Acclimatization of Micropropagated American Ginseng Plantlets for the Replenishment of its Wild Populations and its Production in Forest Farming Systems

Rajae Sfendla [1], Hélène Desilets [1], Sylvie Laliberté [2] and Alain Olivier [1]

[1] Département de phytologie, Université Laval, Québec (Qc), G1K 7P4, Canada

[2] Département des sciences biologiques, UQÀM, Montréal (Qc), H3C 3P8, Canada

ABSTRACT

American ginseng (*Panax quinquefolius* L.) plantlets produced by somatic embryogenesis were pre-acclimatized for four weeks inside growth chambers in a factorial design. The three factors of the experiment were CO_2 concentration (400 and 3000 µL L^{-1}), photosynthetic photon flux (*PPF*, 30 and 50 µmol m^{-2} s^{-1}) and sucrose concentration (0 and 15 g L^{-1}) in the culture medium. The plantlets were subsequently transferred into a greenhouse where they were acclimatized for two weeks. The results show that regardless of *PPF* and sucrose levels, CO_2 enrichment during the pre-acclimatization phase significantly increased (6.3 %) the fresh mass (FM) of American ginseng plantlets in the growth chambers. This positive effect resulted in a 26.4 % increase in FM after the acclimatization phase in the greenhouse. CO_2 enrichment during the pre-acclimatization phase also significantly increased (32 %) leaf area after acclimatization, but had no significant effect on dry mass, height and survival. Increased light irradiance and reduced sucrose concentration had no significant effects on the growth parameters and survival of plantlets. Thus, despite the biological and ecological constraints that limit American ginseng growth and development, CO_2 enrichment could enhance its acclimatization. This could accelerate and ameliorate the production of plantlets for forest farming systems and supply plants to replenish wild populations.

Introduction

In vitro micropropagation allows the rapid production of genetically uniform and disease-free plantlets from numerous plant species (Jeong et al., 1995). However, the acclimatization of plantlets to the ex vitro environment is a critical phase for many species. Anatomical and physiological modifications resulting from tissue culture can delay the transitional process from heterotrophic to autotrophic nutrition (Desjardins et al., 1988), resulting in slow growth and high mortality levels.

Various techniques have been developed to facilitate the acclimatization of the plantlets to the ambient environments. In vitro CO_2 enrichment and supplemental lighting have been the most studied. These techniques have reduced the length of the acclimatization period and improved the quality of strawberry, raspberry and asparagus plantlets (Desjardins et al., 1988). Plantlet survival and growth were improved through in vitro

CO_2 enrichment in carrot (Tsuji et al., 1992), red raspberry (Deng and Donnelly, 1993) and tobacco (Pospisilova et al., 1999), among others. Better growth and higher photosynthetic capacity were also observed in many species as a result of increased in vitro photosynthetic photon flux (Kozai, 1991 ; Itsutsa et al., 1994). Partial or complete removal of sucrose from the culture medium is another technique that increased photosynthetic activity (Hdider and Desjardins, 1994) and improved vigor (Kozai, 1996) and growth rate (Heo et al., 1996) of plantlets by stimulating autotrophy. Although the cost of these techniques is high, in vitro micropropagation remains viable for high-value crops, especially when traditional techniques do not allow the production of adequate quantities of disease-free plants, as is the case with American ginseng (*Panax quinquefolius*).

American ginseng is a native species from the deciduous forests of the Eastern part of North America. It is closely related to Asian ginseng (*Panax ginseng*), which has been used in Asia for over 4000 years because of its medicinal properties. Extensive stands were found when it was discovered at the beginning of the 18th century. Currently, few wild populations remain (Nault, 1998) with supply relying on cultivated production. The biological cycle of this perennial species is very slow, making multiplication through conventional methods a long process (Wang, 1990). Moreover, American ginseng is very sensitive to many pathogens, reducing seed quality and number of viable propagules.

Micropropagation could accelerate and ameliorate the production process and supply plants to replenish wild populations. Micropropagated plantlets of American ginseng have been obtained from root (Wang, 1990; Tirajoh and Punja, 1995; Tirajoh et al., 1998), epicotyle (Tirajoh and Punja, 1995; Tirajoh et al., 1998) and zygotic embryos (Campeau et al., 1998; Wang et al., 1999 ; Laliberté et al., 2000). Using micropropagation has been hampered because acclimatization of American ginseng is difficult and high mortality rates are observed when plantlets are transferred to the ex vitro environment.

