logical control of postharvest diseases—Theory and practice. CRC Press, Boca Raton, Fla.

IES Lighting Handbook. 1981. Non-visual effects of radiant energy—Effects on microorganisms.

Liu, J., C. Stevens, V.A. Khan, J.Y. Lu, C.L. Wilson, O. Adeyeye, M.K. Kabwe, P.L. Pusey, E. Chalutz, T. Sultana, and S. Droby. 1993. The effect of ultraviolet-C light on storage rots and ripening of tomatoes. *J. Food Protect.* 56:868–972.

Lu, J.Y., C. Stevens, P. Yakabu, P.A. Lorentan, and D. Eakin. 1987. Gamma, electron beam and ultraviolet radiation on control of storage rots and quality of Walla Walla onions. *J. Food Process Pres.* 12:53–62.

Luckey, T.D. 1980. Hormesis with ionizing radiation. CRC Press, Boca Raton, Fla.

Mercier, J., J. Arul, and C. Julien. 1993a. Effect of UV-C on phytoalexin accumulation and resistance to *Botrytis cinerea* in stored carrots. *J. Phytopathol.* 139:17–25.

Mercier, J., J. Arul, R. Ponnampalam, and M. Boulet. 1993b. Induction of 6-methoxymellein and resistance to storage pathogens in carrot slices by UV-C. *J. Phytopathol.* 137:44–54.

National Research Council Board of Agriculture. 1987. Regulating pesticides in food—The Delaney paradox. National Academy Press, Washington, D.C.

National Research Council Board of Agriculture. 1993. Pesticides in the diets of infants and children. National Academy Press, Washington, DC.

Stevens, C., C.L. Wilson, J.Y. Lu, V.A. Kahn, E. Chalutz, S. Droby, M.K. Kabwe, Z. Haug, O. Adeyeye, L.P. Pusey, M.E. Wisniewski, and M. Went. 1996. Plant hormesis induced by ultraviolet light-C for controlling postharvest diseases of tree fruits. *Crop Protect.* 15:129–134.

Stevens, C., V.A. Khan, A.Y. Tang, and J. Lu. 1990. The effect of ultraviolet radiation on mold rots and nutrients of stored sweet potatoes. *J. Food Protect.* 53:223–226.

Stevens, C., J.Y. Lu, V.A. Khan, C.L. Wilson, E. Chalutz, and S. Droby. 1991. Ultraviolet light induced resistance against postharvest diseases in vegetables and fruits. In: C.L. Wilson and E. Chalutz (eds.). *Biological control of postharvest diseases of fruits and vegetables*. Proc. Workshop USDA-ARS-92, Shepherdstown, W.Va.

U.S. Department of Agriculture—Agriculture Research Service. 1965. Losses in agriculture. U.S. Dept. Agr. Hdbk. 291.

Wilson, C.L. and M.E. Wisniewski. (eds.). 1995. *Biological control of postharvest diseases of fruits and vegetables—Theory and practice*. CRC Press, Boca Raton, Fla.

Wilson, C.L., A. El-Ghaouth, E. Chalutz, E. Droby, C. Stevens, J.Y. Lu, V. Khan, and J. Arul. 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. *Plant Dis.* 78:837–844.

Wilson, C.L. and A. El Ghaouth. 1993. Multifaceted biological control of postharvest diseases of fruits and vegetables, p. 181–185. In: R.D. Lumsden and J.L. Vaughn (eds.). *Pest management: Biologically based technologies*. Amer. Chem. Soc., Washington, D.C.

Using Ultra-high Carbon Dioxide Levels Enhances Plantlet Growth In Vitro

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SUMMARY. A continuous CO₂ flow system was used to study the growth of carrot (*Daucus carota* L.), citrus (*Citrus macrophylla* L.), kale (*Brassica oleracea* L.), lettuce (*Lactuca sativa* L.), radish (*Raphanus sativus* L.), and tomato (*Lycopersicon esculentum* L.) cultures in vitro under photoautotrophic, photomixotrophic, and heterotrophic conditions. Lettuce plantlets were grown on Murashige and Skoog medium with 0%, 0.3%, 1%, and 3% sucrose within flow chambers containing 350, 750, 1500, 3000, 10,000, 30,000, and 50,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂. Increasing the levels of CO₂, especially at the ultra-high levels (i.e., $\geq 3,000 \mu\text{L}\cdot\text{L}^{-1}$ CO₂), increased fresh weight, shoot length, leaf number, leaf length, leaf width, root number, and root length for plantlets grown regardless of sucrose levels tested compared to plantlets grown at normal atmospheric CO₂ levels, i.e.,

