In Vitro Hardening of Red Raspberry by CO₂ Enrichment and Reduced Medium Sucrose Concentration

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Abstract. Micropropagated 'Festival' red raspberry (*Rubus idaeus* L.) shoots were rooted in specially constructed plexiglass chambers in ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO₂ conditions on a medium containing 0, 10, 20, or 30 g sucrose/liter. Plantlet growth and leaf ¹⁴C-fixation rates were evaluated before and 4 weeks after ex vitro transplantation. In vitro CO₂ enrichment promoted in vitro hardening; it increased root count and length, plantlet fresh weight, and photosynthetic capacity but did not affect other variables such as plantlet height, dry weight, or leaf count and area. No residual effects of in vitro CO₂ enrichment were observed on 4-week-old transplants. Sucrose in the medium promoted plantlet growth but depressed photosynthesis and reduced in vitro hardening. Photoautotrophic plantlets were obtained on sucrose-free rooting medium under ambient and enriched CO₂ conditions and they performed better ex vitro than mixotrophic plantlets grown with sucrose. Root hairs were more abundant and longer on root tips of photoautotrophic plantlets than on mixotrophic plantlets. The maximum CO₂ uptake rate of plantlet leaves was 52% that of greenhouse control plant leaves. This did not change in the persistent leaves up to 4 weeks after ex vitro transplantation. The photosynthetic ability of persistent and new leaves of 4-week-old ex vitro transplants related neither to in vitro CO₂ nor medium sucrose concentration. Consecutive new leaves of transplants took up more CO₂ than persistent leaves. The third new leaf of transplants had photosynthetic rates up to 90% that of greenhouse control plant leaves. These results indicate that in vitro CO₂ enrichment was beneficial to in vitro hardening and that sucrose may be reduced substantially or eliminated from red raspberry rooting medium when CO₂ enrichment is used.

Micropropagation has become an important technique for the commercial mass propagation of red raspberry (Donnelly and Daubeny, 1986). In vitro red raspberry plantlets exhibit the culture-induced phenotype (CIP) typical of temperate-species plants in vitro (Donnelly et al., 1985). This CIP includes small thin leaves, fewer trichomes, less-developed support tissues (collenchyma and sclerenchyma), a higher water content percentage, permanently opened stomata, and low photoautotrophic capacity. The CIP impedes the normal growth of micropropagules transferred directly from culture to ambient greenhouse or field conditions and necessitates a period of acclimatization. During this period, ex vitro plants usually are exposed to high relative humidity (RH) and low light intensity for several days or weeks, followed by a gradual adjustment of RH and light to ambient levels. During ex vitro

acclimatization of *Rubus*, greenhouse- or field-grown (control) type anatomy and physiology developed gradually (Donnelly and Vidaver, 1984a, 1984b; Donnelly et al., 1984, 1985). Ex vitro acclimatization can be expensive in terms of labor, controlled environment facilities, and plant losses. Manipulating the culture environment to alter the CIP toward photoautotrophy and hardening could abbreviate or eliminate the ex vitro acclimatization period and reduce the overall costs of

micropropagation [Donnelly and Tisdall, 1993; reviewed by Kozai (1991)].

Several species have been cultured on sucrose-free medium. Most of the previous studies examined the effect of in vitro CO₂ on plant growth separately from the effect of various sucrose concentrations in the medium. Increasing in vitro CO₂ concentration improved the growth and photosynthesis of carnation (*Dianthus caryophyllus* L.) (Kozai and Iwanami, 1988; Kozai et al., 1987), cauliflower (*Brassica oleracea* L. var. *botrytis*) (Grout and Donkin, 1987), Chinese mustard (*Brassica campestris* L.) (Kozai et al., 1991b), jackfruit (*Artocarpus heterophyllus* Lam.) (Rahman and Blake, 1988), orchid (*Cymbidium* spp.) (Kozai et al., 1987), potato (*Solanum tuberosum* L.) (Kozai et al., 1988), and strawberry (*Fragaria × ananassa* Duch.) (Fujiwara et al., 1988; Kozai et al., 1991a) plantlets. However, in vitro CO₂ concentrations were not optimized. The benefits of reducing or omitting sucrose in the medium include the promotion of autotrophy, cost savings on materials, and reduced biological contamination (Kozai, 1991). It is not known to what extent in vitro hardening can be achieved by CO₂ enrichment and sucrose reduction or omission.

