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Influence of modified oxygen and carbon dioxide atmospheres on mint and thyme plant growth, morphogenesis and secondary metabolism in vitro

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Abstract Growth (fresh weight) and morphogenesis (production of leaves, roots and shoots) of mint (*Mentha sp*. L.) and thyme (*Thymus vulgaris* L.) shoots were determined under atmospheres of 5%, 10%, 21%, 32%, or 43% O_2 with either 350 or 10,000 µmol mol^{-1} CO_2. Plants were grown in vitro on Murashige and Skoog salts, 3% sucrose and 0.8% agar under a 16/8-h (day/night) photoperiod with a light intensity of 180 µmol s^{-1} m^{-2}. Growth and morphogenesis responses varied considerably for the two plant species tested depending on the level of O_2 administered. Growth was considerably enhanced for both species under all O_2 levels tested when 10,000 µmol mol^{-1} CO_2 was added as compared to growth responses obtained at the same O_2 levels tested with 350 µmol mol^{-1} CO_2. Mint shoots exhibited high growth and morphogenesis responses for all O_2 levels tested with 10,000 µmol mol^{-1} CO_2. In contrast, thyme shoots exhibited enhanced growth and morphogenesis when cultured in ≥21% O_2 with 10,000 µmol mol^{-1} CO_2 included compared to shoots cultured under lower O_2 levels. Essential oil compositions (i.e. monoterpene, piperitenone oxide from mint and aromatic phenol, thymol from thyme) were analyzed from CH_2Cl_2 extracts via gas chromatography from the shoot portion of plants grown at all O_2 levels. The highest levels of thymol were produced from thyme shoots cultured under 10% and 21% O_2 with 10,000 µmol mol^{-1} CO_2, and levels were considerably lower in shoots grown under either lower or higher O_2 levels. Higher levels of piperitenone oxide were obtained from mint cultures grown under ≥21% O_2 with 10,000 µmol mol^{-1} CO_2 compared to that obtained with lower O_2 levels.

Keywords CO_2 enrichment · Lamiaceae · *Thymus sativa* · *Mentha sp.* · Thymol

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Introduction

Oxygen is consumed by plants in respiration and generated in photosynthesis. For most C_3 plants, high levels of O_2 inhibit photosynthesis through enhanced photorespiration, while low O_2 levels may or may not have any effect on the rate of photosynthesis (D'Aoust and Canvin 1973; Sharkey and Vassey 1989). While numerous theories have been presented to explain these observations (D'Aoust and Canvin 1973; Sharkey and Vassey 1989), the influence of low O_2 levels on photosynthesis and growth is still unclear. O_2 levels directly affect such processes as respiration, ion uptake, stomatal function and general metabolic activity (Salisbury and Ross 1969), and they may influence plant growth and morphogenesis (Fukuyama et al. 1975; Imamura and Harada 1981; Lenz et al. 1983; Mirjalili and Linden 1995; Shimada et al. 1988; Tate and Payne 1991). O_2 and/or CO_2 concentrations in the tissue may control the differentiation of xylem and other vascular elements (Salisbury and Ross 1969). High O_2 levels were observed to reduce soybean (*Glycine max*) and safflower (*Carthamus tinctorius*) seed germination (Ohlrogge and Kernan 1982). In addition, the effects of O_2, temperature and moisture may act synergistically to depress germination (Ohlrogge and Kernan 1982). Reduced O_2 levels (2.5% or 5%) have been employed as a physiological stress in vitro to stimulate tobacco (*Nicotiana tabacum*) pollen embryogenesis (Imamura and Harada 1981). Food processors store fruits under high CO_2 and low O_2 levels in cold storage to extend the shelf-life (Salisbury and Ross 1969). These reduced O_2 levels are presumed to lower metabolic

activity in an in vitro heterotrophic environment (Tate and Payne 1991); however varying O_2 levels from 1% to 60% were observed to have no effect on the growth rates of either *Catharanthus roseus* or *Daucus carota* suspension cultures growth rates (Tate and Payne 1991). A combination of high temperature (37°–39°C), low O_2 level (5% O_2) and high CO_2 level (900 µmol mol^{-1} CO_2) aided in eliminating viruses from cultured *Prunun avium* shoot tips (Lenz et al. 1983). Low O_2 inhibits fungi and bacteria growth, thereby reducing contamination levels in plant tissue cultures (Shimada et al. 1988). Mirjalili and Linden (1995) reported that low O_2 levels of 10% promote taxol formation in *Taxus cuspidata* suspension cultures.

