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REGULAR ARTICLE

Elevated CO_2 affects plant responses to variation in boron availability

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Abstract
Aim Effects of elevated CO_2 on N relations are well studied, but effects on other nutrients, especially micronutrients, are not. We investigated effects of elevated CO_2 on response to variation in boron (B) availability in three unrelated species: seed geranium (*Pelargonium* x *hortorum*), barley (*Hordeum vulgare*), and water fern (*Azolla caroliniana*).
Methods Plants were grown at two levels of CO_2 (370, 700 ppm) and low, medium, and high B. Treatment effects were measured on biomass, net photosynthesis (P_n) and related variables, tissue nutrient concentrations, and B transporter protein BOR1.
Results In geranium, there were interactive effects ($P<0.05$) of B and CO_2 on leaf, stem, and total plant mass, root:shoot ratio, leaf [B], B uptake rate, root [Zn], and P_n. Elevated CO_2 stimulated growth at 45 μM B, but decreased it at 450 μM B and did not affect it at 4.5 μM B. P_n was stimulated by elevated CO_2 only at 45 μM B and chlorophyll was enhanced only at 450 μM B. Soluble sugars increased with high CO_2 only at 4.5 and 45 μM B. High CO_2 decreased leaf [B] and B uptake rate, especially at 450 μM B. Though CO_2 and B individually affected the concentration of several other nutrients, B *x* CO_2 interactions were evident only for Zn in roots, wherein [Zn] decreased under elevated CO_2. Interactive effects of B and CO_2 on growth were confirmed in (1) barley grown at 0, 30, or 1,000 μM B, wherein growth at high CO_2 was stimulated more at 30 μM B, and (2) *Azolla* grown at 0, 10, and 1,000 μM B, wherein growth at high CO_2 was stimulated at 0 and 10 μM B.
Conclusion Thus, low and high B both may limit growth stimulation under elevated vs. current [CO_2], and B deficiency and toxicity, already common, may increase in the future.

Keywords *Azolla* · Barley · Boron stress · Boron transporter protein (BOR1) · Geranium · Nutrients · Photosynthesis

Introduction

The growth of most plants is enhanced at elevated, relative to current, levels of atmospheric CO_2, and this enhanced growth results in greater demand for mineral nutrients (e.g., Campbell and Sage 2002; Hagedorn et al. 2002). If nutrient availability or plant uptake does not increase to meet this enhanced nutrient demand, then decreases in the concentrations of nutrients will occur in at least some tissues of plants grown at elevated CO_2. Consistent with these

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expectations, several past studies have reported that high-CO_2-stimulation of plants under nutrient-deficient conditions is less, or even absent, when compared to nutrient-sufficient conditions (Cure et al. 1988; Coleman et al. 1993; Ziska 2003). Because elevated CO_2 is expected to alter plant tissue nutrient concentrations, many studies have examined effects of elevated CO_2 on nutrient relations, but most previous studies have focused on macro-nutrients, especially N, (e.g., Ehleringer et al. 2002; Ellsworth et al. 2004; Sicher 2005; Tang et al. 2006; Taub and Wang 2008 and references therein), and only a few have examined CO_2 effects on micro-nutrient relations (Norby et al. 1986; O'Neill et al. 1987; Manderscheid et al. 1995; Fangmeier et al. 1997; Peñuelas et al. 1997, 2001; Prior et al. 1998; Blank and Derner 2004; Pal et al. 2004; Luomala et al. 2005; Jin et al. 2009).

In general, growth of plants under elevated (vs. current) CO_2 typically decreases the concentration of N, especially in leaves, due largely to declines in rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) levels in leaves, and high-CO_2 decreases are usually greater in C_3 than C_4 species (e.g., ca. 20 vs. 5% decreases respectively for leaf %N; Ehleringer et al. 2002). Other macro-nutrients also often decrease in concentration with growth in elevated CO_2, though responses can differ among nutrients within a species (Table 1, for examples). Though few studies to date have examined effects of elevated CO_2 on tissue micronutrient concentrations, the limited results from these studies, summarized in Table 1, indicate (1) that CO_2 effects will be variable among species, tissues, and micro-nutrients; (2) that high CO_2 will often decrease micro-nutrient concentrations; and (3) decreases in micro- (and macro-) nutrient concentrations may be more prevalent in seeds compared to leaves. For example, the responses among related species (barley vs. wheat), and among cultivars within a species (within barley and wheat separately), to micronutrient stress and elevated CO_2 differed (Manderscheid et al. 1995).

