

Techniques to Improve Growth, Morphogenesis and Secondary Metabolism Responses from Lamiaceae Species in Vitro

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Abstract

Cultural procedures to improve growth (fresh weight) and morphogenic (leaves, shoots and roots) development in the mint family (Lamiaceae) species (e.g. basil, catnip, oregano and spearmint) in vitro are presented. Novel plant tissue culture systems were employed to increase biomass and shooting. A survey study with several species is presented testing a wide range of CO₂ levels. Employment of ultra-high levels of CO₂ (i.e. $\geq 3,000 \mu\text{L CO}_2 \text{ L}^{-1}$) promoted greater growth and morphogenesis in most mint species compared to that obtained employing ambient air ($350 \mu\text{L CO}_2 \text{ L}^{-1}$). Employment of a sterile hydroponics system, i.e. an automated plant culture system (APCS), greatly enhanced spearmint biomass and shooting over that obtained employing an agar medium. Analysis of essential oil composition in spearmint cultures grown in various culture systems reveals that high secondary metabolite production is often associated with high growth and morphogenesis responses.

INTRODUCTION

Members of the mint family (Lamiaceae) species are multiplied in vitro for mass distribution of disease-free and/or superior germplasm clones (e.g., Summit Plant Labs Inc., Fort Collins, CO; National Clonal Germplasm Repository, Corvallis, OR). We explored the possibility of employing alternative tissue culture techniques such as high CO₂ and the automated plant culture system (APCS) to improve growth and morphogenesis and secondary metabolism in various Lamiaceae species in vitro. Plants grown under elevated CO₂ levels in vitro may exhibit enhanced plant growth, morphogenesis and development (Buddendorf-Joosten and Woltering, 1996; Cournac et al., 1991; Figueira et al., 1991; Mitra et al., 1997; 1998; Tisserat and Silman, 2000; Tisserat and Vaughn, 2001). Overall plant development improves under elevated CO₂ levels through the conversion of leaves from a heterotrophic or photomixotrophic state to a photoautotrophic state coupled with the attainment of a more "normal" plantlet phenotype (Cournac et al., 1991; Mitra et al., 1997; 1998; Tisserat and Silman, 2000; Tisserat and Vaughn, 2001). The APCS or sterile hydroponics system allows for large scale biomass production (i.e. fresh weight) coupled with enhanced morphogenesis and development (i.e. shooting, rooting, leaf production, leaf enlargement, etc.) (Tisserat, 1991; 1996). Within the APCS, a plant culture is established and maintained in a single chamber while media is periodically introduced and removed on a daily basis. Both methods are attractive to the plant propagator as they are relatively easy to administer. In addition, since Lamiaceae species are rich sources of essential oils, we also determined the relationship between culture systems and secondary metabolism in vitro.

MATERIALS AND METHODS

Medium and Plant Culture

The basal medium (BM) consisted of Murashige and Skoog (1962) salts plus (per liter): 0.5 mg thiamine·HCl, 100 mg myo-inositol, 30 g sucrose and 10 g agar (Difco

Laboratories, Detroit, MI). Medium pH was adjusted to 5.7 ± 0.1 with 0.1 N HCl or NaOH before the addition of agar, then melted and dispensed in 25-ml aliquots into 25 X 150 mm borosilicate glass culture tubes. Tubes were capped with translucent polypropylene closures (Sigma Chemical Co., St. Louis, MO). Liquid BM was adjusted to 5.0 ± 0.1 and distributed in 1-L aliquots in 1-L Pyrex bottles. Medium was autoclaved for 15 min at 1.05 kg cm^{-2} at 121°C . Agar medium was then slanted at a 45° angle while cooling. Stocks of shoots of catnip (*Nepeta cataria* L.), oregano (*Origanum vulgare* L.), spearmint (*Mentha spicata* L.), and sweet basil (*Ocimum basilicum* L.) were maintained on BM under ambient air prior to testing.