The influence of O_2 on the growth and morphogenesis of plant tissue cultures is little understood. In the investigation reported here, we sought to ascertain the influence of various O_2 levels employed with either ambient or ultra-high CO_2 levels on in vitro shoot growth, morphogenesis and secondary metabolism in two species of the mint family (Lamiaceae). Information obtained could aid researchers in effectively employing optimal physiological atmospheres in future biotechnological research.

Materials and methods

O_2 and CO_2 flow systems

The O_2 and CO_2 flow-through-testing chamber consists of a transparent polycarbonate Carb-X tote box and lid (Consolidated Plastics, Twinsburg, Ohio) [45 cm (width) × 65 cm (length) × 37.5 cm (depth); 94.5-l capacity] (Tisserat and Silman 2000). A silicone tape gasket [6.3 mm (width) × 112 cm (length) × 3.2 mm (depth)] (Furon, New Haven, Conn.) was attached to the lid. The box was modified by mounting three polypropylene spigots (Ark-Plas Products, Flippin, Ark.) attached to 0.45-µm air vents (Gelman Science, Ann Arbor, Mich.). The box and lid were clamped with 12 equally spaced stationary binding clips (50 mm long). CO_2, N_2 and O_2 rated 99.8% pure were provided by gas cylinders (BOC Gases, Edison, N.J.). CO_2 was mixed with an ambient air – i.e. 350 µmol mol^{-1} CO_2 – flow produced by an aquarium air pump (Whisper 2000, Carolina Biological Supply, Burlington, N.C.) with a flow meter (Cole Parmer Instrument, Niles, Ill.) to provide elevated CO_2 – i.e. 10,000 µmol mol^{-1} CO_2. O_2 levels were adjusted to 5%, 10%, 21%, 32% or 43% O_2 by mixing N_2 and O_2 gases with the aid of an O_2 sensor (Figaro USA, Wilmette, Ill.). The 10,000 µmol mol^{-1} CO_2 level was adjusted using a LIRA infrared gas analyzer, (model no. 3000, Mine Safety Appliances, Pittsburgh, Pa.). The CO_2 and air flows were added at 2,000 ml min^{-1} during the day photoperiod. Control cultures were given a stream of ambient air generated by the aquarium pump only. Gas applications were administered constantly during the day and night, unless noted otherwise.

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 8 g agar (Difco Laboratories, Detroit, Mich.). The pH of the medium was adjusted to 5.7 ± 0.1 with 0.1 *N* HCl or NaOH before the addition of agar. Following addition and subsequent melting of the agar, the medium was dispensed in 25-ml aliquots into 25×150-mm borosilicate glass culture tubes, which were then capped with translucent polypropylene closures (Sigma Chemical, St. Louis, Mo.). The medium was autoclaved for 15 min at 103 kPa and 121°C and then slanted at a 45° angle while cooling. Stocks of mint (*Mentha sp.* L.) and thyme (*Thymus vulgaris* L.) shoots were maintained on BM containing 3% sucrose under ambient air prior to testing with various O_2 levels. For the experiments, a single 3-cm-long shoot was planted per vessel.

