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Recent and projected increases in atmospheric carbon dioxide and the potential impacts on growth and alkaloid production in wild poppy (*Papaver setigerum* DC.)

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Abstract In the current study, we quantified changes in the growth and alkaloid content of wild poppy, (*Papaver setigerum*) as a function of recent and projected changes in global atmospheric carbon dioxide concentration, [CO_2]. The experimental [CO_2] values (300, 400, 500 and 600μmol mol^{-1}) correspond roughly to the concentrations that existed during the middle of the twentieth century, the current concentration, and near and long-term projections for the current century (2050 and 2090), respectively. Additional carbon dioxide resulted in significant increases in leaf area and above ground biomass for *P. setigerum* at all [CO_2] relative to the 300μmol mol^{-1} baseline. Reproductively, increasing [CO_2] from 300 to 600μmol mol^{-1} increased the number of capsules, capsule weight and latex production by 3.6, 3.0 and 3.7×, respectively, on a per plant basis. Quantification of secondary compounds (i.e. those not involved in primary metabolism) included the alkaloids morphine, codeine, papaverine and noscapine. The amount of all alkaloids increased significantly on a per plant basis, with the greatest relative increase occurring with recent increases in atmospheric carbon dioxide (e.g. from 300 to 400μmol mol^{-1}). Overall, these data suggest that as atmospheric [CO_2] continues to increase, significant effects on the production of secondary plant compounds of pharmacological interest (i.e. opiates) could be expected.

1 Introduction

Botanists have long recognized that plants are able to manufacture a range of secondary chemicals that function as toxicological barriers to viral diseases, fungal pathogens and herbivory. However, many of these secondary compounds are also acknowledged as having

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a significant impact on human physiology. The use of selected plant species and associated chemistries as global remedies for human ailments is ubiquitous throughout human culture (e.g. Schultes and Reis 1995). Globally the World Health Organization (WHO) estimates that more than 3.5 billion people in developing countries rely on plant derived sources of medicine as part of their primary health care (WHO 2002). Even in developed countries, a significant portion of prescriptions written (15–20%) contain plant extracts or active principles prepared from plants (e.g. codeine, see Farnsworth et al. 1985).

Medicinal plants, as with all plant species, rely on four abiotic resources: water, light, nutrients and carbon dioxide in order to complete their life cycle. Consequently, any change in the availability of these resources may, in turn, alter their growth and chemistry. Currently, as a result of anthropogenic activities, primarily fossil fuel burning and deforestation, the amount of carbon dioxide in the atmosphere has risen from 312 to 380μmol mol^{-1}, (~21%) since 1960 (Houghton et al. 2001). The current annual rate of CO_2 increase (~0.5%) from these sources is expected to continue with concentrations projected to exceed 600μmol mol^{-1} by the end of the current century (Schimel et al. 1996).

Surprisingly, in spite of the importance of plant derived secondary metabolites in human health, only a few studies have quantified how the ongoing increase in atmospheric carbon dioxide is likely to affect their production. Exceptions include an increase in the antidepressants, hypericin and pseudohypericin in St. Johns wort (*Hypericum perforatum* L.) and the cardiac glycoside, digoxin, in wooly foxglove (*Digitalis lanata* Ehrh.) at 1,000μmol mol^{-1} relative to current ambient [CO_2] (Stuhlfauth and Fock 1990; Zobayed and Saxena 2004). Recent work with jimson weed (*Datura stramonium*) has also indicated that rising [CO_2] could increase the production or concentration of the anticholinergics atropine and scopolamine (Ziska et al. 2005).

Among medicinal plants, the therapeutic uses of opiate alkaloids from poppy (*Papaver* spp.) have long been recognized (e.g., Sumerians at the end of the third millennium B.C. referred to poppy as hul gil or "joy plant"). Among the Papaveraceae family, the genus *Papaver* comprises about 110 species of both annual and perennial herbs; however, only *Papaver somniferum* and *Papaver setigerum* are known to produce opiates, with the concentration of these opiates varying considerably between species (e.g. Garnock-Jones and Scholes 1990; La Valva et al. 1985). However, almost all commercial poppy production is by *P. somniferum* since the small plant and capsule size of *P. setigerum* make it unsuitable for large scale commercial operations. The overall goal of the current investigation was to evaluate the growth and production of opiates for a broad range of recent and projected atmospheric carbon dioxide concentrations using wild poppy (*P. setigerum*) as a surrogate for *P. somniferum*.