This work is part of a program that aims at developing ginseng forest farming systems adapted to Canadian maple forests, while helping to protect its endangered wild populations. Its purpose was to improve the acclimatization process for American ginseng plantlets by investigating the effects of CO_2 enrichment, supplemental lighting and removal of sucrose from the culture medium on the survival and growth of plantlets developed through somatic embryogenesis.

Material and Methods

Plant material and culture medium. Somatic embryo derived American ginseng plantlets (Campeau et al., 1998; Laliberté et al., 2000) were transferred to Magenta™ GA_7 vessels (Magenta Corp., Chicago) containing 60 ml of MS (Murashige and Skoog, 1962) modified by adding, per liter, 100 mg casein hydrolysate, 100 mg myo-inositol, 4 g Gel-gro (ICN Biomedicals, inc.) and 0 or 15 g sucrose at pH 5.8. Magenta™ vessels were

modified by adding a 1 cm, 0.2 µ CO_2 membrane to facilitate gas exchange. Each vessel contained four American ginseng plantlets.

Pre-acclimatization in growth chambers.

Magenta™ vessels containing American ginseng plantlets were placed in four 65 L growth chambers especially made for controlling internal CO_2 concentration, temperature and relative humidity (Desjardins et al., 1988). Tubes through the polypropylene walls of each growth chamber supplied a mixture of CO_2 (liquid) and compressed air at a rate of 12 L min^{-1}. Mass debit controls made it possible to obtain two different CO_2 concentrations (400 µL L^{-1} and 3000 µL L^{-1}). Fluorescent lights providing 30 or 50 µmol m^{-2} s^{-1} of *PPF* were installed above the chambers. The temperature was controlled with the help of an electronic device coupled to a heat exchanger (air-water) (Laforge et al., 1990) to provide specific day (20-23 °C) and night (18-20 °C) thermoperiods.

Greenhouse acclimatization.

After four weeks of pre-acclimatization in the miniature growth chambers, plantlets were transferred in 4 inches diameter pots (4 plantlets per pot) containing a mix of Vermiculite (20 %), Turface (20 %) and Pro-mix (60 %). In the greenhouse, plantlets were first acclimatized under a plastic tunnel covered by a polypropylene shade cloth that filtrated 80 % of ambient light. They were progressively exposed to the normal atmosphere, from the third day of the acclimatization process, by removing the plastic tunnel. Plantlets were watered regularly to maintain a saturated humidity environment favoring acclimatization. A nutrient solution consisting of calcium nitrate and 8N-8.7P-24.9K was supplemented starting the third day of acclimatization.

Parameters measured.

After pre-acclimatization in growth chambers, survival rates were determined for each treatment, as well as fresh mass (FM), dry mass (DM) (after 72 h at 60 °C), height and leaf area (four plants per treatment). The same measures were taken after two weeks of greenhouse acclimatization.

Experimental design and statistical analysis.

The experimental design was a strip split plot. The treatments were arranged in a 2 x 2 x 2 factorial with CO_2 concentration (400 et 3000 µL L^{-1}) as the main plot, *PPF* (30 et 50 µmol m^{-2} s^{-1}) as the sub-plot and sucrose concentration (0 and 15 g L^{-1}) as sub-sub-plot. Each experimental unit consisted of 4 plantlets in the same Magenta™ vessel. The experiment was conducted 4 times, from Sept. 1999 to June 2000. Analysis of variance was performed using SAS 6.12 (SAS Institute, Inc., NC, USA). A simple comparison of the means was performed with the help of an F test.

Results

There was no significant interaction between CO_2 enrichment, light irradiance and sucrose concentration. Therefore, results regarding the individual treatments effects are presented separately.

Survival rate. In vitro CO_2 enrichment (3000 µL L^{-1}) had no significant effect on the survival rate of American ginseng plantlets after pre-acclimatization ($P = 0.0801$) (Table 1). Increasing *PPF* (50 µmol m^{-2} s^{-1}) or removing sucrose from the culture medium also failed to improve survival percentage ($P = 0.4447$ and $P = 0.2306$, respectively). The survival rate of the American ginseng plantlets after pre-acclimatization was high for every treatment, ranging from 94 to 100 %.

