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EFFECTS OF ATMOSPHERIC CO_2 ENRICHMENT ON THE GROWTH AND DEVELOPMENT OF *HYMENOCALLIS LITTORALIS* (AMARYLLIDACEAE) AND THE CONCENTRATIONS OF SEVERAL ANTINEOPLASTIC AND ANTIVIRAL CONSTITUENTS OF ITS BULBS[1]

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Two 2-yr crops of tropical spider lily (*Hymenocallis littoralis*) plants were grown in field soil in clear-plastic-wall open-top enclosures in the Sonoran Desert environment of central Arizona. Half of the plants were exposed to ambient air of 400 ppm atmospheric CO_2 concentration and half of them were exposed to air of 700 ppm CO_2. This 75% increase in the air's CO_2 content resulted in a 48% increase in aboveground plant biomass and a 56% increase in belowground (bulb) biomass. It also increased the concentrations of five bulb constituents that have been demonstrated to possess anticancer and antiviral activities. Mean percentage increases in these concentrations were 6% for a two-constituent (1:1) mixture of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine, 8% for pancratistatin, 8% for trans-dihydronarciclasine, and 28% for narciclasine, for a mean active ingredient percentage concentration increase of 12%. Combined with the 56% increase in bulb biomass, these percentage concentration increases resulted in a mean active ingredient increase of 75% for the 75% increase in the air's CO_2 concentration used in our experiments.

Key words: Amaryllidaceae; antineoplastic agents; antiviral agents; cancer; carbon dioxide; global change; *Hymenocallis littoralis*; pancratistatin.

Out of concern and curiosity about the ongoing rise in the air's carbon dioxide concentration, many experiments have been conducted to determine the effects of atmospheric CO_2 enrichment on plant growth and development. In the vast majority of these studies, vegetative productivity has been significantly enhanced by increases in the air's CO_2 content (Kimball, 1983; Cure and Acock, 1986; Poorter, 1993; Idso and Idso, 1994; Koch and Mooney, 1996; Raschi et al., 1997), as has plant water use efficiency (Rogers et al., 1983; Valle et al., 1985; Fernandez et al., 1998). In addition, a number of physiological processes have been altered in ways that enhance plant performance in both natural and agroecosystems, including photosynthetic acclimation (Gesch et al., 1998; Rey and Jarvis, 1998), dark respiration (Griffin, Ball, and Strain, 1996), light use efficiency (Gifford, 1992), nitrogen use efficiency (Drake, Gonzales-Meler, and Long, 1997), osmoregulation (Picon, Ferhi, and Guehl, 1997), and photorespiration (Long, 1991).

One important subject that has been largely neglected is the effect of elevated levels of atmospheric CO_2 on the growth of medicinal plants and their production of secondary metabolites of therapeutic value. Although several studies have investigated the effects of atmospheric CO_2 enrichment on the production of various carbon-based compounds (Penuelas and Estiarte, 1998) and antioxidants (Badiani et al., 1993, 1996, 1997; Rao, Hale, and Ormrod, 1995; Schwanz et al., 1996), and some have evaluated the effects of elevated CO_2 on plant vitamin and mineral contents (Madsen, 1975; Knecht and O'Leary, 1983; Tajiri, 1985; Penuelas et al., 1997), few have considered the consequences of atmospheric CO_2 enrichment for specific plant compounds of direct medicinal value. Hence, we decided to conduct such a study on the tropical spider lily *Hymenocallis littoralis* (Jacq.) Salisb., the bulbs of which contain several substances that show promise of becoming important agents in the battle against a number of human cancers and viral infections.

The tropical spider lily has been known since ancient times to possess antitumor activity. The first chemical investigation of this plant was conducted by Gorter (1920a, b), leading ultimately to the isolation of lycorine (Pettit et al., 1986), which was subsequently proven to have both antineoplastic and antiviral properties (Renard-

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Noiake et al., 1989). About the same time it was also found that the bulbs of *H. littoralis* (originally identified as *Pancratium littorale*) contained a new phenanthridone biosynthetic product that its discoverers named pancratistatin (Pettit et al., 1986).