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350 $\mu\text{L}\cdot\text{L}^{-1}$. For example, fresh weights of lettuce plantlets grown on medium containing 0% or 3% sucrose increased 11- and 13-fold, respectively, when supplemented with 30,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 compared to growth of lettuce plantlets grown on the same media without CO_2 enrichment. Similar fold increases in growth responses were obtained with carrot, citrus, kale, radish, and tomato plantlets grown in atmospheres enriched with high CO_2 levels, elevated from 3000 to 30,000 $\mu\text{L}\cdot\text{L}^{-1}$. Optimum CO_2 concentration varied among species, suggesting a species-related response. Varying the rate of CO_2 application between 250, 500, 1500, or 2000 $\text{mL}\cdot\text{min}^{-1}$ did not effect the rate of growth of lettuce plantlets. The passive diffusion continuous flow-through system presented in this paper is inexpensive, easily constructed, and allows for testing ultra-high CO_2 levels on plant culture growth in vitro.

Supplementation of photosynthesis in vitro by CO_2 enrichment has been reported by several researchers for a variety of plants, including asparagus (Desjardins et al., 1988), avocado (Prusky et al., 1996), cacao (Figueira et al., 1991), carnation (Kozai, 1988), orchid (Kozai et al., 1987), potato (Buddendorf-Joosten and Woltering, 1996; Cournac et al., 1991; Fujiwara et al., 1992; Yorio et al., 1995), raspberry (Desjardins et al., 1988), and strawberry (Desjardins et al., 1988; Fujiwara et al., 1988). Early attempts to increase CO_2 levels used forced ventilation, i.e., direct pumping of gas mixtures into specially constructed culture vessels (Kozai, 1988). Passive diffusion of CO_2 into potato tissue cultures was conducted by elevating CO_2 in enclosed rooms or chambers containing culture vessels (Buddendorf-Joosten and Woltering, 1996; Cournac et al., 1991; Jeong et al., 1993). Special membrane filters and vessels have been used to grow potato cultures in elevated CO_2 environments (Buddendorf-Joosten and Woltering, 1996). Kozai (1988) found that 1000 to 1500 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 increased carnation dry weight 2-fold on a medium without sucrose (photoautotrophic condition) compared to dry weights obtained by growing cultures on the same medium without any CO_2 enrichment (heterotrophic condition).

Higher growth rates were obtained when cultures were grown on a medium containing 1% sucrose coupled with an atmosphere enriched with 1000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 (photomixotrophic) over that obtained from cultures grown on the same medium without any CO_2 enrichment or on medium devoid of sucrose but enriched with 1000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 (Kozai, 1988). Fujiwara et al. (1992) and Fujiwara and Kozai (1995) used 850 to 1000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 to double dry weights of potato cultures. At any given CO_2 level tested, dry weight increases were also related to sucrose concentrations suggesting that some combination of sucrose concentration coupled with a certain CO_2 level will give optimum growth (Fujiwara et al., 1992; Fujiwara and Kozai, 1995). Potato shoot cultures cultured on medium devoid of sucrose exhibited up to a 9-fold dry weight increase at CO_2 levels of 10,000 to 50,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 using a passive diffusion flow system (Buddendorf-Joosten and Woltering, 1996; Cournac et al., 1991).

The optimum level of CO_2 for maximum tissue culture growth remains unclear. Based on prior research reports, suggestions as to the proper level of CO_2 to use in vitro varies as much as 10- to 50-fold. This study was conducted to address the influence of various levels of CO_2 on the growth of

diverse crop plants cultured in vitro in order to determine optimum levels of CO_2 and sucrose for growth. Also, a low-cost, easily constructed CO_2 passive diffusion continuous flow through system is described that allows for the use of traditional tissue culture vessels to be used in an enriched CO_2 environment. This system could be easily employed by commercial and research laboratories to apply ultra-high (≥ 3000 $\mu\text{L}\cdot\text{L}^{-1}$) CO_2 levels in vitro.

Materials and methods

CARBON DIOXIDE FLOW SYSTEMS.