CO_2 enrichment ($P = 0.4226$), *PPF* level ($P = 0.3206$) and sucrose concentration ($P = 0.3557$) during the pre-acclimatization phase did not improve survival of American ginseng plantlets after the acclimatization phase in the greenhouse. Survival rates after two weeks of acclimatization varied from 73.42 % for the control to 84.13 % for the plantlets pre-acclimatized in CO_2-enriched atmosphere, increased *PPF* and sucrose-containing culture medium.

Table 1. Results of the analysis of variance (*P* values) on the effects of in vitro CO_2 enrichment, *PPF* and removal of sucrose from the culture medium on the survival rate (SR), FM, DM, height (ht) and leaf area (LA) of American ginseng plantlets after four weeks of pre-acclimatization in growth chambers and two weeks of acclimatization in a greenhouse.

Pre-acclimatization	SR	FM	DM	ht	LA
CO_2	0.0801	0.0457 *	0.7287	0.4910	0.6859
PPF	0.4447	0.4037	0.3017	0.9779	0.4354
Sucrose	0.2306	0.5432	0.4309	0.2883	0.9868
CO_2 x *PPF*	0.4127	0.3745	0.5118	0.9561	0.8183
CO_2 x Sucrose	0.6397	0.2651	0.2496	0.2186	0.2890
PPF x Sucrose	0.6897	0.6242	0.4540	0.4204	0.4780
CO_2 x *PPF* x Sucrose	0.3822	0.9417	0.8363	0.0867	0.6292

Acclimatization	SR	FM	DM	ht	LA
CO_2	0.4226	0.0173 *	0.4217	0.8180	0.0282 *
PPF	0.3206	0.2804	0.4239	0.2394	0.4720
Sucrose	0.3557	0.1579	0.6368	0.9408	0.0962
CO_2 x *PPF*	0.6958	0.9101	0.2437	0.9890	0.0824
CO_2 x Sucrose	0.5380	0.1902	0.2092	0.9474	0.4721
PPF x Sucrose	0.8664	0.7243	0.8161	0.7639	0.7599
CO_2 x *PPF* x Sucrose	0.5716	0.4657	0.3254	0.4755	0.9108

* significant at $P < 0.05$

Fresh mass.

CO_2 enrichment significantly increased the FM of American ginseng plantlets after both pre-acclimatization (6.3 %) ($P = 0.0457$) and acclimatization (26.4 %) ($P = 0.0173$) (Fig.

1a). Increasing *PPF* (Fig. 2a) or removing sucrose from the culture medium (Figure 3a), however, did not have any significant effect on this variable after pre-acclimatization ($P = 0.4037$ and $P = 0.5432$, respectively) or acclimatization ($P = 0.2804$ and $P = 0.1579$, respectively).