There is evidence that elevated CO_2 has interactive effects with other aspects of nutrition, though this has not often been examined. For example, in an interactive-effect study in wheat, Fangmeier et al. (1997) observed complex interaction among CO_2, nitrogen availability, and ozone in spring wheat. Similarly, Coleman et al. (1993) observed CO_2 *x* N effects in *Abutilon theophrasti* and *Amaranthus retroflexus* that were often mediated by effects on development. Hagedorn et al. (2002) found that soil fertility and CO_2 may have interactive effects and these interactions may be species dependent. Specifically, on an acidic loam soil, CO_2 enrichment suppressed net accumulation (total content in biomass) of nine (of 11) investigated mineral nutrients in beech trees (significant only for P, S, Zn), but stimulated it for 10 of 11 nutrients in spruce trees (significant only for Fe, Zn); in contrast, on nutrient-rich calcareous sand, increased atmospheric CO_2 enhanced nutrient accumulation in both species significantly (Hagedorn et al. 2002). Similarly, Blank and Derner (2004) observed interactive effects between soil fertility (low- and high-fertility soils) and CO_2 on various aspects of plant and soil properties in *Lepidium latifolium*, including effects on plant nutrient concentrations that varied among the nutrients examined. To our knowledge, only one previous study has examined interactive effects of CO_2 and micronutrients on plant growth and function: i.e., Jin et al. (2009) examined interactive effects of CO_2 and Fe species (FeEDTA vs. Fe(III) oxide) on tomato, and found that the combination of elevated CO_2 and low Fe increased Fe uptake ability, and that CO_2 affected [Fe] only with Fe oxide.

Notably, we can find only six previous studies wherein effects of elevated CO_2 on tissue B concentrations were investigated (Table 1; Norby et al. 1986; O'Neill et al. 1987; Peñuelas et al. 1997, 2001; Luomala et al. 2005; Liu et al. 2007). As chance would have it, each of these studies (four reports on trees and two on shrubs) examined CO_2-responses in plants grown in soil characterized by the authors as "nutrient poor" or "low in N", and B soil availability was undetermined and un-manipulated in these studies. The paucity of previous research on B *x* CO_2 effects is striking, since (1) among all essential plant nutrients, it is thought that B has perhaps the narrowest range of tissue concentrations over which B levels are adequate and not stressful (i.e., not limiting or toxic) (e.g., Marschner 1995), and (2) B stress is common and economically important in agriculture world-wide (Shorrocks 1997). Though B requirements, as well as thresholds for B deficiency and toxicity, vary significantly among species and categories (e.g., grasses vs. dicots; Blevins and Lukaszewski 1998), available B levels below ca. 2–5 μM usually cause B deficiency (e.g., El-Shintinawy 1999; Wimmer et al. 2005) and levels above 1,000 μM typically induce toxicity

Table 1 Summary of past studies examining effects of elevated (relative to current) CO_2 on the concentration of micro-nutrients