In initial testing of pancratistatin, it was found to be very effective in vivo against murine P-388 lymphocytic leukemia and M-5076 ovary sarcoma (Pettit et al., 1986). In further testing it was found to exhibit cytotoxicity against the U.S. National Cancer Institute's panel of 60 human cancer cell lines, demonstrating greatest effectiveness against melanoma subpanel lines, followed by certain brain, colon, lung, and renal cancer lines (Pettit et al., 1993). In addition, pancratistatin was found to exhibit strong RNA antiviral activity against Japanese encephalitis and yellow, dengue, Punta Tora, and Rift Valley fevers (Gabrielsen et al., 1992a, b). More recently, work has progressed on the extraction and study of several related cell-growth inhibitory isocarbostyrils: narciclasine, trans-dihydronarciclasine, 7-deoxynarciclasine, and 7-deoxy-*trans*-dihydronarciclasine (Pettit et al., 1995a, b). It is the effect of atmospheric CO_2 enrichment on these secondary metabolites, as well as pancratistatin, that we investigate in this paper.

MATERIALS AND METHODS

Plant material—Bulbs of *Hymenocallis littoralis,* originally collected in Hawaii, were multiplied by tissue culture (Backhaus et al., 1992) and successfully cultivated in the Sonoran Desert environment of the American Southwest in experimental plots established at Tempe, Arizona.

Field studies—Two experiments designed to investigate the effects of atmospheric CO_2 enrichment on the growth and development of the plants in a natural field soil—a fine-loamy, mixed (calcareous), hyperthermic Anthropic Torrifluvent—were conducted at Phoenix, Arizona, in four clear-plastic-wall open-top enclosures. The air in two of these enclosures was maintained at the 24-h mean ambient *urban* CO_2 concentration (Idso, Idso, and Balling, 1998; Idso et al., 1998) of ~400 parts per million by volume (ppm), while the air in the other two enclosures was maintained at ~700 ppm by means of the CO_2-sensing, regulation and supply systems described by Kimball et al. (1983, 1992) and Idso, Kimball, and Clawson (1984).

Within each of these enclosures, we planted ten "mother" bulbs of known initial fresh mass (separated from each other by a distance of ~1 m) on 11 February 1993 and 11 others on 26 April 1995. Over the following two 2-yr-long growing seasons (two years being chosen, instead of one, to allow for the production of as much new bulb material as possible), the plants were left undisturbed, except for regular supplements of irrigation water needed to prevent them from drying out and dying in the normally arid environment. Then, in mid-October of 1994 and 1996, the time of year when the pancratistatin concentration of *H. littoralis* bulbs typically reaches its highest level in central Arizona (Pettit et al., 1995a), we harvested and weighed all above- and belowground plant material, including all foliage and all mother and "daughter" bulbs.

Chemical analysis of bulbs—All roots and extra stem materials were trimmed from the bulk-harvested mother and daughter bulbs from each field enclosure, immediately after which they were weighed, finely chopped, and immediately analyzed for the targeted therapeutic substances, as described in detail by Pettit et al. (1993, 1995a). Briefly, after separately pooling all mother bulbs and all daughter bulbs obtained from each chamber, and following extraction with a 1:1 dichloromethane-methanol solution and removal of solvent from the filtrate, the residue was dissolved in 500 mL of water and partitioned with *n*-butanol (1×80 mL, 2×40 mL). The alcohol extracts were combined and solvent removed in vacuo. Part of the residue dissolved in methanol (5 mL), leaving an off-white solid. The solid was collected, and 60 mL of acetone was added to the filtrate. A cloudy white precipitate formed, and the suspension was placed in a refrigerator overnight to ensure maximum precipitation. The solid component was collected and solvent removed from the filtrate to yield a brown residue, which was dissolved in methanol (~2 mL) and chromatographed on a column of Sephadex LH-20 (Pharmacia, particle size 25–100 μm) using methanol as the eluent.

Thin layer chromatography (TLC) was performed on 0.25-mm-thick GHLF uniplates, using dichloromethane-methanol (9:1) as the mobile phase and iodine for development. The identity of each compound was established by comparison with standards on TLC and 300-Mhz proton-nuclear magnetic resonance (^{1}H-NMR) spectra, which were recorded with a Varian-Gemini 300-Mhz spectrometer using deutierated (d_6) dimethyl sulfoxide (d_6-DMSO) with the residual DMSO peak as the internal standard.