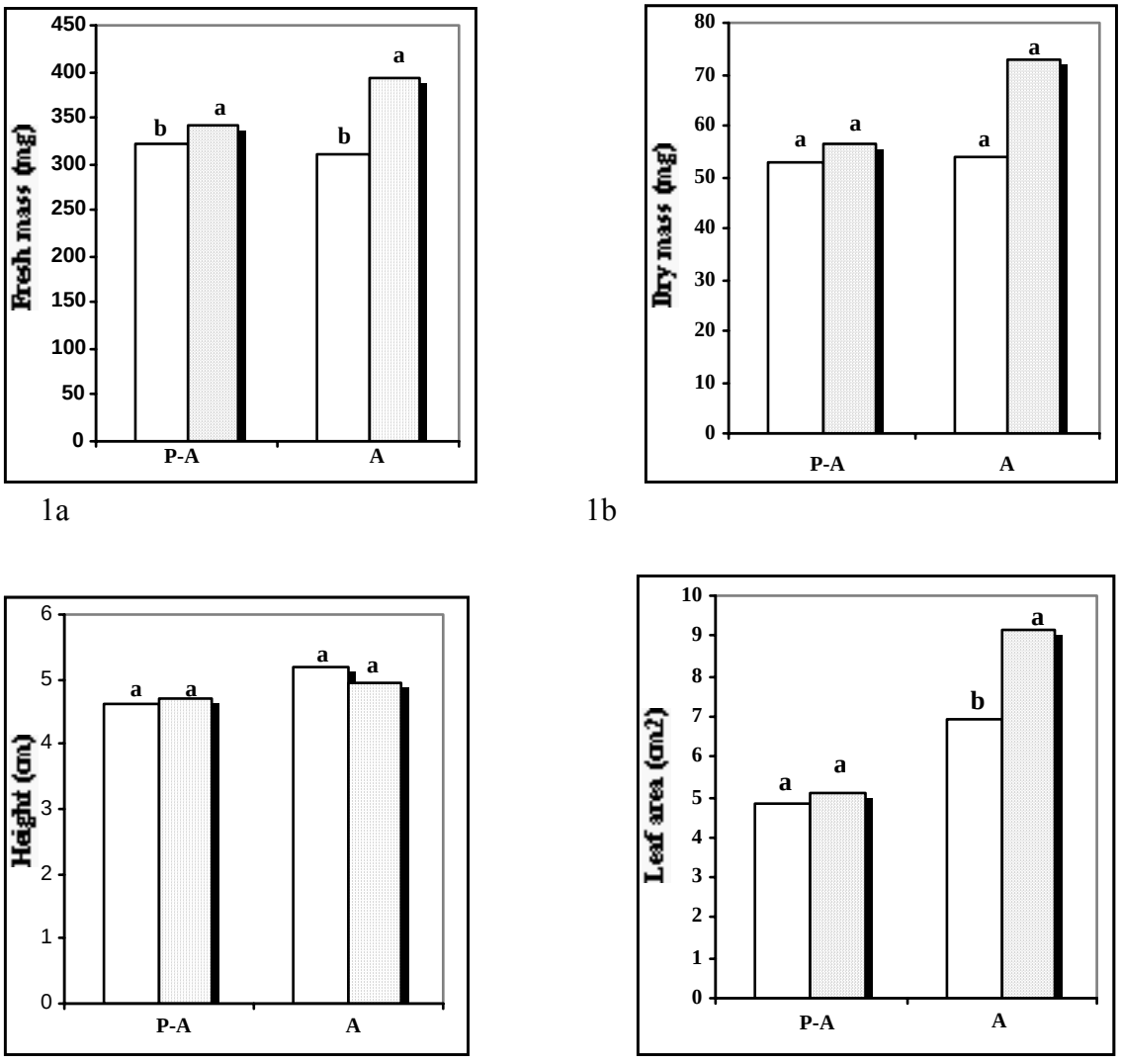
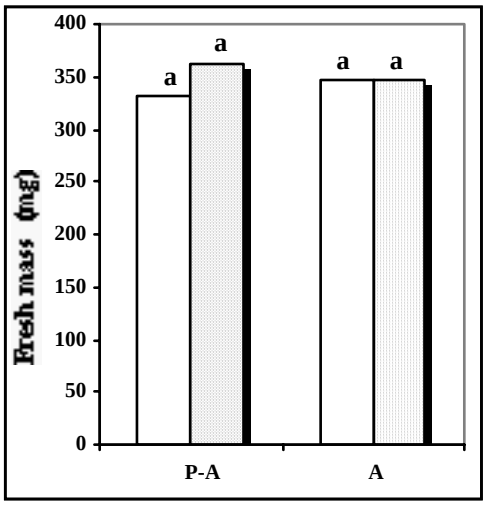


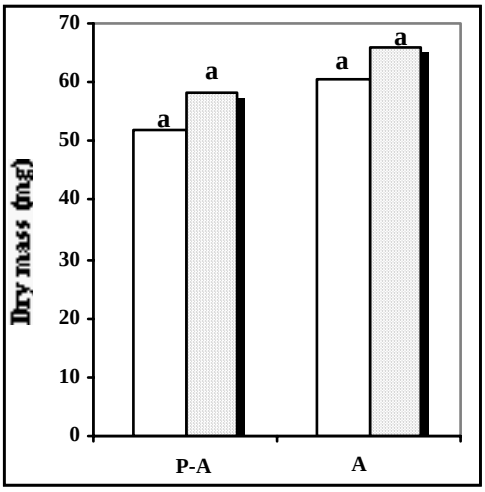
Fig. 1. The effects of in vitro CO_2 enrichment on : a) FM ; b) DM ; c) height ; and d) leaf area of American ginseng plantlets after four weeks of pre-acclimatization (P-A) in growth chambers and two weeks of acclimatization (A) in a greenhouse. Contiguous columns with the same letters represent treatments that are not significantly different at $P = 0.05$.

Dry mass.

