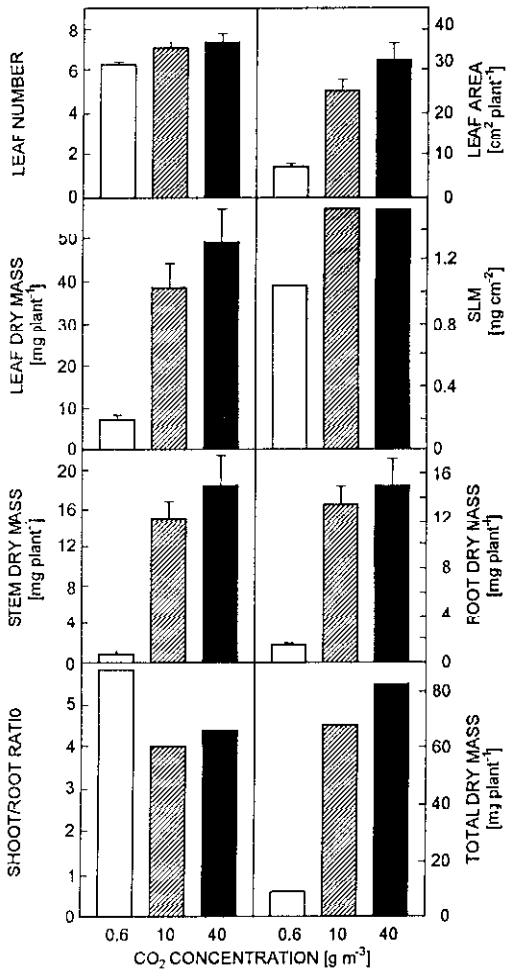

Fig. 1. Effect of CO_2 enrichment on growth parameters of tobacco plantlets grown *in vitro* on medium with 2 % saccharose. Means ± S.E., $n = 10$.

Results

Increase in CO_2 concentration in the growth cabinet from 0.6 to 10 or 40 g m^{-3} during *in vitro* cultivation of tobacco plantlets on the medium with 2 % saccharose resulted in much more rapid growth of the plantlets (Fig. 1). CO_2 enrichment greatly increased leaf, stem and root dry masses and leaf area and to a lesser extent also leaf number per plantlet and specific leaf mass (SLM). The larger size of plantlets grown *in vitro* under CO_2 enrichment persisted during their further growth *ex vitro* (Fig. 2). However, CO_2 enrichment from 0.6 to 10 or 40 g m^{-3} after transplantation *ex vitro* stimulated growth only of those plantlets grown *in vitro* under low CO_2