Species	Tissue	[Nutrient] increase	[Nutrient] decrease	[Nutrient] no change	Source
Herbaceous					
Hordeum vulgare	Leaves	S	N	Ca,Fe,K,Mg,Mn,P,Zn	Manderscheid et al. 1995
	Seeds	K	Fe,N,S,Zn	Ca,Mg,Mn,P	Manderscheid et al. 1995
Triticum aestivum	Leaves	P,S	K,Mg	Ca,Fe,Mn,N,Zn	Manderscheid et al. 1995
	Seeds	K	Ca,Fe,Mg,Mn,N,S,Zn	P	Manderscheid et al. 1995
Triticum aestivum	Leaves		Ca,K,Mg,Mn,N,P,S,Zn	Fe	Fangmeier et al. 1997
	Seeds		Ca,Fe,K,Mg,Mn,N,P,S,Zn		Fangmeier et al. 1997
Gossypium hirsutum	Leaves			Ca,Fe,K,Mg,Mn,N,P,S,Zn	Prior et al. 1998
	Seeds		Cu,Fe,K,N,Zn	Ca,Mg,Mn,P	Prior et al. 1998
Lepidium latifolium	Shoots	Mg	Ca,Fe,K,Mg,Mn,N,P,S		Blank and Derner 2004
Trifolium alexandrium	Leaves	P	N	Ca,Fe	Pal et al. 2004
Lycopersicon esculentum	Shoots	Fe (with Fe(III) oxide)		Fe (with FeEDTA)	Jin et al. 2009
	Roots	Fe (with Fe(III) oxide)		Fe (with FeEDTA)	
Shrubs					
Citrus aurantium	Leaves	B	Ca,N,Mg,Mn	Cu,Fe,K,Na,P,S,Zn	Peñuelas et al. 1997
Erica arborea	Leaves	K,S	Ba,B,Sr	Al,Ca,Cd,Co,Cr,Cu,Fe,Mg, Mn,Mo,N,Na,Ni,P,Pb,Si, Ti,V,Zn	Peñuelas et al. 2001
Myrtus communis	Leaves	Mg,Mn,S	Ba,B,N,Sr	Al,Ca,Cd,Co,Cr,Cu,Fe,K, Mo,Na,Ni,P,Pb,Si,Ti,V,Zn	Peñuelas et al. 2001
Juniperus communis	Leaves	Al,Ca,Fe,K,Mg,Mn, S,Ti	Ba,Co	B,Cd,Cr,Cu,Mo,N,Na, Ni,P,Pb,Si,Sr,V,Zn	Peñuelas et al. 2001
Trees					
Quercus alba	Whole-seedling	Fe	B,Ca,Mg,Mn,N,S,Zn	Al,Cu,K,P	Norby et al. 1986
Liriodendron tulipifera	Whole-seedling		B,N,S	Al,Ba,Ca,Cu,Fe,K,Mg, Mn,P,Sr,Zn	O'Neill et al. 1987
Pinus sylvestris	Leaves	Mn	Cu,N,P,S	B,Ca,Fe,K,Mg,	Luomala et al. 2005
Populus tremula	Leaves	K,P	N,B	Ca,S,Mg,Mn,Cu,Fe,Zn	Liu et al. 2007
Betula papyrifera	Leaves	K,P	N,B	Ca,S,Mg,Mn,Cu,Fe,Zn	Liu et al. 2007

(though levels as low as 200 μM have been reported to be stressful in some species) (Reid et al. 2004).

To date, the most-widely-accepted role of B in plants is that of a structural function in plant cell walls (Brown et al. 2002; Goldbach 1997; Kobayashi et al. 1996; Matoh 1997; Power and Woods 1997). This structural role of B in cell walls is due to its capacity to form diester bridges between adjacent *cis*-hydroxyl containing molecules, such as mono-, oligo-, and polysaccharides, and diols and hydroxyacids (Power and Woods, 1997). B also is involved in plant reproduction, which may or may not be related solely to the structural role of B in cell walls (Blevins and Lukaszewski, 1998; Marschner 1995). Other specific functions of B have been postulated as well (Blevins and Lukaszewski 1998; Bolaños et al. 2004; Dordas and Brown 2000), and boron deficiency can affect several metabolic processes; e.g., cell division and elongation, metabolism of nucleic acids, protein synthesis, metabolism and transport of carbohydrates,

synthesis and metabolism of phenolics, and photosynthesis (Blevins and Lukaszewski 1998; Goldbach 1997; Kouchi 1977; Mishra et al. 2009).

Recently, the first two B-transport membrane proteins have been identified and characterized: one involved in active transport, BOR1, and one involved in facilitated diffusion, the NOD26-like intrinsic protein (NIP), NIP5;1 (Miwa et al. 2009). BOR1 is a B efflux transporter expressed in roots and leaves and is up-regulated under B-deficiency conditions (Takano et al. 2002). The channel protein NIP5;1 is crucial for B uptake in plants under B limitation (Takano et al. 2006). BOR1 increases B supply to the shoots by loading B from the xylem parenchyma into the xylem (Takano et al. 2002). Under toxic concentrations of boron, BOR1 is degraded via endocytosis (Takano et al. 2005). Under elevated CO_2, one might expect that expression levels of BOR1 and/or NIP5 proteins in roots will change, if elevated CO_2 is altering nutrient demand.