Carbon dioxide flow-through testing chambers were constructed from a 94.5-L transparent polycarbonate Carb-X tote box and lid (Consolidated Plastics, Twinsburg, Ohio) (45 cm wide $\times$ 65 cm long $\times$ 37.5 cm in diameter; 94.5-L capacity) (Fig. 1). A silicone tape gasket (112 cm long $\times$ 6.3 mm wide $\times$ 3.2 mm thick) (Furon, New Haven, Conn.) was attached to the lid (Fig. 1). The box was modified by mounting three polypropylene spigots to allow for the inflow and evacuation of gases. Two 0.45- μm air vents (Gelman Science, Ann Arbor, Mich.) were attached to two of these spigots

Fig. 1. The plant tissue culture/ CO_2 enrichment system. Mixing CO_2 and air generated by air pump is conducted in the flowmeter.

with silicone tubing to 1.6-mm I.D. female barbed fittings (Ark-Plas Products, Flippin, Ark.). The box and lid were clamped with 12 equally spaced stationary binding clips (50 mm long). The CO₂ testing chamber was attached to a water reservoir with silicon rubber tubing. The water reservoir consisted of a 2.25-L polycarbonate bottle containing 1.5-L distilled water. Carbon dioxide was provided by a gas cylinder (National Welding Supply Company, Inc., Bloomington, Ill.) rated 99.8% pure and was mixed with room air flow produced by an aquarium pump (Whisper 1000, Carolina Biological Supply Co., Burlington, N.C.) with a flowmeter (Cole Parmer Instrument Co., Niles, Ill.) to provide 350, 750, 1500, 3000, 10,000, 30,000, and 50,000 $\mu\text{L}\cdot\text{L}^{-1}$. Carbon dioxide ranges $\geq 10,000$ $\mu\text{L}\cdot\text{L}^{-1}$ were adjusted using an infrared gas analyzer (model 3000 LIRA; Mine Safety Appliances Co., Pittsburgh) and CO₂ ranges $\leq 3,000$ $\mu\text{L}\cdot\text{L}^{-1}$ were adjusted with the aid of a CO₂/H₂O infrared gas analyzer (LI-6262; LICOR, Lincoln, Nebr.). The CO₂ and air streams were added at about 1500 mL $\cdot\text{min}^{-1}$ for a 16-h photoperiod. Control cultures were given a stream of room air generated by the aquarium pump and hydrated with a water reservoir. In flow experiments, air flow rates were adjusted with gang valves and flowmeters to 250, 500, 1000, 1500, and 2000 mL $\cdot\text{min}^{-1}$. Flow experiments were conducted using 17.6-L transparent polycarbonate boxes (Consolidated Plastics, Twinsburg, Ohio) (32.5 cm long $\times$ 26.3 cm wide $\times$ 30 cm high; 17.6-L capacity) equipped with a silicone gaskets, vents, and water reservoirs as described above. In some cases, this system was modified by including a 905 mL $\cdot\text{min}^{-1}$ VAC fan (5.7 $\times$ 3.8 cm).

DIFFUSION TESTS. Diffusion tests were conducted on the following tissue culture vessels: culture tubes (25 mm in diameter $\times$ 150 mm high or 38 mm in diameter $\times$ 200 mm high) capped with a polypropylene natural closure (Corning, Boston); Magenta GA-7 container (77 mm wide $\times$ 77 mm long $\times$ 97 mm high) capped with polypropylene closure (Sigma Chemical Co., St. Louis); and a Phytacoon (116 mm in diameter $\times$ 108 mm high) (Sigma chemical Co.) capped with a polypropylene cover. An O₂ sensor (Figaro USA, Wilmette, Ill.) was placed inside the culture vessel to be tested

Table 1. Oxygen and carbon dioxide exchange rates (N) for various tissue culture vessels.

Vessel type	Capacity (mm ³)	N (h ⁻¹)	
		O ₂	CO ₂
Culture tube (25 $\times$ 150 mm)	55	2.2	2.1
Culture tube (38 $\times$ 200 mm)	175	1.6	1.6
Magenta GA-7 container	350	0.4	0.3
Phytacoon container	470	0.8	0.9

either by passing the wires from the sensor through an opening obtained by drilling a hole in the closure or by inserting a tube into the vessel and sealing the wires with silicone sealer. For the 25 mm $\times$ 150-mm tube a sidearm was added to the tube for the O₂ sensor. A CO₂ electrode (Diamond General, Ann Arbor, Mich.) was used to measure CO₂ and was inserted into the vessel through a hole drilled in the closure. Nitrogen/CO₂ (80%/20%) gas was passed into the vessel through a tube either inserted into the closure or attached to the vessel. Once zero O₂ was recorded within the vessel, gas input was terminated and O₂ and CO₂ levels were recorded until normal atmospheric O₂ (i.e., 21%) or CO₂ level (i.e., 0.03%) was obtained. The recorded sensor outputs were converted to the estimated number of air exchanges per hour (N) using the following formula: $N = (1/t_2 - t_1) \cdot \ln((C_1 - C_{\text{out}})/(C_2 - C_{\text{out}}))$ where t_1 = time when vessel concentration = C_1 ; t_2 = time when vessel concentration = C_2 ; and C_{out} = concentrations outside vessel (Fujiwara and Kozai, 1995). Air exchange rates were also conducted by inserting a CO₂ probe into a capped 25 $\times$ 150-mm culture tube within a flow-through system with and without a fan using 30,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂.