The objectives of this study were to compare the effects of in vitro CO₂ at ambient and ≈4.5 times ambient concentrations and medium sucrose at 0, 10, 20, or 30 g-liter⁻¹ on the growth and leaf ¹⁴C-fixation of red raspberry plantlets before and after ex vitro transplantation. The appropriate criteria to define in vitro-hardened plantlets and ex vitro-acclimatized transplants also were explored.

Materials and Methods

'Festival' red raspberry was micropropagated as described by Donnelly and Vidaver (1984a). Shoots with two to three leaves and fresh weights of 23 ± 4 mg were rooted under either ambient (340 ± 20 ppm) or enriched (1500 ± 50 ppm) CO₂ conditions on Murashige and Skoog (1962) basal salt medium with either 0, 10, 20, or 30 g sucrose/liter, supplemented with 1.2 μM thiamine·HCl, 550 μM

Fig. 1. The experimental setup showing the plexiglass incubation chambers and CO₂ control systems. Tight-fitting plexiglass lids were secured to the chambers (C) with elastic bands (E) wrapped around paired hooks fastened to the box and lid. Carbon dioxide gas mixtures traveled from compressed gas cylinders through a flowmeter (Fm) and a series of filters (F₁ = in line, type 407, ADC; F₂ = 0.4-μm syringe type; and F₃ = 0.2-μm syringe type) and were humidified in a large Erlenmeyer flask (Hu) before entering a buffering chamber (B), which preceded the incubation chambers. Air entered each incubation chamber through two air inlets (I) and flushed out through two air outlets (O) on the opposite side. The temperature and relative humidity (RH) in the chambers were monitored through the two temperature probe ports (T) and one RH probe port (H).

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inositol, 2.45 μM indole-3-butyric acid (IBA), and 5.5 g bacteriological agar (Anachemia Canada, Montreal)/liter adjusted to pH 5.7.

The experiment was carried out in specially designed sterile plexiglass incubation chambers (55 $\times$ 30 $\times$ 15 cm) with tightly fitting plexiglass lids secured with elastic bands wrapped around paired hooks (Fig. 1). The premixed and analyzed gas mixtures of either ambient or enriched CO_2 from compressed cylinders were supplied continuously to the chambers at a flow rate of 15 $\text{ml}\cdot\text{min}^{-1}$ through a flowmeter and scrubbed with a series of filters before they were humidified in a 4.5-liter Erlenmeyer flask and equilibrated (buffered) in small (250-ml) Erlenmeyer flasks that preceded the incubation chambers. Each chamber had two air inlets and two air outlets on the opposite side and three sensor probe ports: two for temperature and one for humidity. The chambers and tubing systems were surface-sterilized with 10% bleach and 70% ethanol, respectively. The temperatures inside the chambers were monitored at intervals with a telethermocouple (model 8500-40; Cole-Parmer, Chicago) inserted through probe ports in the plexiglass chambers. The two concentrations of CO_2 and four concentrations of medium sucrose were arranged as a 2 $\times$ 4 factorial experiment according to a split-plot design, with CO_2 as the main plot factor and medium sucrose as the subplot factor with four replications. Each replication consisted of one container with nine shoots.

After 4 weeks of incubation, two randomly selected plantlets from each replication were harvested destructively to determine root count, total root length, leaf count, total leaf area, plantlet height, and fresh and dry weights. Five additional plantlets, each with its youngest leaf tagged, were transferred to cell packs containing a substrate of 2 Promix (BX; Les Tourbieres Premiere, Riviere DuLoup, Canada) : 1 loam soil (pasteurized) and incubated in a growth chamber at 25°C under cool-white fluorescent light of 120 $\pm$ 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (400 to 700 nm) and a 16-h photoperiod for 4 weeks. The cell packs were covered with transparent plastic for 10 days to maintain a high RH.

Two 4-week-old transplants from each replication were sampled to evaluate growth. Dry weights were obtained after drying the plant tissues in an oven at 70°C for at least 48 h until constant weight. Five root tips, $\approx$ 1 cm long, were excised from each replication and stained with aqueous safranin (1%) for 1 min before being examined with a light microscope. Two plantlets; two 4-week-old ex vitro transplants from each replication; and five control leaves from 1-year-old, tissue-culture-derived, greenhouse-grown plants were subjected to a $^{14}\text{CO}_2$ fixation assay modified from Donnelly et al. (1984). The labeling apparatus consisted of a closed gas circuit with a sample chamber (15 cm^3) under cool-white fluorescent light at 100 $\pm$ 10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (400 to 700 nm). Each plant was exposed to the $^{14}\text{CO}_2$ (370 kBq) gas mixture for 5 min $\pm$ 10 sec. Excess $^{14}\text{CO}_2$ was absorbed in 200 ml 2 N NaOH at the end of each exposure. After exposure, leaf disks (29.5 mm^2), excluding the major veins, were taken