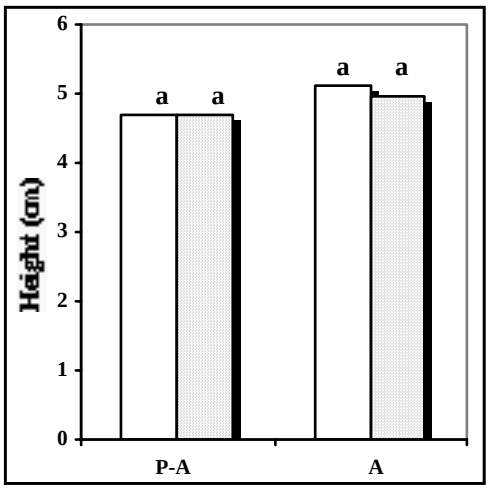
CO_2 enrichment (Fig. 1b), *PPF* level (Fig. 2b) and sucrose concentration (Fig. 3b) did not have any significant effect on the DM of American ginseng plantlets after the pre-acclimatization ($P = 0.7287$, $P = 0.3017$ and $P = 0.4309$, respectively) or the acclimatization phase ($P = 0.4217$, $P = 0.4239$ and $P = 0.6368$, respectively).



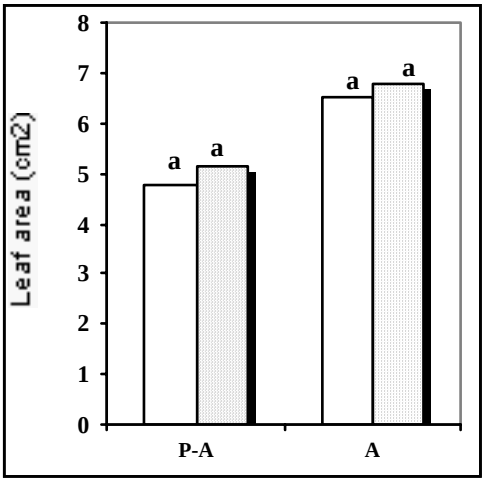
2a



2b



2c



2d

Fig. 2. The effects of in vitro *PPF* on : a) FM ; b) DM ; c) height ; and d) leaf area of American ginseng plantlets after four weeks of pre-acclimatization (P-A) in growth chambers and two weeks of acclimatization (A) in a greenhouse. Contiguous columns with the same letters represent treatments that are not significantly different at $P = 0.05$.

Height.

CO_2 enrichment (Fig. 1c), *PPF* level (Fig. 2c) and sucrose concentration (Fig. 3c) did not have any significant effect on the height of American ginseng plantlets after the pre-acclimatization ($P = 0.4910$, $P = 0.9779$ and $P = 0.2883$, respectively) or the acclimatization phase ($P = 0.8180$, $P = 0.2394$ and $P = 0.9408$, respectively).