MEDIUM AND PLANT CULTURE. The basal medium (BM) consisted of Murashige and Skoog (MS) salts plus (per liter): 0.5 mg thiamine HCl, 100 mg myo-inositol, and 10 g agar (Difco Laboratories, Detroit). BM with 0%, 0.3%, 1%, 3%, and 5% sucrose was tested. The pH was adjusted to 5.7 $\pm$ 0.1 with 0.1 N HCl or NaOH before the addition of agar, then melted and dispensed in 25-mL aliquots into 25 $\times$ 150-mm borosilicate glass culture tubes and capped with translucent polypropylene closures (Sigma Chemical Co., St. Louis). The medium was autoclaved for 15 min at 1.05 kg $\cdot\text{cm}^{-2}$ at 121 $^{\circ}\text{C}$. Agar medium was then slanted at a 45 $^{\circ}$ angle while cooling. Seeds of

carrot (*Daucus carota* L. 'Danver's Half Long'), kale (*Brassica oleracea* L. unknown cultivar), lettuce (*Lactuca sativa* L. 'Grand Rapids'), radish (*Raphanus sativus* L. 'Scarlet Globe'), and tomato (*Lycopersicon esculentum* L. 'Cherry Red') were surface sterilized in a 2.6% sodium hypochlorite solution (containing 2 drops of Tween-20 emulsifier per 100 mL solution) for 20 min and placed on the surface of BM. Two seeds were cultured per vessel. Stock shoots of citrus (*Citrus macrophylla* L. unknown cultivar) were maintained as proliferating axillary buds on BM. A single 2-cm-long shoot was cultured per vessel.

PLANT TISSUE CULTURE EXPERIMENTS. Lettuce seeds were planted in BM containing 0%, 0.3%, 1.0%, 3.0%, or 5.0% sucrose and grown under 350, 750, 1500, 3000, 10,000, 30,000, and 50,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ in 94-L transparent containers. Carrot, citrus, kale, radish, and tomato were similarly grown at the same concentration but on BM with and without 3% sucrose. The influence of the CO₂ flow rates on lettuce seedling growth was tested by growing lettuce seeds on BM with sucrose under 10,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO₂ using 250, 500, 1500, and 2000 mL $\cdot\text{min}^{-1}$ within 17.6-L transparent containers. Cultures were grown in a culture room maintained at 25 $\pm$ 1 $^{\circ}\text{C}$ and using a photoperiod of 16 h light/8 h dark. Light was supplied by a combination of cool-white fluorescent tubes, metal-halide lamps, and incandescent lamps

Fig. 2. Growth responses of lettuce plantlets to various concentrations of CO₂ and sucrose. Two seeds were cultured per tube. Data were averaged for 10 replications/treatment. Experiments were repeated three times and a single representation is presented. Mean separation by Student-Newman-Keuls multiple range test ($P < 0.1$). Columns in the same sucrose concentration with the same letter were not significantly different.

Fig. 3. Growth responses of lettuce plantlets given various CO₂ levels in vitro after 8 weeks in culture. (A) Plantlets grown on medium without sucrose treated with 350 and 10,000 µL·L⁻¹ CO₂. Note the difference in the size of plantlets depending on whether CO₂ was enhanced or not. (B) Plantlets treated with 10,000 µL·L⁻¹ CO₂ cultured in various concentrations of sucrose. Note that the sucrose levels do not alter growth responses at this level of CO₂ tested. Scale bar = 16 mm.

at a total photosynthetic photon flux density (PPFD) of 260 µmol·m⁻²·s⁻¹ at the vessel periphery.

Ten or twenty replicates were planted originally, and experiments were repeated at least twice. After 8 weeks of incubation, data on culture dry weight, fresh weight, shoot height, leaf number, leaf length, leaf width, root number, and root length were recorded and analyzed with Student-Newman-Keuls multiple range test as appropriate.

Results and discussion

Diffusion results in terms of the O₂ and CO₂ exchange rates (N) for the various culture vessels are given in Table 1. For the same vessel type, O₂ and CO₂ exchange rates were similar. The 25 × 150-mm tube gave the best air exchange rates followed by the 38 × 200-mm tube, the Sigma vessel, and the Magenta vessel, respectively. The choice to use 25 × 150-mm test tubes in this study was made based on this test because faster air exchanges could be obtained with these vessels and closures compared to any other commonly used tissue culture vessels. Using a flow-through system with a fan

in 60 min using a system with a fan will require 85 min in a system without a fan. This increased time is negligible over a daily 16-h period of CO₂ application, and a fan was not used in subsequent tests.