from each leaf using a paper punch. The labeled tissues were placed in 80% ethanol within 5 sec and soaked for 2 to 3 days to extract the ^{14}C -labeled compounds. After extraction, two 100- μl subsamples were used to determine ^{14}C activity in 5 ml liquid scintillation cocktail (Universol; ICN Biomedical, Costa Mesa, Calif.) in a liquid scintillation spectrometer (model LS-5801; Beckman, Fullerton, Calif.). The ^{14}C activity was measured in the 0 to 670 energy window at an efficiency of 90% to 95% (H-number between 60 to 80), corrected for background activity, and adjusted for ethanol extract volume. The specific activity of labeled tissues was expressed as becquerel per square centimeter.

Analysis of variance was performed using SAS's General Linear Model procedure (GLM) (SAS Institute, 1985) on the means of each experimental unit. Homogeneity of variance was tested using the Bartlett's test (Steel and Tome, 1980), and appropriate transformations were used where necessary (Gomez and Gomez, 1984).

Results

All plantlets survived the 4-week in vitro incubation period regardless of CO_2 or medium sucrose concentration. Both CO_2 and sucrose affected in vitro plantlet growth independently. In vitro CO_2 enrichment significantly increased root count, root length, and total plantlet fresh weight compared with those of plantlets grown under ambient CO_2 (Table 1). Plantlets grown under enriched CO_2 also had a healthier appearance than those grown under ambient CO_2 . However, other characteristics, such as plantlet height, percent dry weight, and leaf count and area, were similar among plantlets grown under the two CO_2 concentrations (data not shown).

Plantlets grew successfully on sucrose-free medium, a result suggesting that photoautotrophy was established. These photoautotrophic plantlets had shorter total root lengths (3.2 $\pm$ 0.6 cm) and lower fresh weights (225 $\pm$ 16 mg) than the mixotrophic plantlets that were grown with sucrose (7.3 $\pm$ 0.9 cm and 337 $\pm$ 25 mg, respectively). Plantlet fresh weight increased with increasing concentration of medium sucrose (Fig. 2); this trend was not observed for the other characteristics. Although root hairs were present on all root tips examined, those on root tips of plantlets grown on sucrose-free medium were significantly longer and consistently stained darker than those on root tips of plantlets grown with sucrose, regardless of the amount of sucrose in the medium (Fig. 3). In addition, the root hair zone on

root tips of plantlets grown on sucrose-free medium began closer to the tip and hairs were more abundant compared with those of plantlets grown with sucrose.

The ^{14}C activity in cultured plantlet leaves was negatively related to sucrose concentration in the medium under enriched, but not ambient, CO_2 conditions (Fig. 4). The mean ^{14}C activity in cultured plantlet leaves grown under enriched CO_2 was higher (0.28 $\pm$ 0.02 $\text{kBq}\cdot\text{cm}^{-2}$) than that in plantlets grown under ambient CO_2 conditions (0.21 $\pm$ 0.01 $\text{kBq}\cdot\text{cm}^{-2}$), a result indicating higher photosynthetic capacity.

Four weeks after ex vitro transplantation, transplants from photoautotrophic plantlets had developed significantly larger leaf areas (4.1 $\pm$ 0.4 cm^2) and had higher fresh weights (983 $\pm$ 76 mg) than transplants from mixotrophic plantlets (2.6 $\pm$ 0.2 cm^2 and 643 $\pm$ 59 mg, respectively). However, all other growth characteristics, i.e., root count, total root length, leaf count (persistent and new leaves), and plantlet height, were similar in all transplants (data not-shown).

The ^{14}C activity in persistent and new leaves on 4-week-old transplants was not related to in vitro CO_2 or medium sucrose concentrations

Fig. 2. Effects of sucrose concentration on total plantlet fresh weight after 4 weeks in culture. Values are the means of 48 samples. Regression equation: $Y = 236.6 + 4.83X$, $R^2 = 0.42$, $P \leq 0.01$.

Fig. 3. Light micrographs of representative root tips of (A) photoautotrophic plantlets showing abundant and long root hairs and (B) mixotrophic plantlets showing fewer and shorter root hairs. Bar = 0.5 mm.

Table 1. The effects of ambient or enriched CO_2 concentrations in vitro on root count and length and fresh weight of red raspberry plantlets.