Fractions were monitored by TLC, and those containing components at R_f 0.12 were combined, as were those containing components at R_f 0.16. The solutions were concentrated via rotary evaporator, dried, and weighed. The relative amounts of each compound in the two mixtures were determined by the comparison of the integration of known NMR peaks. The R_f 0.16 component corresponded to a mixture of pancratistatin, narciclasine, and trans-dihydronarciclasine, while the two-constituent mixture (1:1) at R_f 0.12 contained 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine.

RESULTS

Fresh mass yields—At the end of the first 2-yr experiment, the total (pooled) aboveground fresh mass of spider lily tissue—composed of leaves, flower stalks, and flowers—produced by the CO_2-enriched plants was determined to be 46% greater than that produced by the plants growing in ambient air. At the end of the second 2-yr experiment, the total aboveground fresh mass production in the CO_2-enriched plants was found to be 49% greater.

Bulb production results were explored in more detail, particularly in the second of our two 2-yr experiments. In the first experiment, we harvested and weighed (as two pooled groups per CO_2 treatment) the original mother bulbs and the new daughter bulbs they had produced; while in the second experiment we determined these results for each of the two open-top chambers (replications) comprising each CO_2 treatment, obtaining the results presented in Table 1.

Based on the primary mean results of Table 1 for experiment 2, and the similar directly determined (pooled) results for experiment 1, we calculated the grams of mother bulb, daughter bulb, and total bulb production per gram of original mother bulb tissue for both experiments, obtaining the results presented in Table 2, where we have also included the percent change in these three bulb production categories due to the 300 ppm difference in atmospheric CO_2 concentration maintained between the chambers of the two CO_2 treatments.

In viewing the results of Table 2, it can be seen that the mother bulbs of the first experiment, which had an average initial fresh mass of 354 g, lost almost half their original mass over the two growing seasons of the experiment in both CO_2 treatments. In the second experiment, however, where the average initial fresh mass of

TABLE 1. Mean original and final (harvested) fresh masses of spider lily bulbs in the two replications of the two CO_2 treatments of experiment 2, together with the treatment means and standard errors, in units of grams per bulb.

Material	400 ppm				700 ppm			
	Rep. 1	Rep. 2	Mean	SE	Rep. 1	Rep. 2	Mean	SE
Original mother bulbs	84.78	84.69	84.74	.04	84.75	84.69	84.72	.03
Harvested mother bulbs	167.7	197.5	182.6	14.9	251.8	279.3	265.5	13.8
Harvested daughter bulbs	32.17	60.63	46.4	14.2	79.26	141.2	110.2	31.0
All harvested bulbs	199.8	258.1	229.0	29.1	331.0	420.6	375.8	44.8

each mother bulb was only 85 g, the bulbs exposed to ambient air more than doubled their original weight, while those exposed to the CO_2-enriched air more than tripled what they weighed originally.

Daughter bulb production was positive in all treatments in both of the 2-yr experiments. In the first study, each original fresh mass gram of mother bulb material produced 0.235 g of fresh mass daughter bulb material in the ambient CO_2 enclosures and 0.676 g of fresh mass daughter bulb material in the CO_2-enriched enclosures, resulting in a CO_2-induced fresh mass enhancement of 188%. In the second study, the corresponding numbers were 0.548 g, 1.302 g, and 138%.

In terms of total mother and daughter bulb production, the ambient treatment plants of the first 2-yr study actually lost mass. For each original gram of mother bulb material, only 0.80 g of combined mother and daughter bulb material were present at the end of the experiment. In the CO_2-enriched treatment, on the other hand, net growth was positive; and for each original gram of mother bulb material, 1.18 g of combined mother and daughter bulb material were present at the end of the study, representing a 48% improvement over net total growth under ambient CO_2 conditions.

In the second 2-yr study, growth was more robust, with each original gram of mother bulb material resulting in 2.70 g of combined mother and daughter bulb material in ambient air and 4.44 g of such material in CO_2-enriched air, for an improvement of 64% in net total growth under CO_2-enriched conditions. Hence, for the entire 4 yr of growth in the field, the 75% increase in the air's CO_2 content resulted in an average 56% increase in total fresh mass bulb production.