Levels of CO₂, especially above the 1500 µL·L⁻¹ CO₂ level, resulted in significant lettuce plantlet growth increases in BM containing 0% to 3% sucrose (Fig. 2). However, 5% sucrose was inhibitory to plantlet growth for all CO₂ concentrations tested compared to lower sucrose concentrations tested and will not be considered in the following discussion hereafter (data not shown). Invariably, administration of air containing 350 µL·L⁻¹ CO₂ gave the least growth response from cultured lettuce seedlings, followed by 750 µL·L⁻¹ CO₂, regardless of the sucrose levels tested. Highest growth responses were obtained using 10,000, 30,000, and 50,000 µL·L⁻¹ CO₂ compared to 350 µL·L⁻¹ CO₂ (Figs. 2 and 3). The greatest fresh weights obtained were from lettuce grown in 30,000 µL·L⁻¹ CO₂, regardless of the levels of sucrose used. For example, lettuce plantlets grown in 0% and 3% sucrose under 30,000 µL·L⁻¹ CO₂ exhibited 13.8- and 7.2-fold increases, respectively, in growth over cultures grown in air only on the same media. The high photomixotrophic condition (3% sucrose with high CO₂ levels) usually gave similar results compared to the photoautotrophic condition (0% sucrose with high CO₂ levels) or the low photomixotrophic condition (0.3% or 1% sucrose with high CO₂ levels). All of these treatments were superior to the heterotrophic condition (0.3% to 3% sucrose without any CO₂ enrichment). Low sucrose levels coupled with

allowed for faster air exchange than without a fan, 2.07 and 1.39 h⁻¹, respectively. Without a fan, air exchange was only about 70% as fast in the flow-through system than with a fan. In other words, the time to achieve a given concentration

enriched CO₂ environments have been reported to give higher dry weights from carnation, orchids, and potato cultures than those obtained from cultures grown on no or higher sucrose levels devoid of CO₂ enrichment (Fujiwara et al., 1992; Kozai, 1988). With lettuce this pattern did not occur; however, considerably higher levels of CO₂ were used in the present study (Figs. 2 and 3). We found that greater increases in growth (i.e., fresh weight) occurred from lettuce cultures with 30,000 µL·L⁻¹ CO₂-treated cultures at the 0% sucrose levels than with any other sucrose levels used (Fig. 2). No notable beneficial effect occurred using BM containing 0.3% or 1% sucrose compared to using BM containing 0% or 3% sucrose for any of the growth responses recorded. Shoot lengths of lettuce plantlets were similar when cultured under 3000 to 50,000 µL·L⁻¹ CO₂, regardless of the sucrose levels used. The highest number of leaves per culture, leaf lengths, and leaf widths occurred using the 30,000 to 50,000 µL·L⁻¹ CO₂ levels compared to the lower CO₂ levels, regardless of the sucrose levels used (Fig. 2). Similarly, Buddendorf-Joosten and Woltering (1996) found that leaf number and leaf area significantly increased growing potato shoots at 50,000 µL·L⁻¹ CO₂ compared to the cultures grown in air only. Cacao shoot cultures cultured on 20,000 µL·L⁻¹ CO₂ exhibited enhanced leaf area production and shoot elongation compared to those cultured at 800 µL·L⁻¹ CO₂ (Figueira et al., 1991).

Carbon dioxide enrichment in vitro has been associated with growth responses such as increases in dry weight (Cournac et al., 1991; Fujiwara et al., 1992; Kozai et al., 1987), plant height (Cournac et al., 1991; Figueira et al., 1991), fresh weight (Buddendorf-Joosten and Woltering, 1996), or leaf area (Buddendorf-Joosten and Woltering, 1996; Figueira et al., 1991). Lettuce plantlet root responses, i.e., root number and root lengths, were significantly improved when cultured under ultra-high levels of CO₂ (≥3,000 µL·L⁻¹) for all sucrose levels tested (Figs. 2 and 3). The beneficial effect of CO₂ on rooting in vitro has not been mentioned in previous reports.

