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Carbon dioxide improves the growth of hairy roots cultured on solid medium and in nutrient mists

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Summary. The effect of varying CO_2 concentrations on the growth of beet and safflower hairy roots was measured for tissues cultured in nutrient mists and on solid media in chambers fed mixtures of humidified air supplemented with different CO_2 concentrations. Hairy root tissue grown on solid media in air enriched with CO_2 showed increased growth, as measured by dry weight increases vs air-fed controls. Growth increased with CO_2 enrichment as much as 2.5 times more than the air-fed control for safflower at 1.0% CO_2 and 1.4 times more than the air-fed control for beets at 1.5% CO_2 over a 12-day period. Beet hairy root tissue was also cultured aeroponically in nutrient mists. Beet hairy root cultured in nutrients mists enriched with 1.0% CO_2 showed a 15% increase in biomass over a 7-day period vs tissue cultured in nutrient mists (with ambient air) or in shake flasks. The stimulation of root growth via CO_2 enrichment reduced the time required for biomass accumulation.

Introduction

Nutrient mist technology has proven successful for micropropagation of a variety of plant species. For example, *Daucus carota* callus increased in dry weight 3.5 times faster in nutrient mists than in conventional tissue culture using agar, while shooting of both *Ficus* and *Cinchona* increased by 14% (Weathers and Giles 1988). Increases in shoot length and number for a number of ornamental species were obtained using ultrasonically generated nutrient mists (Weathers et al. 1988). Nutrient mists also improved rooting for a recalcitrant asparagus cultivar vs conventional tissue culture methods (Cheetham et al. 1990).

Carbon dioxide uptake by excised root tissue occurs both in the presence (Flores et al. 1988) and absence of light (Arteca et al. 1979). Photosynthetic roots of transformed *Bidens sulphureus,* obtained by exposure to light, can be cultured with as little as 0.3% sugar in 1–2% CO_2 (Flores et al. 1988). In the absence of light, CO_2 is thought to be assimilated into the tricarboxylic acid cycle to replenish oxaloacetate removed in the synthesis of aspartate. This latter process was demonstrated using radiolabeled bicarbonate, which was recovered in root cultures as malate and other organic acids (Arteca et al. 1979). Treatment with CO_2 also stimulated the uptake of calcium, potassium and magnesium ions (Arteca et al. 1979). Growth of excised tomato roots in the dark in liquid culture increased by 50% when ambient air was bubbled through a solution containing these roots vs roots grown in ambient air without CO_2 (Splittstoesser 1966). Applications of CO_2 to the root zone of potato seedlings promoted shoot as well as root growth (Arteca et al. 1979). Similar applications to tomato seedlings for a 12-h period (0.5–5.0%) produced a 50% increase in dry weight vs controls fed ambient air (Yurgalevitch and Janes 1988). The effect on pH of bubbling CO_2 through the liquid medium was accounted for by adding HCl to the culture fed ambient air. However, dissolved CO_2 has also been shown to affect rubidium uptake (Yurgalevitch and Janes 1988) and membrane bound K^+-ATPase activity (Malhotra and Spencer 1974). Whether these effects were a direct effect of CO_2 uptake or CO_2 alteration of the local medium environment is not known.

In terms of excised root tissue, CO_2 enrichment of wheat roots improved growth, measured as dry weight increases, when ambient air enriched with CO_2 (5–10% v/v) was bubbled through the liquid culture medium (Talbot and Street 1968). In our study, CO_2 enrichment was applied to transformed root cultures grown on solid medium, to reduce the complications of dissolved CO_2 in liquid media. The negative geotropism exhibited by transformed roots allowed good contact between the gas and the aerial roots growing on solid media. The dry weight increases were compared to controls fed ambient air.

In order to exploit one of the the key advantages of nutrient mist culture, control over the gaseous atmo-

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sphere, the effect of CO_2 enrichment on the growth of transformed roots was tested for tissue growing first on solid medium in order to estimate the potential benefits of CO_2 enrichment. Then, based on the effective concentration range determined from these experiments, CO_2 enrichment could be applied to nutrient mist cultures. The effect of CO_2 enrichment on transformed beet root tissue in a nutrient mist bioreactor was compared to control tissues cultured in shake flasks with ambient air.