The present study aimed to investigate effects of elevated CO_2 on growth, photosynthesis, and nutrient (especially B) relations in geranium (*Pelargonium hortorum* cv. Maverick White; a dicot) plants grown for 30 days while supplied with one of three different B concentrations, ranging from potentially sub-optimal (4.5 μM) to near-optimal (45 μM) to potentially supra-optimal (450 μM). We tested the *apriori* hypothesis that elevated CO_2 would (1) exacerbate B deficiency at low levels of B availability, and (2) decrease B toxicity at high levels of B; in both cases, by enhancing plant growth and thus increasing the dilution of B in tissues. To determine if the CO_2 *x* B effects observed in geranium are common in other species, we examined effects of CO_2 in (1) barley (*Hordeum vulgare*; a monocot) grown at 30 μM B and transferred to 0, 30 or 1,000 μM B and (2) water fern (*Azolla caroliniana*) grown at 10 μM B and transferred to 0, 10, or 1,000 μM B. For these latter two species, we expanded the range of B levels to increase the severity of B deficiency or toxicity.

Materials and methods

Plant material and B and CO_2 treatments

Seeds of geranium plants (*Pelargonium* x *hortorum* cv. Maverick White) were sown into foam cubes (15-mm×15-mm×30-mm each; LC1-type, Smithers-Oasis North America, Kent, Ohio) and irrigated with complete fertilizer solution (Hoagland's). After 40 days, when seedlings had 3-to-4 true leaves, seedlings were transferred to opaque 4 L plastic tubs filled to volume with complete Hoagland's solution, as in Mishra et al. (2009). The tubs had opaque lids with two evenly-spaced 2-cm-diameter holes through which seedlings were suspended (by wrapping with thin strips of foam around the root-shoot interface), such that the root system was enclosed in the tubs and shoots were above the level of the tub lids. Seedlings were then allowed to acclimate for 10 days to minimize transplant stress. Plants were germinated and grown in a greenhouse, under a 20–28°C temperature range and with supplemental light to extend the photoperiod to 14 h (6 am–8 pm; minimum PAR=200 μmol m^{-2} s^{-1}).

For B and CO_2 treatments, plants were transferred to (otherwise) complete nutrient solutions containing one of three levels of B (4.5, 45, or 450 μM, based on results in Mishra et al. 2009; three replicate tubs per B level), and then 9 tubs containing 2 plants per tub were kept under two different concentrations of CO_2 (thus 18 tubs total) in controlled-environment chambers [one at 370±20 ppm (ambient) and one at 700±20 ppm (elevated) CO_2]. Each tub contained two plants, and these two plants were averaged to generate the value for the tub, with mean tub values being the experimental replicates. Nutrient solutions, checked regularly and maintained at pH 5.6 with addition of 1 N HCl or KOH, were changed weekly, which was determined in preliminary experiments to be frequent enough to prevent depletion of nutrients. Each tub was aerated by constant bubbling of nutrient solution to make it homogeneous. Plants were grown at 23°C day/19°C night with uncontrolled humidity (typically>50%), under a 16-h photoperiod, and at a light intensity of 300 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation), which provided 17.28 mol m^{-2} d^{-1} of PAR. This light level is optimal for this geranium cultivar (Mishra et al. 2009). Light levels were monitored twice weekly with a line quantum sensor (model LQSV-E, Apogee Instruments, Inc. Logan, Utah); chamber CO_2 and temperature levels were monitored several times a day with calibrated and independent sensors; plants were rotated within chambers every other day. Plants were grown in the above growth conditions for 30 d, during which time, plant biomass increased in all treatments.