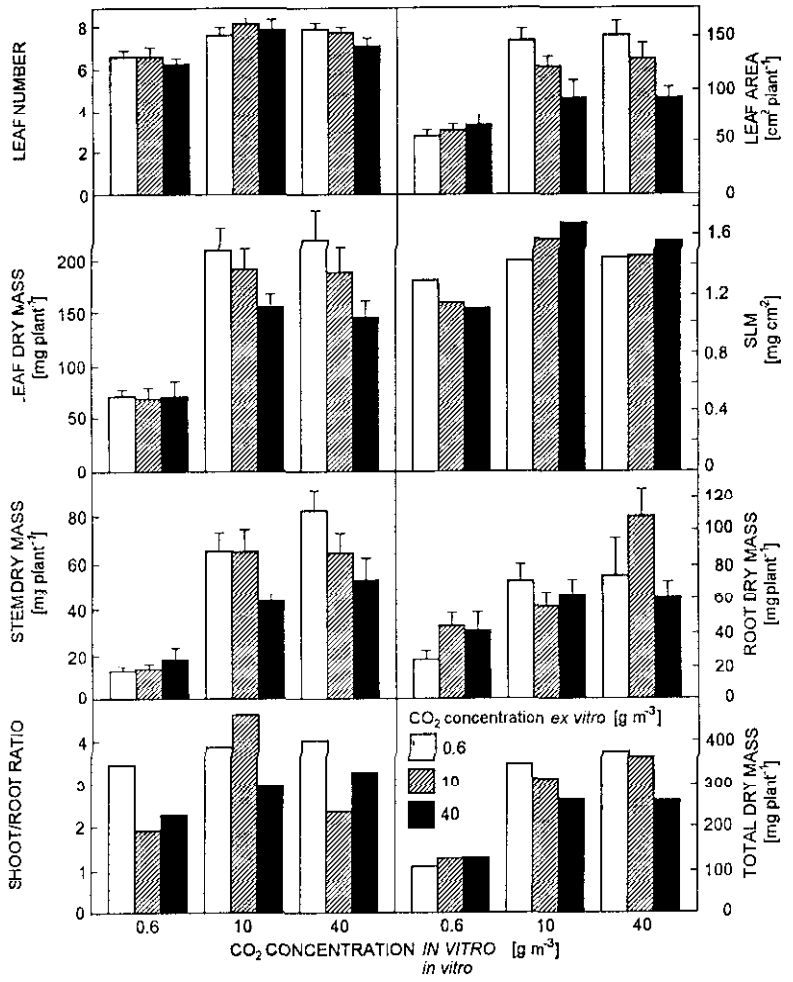

Fig. 2. Effect of different CO_2 concentrations (0.6, 10 and 40 g m^{-3}) on growth parameters of tobacco plants during their acclimation to *ex vitro* conditions. Plantlets were initially grown *in vitro* on medium with 2 % saccharose at CO_2 concentrations 0.6, 10 or 40 g m^{-3}. Growth parameters were measured 3 weeks after transplantation. Means ± S.E., n = 10.

concentration. Plantlets grown *in vitro* at CO_2 concentration 0.6 g m^{-3} and *ex vitro* at CO_2 concentrations 10 and 40 g m^{-3} had higher dry masses of stems and roots and leaf area than those grown *ex vitro* under CO_2 concentration 0.6 g m^{-3} but the leaf dry mass and number of leaves per plant were not affected (Fig. 2). On the other hand, plantlets cultivated *in vitro* at CO_2 concentration 10 or 40 g m^{-3} had the highest leaf area, and leaf and stem dry masses when cultivated *ex vitro* at CO_2 concentration 0.6 g m^{-3}. CO_2 enrichment during *ex vitro* cultivation even had negative effects on growth of these plantlets (Fig. 2). Nevertheless, the plants grown during the whole experimental period (3 weeks *in vitro* and 3 weeks *ex vitro*) under CO_2 concentration enriched to 10 or 40 g m^{-3} were clearly more vigorous than the plants grown during the whole experimental period at CO_2 concentration 0.6 g m^{-3}.

CO_2 enrichment to 40 g m^{-3} during *in vitro* cultivation also stimulated growth of carnation plantlets. This stimulation was observed in those grown on the medium without saccharose as well as with saccharose, only the effect on plantlets grown with saccharose was less marked (Fig. 3). CO_2 concentration decreased near to zero inhibited plantlet growth, and growth of plantlets on the medium without saccharose was very low and the plantlets did not develop roots. Presence of saccharose in the medium had positive effects on plantlet growth not only under low CO_2