CO_2 concn (ppm)	Root		Fresh wt (mg)
	Count	Length (cm)	
340	4.9	4.4	276
1500	7.3	8.2	346
Significance	**	**	**

**Significant at $P \leq 0.01$.

Fig. 4. Effects of CO_2 at (A) ambient or (B) enriched concentrations and medium sucrose concentrations from 0% to 3% on ^{14}C activity levels in plantlet leaves (numbered basipetally). Value is the mean of 12 samples. Leaf 1 (●): $Y = 19.10 - 1.340X + 0.082X^2 - 0.0015X^3$, $R^2 = 0.88$, $P \leq 0.01$; leaf 2 (○): $Y = 22.03 - 1.105X + 0.081X^2 - 0.0018X^3$, $R^2 = 0.71$, $P \leq 0.01$; leaf 3 (Δ): $Y = 24.69 - 1.078X + 0.068X^2 - 0.0014X^3$, $R^2 = 0.70$, $P \leq 0.01$; leaf 4 (◇): $Y = 21.15 - 1.615X + 0.102X^2 - 0.0020X^3$, $R^2 = 0.64$, $P \leq 0.01$.

(data not shown). The ^{14}C activity in persistent leaves of 4-week-old transplants was obtained from tagged leaves that had completed 10% to 50% of their final expansion at the time of transplantation. The overall average ^{14}C activity in these persistent leaves was similar to that of plantlet leaves or $\approx 53\%$ that of control plant leaves (Table 2). Consecutively developed new leaves had gradually higher $^{14}\text{CO}_2$ uptake capacity than persistent leaves (Table 2). The $^{14}\text{CO}_2$ uptake ability of the third ex vitro developed leaf reached almost 90% that of control leaves (Table 2).

Discussion

Photosynthetic capacities of cultured plantlets have ranged from negative or zero (net respirers) in photosynthetically incompetent species such as cauliflower (Grout and Aston, 1978) and strawberry (Grout and Millam, 1985) to slightly positive ($< 5 \text{ mg CO}_2/\text{dm}^2 \text{ per h}$) in photosynthetically competent species such as red raspberry (Donnelly and Vidaver, 1984b), *Actinidia deliciosa* (Chev.) Liang & Ferguson (Infante et al., 1989), tobacco (*Nicotiana tabacum* L.) (Kozai et al., 1990b), orchid (Kozai et al., 1990a), and Asian white birch [*Betula platyphylla* var. *szechuanica* (Schneid.) Rehd.] (Smith et al., 1986) when measured under higher light intensity than levels commonly used in vitro. The low photosynthetic capacities of cultured plantlets was attributed mainly to the low CO_2 concentrations inside the culture vessels [reviewed by Kozai (1991)].

In vitro CO_2 enrichment promoted root

Table 2. ^{14}C Carbon activity of plantlet leaves, persistent and new leaves of ex vitro transplants, and greenhouse control plant leaves after the plants were exposed to $370 \text{ kBq } ^{14}\text{CO}_2$ for 5 min. Values represent the mean of 64 leaves, except for control values, which represent the mean of five leaves.

Ex vitro time(wk)	Leaves	Activity ($\text{kBq}\cdot\text{cm}^{-2}$)	Percentage of control
0	Plantlet	0.24 ± 0.02	51
4	Persistent	0.25 ± 0.02	53
	New 1	0.24 ± 0.03	51
	New 2	0.37 ± 0.03	79
	New 3	0.42 ± 0.03	89
	Control	0.47 ± 0.02	100

formation and increased the fresh weight of red raspberry, a result supporting the observations on strawberry (Kozai et al., 1991a), tobacco (Kozai et al., 1990b), carnation (Kozai and Iwanami, 1988), orchid (Kozai et al., 1987), and potato (Kozai et al., 1988). Increased growth seemed to relate to high photosynthetic ability. The CO_2 uptake rates of red raspberry plantlets were higher under enriched than under ambient CO_2 conditions, a result confirming that CO_2 concentrations were limiting during conventional micropropagation. Improved photosynthesis corresponding to higher vessel CO_2 concentrations also was observed in cultured plantlets of strawberry (Desjardins, 1990; Kozai et al., 1991a) and *Actinidia deliciosa* (Infante et al., 1989).

The effects of in vitro CO_2 enrichment of red raspberry did not carry over to 4 weeks ex vitro, unlike results with strawberry (Desjardins, 1990). Improved transplant growth using ex vitro CO_2 enrichment has been reported for grape (*Vitis* L. hybrid) (Lakso et al., 1986) and strawberry (Desjardins et al., 1987). The potential benefits of CO_2 enrichment probably are achieved best by extending this treatment ex vitro.