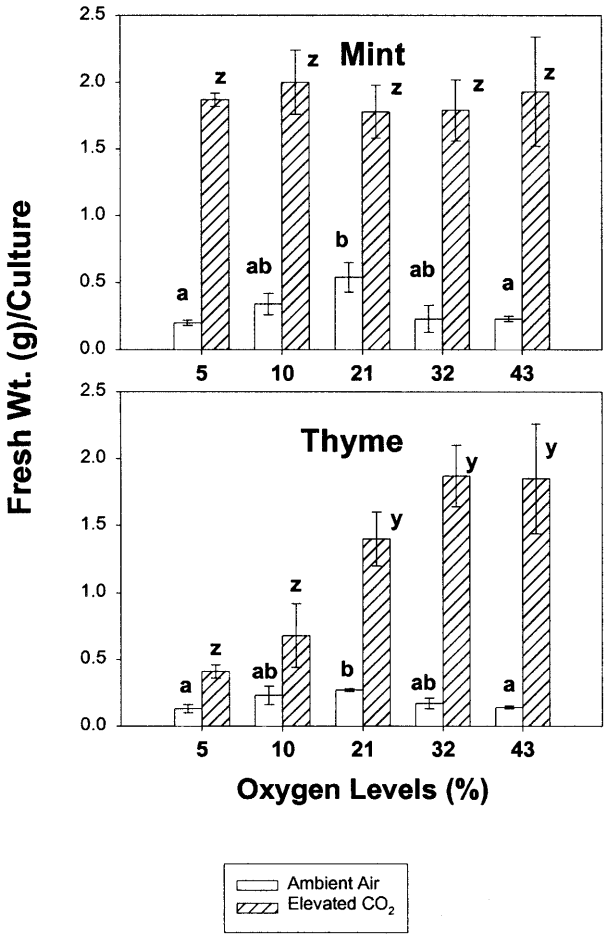
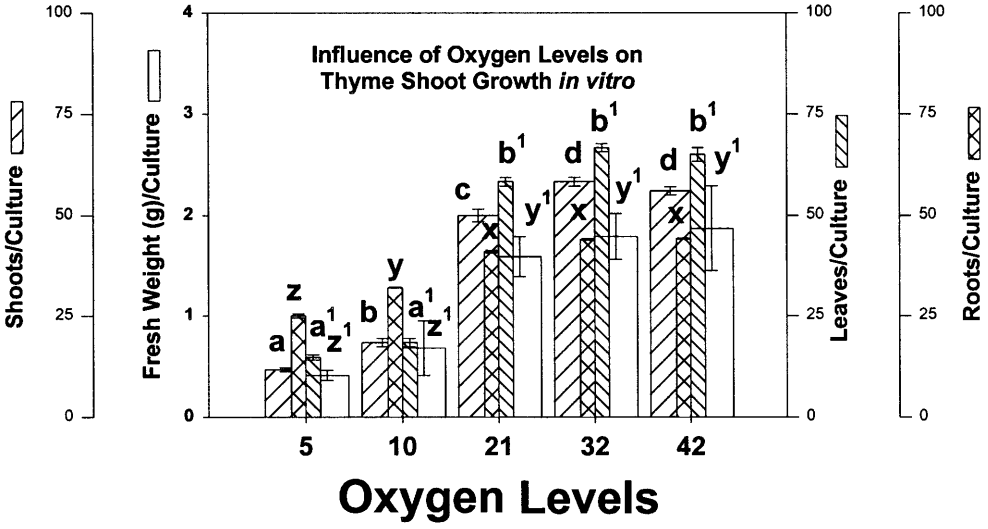


Fig. 1 Growth response from mint and thyme shoots grown under various O_2 levels with either 350 or 10,000 µmol mol^{-1} CO_2. Cultures were grown in 25×150-mm culture tubes for 8 weeks. Data were averaged for 20 replications per treatment. Experiments were repeated three times, and a single representation is presented. Mean separation is by Student-Newman-Keuls multiple range test (P<0.1). *Within* CO_2 levels, treatments with the *same letter* were not significantly different

Plant tissue culture experiments

To determine the influence of O_2 levels on growth and morphogenesis, we planted mint and thyme shoots on BM in culture tubes and placed the tubes within 94-l transparent containers under 5%, 10%, 21%, 32%, or 43% O_2 with either 350 or 10,000 µmol mol^{-1} CO_2. Subsequent experiments were conducted only in order to determine the influence of O_2 on growth, morphogenesis and secondary metabolite formation by growing mint and thyme shoots on BM under various O_2 levels containing 10,000 µmol mol^{-1} CO_2. During the day, 10,000 µmol mol^{-1} CO_2 was applied to all O_2 treatments, while at night, only ambient CO_2 levels (350 µmol mol^{-1} CO_2) were given.