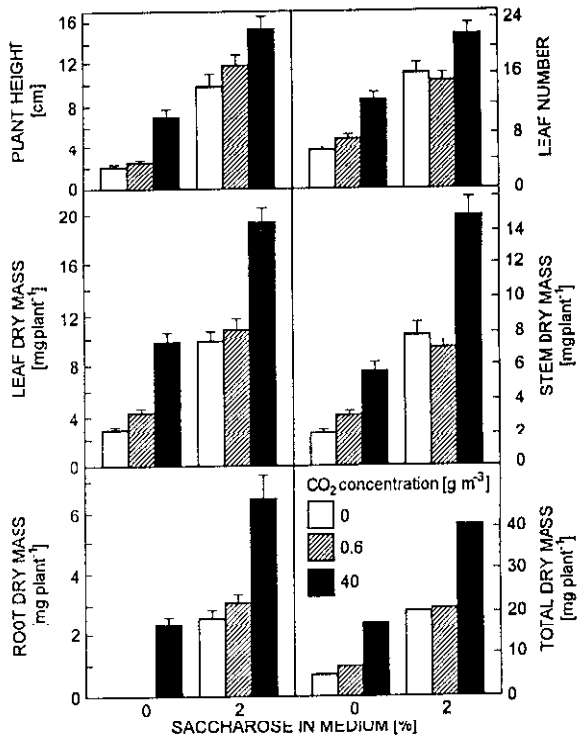

Fig. 3. Effect of different CO_2 concentrations (0, 0.6 and 40 g m^{-3}) on growth parameters of carnation plantlets grown *in vitro* on medium with or without 2 % saccharose. Means ± S.E., $n = 25$.

concentrations but also under CO_2 enrichment. Larger sizes of plantlets grown on medium with saccharose also persisted during *ex vitro* cultivation (Fig. 4). After 6 weeks the biggest plants were those cultivated *in vitro* on medium with 2 % saccharose and CO_2 concentration 0.6 g m^{-3} and *ex vitro* at CO_2 concentration 40 g m^{-3}. The size of plants grown during the whole experimental period at CO_2 concentrations 0.6 or 40 g m^{-3} was comparable.

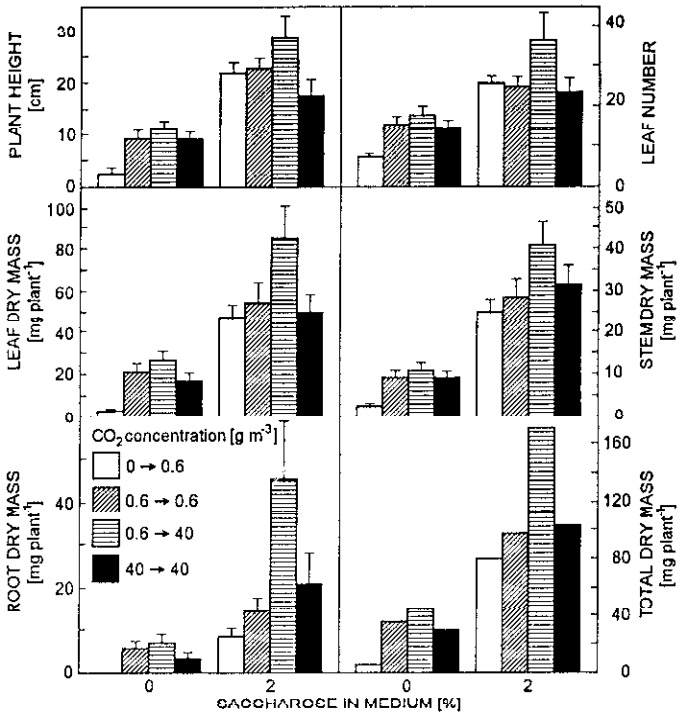

Fig. 4. Effect of different CO_2 concentrations (0, 0.6 and 40 g m^{-3}) on growth parameters of carnation plants during their acclimation to *ex vitro* conditions. Plantlets were initially grown *in vitro* on medium with or without 2 % saccharose and at CO_2 concentrations 0, 0.6 or 40 g m^{-3}. Growth parameters were measured 3 weeks after transplantation. Means ± S.E., $n = 25$.