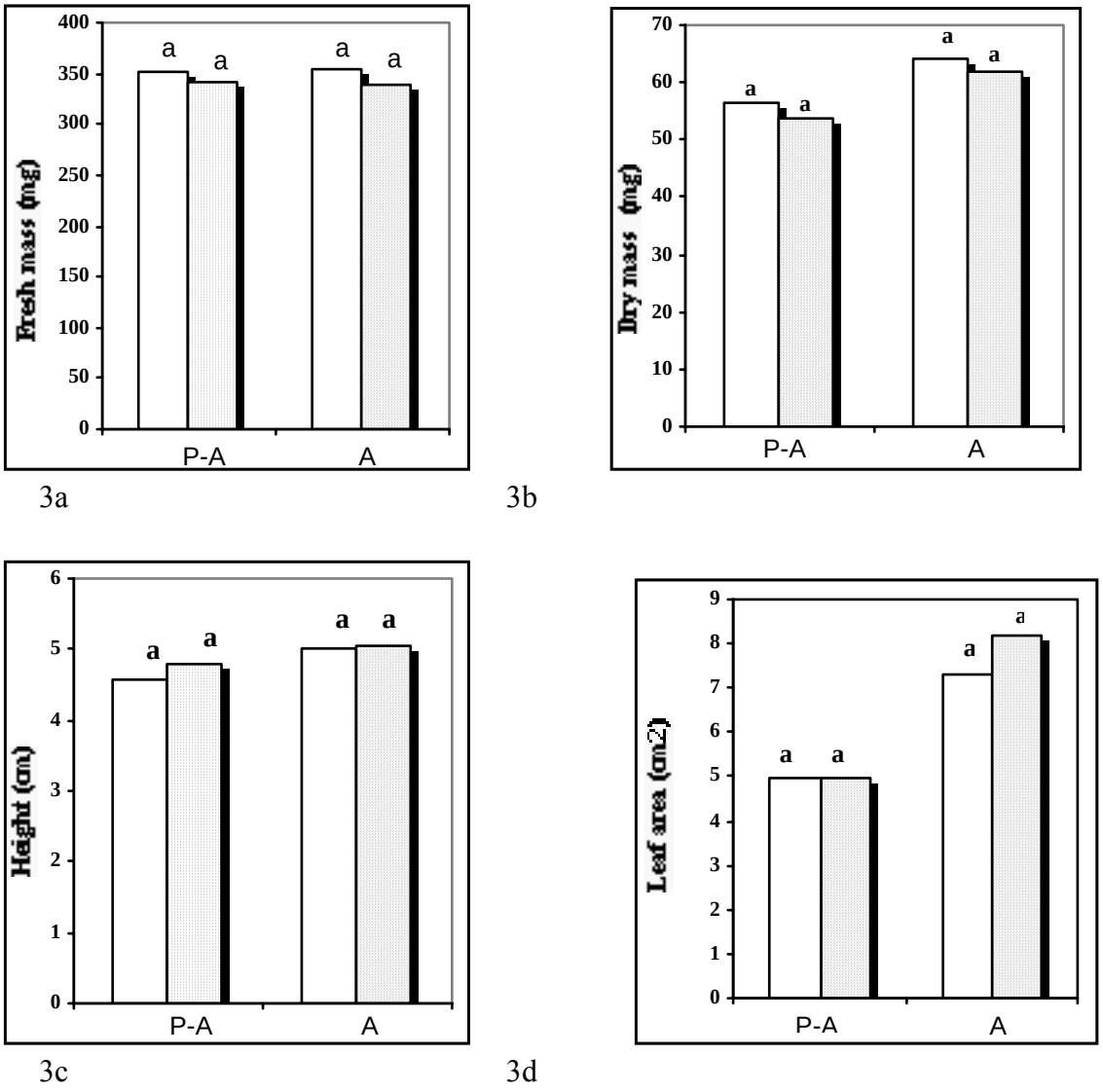


Fig. 3. The effects of the removal of sucrose from the in vitro culture medium on : a) FM ; b) DM ; c) height ; and d) leaf area of American ginseng plantlets after four weeks of pre-acclimatization (P-A) in growth chambers and two weeks of acclimatization (A) in a greenhouse. Contiguous columns with the same letters represent treatments that are not significantly different at $P = 0.05$.

Leaf area.

CO_2 enrichment (Fig. 1d), *PPF* level (Fig. 2d) and sucrose concentration (Fig. 3d) did not have any significant effect on the leaf area of American ginseng plantlets after the pre-acclimatization phase ($P = 0.6859$, $P = 0.4354$ and $P = 0.9868$, respectively).

However, CO_2 enrichment significantly increased (32 %) the leaf area of American ginseng plantlets after acclimatization ($P = 0.0282$). Increasing light irradiance or removing sucrose from the culture medium did not have any significant effect ($P = 0.4720$ and $P = 0.0962$, respectively).

Some plantlets submitted to both CO_2 enriched environment and increased *PPF* initiated flowering, indicating a more precocious physiological maturity.

Discussion

In vitro CO_2 enrichment is known as one of the ways of enhancing ex vitro survival rate, growth and quality of micropropagated plantlets of a variety of plant species (Kozai, 1991). Until now, little has been published, however, regarding acclimatization of American ginseng, that still requires improvement (Brown et al., 2001). As far as we know, the present study is the first to demonstrate the positive impact of in vitro CO_2 enrichment on acclimatization of this species.

Two growth factors, FM and leaf area, were particularly affected by CO_2 enrichment. Following four weeks of pre-acclimatization in growth chambers with 3000 $\mu L\ L^{-1}$ of CO_2, FM of American ginseng plantlets increased significantly (6.3 %) as compared to control. This effect was even more pronounced (26.4 %) after two weeks of acclimatization in the greenhouse. The positive impact of CO_2 enrichment (1650 and 3000 $\mu L\ L^{-1}$) on FM has already been reported for other crops such as asparagus (Desjardins et al., 1988). Increased leaf area as an effect of CO_2 enrichment (1000 $\mu L\ L^{-1}$) has also been observed in plant species such as *Cymbidium* sp. (Heo et al., 1996). In the present experiment, however, the positive effect of CO_2 enrichment on leaf area was notable only after the acclimatization phase. This seems to indicate the existence of a residual effect.