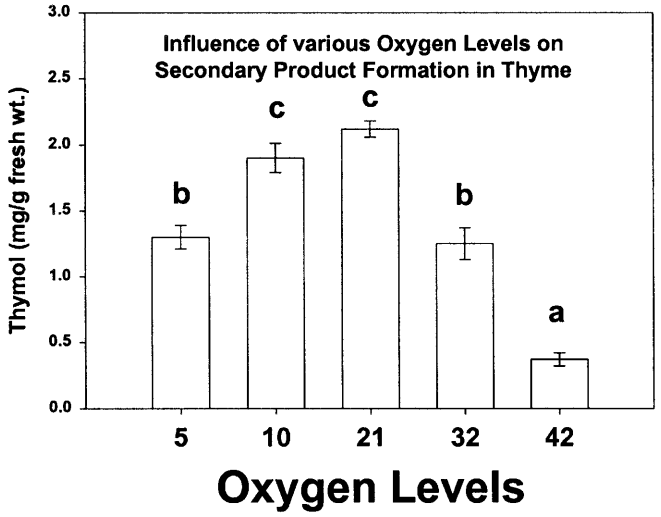


Fig. 2 Growth, morphogenesis and secondary metabolite responses from thyme shoots under various O_2 levels with 10,000 µmol mol^{-1} CO_2. Cultures were grown in 25×150-mm culture tubes for 8 weeks. Data were averaged for 20 replications per treatment. Experiments were repeated three times, and a single representation is presented. Mean separation is by Student-Newman-Keuls multiple range test (P<0.1). Growth, morphogenesis or secondary metabolite responses from various O_2 treatments with the *same letter* were not significantly different

Cultures were grown at 25°±1°C under a 16/8-h (light/dark) photoperiod. Light was supplied by a combination of cool-white fluorescent tubes and metal-halide and incandescent lamps at a total photosynthetic photon flux density of 180 µmol m^{-2} s^{-1} at the vessel periphery.

Twenty replicates were planted originally, and experiments were repeated at least three times. After 8 weeks of incubation, data on culture fresh weight, leaf number, root number and shoot number were recorded and analyzed with the Student-Newman-Keuls multiple range test.

Extract preparation

Whole shoot tissues were ground in a coffee grinder to a fine powder to facilitate extraction. Secondary metabolites were extracted from 1- to 4-g samples of shoot tissues in CH_2Cl_2 using a Soxhlet apparatus. The extracts were concentrated by rotovaporation at low temperatures to prevent the loss of volatiles.

Chromatography and spectroscopy

Routine gas chromatography was performed using a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a HP 3396 integrator. Mass spectra were produced by a HP 5972A Series Mass selective Detector. The columns used were fused silica HP-5MS capillaries [0.25-µm film thickness, 30 m (length) × 0.25 mm (internal diameter)].

Results and discussion

Vegetative growth and morphogenesis responses to O_2

Our results show that neither low (5% and 10%) nor high O_2 (32% or 43%) levels were phytotoxic to mint or thyme growth. These results confirm those of Tate and Payne (1991) with *Catharanthus roseus* and *Daucus carota* suspension culture growth. Differences in the growth responses of mint and thyme cultures were obtained employing various O_2 levels with ambient CO_2 levels (350 µmol mol^{-1} CO_2) (Fig. 1). An O_2 level of 21% with ambient CO_2 gave the highest fresh weights relative to growth obtained from cultures grown under a regime of either higher and lower O_2 levels with ambient CO_2 (Fig. 1). No significant difference in growth occurred at the 10%, 21% or 32% O_2 levels with ambient