Discussion

CO_2 enrichment during *in vitro* cultivation greatly stimulated growth of tobacco and carnation plantlets. Previous experiments (Solárová *et al.* 1989) showed that the development of the photosynthetic apparatus in tobacco plantlets was only slightly dependent on the type of carbon supply (medium with 2 % saccharose or without saccharose and CO_2 concentration 0.6, 10 or 40 g m^{-3}). Under natural CO_2 supply, the CO_2 concentrations inside the vessels increased during the dark period and decreased during the light period due to plantlet respiration and photosynthesis (see

Solárová 1989 for details). Under CO_2 concentrations elevated to 10 or 40 g m^{-3}, the CO_2 in the vessels during the light period was never depleted to concentrations lower than those saturating plantlet photosynthesis (1.0 - 1.4 g m^{-3}) (Solárová *et al.* 1989, 1996). Therefore increased biomass accumulation was mostly a consequence of increased photosynthetic rate of plantlet *in situ* due to control of environmental conditions.

The growth enhancement under CO_2 enrichment was more strongly expressed in tobacco than in carnation plantlets. In addition to species-specific responses, the higher proportion of leaves in the total biomass in tobacco than in carnation might be important.

The effect of CO_2 enrichment was slightly more marked on carnation plantlets grown on medium without saccharose than on those grown on medium with 2 % saccharose. Nevertheless, growth parameters of carnation plantlets were higher in those grown mixotrophically than in those grown photoautotrophically under all CO_2 concentrations, as was found previously in tobacco plantlets (Solárová *et al.* 1989, Tichá 1996).

Larger sizes of tobacco and carnation plantlets grown *in vitro* under CO_2 enrichment persisted during their further growth *ex vitro*. Therefore the promotion of growth of *Asparagus, Eucalyptus, Fragaria* and *Rubus* plantlets during acclimation to *ex vitro* conditions, as an after-effect of increased CO_2 concentration during *in vitro* cultivation (Laforge *et al.* 1991, Deng and Donnelly 1993, Kirdmanee *et al.* 1995), was confirmed also in tobacco and carnation plantlets.

The CO_2 enrichment after transplantation to *ex vitro* conditions strongly stimulated growth of tobacco and carnation plantlets grown *in vitro* under low CO_2 concentration. After 6 weeks the size of plants was the largest when they were cultivated *in vitro* at CO_2 concentration 0.6 g m^{-3} and *ex vitro* at CO_2 concentration 40 g m^{-3}. Therefore the usefulness of CO_2 enrichment during the acclimation period of transplanted strawberry plants found by Desjardins *et al.* (1987) and Hayashi and Kozai (1987) was confirmed also with tobacco and carnation plants.

On the other hand, plantlets cultivated during both growth phases under CO_2 enrichment were smaller. So the long-term effect of high CO_2 concentration was less effective than the short-term effect. This might be because of down-regulation of photosynthesis found in plants growing for a long period under CO_2 enrichment (for review see, *e.g.*, Reining 1994).

References

Buddendorf-Joosten, J.M.C., Woltering, E.J.: Components of the gaseous environment and their effects on plant growth and development *in vitro*. - Plant Growth Regul. **15**: 1-16. 1994.

Deng, R., Donnelly, D.J.: *In vitro* hardening of red raspberry through CO_2 enrichment and relative humidity reduction on sugar-free medium. - Can. J. Plant Sci. **73**: 1105-1113. 1993.

Desjardins, Y., Gosselin, A., Yelle, S.: Acclimatization of *ex vitro* strawberry plantlets in CO_2-enriched environments and supplementary lighting. - J. amer. Soc. hort. Sci. **112**: 846-851, 1987.

Figueira, A., Whipkey, A., Janick, J.: Increased CO_2 and light promote *in vitro* shoot growth and development of *Theobroma cacao*. - J. amer. Soc. hort. Sci. **116**: 585-589, 1991.

Fournioux, J.C., Bessis, R.: Use of carbon dioxide enrichment to obtain adult morphology of grapevine *in vitro*. - Plant Cell Tissue Organ Cult. **33**: 51-57, 1993.