Materials and methods

Cultures. After transformation of *Beta vulgaris* (Detroit Dark Red) with *Agrobacterium rhizogenes* ATCC no. 15834, excised beet hairy roots were cultured on B5 (Gamborg et al. 1968) medium containing 3% sucrose, 0.2% Gel-rite and 0.025% carbenicillin (Flores et al. 1988). Axenic cultures were obtained by subsequent immersion in 10% commercial bleach (Clorox) for up to 10 min. Cultures were maintained on B5 media containing 3% sucrose and 0.2% Gel-rite in the dark at 25° C.

Hairy root cultures of safflower (*Carthamus tinctorius*, courtesy of H. Flores, Pennsylvania State University) were maintained on MS salts (Murashige and Skoog 1962) containing 3% sucrose and 0.2% Gel-rite. Inocula for all experiments were from 2-week-old cultures grown on solid media in the dark. Shake-flask cultures were inoculated with 0.3 g fresh weight of root tips (unless otherwise noted) and cultured at 25° C on a rotary shaker at 117 rpm. Wet to dry weight correlations of the inoculum were determined before each experiment by measuring fresh weights of tissue taken from plates, drying the tissue at 60° C overnight and determining the percentage moisture. Typical values for beet and safflower hairy roots were $94.3 \pm 0.5\%$ and $93 \pm 1\%$, respectively (average of ten trials). The mass increase over the culture period was defined as the mass ratio of the final dry weight (X_1) to the initial dry weight (X_0) in arbitrary units. The medium pH was adjusted within the range 3.5–6.0 using 2 M NaOH. All reagents were obtained from Sigma (St. Louis, Mo., USA), unless otherwise noted.

Opine assay. Verification of transformation was by the detection of opines (Firmin 1990).

Aeration control. To avoid dehydration of the tissue on solid media during the 12-day culture period, an automated gas feeding system was designed for delivering humidified gas. A ChronTrol (Fisher Scientific), four-port controller was used to cycle the air feed (Whisper 500 aquarium pump obtained from local pet supply stare) and CO_2 feed (Valcor Scientific, Springfield, NJ, USA, SV23-C-12 pinch valve in line with a compressed CO_2 supply) to establish a 10-min gas feeding cycle every 2–8 h. During this time, using empty culture chambers (1-l, cubical, screw capped, polycarbonate bottles; a gift of Mr. Tomi Iwamura, Gifu, Japan), the actual CO_2 concentration was determined to have remained constant by analysis of the culture chamber contents using gas chromatography (Patriquin and Knowles 1974). The total volume of gas removed daily for sampling was 800 µl, which was 0.08% of the available chamber volume. All sampling was done in duplicate and means were determined with less than 5% drift in gas analyses. The presence of ehtylene was tested by analyzing 5 ml of gas from the sealed chamber (Patriquin and Knowles 1974) at the end of the 12-day growth period. Ethylene detection was sensitive to 50 µl/l (62.5 µM).

Apparatus for CO_2 enrichment. For each CO_2 concentration tested, two identical culture chambers were constructed for simultaneous use. Compressed CO_2 and ambient air were mixed in the desired ratios using a Matheson gas mixer. The mixture passed through a 0.2-µ Gelman filter and then through 300 ml sterile deionized water (for humidification) before entering the chambers through the airtight screw cap on the side of the chamber. All tubing and apparatus downstream of the sterile 0.2-µ filter were sterile. The gas outlet, a sterile drying tube filled with sterilized glass wool, was placed on top of the chamber adjacent to the sampling port allowing for good mixing and accurate sampling for monitoring the CO_2 concentration. The pH of the solid medium after CO_2 enrichment was determined by making a slurry of the Gel-rite and using a Sensorex (Stanton, CA, USA) 450C surface pH electrode to determine the pH of the culture medium.