To confirm B *x* CO_2 effects on biomass observed in the above experiment, we conducted two additional experiments, wherein we grew barley (*Hordium vulgare*) and aquatic fern (*Azolla caroliniana*), respectively. Based on results from the geranium experiment, in barley and *A. caroliniana*, the severity of both low- and high-B stress was increased by decreasing [B] in the low-B treatment and increasing [B] in the high-B treatment. Barley plants were grown hydroponically as above under three concentrations of B (0, 30 and 1,000 μM) and two levels of CO_2 (370 and 700 ppm). Seeds were sown into soil, and after 15 d, when seedlings had three to four true leaves, they were rinsed to remove soil on roots and transferred to hydroponic tubs containing complete nutrient solution (including 30 μM B) and allowed to acclimate for 7 d to minimize transplant stress. At this time, subsets of plants were transferred to nutrient solutions containing 0 or 1,000 μM B (in otherwise complete nutrient solution), while control plants continued to receive 30 μM B. Three replicate plants (in separate tubs) at each B level were grown under ambient (370 ppm) or elevated CO_2 (700 ppm) in growth chambers. Plants were grown at 25°C day/20°C night, under a 16 h photoperiod, and at a light intensity 800 μmol m^{-2} s^{-1} PAR. Plants were kept for 30 days of treatment prior to harvest. Nutrient solutions were changed weekly, and plants were rotated within chambers every other day. For *Azolla*, plants were grown for >2 weeks in a nutrient solution designed for algae (WC medium: 250 μM $CaCl_2$, 150 μM $MgSO_4$, 50 μM K_2HPO_4, 11.7 μM Fe-EDTA, 0.9 μM $MnCl_2$, 0.08 μM $ZnSO_4$, 0.05 μM $CoCl_2$, 0.04 μM $CuSO_4$, 10 μM H_3BO_3, and 0.0037 μM $(NH_4)_6Mo_7O_{24}$). Plants were transferred to plastic-tubs (600 ml) with the above nutrient solution and one of three B concentrations (0, 10, or 1,000 μM). Four replicates of each B treatment (=12 tubs) were kept under ambient CO_2 (370 ppm) and another 12 tubs under elevated CO_2 (700 ppm) in controlled-environment chambers. Prior to harvest, plants were grown for 10 days at 25°C/20°C (day/night), under a 16 h photoperiod and 200 μmol m^{-2} s^{-1} PAR light intensity.

Growth and nutrient analysis

Entire plants were harvested and then immediately separated into roots, stems, and leaves for geranium and roots and shoots for barley; intact plants were analyzed for *Azolla caroliniana*. Tissues were oven dried at 70°C for 72 h (to constant mass) and then weighed. To determine tissue nutrient content, we followed our previously−reported method (Mishra et al. 2009). Briefly, all harvested tissues were rinsed with 0.1 N HCl, rinsed again with distilled water, and then oven dried in a forced-air oven at 55°C for 72 h. Tissue was ground by mortar and pestle into a powder and 0.15 g was digested in a microwave digester (MARS Express II, CEM Corp., Matthews, North Carolina), using a modified EPA method (EPA method 3051, Nelson 1988; HNO_3 digestion at 200°C with an additional peroxide digestion step). Nutrient concentration (B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) was determined with inductively-coupled-plasma optical-emission spectroscopy (ICP-OES; Model IRIS Intrepid II, Thermo Corp., Waltham, MA).

Photosynthesis

Steady-state net photosynthesis (P_n; net CO_2 exchange) of recently fully-expanded intact leaves of geranium, which had developed after the exposure to experimental treatments, was measured with a portable photosynthesis system with an infrared gas analyzer (model 6400, LiCOR, Lincoln, Nebraska, USA), equipped with a 250-mm^3 leaf chamber and CO_2, light, and temperature control (as in Mishra et al. 2009). Measurements were made within one min of insertion of leaves in to the cuvette, and after stabilization of CO_2 and H_2O flux, to ensure that photosynthetic responses reflected those within the growth chambers. Net photosynthesis of plants was measured at the same CO_2 levels at which the plants were growing (either 370 or 700 ppm CO_2) at a light level of 300 μmol m^{-2} s^{-1} PAR.

Chlorophyll and carbohydrate content

Chlorophyll and carbohydrate content was measured as in Mishra et al. (2009). Briefly, chlorophyll content (per fresh mass) in leaves was estimated spectrophotometrically after extraction in dimethyl sulfoxide (DMSO), using the equations of Barnes et al. (1992). Leaf samples were incubated at 65°C for 1 h and then cooled to room temperature in the dark prior to measurements. Total soluble carbohydrate content in root tissue was estimated by using the phenol-sulfuric

acid method of Dubois et al. (1956), with minor modification. Fresh tissues (50 mg dry mass) were ground in liquid N_2, and then mixed with 2 mL of 0.1 M phosphate buffer (pH 7.2) and re-ground. The homogenate was centrifuged at 21,000 g, and then 1 mL of supernatant was taken and mixed with 1 mL of 5% aqueous phenol. Concentrated sulfuric acid (5 mL) was added, and absorbance at 470 nm was determined after 20 min. Glucose was used for generating a standard curve.