Based on the previous experiments, ultra-high levels of CO₂ (≥3,000 µL·L⁻¹) were found to be beneficial to lettuce plantlet growth. Several differ-

ent species were cultured on the these same elevated levels of CO_2 on BM with or without sucrose, and high concentrations of CO_2 aided in the growth of all species studied (Fig. 4). However, an optimum concentration of CO_2 appeared to exist for each species where above or below this concentration less growth occurs (i.e., fresh weight). For example, citrus shoots grown in 0% sucrose exhibit maximum growth at the $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ level while kale seedlings grown in 0% sucrose exhibit maximum growth at the $30,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ level. Also, it was observed that maximum growth increases (i.e., fold increases) were not always the same when grown in 0% or 3% sucrose. For example, radish and

tomato exhibited higher growth rates on BM with sucrose than on BM devoid of sucrose, regardless of the CO_2 concentration, while kale and citrus exhibited higher growth rates on medium devoid of sucrose. Apparently, a species-related response to CO_2 levels coupled with the sucrose concentration occurred to modify the growth responses observed. Except for tomato, greater fresh weight increases occurred on BM without sucrose at a certain optimum level of CO_2 . This observation agrees with that observed by Kozai (1988) with other species. The optimum CO_2 level differed somewhat among species and media sucrose concentration but, generally, 3000 to $30,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ levels were found

to give the largest fresh weight increases (Fig. 4). Carrot exhibited maximum fresh weight increase of 9.5-fold on BM without sucrose with $30,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$; on BM with sucrose, a maximum fresh weight of only 1.7-fold occurred with $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$. The effect of ultra-high levels of CO_2 on the different species (e.g., carrot) was visibly obvious when compared to growth obtained at $350 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ (Fig. 5). Similarly, kale plantlets exhibited their maximum fresh weight response, a 6.5-fold increase, on BM without sucrose on $30,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$; on BM with sucrose, only a 1.7-fold increase in fresh weight occurred on $3000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$. Citrus shoots exhibited a maximum fresh weight increase of 4.7-fold on BM without sucrose with $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$, but on BM with sucrose only a maximum of 1.3-fold increase with $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$. Radish plantlets exhibited a maximum fresh weight increase of 6.3-fold on BM without sucrose with $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ and only a 3.3-fold increase on BM with sucrose with $30,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$. Tomato plantlets exhibited a maximum fresh weight increase of 0.8-fold on BM without sucrose with $3000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$ and a 1.2-fold on BM with sucrose with $10,000 \mu\text{L}\cdot\text{L}^{-1} \text{CO}_2$. Associated with increasing fresh weights are

Fig. 4. Growth responses of several plants to various levels of CO_2 in vitro after 8 weeks in culture. Two seeds each of carrot, kale, radish, and tomato were cultured per tube. For *Citrus* one 2-cm-long shoot culture was planted. Data were averaged for 10 replications/treatment. Experiments were repeated three times and single representation is presented. Mean separation by Student-Newman-Keuls multiple range test ($P < 0.1$). Columns for the same sucrose concentration with the same letter were not significantly different.

Fig. 5. Growth response of carrot seedlings grown on basal medium (BM) without sucrose and given ultra-high levels of CO₂ for 8 weeks. Note the enhanced growth of plantlets on medium containing the 10,000 to 50,000 µL·L⁻¹ CO₂ compared to plantlets grown on medium without CO₂ enrichment (350 µL·L⁻¹ CO₂). Scale bar = 15 mm.

also increases in shoot length, leaf number, leaf length, leaf width, root number, and root length (data not shown).

Differences in growth responses of various species cultured under various levels of CO₂ have been reported previously (Fujiwara et al., 1988; 1992; Jeong et al., 1993; Kozai et al., 1987). Our results indicate that certain levels of CO₂ cause optimum growth responses depending on the species and medium sucrose concentration. It can be concluded from these experiments that CO₂ administered at a wide range of concentrations aids in the growth of sterile seedlings; however ultra-high levels of CO₂ ($\geq 3,000$ µL·L⁻¹) are particularly advantageous in the *in vitro* growth of most plantlets regardless of the inclusion or omission of carbohydrates from the BM. These results contrast to those reported from previous greenhouse studies that suggest 1000 µL·L⁻¹ CO₂ is optimum for most plants (Enoch and Kimball, 1986). The use of CO₂ >1000 µL·L⁻¹ CO₂ level is considered unnecessary and is often detrimental to the growth of plants (Enoch and Kimball, 1986). Similarly, Kozai et al. (1987) found

that 1000 µL·L⁻¹ CO₂ levels gave high growth rates with a number of different plants (e.g., carnation, orchid, and potato) *in vitro*. However, other researchers have used higher levels of CO₂ (10,000 to 50,000 µL·L⁻¹ CO₂) to enhance plant growth (Buddendorf-Joosten and Woltering, 1996; Cournac et al., 1991; Figueira, et al., 1991; Treat et al., 1989). In our study, the merits of using high CO₂ (750 and 1500 µL·L⁻¹) and ultra-high CO₂ (≥ 3000 µL·L⁻¹) concentrations were evaluated on six species. Both ranges of CO₂ enhanced the growth of plantlets *in vitro*. However, the ultra-high concentrations of CO₂ consistently gave better results and will merit serious consideration in further CO₂ testing research with other plants. In addition, the optimum level of CO₂ must be determined empirically for each plant.