2 Materials and methods

Experimental conditions At present, no methodological system exists that is able to provide subambient [CO_2] to plants under field conditions 24h a day (see Mayeux et al. 1993); consequently, the study was conducted using controlled environment chambers (EGC Corporation, Chagrin Falls, OH, USA) at Beltsville, MD, USA with a given chamber set at one of four [CO_2] setpoints (300, 400, 500 and 600μmol mol^{-1}) for 24h day^{-1}. Actual average 24h [CO_2] values (±SE) were 299 ± 15.2, 389 ± 12.1, 504 ± 18, 589 ± 21μmol mol^{-1}. The concentration used approximated atmospheric [CO_2] during the middle of the twentieth century, the current ambient, and that projected for the years 2050 and 2090 (Schimel et al. 1996). For all chambers, day/night temperature was 25/20°C, with an average daily (24h)

value of 22.9°C. Daytime photosynthetically active radiation (PAR, between 400–700nm at 14h, 500μmol m^2 s^{-1}) was supplied by a mixture of high-pressure sodium and metal halide lamps. The [CO_2] of the air within each chamber was controlled by adding either CO_2 or CO_2 free air to maintain the set concentration. Injection of CO_2 and CO_2-free air was controlled by a TC-2 controller using input from an absolute infrared gas analyzer (WMA-2, PP Systems, Haverhill, MA, USA).

Growth and biomass sampling Some Floras treat *P. somniferum* and *P. setigerum* as separate species based on morphological distinctions and chromosome numbers (*P. setigerum* $2n = 44$; *P. somniferum* $2n = 22$); others consider *P. setigerum* a sub-species of *P. somniferum* (see Malik et al. 1979; La Valva et al. 1985; Garnock-Jones and Scholes 1990 for a discussion). In the current experiment, seeds of wild poppy (*P. setigerum* D.C.) were obtained by request from the Institut fur Pflanzengenetik und Kulturpflanzenforschung at Gatersleben, Germany. Seeds were sown by hand in 2.6L pots filled with a 4:1:1 mixture of sphagnum, perlite and vermiculite to provide proper drainage and grown in one of the aforementioned CO_2 concentrations. Following emergence, plants were thinned to one per pot. For each CO_2 treatment, pots were watered to the drip point daily with a complete nutrient solution (Robinson 1984). Floral initiation occurred at approximately 70days after sowing and did not vary as a function of [CO_2] treatment. Scoring of immature pods began about 2weeks after the loss of floral petals. Scoring was done using a razor blade with two to three, 1mm deep incisions for approximately eight to ten capsules per plant for each [CO_2] treatment. For each capsule, latex was collected over a 24h period on aluminum foil, allowed to air dry and then weighed. At maturity (approximately 90–100days after sowing), final capsule number was determined and above ground vegetative biomass was separated into capsules, peduncles, stems and leaves for 10–12 plants. Leaf area was determined for a subset of five leaves per plant with the allometric relationship between leaf area and leaf weight ($r^2 > 0.91$) used to estimate leaf area from leaf dry weight for individual plants. Dry weight of plant organs was determined following drying at 65°C for a minimum of 48h or until dry mass was constant. Latex per plant was determined based on the amount of latex collected per capsule times the number of capsules.