BOR-1 Protein analysis

Total cell protein was extracted from frozen root tissues (400 mg fresh weight) by grinding in liquid N_2 in a mortar and pestle, and then in an extraction buffer containing [0.5 M Tris–HCl (pH 8.0), 50 mM EDTA, 0.1 M KCl, 0.9 M sucrose and 2% β mercaptoethanol]. The homogenates were transferred to a 15 mL tube and the same volume of Tris-buffered phenol (pH 8.0) was added. After incubating for 10 min on a shaker at room temperature, samples were centrifuged at 5,500*g* for 20 min at 4°C to separate the aqueous and organic phase. The upper phenolic phase was recovered and transferred to a fresh tube. This phenol phase was washed with an equal volume of extraction buffer and then centrifuged at 5,500*g* for 20 min at 4°C. The protein-containing phase was transferred to a fresh tube and precipitated with 5 volumes of 0.1 M ammonium acetate in 100% (v/v) methanol and incubated overnight at −20°C. The precipitate was washed three times with 0.1 M ammonium acetate in 100% methanol followed by three times with 80% acetone, and a final time with 100% acetone. The final protein pellet was resuspended in sample buffer (Tris–HCl pH 6.8, 2% SDS, 0.05% β-mercaptoethanol and glycerol). The total protein concentration of each sample was determined in triplicate by the Coomassie-dye-binding method of Ghosh et al. (1988), using bovine serum albumin as a standard. The colorimetric density of protein in sample spots on filter-paper discs was determined using a desktop scanner and densitometry analysis, using National-Institutes-of-Health imaging software (Scion, National Institutes of Health, Bethesda, MD). Proteins were then separated by 1D SDS-PAGE, transferred to nitrocellulose by electro (western) blotting, and subjected to immuno-detection and quantification as in by Mishra et al. (2008). BOR1 protein was detected using a rabbit polyclonal antiserum generated against a conserved peptide (GDYPLSATIMSEYANKKTRG) identified from BOR1 amino-acid sequences available from public databases and the BOR1 sequence identified in geranium (Deng 2009).

Statistical analysis

Results were analyzed statistically by two-way (B *x* CO_2) analysis-of-variance (ANOVA), with B and CO_2 levels as fixed factors, using JMP software (SAS Corp, Cary, NC). Treatment effects were considered significant if $P<0.05$ and marginally significant if $P<0.10$. Following significant main-factor effects by ANOVA, Tukey's test was used to determine significant differences among treatment levels among main factors.

Results

Though geranium plants were measured and harvested after 30 days of experimental treatments, treatment effects were visible sooner (not shown). For example, after 20 days, mild chlorosis was observed in leaves of plants grown under low and high [B] in elevated CO_2 (mostly in the youngest leaves at low B, and in the older leaves with high B). However no distinct treatment effects were observed in roots, except that they appeared more-branched at high B and high CO_2.

There were significant B *x* CO_2 interactive effects on dry mass of leaf, stem, and total mass (similar trend but non-significant effects on flower mass) (Fig.1a–d; Table 2). For example, peak biomass of leaves, stems and whole-plants was observed at 450 μM at ambient CO_2, but at 45 μM B at elevated CO_2. When comparing effects of elevated vs. ambient CO_2 within each B level, stimulation of growth at high CO_2 was observed only at 45 μM B, with no stimulation at 4.5 μM B, and with decreased biomass with high CO_2 at 450 μM B. No B *x* CO_2 effects were observed for roots, though root mass was increased by high CO_2 (Fig. 1e; Table 2). However, there were interactive effects of B and CO_2 on root:shoot ratio, and root:shoot ratio was increased by elevated CO_2 (Fig. 1f; Table 2).

As expected, elevated CO_2 increased net photosynthesis (P_n), by increasing leaf internal CO_2 concentration (C_i), but elevated CO_2 had no effect on stomatal