CO₂ Flow Systems

The CO₂ flow through testing chamber consists of a transparent polycarbonate box and lid (Consolidated Plastics, Twinsburg, OH) (32.5 cm W X 30 cm L X 26.3 cm D; 17.6-liter capacity). A silicone tape gasket (Furon, New Haven, CT) was attached to the lid. The box was modified by mounting three polypropylene spigots (Ark-Plas Products, Flippin, AR) attached to 0.45 μm air vents (Gelman Science, Ann Arbor, MI). The box and lid were clamped with 10 equally spaced stationary binding clips (50 mm L). CO₂ was provided by a gas cylinder (BOC Gases, Edison, NJ) rated 99.8 % pure and was mixed with an ambient air, i.e. 350 $\mu\text{L liter}^{-1}$ CO₂, flow produced by an aquarium air pump (Whisper 2000, Carolina Biological Supply Company, Burlington, NC) via a flow meter (Cole Parmer Instrument Co., Niles, IL) to provide 350, 1,500, 3,000, 10,000, and 30,000 $\mu\text{L CO}_2 \text{ L}^{-1}$. CO₂ ranges $\geq 10,000 \mu\text{L CO}_2 \text{ L}^{-1}$ were adjusted using a LIRA infrared gas analyzer, (model #3000, Mine Safety Appliances Company, Pittsburgh, PA) and CO₂ ranges $\leq 3,000 \mu\text{L CO}_2 \text{ L}^{-1}$ were adjusted with a Li-Cor CO₂/H₂O infrared gas analyzer, (model LI-6262, Li-Cor, Inc., Lincoln, NE). The CO₂ and air streams were added at 2,000 ml min^{-1} during the photoperiod. Control cultures were given a stream of ambient air generated by the aquarium pump only. No CO₂ or air control was applied during the dark. Culture tubes were measured to have 1.4 h^{-1} air exchanges.

Automated Culture System

A polycarbonate bottle (247.6 mm H x 127 mm diam; 2,000 ml cap.) (McMaster-Carr Supply Company, Chicago, IL) fitted with a polypropylene closure was employed as the culture chamber for the APCS (Tisserat, 1996). Two filters were attached via silicone tubing to 90° 1.6 mm I.D. male barbed fittings which were threaded with 1/8-27 threaded holes into polypropylene closures. A third hole was employed as the medium spigot that consisted of a 90° 1.6 mm I.D. male barbed fitting attached to a 1.6 mm I.D. female barbed fitting. A polypropylene tube attached to the female fitting extended to the bottom the culture chamber. To provide culture support a 1-cm layer of glass gravel (4 to 8 mm diam) was added to bottle of bottle. The APCS Bio-Safe container was attached to a 1-L Pyrex bottle reservoir containing one-liter BM. Cultures were soaked 4-x daily for 10 minutes and then the medium was evacuated back to the reservoir employing a peristaltic pump.

Plant Tissue Culture Experiments

To determine the optimum CO₂ levels for optimum growth and secondary metabolism, catnip, oregano, spearmint, and sweet basil, shoots were cultured on 350, 1,500, 3,000, 10,000, and 30,000 $\mu\text{L CO}_2 \text{ L}^{-1}$. A single 3-cm long shoot was cultured per vessel. Three cultivars of spearmint were grown in either culture tubes or in polycarbonate bottles coupled to the APCS. Cultures were grown in a culture room maintained at $25^\circ\text{C} \pm 1^\circ\text{C}$ and employing a photoperiod of 16 hr light/8 hr dark. For CO₂ experiments, light was supplied by a combination of Coolwhite fluorescent tubes, metal-halide and incandescent lamps at a total photosynthetic photon flux density of 180 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the vessel periphery. APCS experimental cultures were exposure to 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ cool white fluorescent lamps. After 8 or 16 weeks of incubation, data on culture fresh weight, shoot length, leaf number, root number, and shoot number were

recorded and analyzed with Student-Newman-Keuls multiple range test as appropriate. Experiments were repeated three times employing 10 to 20 replications/treatment.