Opiate analysis Opium standards were obtained from the reference collection of the Drug Enforcement Administration Special Testing and Research Laboratory (Dulles, VA, USA). All capillary electrophoresis (CE) reagents and run buffer solutions were obtained from Microsolv™ Technology (Eatontown, NJ, USA). High performance liquid chromatography grade methanol was obtained from Burdick and Jackson (Muskegon, MI, USA). High purity, deionized water was obtained from a Millipore Milli-Q-Gradient A10 water system (Bedford, MA, USA). An internal standard stock solution of tetracaine hydrochloride was prepared by weighing 25mg into 100mL volumetric flask and diluted to volume with 1:11 mixture of methanol and 3.75mM phosphate buffer (pH 3.2). To obtain the tetracaine internal standard working solution, 6mL of the tetracaine HCl internal standard stock solution was diluted to volume with 3.75mM phosphate buffer (pH 3.2) in a 50mL volumetric flask. Appropriate amounts of morphine, codeine, thebaine, noscapine and papaverine base standards were weighed into a 100mL volumetric flask in order to obtain an approximate final concentration of 0.025mg mL^{-1} for each compound. Ten milliliter of the internal standard stock solution was pipetted into the above mentioned volumetric flask and diluted to volume with 1:11 mixture of methanol and 3.75mM phosphate buffer. Approximately 500μL of the solution was filtered using 0.45μm regenerated cellulose Titan filter and transferred to a 1.0mL polypropylene CE injection vial.

Appropriate amounts of plant derived latex were weighed into a volumetric flask in order to obtain a concentration of morphine similar to that of the standard. The flask was filled to half volume with methanol and sonicated for 30min at 55°C allowing the complete extraction of alkaloids. The flask was then allowed to cool, and diluted to volume with 3.75mM phosphate buffer (pH 3.2). Four hundred microliter of the above solution was added to 2.0mL of the internal standard working solution. Approximately 500μL of the above solution was filtered using a 0.45μm regenerated cellulose Titan filter and transferred to a 1.0mL polypropylene CE injection vial. An Agilent Model HP^{3D}CE capillary electrophoresis system equipped with a diode array detector (Waldbronn, Germany) was used for alkaloid quantification as described by Lurie et al. (2003). All experiments were carried out with fused silica 32cm (23.5cm to detector window) × 50μm I.D pre-made capillaries obtained from Agilent Technologies (Part No: G1600-63211).

Design, data analysis Given the limited number of chambers, and because pots do not represent valid replications, a randomized complete block design was utilized with runs over time as replications (blocks). Each of two chambers was assigned one of the four CO_2 treatments with all CO_2 treatments occurring in each chamber. At the end of a given run, CO_2 treatments were reassigned to another chamber and the entire experiment was repeated. PAR, humidity and temperature were quantified prior to, and the end of each run in order to determine within chamber and between chamber variability. Temperature, PAR, humidity and [CO_2] were also recorded every 15min throughout a given run, and daily averages determined for each chamber. Temperature, PAR and humidity did not differ between chambers during the experiment. Individual plants were rotated every 2weeks inside the chambers until the final destructive harvest. The experiment was repeated twice, with each run considered a replicate. All plant parameters and alkaloid concentrations were analyzed using an analysis of variance with [CO_2] and cohort as the classification variables (Statview, Cary, NC, USA). Treatment comparisons were made using a Fisher's protected least significant difference. Unless otherwise stated, differences for any measured parameter were considered significant at the $P \leq 0.05$ level.

3 Results

Increasing carbon dioxide had a significant effect on both leaf area and vegetative biomass for *P. setigerum* with the greatest increase observed at 400μmol mol^{-1} relative to the 300μmol mol^{-1} baseline at maturity (Fig. 1). Similarly, the number of capsules produced, the size of the capsule (i.e. weight) and the latex obtained, increased significantly as a function of [CO_2]; again with the greatest relative increase (~2×) occurring from 300 to 400μmol mol^{-1} (Table 1). No significant change in reproductive effort, the ratio of reproductive to vegetative biomass, was observed with [CO_2] (data not shown).

Carbon dioxide increases had no stimulatory effect on alkaloid concentration for codeine, papaverine and noscapine; although significant effects of [CO_2] on morphine were observed (Table 2). However, whole plant production of all opiates increased significantly in response to rising carbon dioxide because the stimulatory effect of [CO_2] on capsule size and number compensated for any CO_2-induced change in concentration (Fig. 2). In addition, the relative proportion of morphine (to the other alkaloids) increased significantly up to a [CO_2] of 500μmol mol^{-1}(10.4%, 11.7%, 12.9% and 12.4% respectively for the 300, 400, 500 and 600μmol mol^{-1} CO_2 treatments).