The CO₂ flow rate when varied from 250 to 2000 mL·min⁻¹ was not critical to the growth of lettuce plantlets (Table 2). There was no effect of CO₂ flow rate on the growth of lettuce seedlings over the flow rates tested. Flow rates administered to cultures have varied in the literature greatly ranging from as low of 10 µL·min⁻¹ (Prusky et al., 1996) to as high of 5000 mL·min⁻¹ (Cournac et al., 1991). The flow rate becomes critical depending on the size of the chamber. In this study, the 250-mL·min⁻¹ rate displaces 17.6 L of chamber air in 70 min. This corresponds to other experiments where 1500 mL·min⁻¹ was applied to the larger 94.5-L chambers, which took 62.6 min to displace this volume. Significant modifications of the gaseous composition occurs during the growth of cultures within vessels (Kozai, 1988). Volatile products such as ethylene and other hydrocarbons may accumulate and inhibit plant growth and development. In the flow-through system presented in this study,

it is likely photosynthesis is enhanced by elevated CO₂ levels coupled with the added benefit of the removal of volatiles (Buddendorf-Joosten and Woltering, 1994).

Remarks

Photoautotrophic and photo-mixotrophic conditions were achieved in a flow-through system and a wide range of CO₂ concentrations was tested on several plant species within traditional tissue culture vessels. It should be pointed out that use of special filters or vessels was not necessary to use an enriched CO₂ environment, provided the culture vessel and closure provides for sufficient ventilation. The typical tissue culture tubes and closures satisfied this requirement. Light intensity is critical to obtain optimum benefit from high CO₂ levels. Our experiments were conducted under 260 µmol·m⁻²·s⁻¹ while the typical tissue culture room uses 40 to 100 µmol·m⁻²·s⁻¹. Cournac et al. (1991) found that 1000 µmol·m⁻²·s⁻¹ caused potato cultures supplemented with 10,000 µL·L⁻¹ CO₂ to grow better than cultures under 230 µmol·m⁻²·s⁻¹ at the same CO₂ levels. Similarly, we found higher growth rates for our cultures when given 260 instead of 120 µmol·m⁻²·s⁻¹ (data not shown). High light intensity coupled with high and ultra-high CO₂ levels used in our experiments were responsible for the autotrophic growth of cultures on BM without sucrose. We conclude that a wide range of CO₂ enhances plantlet growth *in vitro*; however, ultra-high levels of CO₂ ($\geq 3,000$ µL·L⁻¹) consistently gave better results than high CO₂ levels (750 to 1500 µL·L⁻¹). Ultra-high levels of CO₂ may completely eliminate the requirement of an external carbohydrate source for culture survival and optimum growth. Frequently, culture growth on BM without sucrose (photoautotrophic

Table 2. Influence of various flow rates 10,000 µL·L⁻¹ CO₂ on the growth of lettuce plantlets *in vitro*. Cultures were grown in 17.6-L transparent containers. Carbon dioxide was administered for 16 h·d⁻¹ for 8 weeks².

Flow (mL·min ⁻¹)	Leaves per culture	Shoot length (mm)	Culture wt (g)
250	11.0 ± 0.6 a	95.0 ± 15.0 a	5.8 ± 1.0 a
500	11.8 ± 0.3 a	95.0 ± 15.0 a	6.6 ± 0.1 a
1500	11.4 ± 0.4 a	98.3 ± 11.7 a	6.6 ± 1.6 a
2000	12.5 ± 0.9 a	90.0 ± 15.3 a	7.2 ± 0.8 a

²Treatment averages and SE presented. Mean separation within growth response columns by Student-Newman-Keuls multiple range test ($P \leq 0.1$).

condition) exceeded that occurring in BM containing sucrose (photo-mixotrophic condition) for several plants (e.g., carrot and lettuce) if given an adequate CO₂ level (Fig. 4). We conducted our studies with different species and obtained significant growth increases using ultra-high levels of CO₂. This work suggests that other species should now be tested using the inexpensive CO₂ flow-through system presented herein with similar CO₂ levels.