Chemical Analysis

For chemical analysis of spearmint plants, one gram of fresh plant tissues were extracted with 15 ml CH₂Cl₂ for 72 h. After filtering, the extracts were analyzed on a Hewlett-Packard (HP) 5890 gas chromatograph (GC) equipped with a flame ionization detector. GC-mass spectrometry was performed using a HP 6890 Series II gas chromatograph attached to a HP 5972A mass selective detector. Columns used were fused silica HP-5MS capillaries (0.25 µm film thickness, 30 m x 0.25 mm ID). The major compound found in the extracts, (-)-carvone, was identified by comparison with mass spectra of a library database, and concentrations were calculated from a standard curve of (-)-carvone.

RESULTS AND DISCUSSION

Influence of CO₂ Levels on Growth, Morphogenesis, and Secondary Metabolites

Shoots grown under ambient air (i.e. 350 µL CO₂ L⁻¹) usually gave the lowest growth (i.e. fresh weight) and morphogenesis (i.e. leaves, roots and shoots) responses compared to employing higher CO₂ levels (Figs. 1 and 2). Each species tested responded somewhat differently to CO₂ applications. For example, spearmint cultures grew best at 10,000 and 30,000 µL CO₂ L while sweet basil responded well under 1,500 to 10,000 µL CO₂ L. Higher growth and morphogenesis responses (except sweet basil) in variable occurred employing the ultra-high CO₂ levels (i.e. ≥ 3,000 µL CO₂ L⁻¹) compared to that obtained than under ambient CO₂. Similar results have been previously reported with a other C₃ species employing similar CO₂ levels (Buddendorf-Joosten and Woltering, 1994; Figueira et al., 1991; Mitra et al., 1997; 1998; Tisserat and Silman, 2000; Tisserat and Vaughn, 2001). Although fresh weight and rooting increased with ultra-high CO₂ levels shooting and leaf production in sweet basil did not. An overall improvement in the sweet basil shoot phenotype occurred when shoots were given higher CO₂ levels as evidenced by their increased shoot length (data not shown), root formation and fresh weight. Although several secondary products were detected in CH₂Cl₂ extracts of spearmint, only a single secondary metabolite, carvone, was dominate constituting 95 % of the available essential oils. Spearmint carvone levels increased 88.7 % employing 10,000 µL CO₂ L⁻¹ compared to levels occurring in cultures grown under ambient air (Fig. 1). Higher growth and morphogenesis rates were associated with higher secondary metabolism (Tisserat and Vaughn, 2001).

Influence of Culture Systems on Growth

Spearmint cultivars grown for 16 weeks on the APCS exhibited 13.5 to 19.6-fold increases, in fresh weight, compared to cultures grown in culture tubes for 8 weeks (Fig. 3). Spearmint cultures can not be grown for longer than 8 weeks in a culture tube without senescence and culture death. Therefore, only a 8 week old culture grown in agar was employed for comparative purposes. Sixteen week-old treatments were conducted with the APCS because at that time cultures filled the available culture vessel capacity. Spearmint shoots invariably produced higher fresh weights and morphogenesis responses when cultured within the APCS compared to shoots grown in agar tubes. Plant height and leaf size (data not shown) were significantly greater among the plants grown in the APCS compared those grown in the agar tubes. Similar results have been found for a variety of different species (Tisserat, 1991; 1996). Carvone levels were less in cultures grown in the APCS compared to those grown in culture tubes (Fig. 3). However, the total levels of carvone from spearmint plants grown within the APCS vessel was 30-fold greater over that occurring from spearmint shoots grown in culture tubes (Fig. 3). We attributed this increase in carvone to the substantial increase in the biomass/differentiation processes (i.e., shoot length, shoots, leaves and fresh weight) occurring the APCS. Within the same

APCS vessel a shoot culture may manifest a variety of morphological and developmental growth stages simultaneously. Paradoxically, mature large sized shoots produce numerous Axillary immature shoots and leaves resulting in a culture containing a mixture of foliage types with immature shoots predominating.