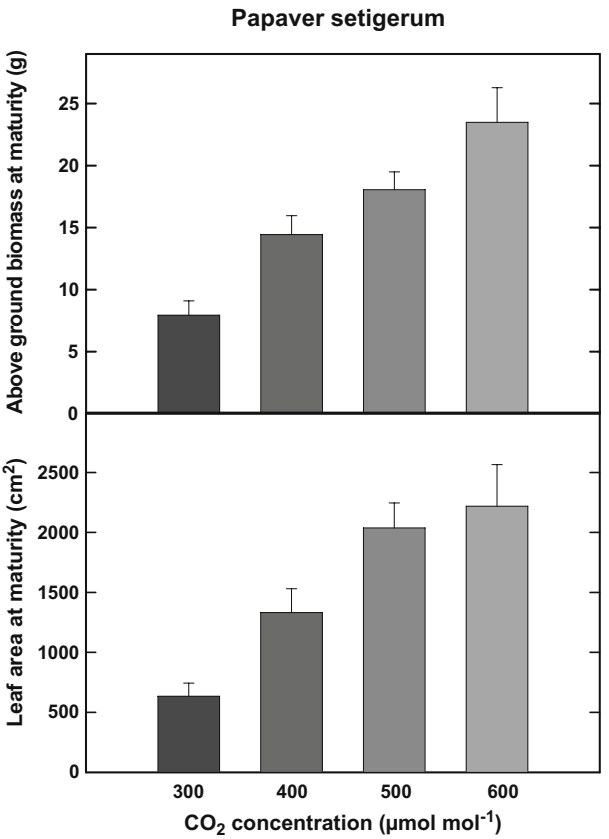

Fig. 1 Total above-ground biomass and leaf area of wild poppy (*P. setigerum*) as a function of atmospheric carbon dioxide concentrations. Significant [CO_2] differences were observed for all treatments relative to the 300 µmol mol^{-1} baseline. The concentrations approximate the atmospheric [CO_2] from 1950, today, and that projected for the years 2050 and 2090, respectively. *Bars* are ±SE

4 Discussion

Synthesis of secondary chemicals (i.e. those not engaged in primary metabolism) by plant species is generally accepted as resulting from the interaction of plants and their major pests, pathogens and predators. Among secondary compounds, alkaloids are recognized as reducing rates of herbivory with subsequent impacts on survivability and insect–plant coevolution (Cheeke 1989; Theis and Lerdau 2003).

Table 1 Averages and P values of the analysis of variance for CO_2 concentration for reproductive characteristics of *P. setigerum*

Variable	Averages				P-value
	300	400	500	600	CO_2 effect.
Capsule no.	14.6 ± 1.5	29.4 ± 1.6	32.9 ± 1.1	52.1 ± 3.0	**
Capsule wt. (g)	1.44 ± 0.16	2.47 ± 0.12	3.55 ± 0.17	4.30 ± 0.35	**
Latex (mg)	97 ± 20	198 ± 17	259 ± 26	363 ± 69	**

Data are per plant. Bars are ±SE.

*$P < 0.05$; **$P < 0.01$

Table 2 Averages and *P* values of the analysis of variance for CO_2 concentration for percent changes in secondary alkaloids of *P. setigerum*

Variable	Averages				*P*-value
	300	400	500	600	CO_2 effect.
Morphine (%)	2.20 ± 0.15	2.34 ± 0.09	2.56 ± 0.16	2.67 ± 0.14	0.06
Codeine (%)	2.11 ± 0.28	2.56 ± 0.13	1.76 ± 0.24	2.27 ± 0.16	*
Papaverine (%)	6.15 ± 0.25	4.82 ± 0.18	5.83 ± 0.27	5.42 ± 0.15	**
Noscapine (%)	10.78 ± 0.50	10.33 ± 0.25	9.77 ± 0.49	11.21 ± 0.37	0.09

Bars are ±SE.