Sucrose was essential in the culture medium for many species and, in some cases, independent growth could not be achieved on medium without sucrose during proliferation (Langford and Wainwright, 1987) or rooting (Grout and Price, 1987). However, strawberry (Kozai et al., 1991a) and carnation (Kozai and Iwanami, 1988) plantlets rooted well on sucrose-free medium. In fact, in cauliflower (Grout and Donkin, 1987), rose (*Rosa multiflora* L.) (Capellades et al., 1991; Langford and Wainwright, 1987), and potato (Kozai et al., 1988), photosynthesis was higher in plantlets cultured on sucrose-free medium than those cultured with sucrose. Our results (Fig. 4) also confirm these observations.

Root function was positively linked to the number and length of root hairs on the root tips (Nobel, 1991). The enormous number of long root hairs on the root tips of photoautotrophic plantlets should improve root function after transplantation. Photoautotrophic plantlets performed better than mixotrophic plantlets after ex vitro transplantation for up to 4 weeks, despite their shorter roots and lower fresh weights at transplantation. It was clear that sucrose was responsible for the differences in root hairs. However, the relationship between

the number and length of root hairs on root tips and the medium sucrose concentration is not understood. The response was not related to differences in water potential of the medium, since root tips appeared similar on plantlets grown on medium containing 10, 20, or 30 g sucrose/liter.

Carbon dioxide uptake measured by infrared gas analysis (IRGA) showed that in vitro red raspberry plantlet leaves had CO_2 uptake rates of only 2 to $3 \text{ mg}\cdot\text{dm}^{-2}\cdot\text{h}^{-1}$, or $\approx 20\%$ that of control plant leaves ($10 \text{ to } 15 \text{ mg}\cdot\text{dm}^{-2}\cdot\text{h}^{-1}$) (Donnelly and Vidaver, 1984b). While this rate was considerably lower than the apparent 62% of control leaves, as indicated by $^{14}\text{CO}_2$ labeling methods (Donnelly et al., 1984), the $^{14}\text{CO}_2$ technique usually yields CO_2 uptake rates 2 to 3 times higher than the IRGA method (Karlsson and Sveinbjornsson, 1981). In our study, the $^{14}\text{CO}_2$ uptake rates of plantlet leaves were 52% of control plant rates and did not change up to 4 weeks ex vitro (Table 2). Red raspberry persistent leaves could last up to 3 months ex vitro during conventional acclimatization (Donnelly and Vidaver, 1984a). These results indicate that the potential photosynthetic contribution of leaves of plantlets and persistent leaves of transplants was still significant 4 weeks after transplantation.

Consecutively developed new leaves had gradually higher CO_2 uptake rates than persistent leaves. Similarly, leaves with transitional phenotypes were observed in *Leucaena leucocephala* (Lam.) De Wit (Dhawan and Bhojwani, 1987), strawberry (Fabbri et al., 1986), cherry (*Prunus cerasus* L.) (Marin et al., 1988), and sweetgum (*Liquidambar styraciflua* L.) (Wetzstein and Sommer, 1982). At least five transitional leaves were produced on red raspberry transplants from conventional micropropagation during conventional ex vitro acclimatization (Donnelly et al., 1984). We found that the CO_2 uptake rates of the third ex vitro new leaves reached 90% that of control leaves.

Plantlets may be considered hardened if they can survive ex vitro ambient conditions with minimal or no extra precautions when transferred; they must have high photosynthetic capacity and narrow stomatal apertures. Hardened plantlets may show many aspects of the CIP and will produce transitional leaves after transplantation. Transplants may be considered fully acclimatized ex vitro if they have functional stomata and photosynthetic capacity comparable to that of control plants. When acclimatization is completed, transitional leaves no longer are formed; CIP-type leaves are produced.

The number of transitional leaves formed on transplants seems to depend on the degree of hardening of the cultured plantlets and the stress of the new ex vitro environment. The fewer transitional leaves, the briefer the acclimatization interval. The fewer transitional leaves produced on transplants in the current study compared with conventionally micropropagated plantlets suggests that our cultured plantlets were hardened to a greater extent; this probably was due to growth under enriched CO_2 conditions.

Photoautotrophic red raspberry plantlets were established on sucrose-free medium under either ambient or enriched CO₂ conditions, and their ex vitro performance was superior to that of mixotrophic plantlets. Red raspberry could be rooted on medium without or with less sucrose than used conventionally when the atmosphere was enriched with CO₂. In vitro CO₂ enrichment improved plantlet quality and photosynthetic ability, enhanced ex vitro performance, and reduced the ex vitro acclimatization interval.

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