CO_2 enrichment did not have any effect on DM. This is surprising since FM was affected. In their studies on red raspberry and *Cymbidium* sp., Deng and Donnelly (1993), as well as Heo et al. (1996), noted a positive effect of CO_2 enrichment on both fresh and DM. Desjardins et al. (1988), however, did not observe any impact of CO_2 enrichment on the DM of strawberry, raspberry and asparagus plantlets. According to the authors, the fact that CO_2 enrichment did not stimulate DM accumulation could be the consequence of the substitution of sucrose by autotrophic carbon. In the present study, it could also be due to the fact that the DM of the American ginseng plantlets at the beginning of the experiment was very low and variable. This may have masked eventual differences between treatments in the course of the statistical analyses.

The height of the American ginseng plantlets was also unaffected by CO_2 enrichment. This is in contrast with the results of the experiment done by Deng and Donnelly (1993) on red raspberry plantlets, whose height doubled under CO_2 enrichment (1500 μL^{-1} L). According to the same authors, the survival rate of the plantlets was also enhanced by CO_2 enrichment. In contrast to these results, the survival rate of American ginseng plantlets was not affected by CO_2 enrichment in the present study. It is worth mentioning, however, that the survival rate after pre-acclimatization was very high (94 to 100 %), a very interesting result.

Light intensity is one of the most important factors affecting the growth and the quality of micropropagated plantlets. Increasing light intensity has been used to stimulate the photosynthetic activity of micropropagated plantlets in many plant species (Kozai, 1991). In the present study, however, increasing the *PPF* from 30 to 50 µ mol m^{-2} s^{-1} did not have any effect on the survival rate, FM, DM, height and leaf area of American ginseng plantlets. This could be due to the particular nature of this plant, which is a shade species intolerant to direct sun rays. It is also possible that the difference between the two treatments was too low to observe any effect. In an experiment on lettuce, Kitaya et al. (1998) reported positive effects of increased *PPF* on the growth and quality of plantlets only for *PPF* ranging from 100 to 300 µmol m^{-2} s^{-1}. Similarly, blueberry DM increased only after exposition to *PPF* of around 100 µmol m^{-2} s^{-1} (Isutsa et al., 1994).

Quite often, *PPF* increase and CO_2 enrichment seem to act synergistically. This was reported, for example, for the FM (Laforge et al., 1990) and DM (Desjardins et al., 1988) of strawberry and for the DM of *Pinus radiata* micropropagated plantlets (Kumar et al., 1987). In the present experiment, however, no such synergistic effect has been observed, although an non statistically significant 66.5 % increase of leaf area was noted after the acclimatization phase when combining the highest *PPF* level and CO_2 enrichment. A few cases of flowering were also observed. This could be due to the positive impact of the treatment on the physiology of American ginseng plantlets.

Removing sucrose from the culture medium has also been proposed as a way to enhance the photosynthetic activity of micropropagated plantlets. The positive impact of sucrose removal on growth and photosynthetic activity has been observed in strawberry (Hdider et Desjardins, 1994) and C*ymbidium* sp. (Heo et al., 1996). In American ginseng, however, it had no effect on the mass and survival rate of plantlets, even if a noticeable although insignificant increase of leaf area was observed. Heo et al. (1996) reported that leaf area was the growth factor that was the most affected by the removal of sucrose from the culture medium in *Cymbidium* sp.

The results of this experiment thus demonstrate the potential effect CO_2 enrichment could have on enhancing the acclimatization of American ginseng. Indeed, CO_2 enrichment significantly increased the FM and leaf area of the plantlets. However, the impact of such a treatment on the long-term development of the plantlets is uncertain, especially under the hard conditions encountered in Canadian maple forests.

Acknowledgments
This study was made possible thanks to a grant from the Conseil de recherches en pêche et en agro-alimentaire du Québec (CORPAQ). Special thanks to Nathalie Campeau, from UQÀM, Montréal (Qc), Canada, for her help in providing the micropropagated plantlets of American ginseng.

Literature Cited

Brown D.C., Amyoy L. and Rintoul T. 2001. Micropropagation of North American ginseng (*Panax quinquefolius* L.) from test-tube to the field. In: Punja Z.K. (ed) Proc Intl Ginseng Workshop: Utilization of biotechnological, genetic and cultural approaches for North American and Asian ginseng improvement. Simon Fraser Univ Press, Burnaby, BC, Canada. pp 163-178.