Literature Cited

- Buddendorf-Joosten, J.M.C. and E.J. Woltering. 1994. Components of the gaseous environment and their effects on plant growth and development in vitro. *Plant Growth Regulat.* 15:1-16.
- Buddendorf-Joosten, J.M.C. and E.J. Woltering. 1996. Controlling the gaseous composition in vitro—description of a flow system and effects of the different gaseous components on in vitro growth of potato plantlets. *Sci. Hort.* 65:11-23.
- Cournac, L., B. Dimon, P. Carrier, A. Lahou, and P. Chagvardieff. 1991. Growth and photosynthetic characteristics of *Solanum tuberosum* plantlets cultivated in vitro in different conditions of aeration, sucrose supply, and CO₂ enrichment. *Plant Physiol.* 97:112-117.
- Desjardins, Y., F. Laforge, C. Lussier, and A. Gosselin. 1988. Effect of CO₂ 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.
- Enoch, H.Z. and B.A. Kimball. 1986. Carbon Dioxide Enrichment of Greenhouse Crops. vol. 1. CRC Press, Inc., Boca Raton, Fla.
- Figueira, A., A. Whipkey, and J. Janick. 1991. Increased CO₂ and light promote in vitro shoot growth and development of *Theobroma cacao*. *J. Amer. Soc. Hort. Sci.* 116:585-589.
- Fujiwara, K., S. Kira, and T. Kozai. 1992. Time course of CO₂ exchange of potato cultures in vitro with different sucrose concentrations in the culture medium. *J. Agr. Met.* 48:49-56.
- Fujiwara, K.S. and T. Kozai. 1995. Physical microenvironments and its effects, p. 319-369. In: J. Aitken-Christie, T. Kozai, and M. Lila Smith (eds.). *Automation and environmental control in plant tissue cultures*. J. Kluwer Academic Publishers, Boston.
- Fujiwara, K., T. Kozai, and I. Watanabe. 1988. Development of a photoautotrophic tissue culture system for shoots and/or plantlets at rooting and acclimation stages. *Acta Hort.* 230:153-158.
- Jeong, B.R., K. Fujiwara, and T. Kozai. 1993. Carbon Dioxide enrichment in autotrophic micropropagation: Methods and advantages. *HortTechnology* 3:412-414.
- Kozai, T. 1988. Autotrophic (Sugar free) tissue culture for promoting the growth of plantlets in vitro and for reducing biological contamination, p. 39-51. In: T.W. Flegel, G. Tharun, and Y. Yuthavonh (eds.). *Intl. Symp. Appl. Biotechnol. Small Ind. Dev. Developing Countries*, 21-23 Sept., Bangkok, Thailand.
- Kozai, T., H. Oki, and K. Fujiwara. 1987. Effects of CO₂ enrichment and sucrose concentration under high photosynthetic photo fluxes on growth of tissue-cultured cymbidium plantlets during the preparation stage. *Symp. Florizel on Plant Microprop.* in *Hort. Ind.*, Arlon, Belgium. p. 135-141.
- Prusky, D., H. Hamdan, R. Ardi, and N.T. Keen. 1996. Induction of biosynthesis of epicatechin in avocado suspension cells treated with an enriched CO₂ atmosphere. *Physiol. Mol. Plant Pathol.* 48:171-178.
- Treat, W.J., C.R. Engler, and E.J. Soltes. 1989. Pine in a modified Fermentor using a novel impeller. *Biotech. Bioeng.* 34:1194-1202.
- Yorio, NC., R.M. Wheeler, and R.C. Weigel. 1995. Effect of irradiance, sucrose, and CO₂ concentration on the growth of potato (*Solanum tuberosum* L.) in vitro. *NASA Tech. Mem.* 110654:1-19.

Effect of Natural Organic Nitrogen Fertilizers on Hybrid Bermudagrass Growth

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ADDITIONAL INDEX WORDS. *Cynodon*, turfgrass, golf courses

SUMMARY. Eco, Milorganite, Ringer, and Sustane natural organic fertilizers, alone or combined with the synthetic organic fertilizer isobutylidene diurea (IBDU), were compared with IBDU alone for their effect on a 'Tifdwarf' hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy] golf course putting green. Over the 2-year study period, no consistent differences were observed among the fertilizer treatments on the turfgrass growth parameters of quality, clipping weights, or root weights.

Hybrid bermudagrass (*Cynodon dactylon* x *C. transvaalensis*) grows all year on golf course putting greens in southern Florida. Due to the extensive rainfall received in this subtropical climate, slow-release rather than quick-release nitrogen fertilizer sources are commonly used to reduce nitrogen leaching and maintain turfgrass quality (Sartain, 1982; Snyder et al., 1981). Products developed from urea, such as ureaformaldehydes, sulfur-coated urea, and isobutylidene diurea (IBDU), are examples of slow-release synthetic organic nitrogen fertilizers.

Other alternatives to quick-release nitrogen sources are natural organic nitrogen fertilizers. These fertilizers