Hayashi, M., Kozai, T.: Development of a facility for accelerating the acclimatization of tissue-cultured plantlets and the performance of test cultivations. - In: Ducate, G., Jacob, M., Simpson, A. (ed.): Plant Micropropagation in Horticultural Industries. Pp. 123-134. Arlon 1987.

Hewitt, E.J.: Sand and Water Culture Methods Used in the Study of Plant Nutrition. 2nd Edition. - Commonwealth Agr. Bur., Farnham Royal 1966.

Jeong, B.R., Fujiwara, K., Kozai, T.: Environmental control and photoautotrophic micropropagation. - Hort. Rev. **17**: 123-170, 1995.

Kirdmanee, C., Kitaya, Y., Kozai, T.: Effect of CO_2 enrichment and supporting material *in vitro* on photoautotrophic growth of *Eucalyptus* plantlets *in vitro* and *ex vitro*. - *In Vitro* cell. dev. Biol. Plant **31**: 144-149, 1995.

Kozai, T.: Micropropagation under photoautotrophic conditions. - In: Debergh, P.C., Zimmerman, R.H. (ed.): Micropropagation Technology and Application. Pp. 447-469. Kluwer Academic Publishers, Dordrecht - Boston - London 1991.

Kozai, T., Iwanami, Y.: Effect of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during preparation stage. - J. jap. Soc. hort. Sci. **57**: 279-288, 1988.

Kozai, T., Koyama, Y., Watanabe, I.: Multiplication of potato plantlets *in vitro* with sugar free medium under high photosynthetic photon flux. - Acta Hort. **230**: 121-127, 1988a.

Kozai, T., Kubota, C., Watanabe, I.: Effect of basal medium composition on the growth of carnation plantlets in auto- and mixo-trophic tissue culture. - Acta Hort. **230**: 159-166, 1988b.

Kozai, T., Smith, M.A.L.: Environmental control in plant tissue culture - general introduction and overview. - In: Aitken-Christie, J., Kozai, T., Smith, M.L. (ed.): Automation and Environmental Control in Plant Tissue Culture. Pp. 301-318. Kluwer Academic Publishers, Dordrecht - Boston - London 1995.

Laforge, F., Lussier, C., Desjardins, Y., Gosselin, A.: Effect of light intensity and CO_2 enrichment during *in vitro* rooting on subsequent growth of plantlets of strawberry, raspberry and asparagus in acclimatization. - Scientia Hort. **47**: 259-269, 1991.

Pospíšilová, J., Solárová, J., Čatský, J.: Photosynthetic responses to stresses during *in vitro* cultivation. - Photosynthetica **26**: 3-18, 1992.

Pospíšilová, J., Čatský, J., Šesták, Z.: Photosynthesis in plants cultivated *in vitro*. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 525-540. Marcel Dekker, New York 1997.

Reining, E.: Acclimation of C_3 photosynthesis to elevated CO_2: hypotheses and experimental evidence. - Photosynthetica **30**: 519-525, 1994.

Solárová, J.: Photosynthesis of plant regenerants. Diurnal variation in CO_2 concentration in cultivation vessels resulting from plantlet photosynthetic activity. - Photosynthetica **23**: 130-135, 1989.

Solárová, J., Pospíšilová, J., Čatský, J., Šantrůček, J,: Photosynthesis and growth of tobacco plantlets in dependence on carbon supply. - Photosynthetica **23**: 629-637, 1989.

Solárová, J., Součková, D., Ullmann, J., Pospíšilová, J.: *In vitro* culture: environmental conditions and plantlet growth as affected by vessel type and stopper material. - Zahradnictví (Praha) **23**(2): 51-58, 1996.

Tichá, I.: Optimization of photoautotrophic tobacco *in vitro* culture: effect of suncaps closures on plantlet growth. - Photosynthetica **32**: 475-479, 1996.