Axenic root cultures (0.3 g dry wt.) were placed aseptically in sterilized chambers containing 200 ml of growth media: B5 for beets and MS for safflower. Humidified gas containing ambient air or 0.5–2.0% CO_2 (v/v) in air, was introduced into the chambers at the rate of 900 ml/min for 10 min every 2–8 h for 12 days. The cycle was empirically determined to be 8 h for the first 4 days in culture, and was reduced to 4 h (safflower) and 2 h (beet) after the fourth day. The cycle period was shortened to reduce the build-up of respiratory CO_2 from the growing tissue, thereby maintaining a nearly constant CO_2 concentration.

Root tissue was cultured three different ways:

1. Chambers were fed ambient air at 900 ml/min for the specified cycle time.
2. Chambers were fed air enriched with 0.5 to 2.0% CO_2 at 900 ml/min using the same cycle time.
3. Chambers were sealed allowing no gas exchange.

Two sets of gas samples (200 µl) were taken daily from each chamber and analyzed via gas chromatography. The first set was taken just prior to the beginning of a gas feeding cycle, the second set just after the gas flow stopped. Background light contamination was a maximum of 0.125 W/m^2 for 8 h a day with total darkness for the remaining 16 h during the maintenance period and the gas feeding experiments. Experiments were run in duplicate and repeated.

Nutrient mist culture. Beet root tissue was cultured using a Mistifier aeroponics system (Manostat Corporation, NY, NY, USA). Nutrient mist is adense fog of particles of average size 7–10 µ. The bioreactor modified for hairy root culture was described in part by Weathers et al. 1989. Further bioreactor modifications and the description of culture conditions and inoculation procedures used were as described previously (DiIorio et al. 1992).

Results

With increasing CO_2 concentrations in the chamber atmosphere using solid medium, growth increased steadily to a maximum mass increase of 10.6 times the inoculum size for safflower at 1% CO_2, and 7.0 times the inoculum size for beets at 1.5% CO_2 (Fig. 1). The tissues in the control chambers fed ambient air always exhibited the smallest dry weight increases among the gas-fed chambers; mass increases of 2.5 and 2.3 were observed for safflower and beets, respectively. Although an improvement over the controls was observed, CO_2 concentrations in excess of 1.5% were not as effective as 1.0–1.5% in achieving maximum growth.

Roots placed in sealed chambers, on solid medium, did not grow as well as roots aerated with ambient air. Safflower and beet hairy root tissues grown in sealed chambers exhibited average mass increases of 4.47 and 4.20, respectively. (The mass increase was defined as X_1/X_0, where X_1 and X_0 are the final and initial dry weights of the tissue, respectively.) The CO_2 concentra-

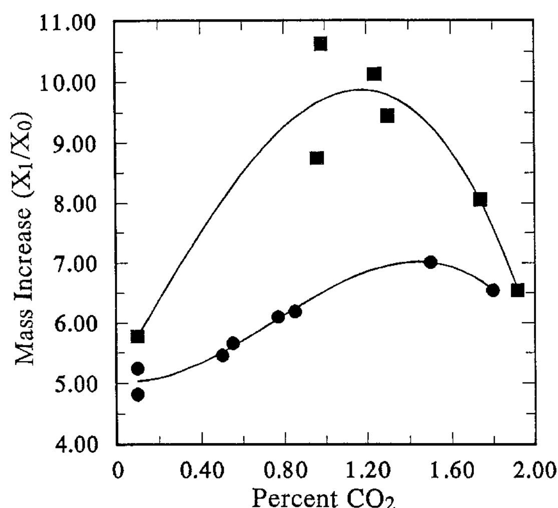

Fig. 1. The effect of CO_2 enrichment on the growth of safflower and beet hairy roots after 12 days. Cultures were fed gas every 2–8 h with the prescribed CO_2 concentration. The mass increase was defined as the mass ratio of final dry weight (X_1) to initial dry weight (X_0): ■, safflower hairy root tissue; ●, beet hairy root tissue