*$P < 0.05$; **$P < 0.01$

While the defensive nature of these chemicals is recognized, their anthropogenic use is also widely acknowledged. The value of plants as a source of such chemicals, and their related use in therapeutic and/or ceremonial purposes has been an integral aspect of civilization, past and present (see Schultes and Reis 1995 for an overview).

Both *P. somniferum* and *P. setigerum* produce raw opium, a complex mixture of secondary alkaloids that contain the analgesics morphine and codeine as well as the antispasmodic noscapine, and the vasodilator papaverine. The ethnobotanical uses of

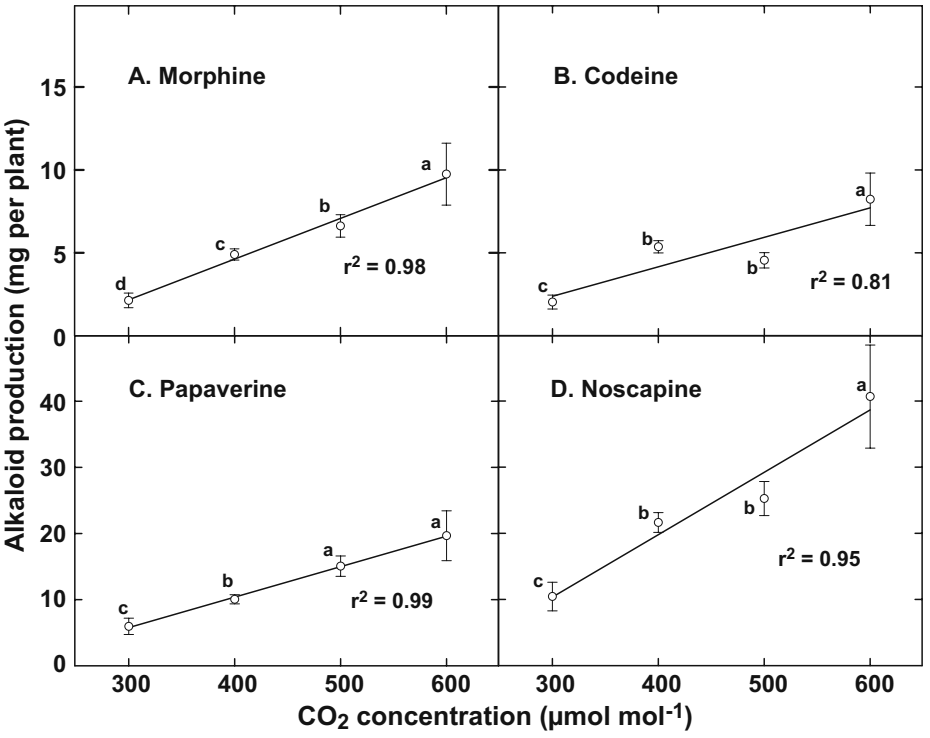

Fig. 2 Whole plant production of four secondary alkaloids of wild poppy (*P. setigerum*) grown to maturity at four different [CO_2]. The concentrations approximate the atmospheric [CO_2] from 1950, today, and that projected for the years 2050 and 2090, respectively. Different *letters* indicate Fisher's protected least significant difference as a function of [CO_2]. *Bars* are ±SE

opiates derived from both species are well documented (Schultes and Reis 1995). The wider pharmacological application of these secondary metabolites to human society are commonly accepted as having both negative (e.g. heroin, the acetylated form of morphine) and positive (e.g. codeine, an analgesic) interactions with respect to public health (Darlacher 2000).

Changes in the abiotic environment (e.g. sunlight, nutrient availability, temperature, etc.) can affect carbon uptake and hence the synthesis and utilization of secondary chemicals in plant species (Topliss et al. 2004; Ziska et al. 2005). Given the universal importance of opiates on human health (for good or ill), how will recent and projected changes in atmospheric carbon dioxide alter their concentration and/or production? In the current study, while the impact of [CO_2] on concentration was variable, on a per plant basis, increasing carbon dioxide above mid-twentieth century levels resulted in an increase in the total production of all alkaloids examined. Interestingly, among the alkaloids tested, morphine exhibited a strong response to [CO_2]; increasing both in concentration, and as a fraction of total alkaloids produced. Given the subtle distinctions that exist between potential for abuse and benefit, carbon dioxide induced changes in alkaloid concentration, proportion, or overall production will certainly influence their efficacy and use in cultures that depend on local sources of *P. setigerum* for medicine (Duke 1973).