Campeau N., Nault A. and Laliberté S. 1998. Somatic embryogenesis in *Panax quinquefolium*: effect of seed stratification and developmental stage of zygotic embryos. Poster presented at the 34th Annu Mtg Can Bot Assn, 27 June – 1 July 1998. Saskatoon, Sask, Canada.

Deng R. and Donnelly D.J. 1993. 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.

Desjardins Y., Laforge F., Lussier C. and Gosselin A. 1988. Effect of CO_2 enrichment and high photosynthetic photon flux on the development of autotrophy and growth of tissue-cultured strawberry, raspberry and asparagus plants. Acta Hort 230:45-53.

Hdider C. and Desjardins Y. 1994. Effects of sucrose on photosynthesis and phosphoenolpyruvate carboxylase activity of in vitro cultured strawberry plantlets. Plant Cell Tissue Organ Cult 36:27-33.

Heo J.W., Kubota C. and Kozai T. 1996. Effects of CO_2 concentration, PPFD and sucrose concentration on *Cymbidium* plantlet growth in vitro. Acta Hort 440:560-565.

Isutsa D.K., Pritts M.P. and Mudge K.W. 1994. Rapid propagation of blueberry plants using ex vitro rooting and controlled acclimatization of micropropagules. HortScience 29:1124-1126.

Jeong B.R., Fujiwara K. and Kozai T. 1995. Environmental control and photoautotrophic micropropagation. Hort Rev 17:125-172.

Kitaya Y., Niu G., Kozai T. and Ohashi M. 1998. Photosynthetic photon flux, photoperiod, and CO_2 concentration affect growth and morphology of lettuce plug transplants. HortScience 33:988-991.

Kozai T. 1991. Acclimatization of micropropagated plants. In: Bajaj Y.P.S (ed) High-tech and micropropagation. Springer-Verlag, Berlin. pp 127-141.

Kozai T. 1996. Environmental control and effects in transplant production under artificial light. J Korean Soc Hort Sci 114:12-16.

Kumar P.R., Reid D.M. and Thorpe T.A. 1987. The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata*. Physiol Plant 69:244-252.

Laforge F., Desjardins Y., Graham M.E.D. and Gosselin A. 1990. Miniature growth chambers for the study of environmental conditions in vitro. Can J Plant Sci 70:825-836.

Laliberté S., Campeau N. and Nault A. 2000. Intra- and interpopulation variability in somatic embryogenesis potential of American ginseng. Poster presented at the 36th Annu Mtg Can Bot. Assn, 24-28 June 2000. London, ON, Canada.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol Plant 15:437-497.

Nault A. 1998. La situation du ginseng à cinq folioles (*Panax quinquefolium* L.) au Québec. Ministère de l'environnement et de la faune du Québec, Direction de la conservation et du patrimoine écologique, Québec, QC. 43 pp.

Pospisilova J., Synkova H., Haisel D., Catsky J., Wilhelmova N. and Sramek F. 1999. Effect of elevated CO_2 concentration on acclimatization of tobacco plantlets to ex vitro conditions. J Expl Bot 50:119-126.

Tirajoh A. and Punja Z.K. 1995. Tissue culture and *Agrobacterium* - mediated transformation of American ginseng (*Panax quinquefolium* L.). In: Bailey W.G., Whitehead C., Proctor J.T.A and Kyle J.T. (eds) Proc Intl Ginseng Conf - 1994. Vancouver, BC, Canada. pp 144-155.

Tirajoh A., Kyung T.S. and Punja Z.K. 1998. Somatic embryogenesis and plantlet regeneration in American ginseng (*Panax quinquefolium* L.). In vitro Cell Dev Biol Plant 34:203-211.

Tsuji K., Nagaoka M. and Oda M. 1992. Promotion of the growth of carrot plantlets in vitro by controlling environmental conditions in culture vessels. Acta Hort 319:297-303.

Wang S.A. 1990. Callus induction and plant regeneration of American ginseng. HortScience 25:571-572.

Wang X., Proctor J.T.A., KrishnaRaj S., Saxena P.K. and Sullivan J.A. 1999. Rapid somatic embryogenesis and plant regeneration in American ginseng: effects of auxins and explants. J Ginseng Res 23: 148-163.