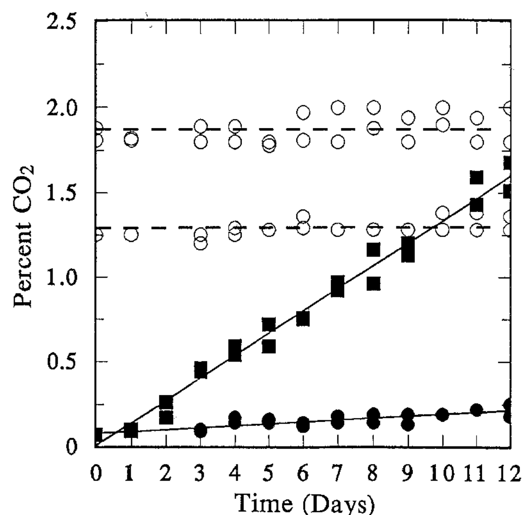

Fig. 2. Time course of CO_2 concentrations in the headspace of the growth chambers during the 12-day culture period of safflower hairy root tissue. Air was enriched with 1.3 and 1.8% CO_2. The data shown are CO_2 concentrations in the chambers just before gassing with the prescribed concentration of CO_2 (before the onset of the cycle). Gas feeding cycles were 10 min at 900 ml/min every 8 h for days 0–4 and every 2 and 4 h for days 5–12 for beet and safflower, respectively. This gas input was sufficient to purge the entire chamber and re-establish the CO_2 concentration within the chamber to that of the gas stream (for both air-fed chambers and chambers fed CO_2-enriched air). Samples from the chambers were taken daily and assayed for CO_2. Similar results were observed for beet hairy root tissue: ■, sealed chambers; ●, air-fed chambers; O, CO_2-enriched chambers

tion in the sealed chambers containing safflower hairy roots increased, and varied from ambient air at day one to 1.7% on day 12 (Fig. 2). The build-up of CO_2 was probably due to accumulation of respiratory CO_2. A similar accumulation of CO_2 occurred in sealed flasks containing beet hairy roots with the final CO_2 concentration reaching 1.5%. No trace of ethylene production was detected in the sealed chambers containing beet or safflower hairy roots at the end of the 12-day growth period. The results for these controls showed that periodic aeration of the growth chambers (relative humidity maintained above 95%) was beneficial to the roots, compared to the conventional tissue culture technique of sealing culture chambers.

Carbon dioxide concentrations in the chambers fed air enriched with CO_2 were maintained within the limits of $0.98 \pm 0.08\%$, $1.29 \pm 0.05\%$, and $1.87 \pm 0.08\%$ in the CO_2-enriched chambers (Fig. 2). For the two chambers fed ambient air, the average CO_2 concentration rose to a maximum of 0.24% at the end of the experiment. It is important to note that after each purging cycle (every 4–8 h), the CO_2 concentration in these chambers was always reduced to that of ambient air, but due to the increased biomass late in the experiment, build-up of respiratory CO_2 was more rapid. In earlier experiments, CO_2 build-up late in the experiment (after 12 days of growth) was up to 0.7%, but the build-up was reduced to only 0.24% (after 12 days of growth) by doubling the purging frequency (beginning on day 5) from three times per day to six times per day. CO_2 accumulation late in growth, although substantial, did not affect growth in the chambers fed ambient air. The increased frequency of aeration from three to six times a day, reduced CO_2 accumulation on day 12 from a maximum of 0.7% down to 0.24%. The effect on growth was minimal: mass increases of 4.69 vs 4.92 for frequencies of three and six times per day, respectively; thus, the air fed chambers were used as controls for comparison with CO_2-enriched chambers.

Since dissolved CO_2 lowers the medium pH, it was important to determine if the growth enhancement was caused by a pH effect in the medium. The pH of the solid medium after CO_2 enrichment was equal to that of the controls (average pH of 5.0 in the culture slurry) negating any pH effect by CO_2 enrichment of tissue on solid medium. Furthermore, the results obtained upon culturing beet hairy root tissue at pH values ranging form 3.5 to 6.0 indicated that lower pH values (which might result from CO_2 enrichment of liquid medium) were inhibitory to growth over a 1-week period (Table 1).

Based on the results obtained using solid medium, the range of effective CO_2 enrichment was determined to be between 1.0 and 1.5% (Fig. 1). Beet hairy roots cultured in the nutrient mist bioreactor with 1.0% CO_2 in the air feed exhibited a 15% increase in biomass after 1 week vs tissues cultured with ambient air or vs tissues cultured in shake flasks (Table 2).