How changes in the abiotic environment alter the production and transfer of secondary alkaloids within *Papaver* is not entirely understood. Although many of these secondary alkaloids probably play a defensive role (sanguinarine in *Papaver* as a fungitoxin, Cline and Coscia 1988), little is known about how carbon dioxide per se might regulate their production. The carbon/nutrient balance hypothesis (Bryant et al. 1983) predicts that as [CO_2] rises, the resulting increase in the C:N of plants will reduce the concentration of nitrogen-based secondary compounds (including alkaloids) since increased vegetative growth will necessitate a larger investment in nitrogen. A similar result could also occur if soil conditions or excess competition limited nutrient availability. This hypothesis is consistent with previous data regarding [CO_2] and secondary alkaloids in jimson weed and tobacco (Ziska et al. 2005); however, in the current study, variation in the C:N ratio of morphine, codeine, noscapine and papaverine (approximately 20:1 for these alkaloids) was insufficient to test this hypothesis.

But will the response of *P. setigerum* to rising atmospheric carbon dioxide observed here reflect response of this species *in situ*? It can be argued for example, that nutrients or some other resource could limit the response of plants to rising [CO_2] under field conditions (e.g. Reich et al. 2006). Yet, it is important to remember that the commercial value of poppy (and other plant species) is such that they will be grown in managed environments where nutrients or other abiotic resources are likely to be supplemented. This does not, of course, mean that interactions between rising [CO_2] and other resources (e.g. water, nutrients) cannot alter the concentration and/or distribution of secondary alkaloids in poppy; this seems likely, even though additional data as to the nature of these interactions is needed. However, it cannot be assumed that poppy growth and reproduction will fail to respond to rising atmospheric carbon dioxide as a result of some other abiotic limitation.

It can also be argued that temperature may modify the response of *Papaver* species to rising atmospheric CO_2 levels. This may be particularly true for temperatures associated with projected CO_2 changes by the end of the current century, rather than recent temperature changes for the latter half of the twentieth century (i.e. the change from 300 to 400μmol mol^{-1}, IPCC 2007). However, previous work for the secondary alkaloids scopolamine and atropine suggested that while increasing temperature did increase concentration, the relative stimulation by [CO_2] did not vary (Ziska et al. 2005). In

addition, temperature projections are highly variable, with increasing temperatures related to a greater degree of warming for winter relative to summer months (e.g. Balling et al. 1998) and not higher mean temperatures per se. For *P. somniferum*, optimal development rates did not vary for day/night temperatures of 20/15°C, 24/19°C and 28/23°C, (i.e. 2.6°C above the mean temperature used in the current experiment; Acock et al. 1997).

The global importance of *P. somniferum*, from a cultural, economic, social, medical and even terrorism perspective, is unquestioned. While *P. somniferum* as such was not examined in the current study, it is not unreasonable to suppose a similar [CO_2] response for both *P. setigerum* and *P. somniferum* given their genetic and morphological similarity. Clearly, if *P. setigerum* is any guide, there should be a strong impetus for specific data on the [CO_2] response of *P. somniferum*. Such data may be particularly germane, given that *P. setigerum* showed the greatest relative response to recent increases in [CO_2] (i.e. 300–400μmol mol^{-1}).

To our knowledge, this is the first study to specifically address the impact of anthropogenic increases in atmospheric CO_2 on opium production. We recognize that the role of rising [CO_2] on the production and concentration of opiates in poppy (wild or cultivated) cannot be elucidated in a single study. Nevertheless, the impact of climate, in particular recent and projected increases in atmospheric carbon dioxide, remains an overlooked factor in regards to opiate production and as such, deserves additional scrutiny. To that end, the preliminary data presented here, will, hopefully, be part of a larger, sustained research effort to quantify the impact of environmental change on plant-based pharmacological compounds.

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