Chemical yields—Chemical constituent production results are presented in Table 3. In the first 2-yr experiment, where narciclasine was not measured, the concentration of pancratistatin in the spider lily bulb tissue was enhanced by ~19% in the plants growing in the CO_2-enriched air, while the concentration of the two-constituent (1:1) mixture of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine was enhanced by 14%. In the second experiment, however, the concentrations of all three of these substances were decreased by ~2% in the plants growing in the CO_2-enriched air. The concentration of trans-dihydronarciclasine, on the other hand, was increased by 8%, while that of narciclasine was increased by 28%.

TABLE 2. Fresh masses of bulbs harvested at the end of the two 2-yr field experiments in grams per gram of original mother bulb fresh mass, plus the percentage changes in bulb fresh mass due to increasing the air's CO_2 concentration from 400 to 700 ppm.

Material	Experiment 1			Experiment 2		
	400 ppm	700 ppm	% change	400 ppm	700 ppm	% change
Mother bulbs	0.566	0.507	−10%	2.155	3.134	+45%
Daughter bulbs	0.235	0.676	+188%	0.548	1.301	+137%
All bulbs	0.801	1.183	+48%	2.702	4.436	+64%

DISCUSSION

A 400–700 ppm (75%) increase in atmospheric CO_2 concentration led to a 48% increase in aboveground growth and a 56% increase in belowground (bulb) growth of *Hymenocallis littoralis.* These responses are similar to those observed in other crop plants in terms of total productivity enhancement and differences in above- and belowground growth stimulation. For root crops such as carrot and radish, for example, the biomass of the primary root storage organ is typically enhanced slightly more than the aboveground biomass (Idso, Kimball, and Mauney, 1988). In contrast, plants such as cotton and soybean, which do not possess root storage organs, generally experience a more equal above- and belowground growth stimulation (Idso, Kimball, and Mauney, 1988), as we have also observed in sour orange trees (Idso and Kimball, 1991).

CO_2-induced changes in bulb chemical constituent concentrations were smaller and more variable. In the first 2-yr experiment, the 75% increase in the air's CO_2 content produced a 19% increase in the concentration of pancratistatin; but in the second experiment, CO_2 enrichment actually reduced the concentration of pancratistatin by ~2%. Similarly, the CO_2-incuded increase in the concentration of the two-constituent (1:1) mixture of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine was

TABLE 3. Chemical constituents in milligrams per kilogram of fresh-mass bulb material removed from the ground at the conclusions of experiments 1 and 2, plus the percentage changes in bulb chemical constituent concentrations due to increasing the air's CO_2 concentration from 400 to 700 ppm.

Code[a]	Mother bulbs			Daughter bulbs			All bulbs
	400	700	% change	400	700	% change	% change
1A.	7.4	11.0	+49	11.5	11.0	−4	+19
1B.	6.0	7.0	+17	13.0	14.5	+12	+14
2A.	25.2	24.7	−2	34.5	33.4	−3	−2
2B.	60.2	64.1	+6	130	116	−11	−2
2C.	6.3	8.2	+30	12.6	11.0	−13	+8
2D.	6.3	8.2	+30	8.8	11.0	+33	+28

[a] 1 = experiment 1; 2 = experiment 2; A = pancratistatin; B = one-to-one mixture of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine; C = trans-dihydronarciclasine; D = narciclasine.

14% in the first experiment, but it too was reduced by 2% in the second. Concentrations of narciclasine and trans-dihydronarciclasine in experiment 2, however, were increased by 28 and 8%, respectively.

Averaging the results for the chemical constituents that were evaluated in both experiments, the 400–700 ppm (75%) increase in atmospheric CO_2 concentration imposed in our study resulted in a 6% increase in the concentration of the two-constituent (1:1) mixture of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine, an 8% increase in the concentration of pancratistatin, an 8% increase in the concentration of trans-dihydronarciclasine, and a 28% increase in the concentration of narciclasine.

In summary, it would appear that atmospheric CO_2 enrichment not only significantly enhances biomass production in *Hymenocallis littoralis,* but that it also slightly increases the concentrations of several therapeutic compounds produced in its bulbs. Multiplying the mean CO_2-induced bulb biomass enhancement factor (1.56) by the mean CO_2-induced concentration enhancement factor of the several therapeutic substances (1.12) suggests that a 400–700 ppm (75%) increase in the air's CO_2 content would boost the total production of these several protomedicines by approximately the same amount, i.e., 75%.

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