Discussion

The use of hairy root tissues cultured on solid media in gas-fed culture chambers facilitated the study of the effect of different CO_2 concentrations on root growth. The proliferation of aerial roots allowed good contact

Table 1. The effect of medium pH on the growth of beet hairy roots

Medium pH	Initial dry wt (g)	Final dry weight (g)	Mass increase[a] (X_1/X_0)
3.5	0.0171	0.0417	2.44
4.1	0.0171	0.0536	3.14
5.0	0.0165	0.0801	4.83
5.5	0.0177	0.0979	5.55
6.0	0.0182	0.0980	5.69

Root tips were cultured for 1 week in B5 medium with 30 g/l sucrose
[a] Defined as the mass ratio of final dry weight (X_1) to initial dry weight (X_0)

Table 2. The effect of CO_2 enrichment on the growth of beet hairy roots cultured in nutrient mists

Culture mode	Inoculum[d] (g wet weight)	Misting cycle	Mass increase (X_1/X_0)[a]
NMB batch[c]	4.5	5\6[b]	3.5
NMB batch[c] with 1% CO_2	4.5	5\6[b]	4.0
Shake flasks[c]	1.5	—	3.4

NMB, nutrient mist bioreactor
[a] Defined as the mass ratio of final dry weight (X_1) to initial dry weight (X_0)
[b] Number of minutes of misting ON\number of minutes of misting OFF. The air flow rate, or combined air/CO_2 flow rate, was 1500 ml/min during misting
[c] Whole portions of roots were cultured in B5 medium with 30 g/l of sucrose at 117 rpm (DiIorio 1991)
[d] Whole portions of transformed roots (tips as well as mature tissue) were used as inoculum (DiIorio et al. 1992; DiIorio 1991)

between the gas and the transformed root tissue. Maintenance of the cultures in a near dark environment (0.125 W/m^2 for 8 h; total darkness for 16 h) precluded growth increases due to photosynthesis, as described by Flores et al. (1988), i.e. there was no greening of the tissue. Finally, strict control over gas concentrations in the experimental chambers made it possible to determine the most effective CO_2 concentration for maximizing growth of transformed roots of beet and safflower for eventual use in the aeroponics system (the nutrient mist bioreactor).

The growth response in aerated vs. sealed cultures can be species specific (Jackson et al. 1991). For this reason, both types of chambers were necessary as controls for this CO_2-enrichment study. Sealed chambers containing soybean callus were growth inhibited relative to aerated controls (Zobel 1987). In contrast, excised wheat roots in sealed (glass stoppered) flasks grew better than in cotton-stoppered flasks (Talbot and Street 1968). The improvements in growth over either control (sealed or aerated chambers) support the use of CO_2 as a growth stimulant for hairy roots. This response appears independent of pH effects. The relatively poor growth of beet and safflower hairy roots in the sealed chambers agrees with the results of Zobel (1987). Certainly O_2 depletion in the sealed chambers also could have contributed to poor growth. No ethylene was detected in the sealed chambers at concentrations greater than 50 μl/l (62.5 μM).

The improvements in growth using CO_2 enrichment for beet and safflower obtained in this study are similar to results obtained by Ohkawa et al. (1989), using gibberellic acid. Hairy roots of *Datura innoxia* were fed varying levels of gibberellic acid (100 ng/l to 10 mg/l) with maximum growth increases of 1.5 times the control (Ohkawa et al. 1989). Gibberellic acid acts as a CO_2 analog for root growth (Zobel 1989). It was also suggested that the Ri plasmid, inserted into the plant genome via *Agrobacterium* transformation, may enhance the action of gibberellic acid on hairy root growth (Ohkawa et al. 1991). However, since similar effects on growth enhancement of excised roots have been reported earlier for non-transformed species (Arteca et al. 1979; Talbot and Street 1968; Zobel 1987), the effect of the transformation, if any, on CO_2 interactions with hairy roots could be independent of the Ri plasmid. The results of both of these studies warrant further study on the interactions of regulators on transformed tissue.

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