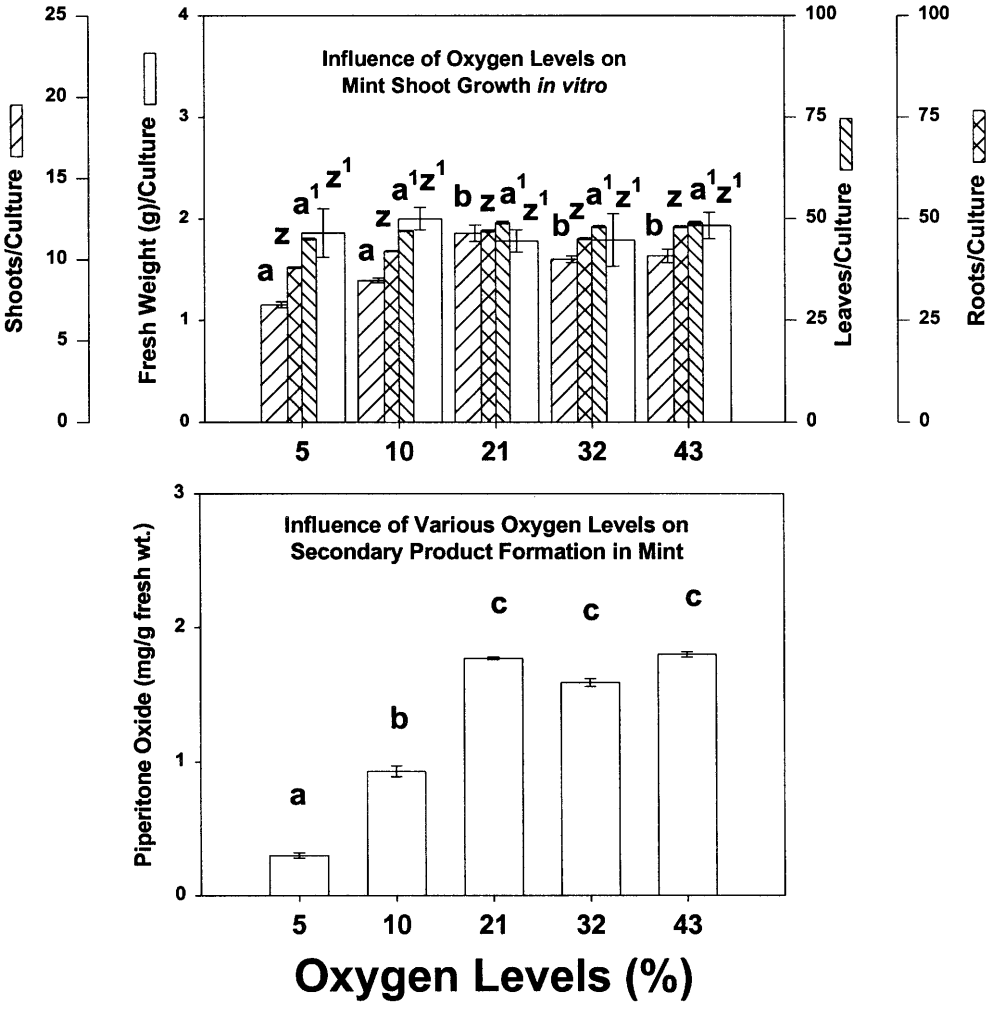


Fig. 3 Growth, morphogenesis and secondary metabolite responses of mint shoots under various O_2 levels with 10,000 µmol mol^{-1} CO_2. Cultures were grown in 25×150-mm culture tubes for 8 weeks. Data were averaged for 20 replications per treatment. Experiments were repeated three times, and a single representation is presented. Mean separation is by Student-Newman-Keuls multiple range test (P<0.1). Growth, morphogenesis or secondary metabolite responses from various O_2 treatments with the *same letter* were not significantly different

CO_2 for either thyme or mint shoots. Substantially higher fresh weights invariably occurred when either thyme or mint shoots were grown under various O_2 concentrations with elevated CO_2 (i.e. 10,000 µmol mol^{-1} CO_2) than with ambient CO_2. Growth rates were two to eight fold higher for thyme or mint cultures when grown under various O_2 levels containing 10,000 µmol mol^{-1} CO_2 as compared to cultures grown under 350 µmol mol^{-1} CO_2. In subsequent O_2 tests, 10,000 µmol mol^{-1} CO_2 was included in all treatments in order to achieve better growth rates, which also provided the sufficient biomass necessary for biochemical analysis. It should also be noted that growth rate trends may be somewhat dissimilar when cultures grown under O_2 levels with ambient CO_2 are compared to cultures grown under elevated CO_2 levels. A synergistic CO_2 effect apparently occurs with some O_2 levels. For example, with mint plants a significant difference in growth occurred when 5% O_2 versus 21% O_2 was used with the ambient level of CO_2, while no significant difference in growth occurred with these same O_2 levels in combination with the elevated CO_2 level. Thyme growth and morphogenesis responses were greater when the plants were cultured under the higher O_2 levels (≥21%) with elevated CO_2 than when cultured under lower O_2 levels (5% or 10%) (Fig. 2). Little difference in growth and morphogenesis occurred in mint cultures regardless of the concentration of O_2 tested with elevated CO_2 (Fig. 3). High growth rates and yields have been obtained with several tissue-cultured plants when ultra-high CO_2 environments (≥10,000 µmol mol^{-1} CO_2) were employed (Seko and Nishimura 1996; Solárová et al. 1996; Tisserat et al. 1997; Tisserat and Silman 2000). In the present study, regardless of the concentration of O_2 tested, elevated CO_2 (10,000 µmol mol^{-1} CO_2) improved growth rates several fold over that obtained with the same O_2 levels with ambient CO_2. Similarly, Mirjalili and Linden (1995) employed ultra-high CO_2 (5,000 µmol mol^{-1} CO_2) with 10% O_2 to obtain optimum *Taxus cuspidata* suspension growth.