CONCLUSION

Both culture systems studied substantially improved the growth, morphogenesis and secondary metabolism of various Lamiaceae species without any media additions or alterations. The APCS benefits cultures primarily by providing increased culture space and additional media volume. Ultra-high CO₂ levels increase available photosynthetic carbon to cultured shoots. Highly differentiated plantlet or shoot cultures attained from these culture systems are a viable source of plant material with which to study secondary metabolism in vitro.

ACKNOWLEDGEMENTS

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Figures

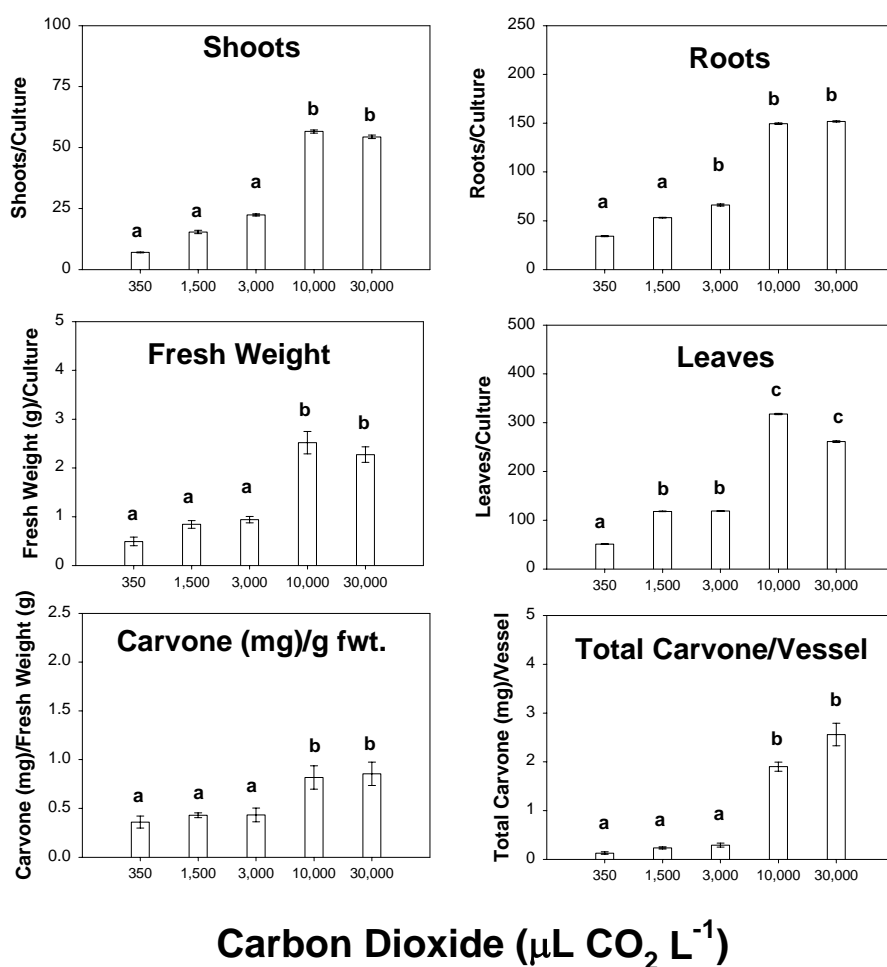


Fig. 1. Influence of CO₂ on the growth, morphogenesis and biochemical responses for *Mentha spicata* L. cv. '557787' (spearmint) cultures after 8 weeks incubation. Physical and Chemical data was averaged for 5 and 3 replications per CO₂ treatment, respectively. Experiments were repeated 3 times and single representation is presented. Mean separation by Student-Newman-Keuls multiple range test (P<0.05). Treatment bars with the same letter on top were not significantly different.