Secondary metabolism in vitro in response to O_2

Although numerous fragrant essential oils occurred in the mint species studied, piperitenone oxide, a monoterpene, was the dominant one. Terpenes are produced in the isoprenoid biosynthesis pathway; their pathways consist of the mevalonic acid and “Rohmer” non-mevalonic

acid pathways. Piperitenone oxide levels varied considerably depending on the O_2 level employed: they were dramatically lower in shoots when the latter were grown in 5% and 10% O_2 compared to shoots grown in ≥21% O_2 (Fig. 3). Piperitenone oxide levels within mint cultures grown under 5% O_2 were 4.9-fold lower than the amount of piperitenone oxide obtained from mint cultures grown under 21% O_2. Thymol is the dominant essential oil in thyme and is an aromatic phenolic produced in the phenylpropanoid biosynthesis pathway. The highest levels of thymol were found in cultures grown under 10% and 21% O_2, and they progressively decreased in amount under either higher or lower O_2 levels (Fig. 2). For example, the thymol level in thyme cultured under 5% O_2 was 1.3 mg thymol/g fresh weight compared to 2.12 mg thymol/g fresh weight under 21% O_2.

Both mint and thyme exhibited significant differences in secondary metabolites that were dependent on the O_2 level administered and the plant tested. Mirjalili and Linden (1995) employed 10% O_2 with 5,000 µmol mol^{-1} CO_2 to attain a high level of taxol, a diterpene, from *T. cuspidata* suspension cultures; however, the influence of other O_2 levels on secondary products was not tested. In our study, monoterpene piperitenone oxide levels in mint were considerably lower in cultures incubated in 10% O_2 than in those cultured in 21% O_2, while no difference in aromatic phenolic thymol levels occurred in thyme plants grown in either 10% or 21% O_2. It has been suggested that suboptimal O_2 levels reduce the level of secondary metabolites from cultured cells (Tate and Payne 1991). We confirmed this premise in our study, in which dramatic reductions in both thymol and piperitenone oxide levels from thyme and mint plants, respectively, occurred under culture conditions with 5% O_2 compared to those obtained from cultures in 21% O_2. Tate and Payne (1991) found that *C. roseus* (Apocynaceae) suspension cultures exhibited lower growth rates when cultured below 10% O_2 or above 50% O_2; when cultured above 70% O_2, growth was dramatically suppressed. We also observed this trend to some degree in our study with species in the mint family Lamiaceae. Low levels of thymol in thyme were observed at 5% and 43% O_2 compared that occurring at 21% O_2.

Our results clearly show that O_2 levels can influence growth, morphogenesis and secondary metabolite production. However, there was no particular advantage in employing high or low O_2 levels over employing ambient O_2 levels with these mint species. Although of the same family, mint and thyme contain different dominant essential oils produced from different metabolic pathways, and they did not respond in the same manner when treated with the same O_2 atmospheric environments. Similarly, growth responses from mint and thyme may be decidedly different when given the same O_2 atmospheric environments. These observations suggest that O_2 atmospheric environments must be tested empirically, employing broad ranges to determine their influence on a plant's growth and secondary metabolism. Prior atmospheric studies employing different plant species can only provide generalizations as to the influence of O_2 and CO_2 on plant growth and secondary metabolism.

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