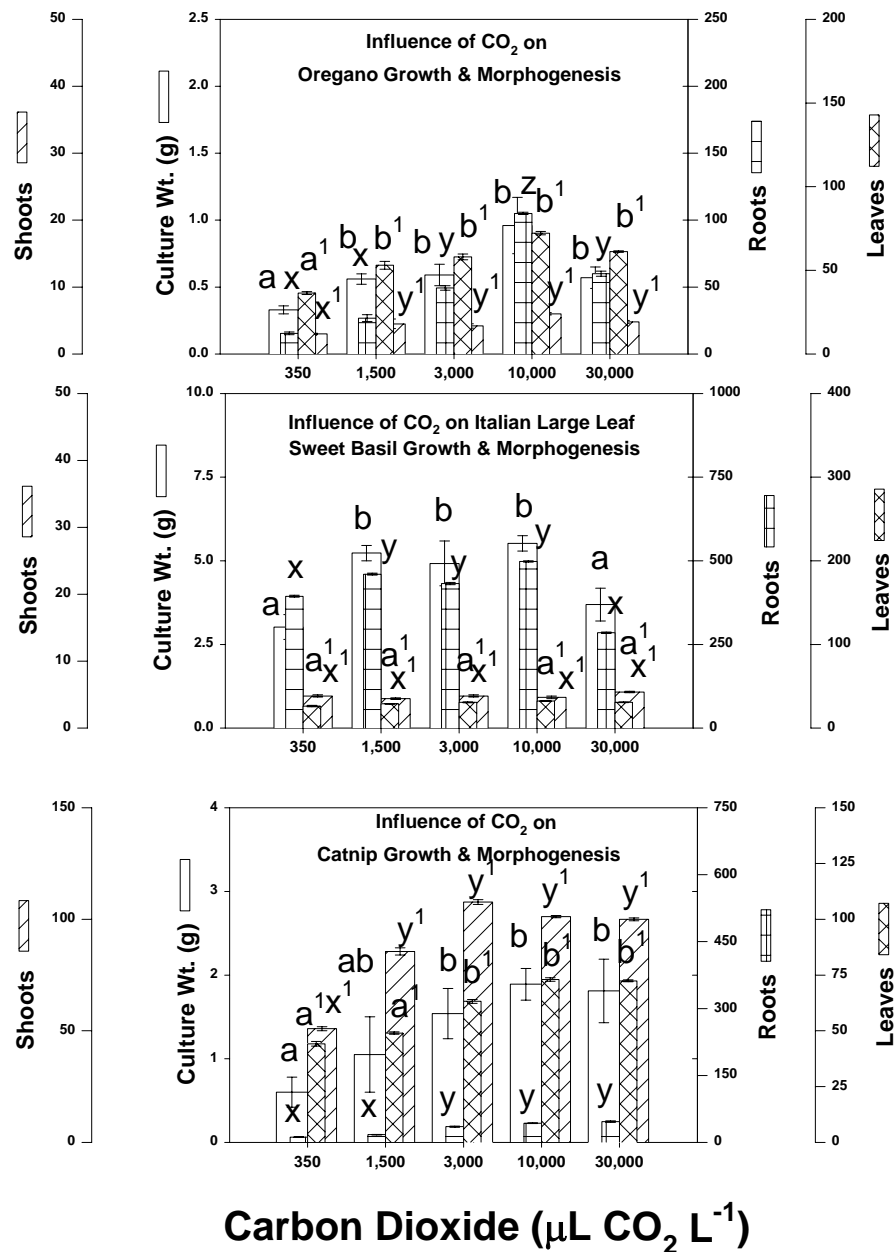
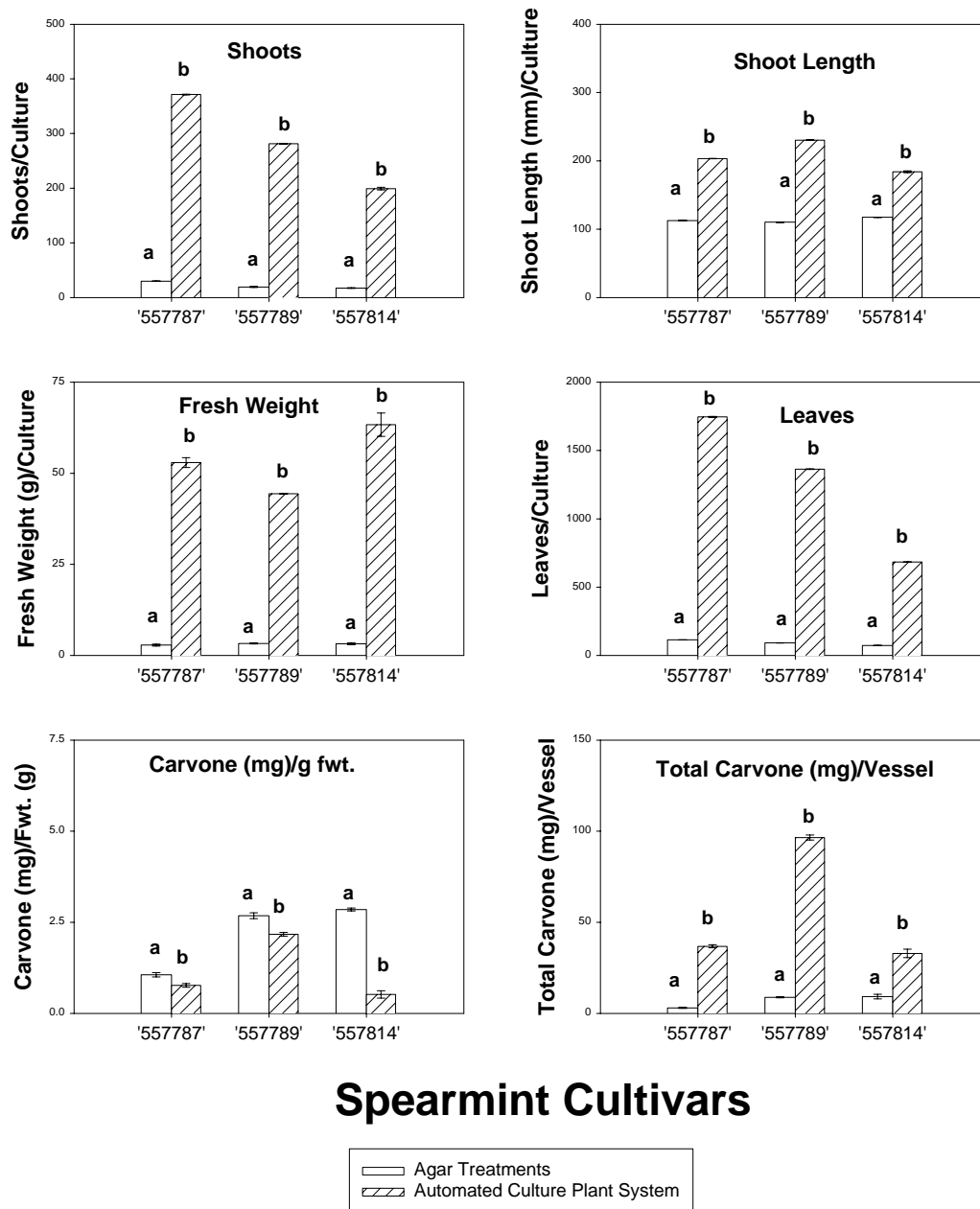


Fig. 2. Influence of CO₂ on the growth and morphogenesis on various Lamiaceae species after 8 weeks incubation. Data was averaged for 5 replications/treatment. Experiments were repeated 3 times and single representation is presented. Mean separation by Student-Newman-Keuls multiple range test (P<0.05). Treatment bars with the same letter on top were not significantly different.



Spearmint Cultivars

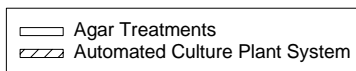


Fig. 3. Comparison of the growth, morphogenesis and biochemical responses for various cultivars of *Mentha spicata* L. (spearmint) grown in either culture tubes containing agar medium or in automated plant tissue culture systems with liquid medium after 8 weeks incubation. Physical and Chemical data was averaged for 5 and 3 replications per CO₂ treatment, respectively. Experiments were repeated 3 times and single representation is presented. Mean separation by Student-Newman-Keuls multiple range test (P<0.05). Treatment bars with the same letter on top were not significantly different.