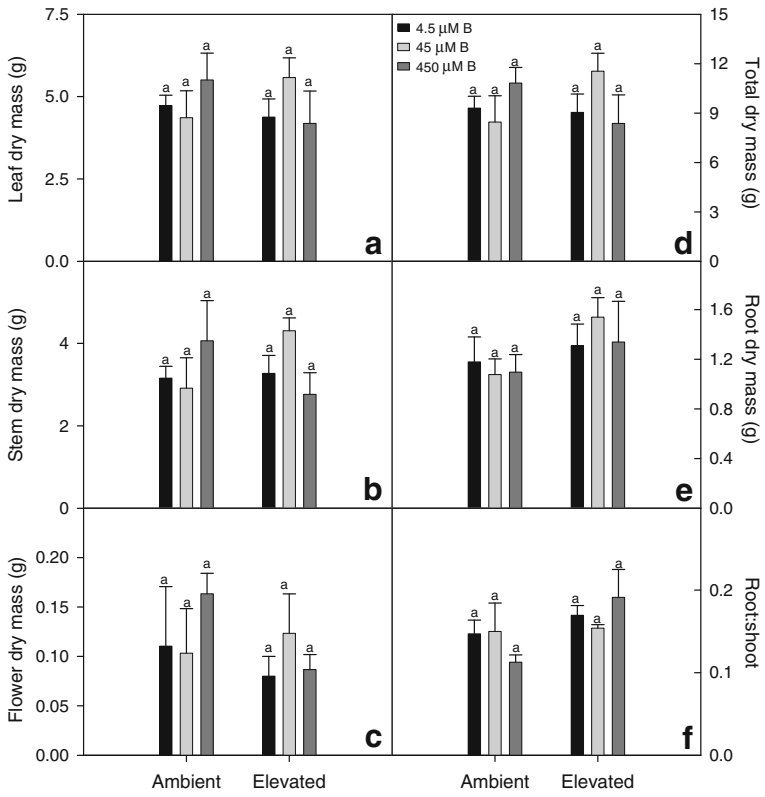

Fig. 1 Effect of B (4.5, 45, and 450 μM) and CO_2 (ambient=370 and elevated=700 ppm) on biomass of different tissues of geranium. Each bar represents the mean (±1 SD) of three independent replicates. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

conductance (G_s) in geranium (Fig. 2a–c; Table 2). However, when comparing P_n in elevated vs. ambient CO_2 within each B level, elevated CO_2 stimulated P_n only at 45 μM B. Also, P_n was greatest at 4.5 μM B at ambient CO_2, while B had no effect on P_n at elevated CO_2. Hence, there were interactive effects of B and CO_2 on P_n. No B *x* CO_2 effects were observed on total chlorophyll (Chl_{tot}), chlorophyll *a:b* (Chl *a:b*), or soluble sugars (Fig. 2d–f; Table 2). Boron did affect Chl_{tot} and Chl *a:b*, with maximum Chl_{tot} at 45 μM B, and with inconsistent effects on Chl *a:b*. Soluble sugar content was enhanced under elevated, compared to ambient, CO_2 both in leaf and root tissues.

As we anticipated, both B and CO_2 had effects on the concentration of B in plant tissues, and there was a significant interactive effect of B and CO_2 on [B] of leaf tissue (Fig. 3a,b; Table 2). Leaf and root [B] increased with increasing B availability (marginally significant in roots), with larger increases in tissue [B] when comparing 450 to 45 μM B than 45 to 4.5 μM. On average, across all B levels in both roots and shoots, elevated CO_2 decreased tissue [B], but within each B level individually, this high-CO_2 decrease was significant only in leaves at 450 μM B, wherein [B] was reduced by 55% by high CO_2; hence, the significant B *x* CO_2 interaction in leaves. Similar patterns for [B] were observed for root-specific uptake rates of B (total g plant B per g of root); i.e., B uptake rate increased with B availability (especially at 450 μM B), decreased at high CO_2, and the high-CO_2-related decrease was significant only at 450 μM B (ambient was 2.5 times that at elevated CO_2), resulting in a significant B *x* CO_2 effect (Fig. 3c; Table 2). Neither B nor CO_2 had significant effects on the relative content of the B transporter, BOR1, though elevated CO_2 tended to increase BOR1 content (Fig. 3d; Table 2). Content of BOR1 remained almost constant in both CO_2 treated plants at 450 μM B. Also under elevated CO_2 among all the treatments of B, BOR1 declined at 450 μM.

Along with B, the concentration of other nutrients (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) in tissues was also measured in leaves and roots. Because effects of elevated CO_2 on nutrient concentrations have been shown previously in multiple studies (e.g., Table 1), we restrict presentation of results here to nutrients

Table 2 Results from statistical analysis (*P* values from ANOVA) of treatment effects of B, CO_2, and their interactions on various response variables. Geranium plants were grown at different levels of B (4.5, 45, and 450 μM) and CO_2 (370, 700 ppm)

Treatment effects			
Variables	CO_2	B	B *x* CO_2
Biomass:			
Leaf	0.66	0.59	0.02*
Stem	0.80	0.053	0.007*
Root	0.011*	0.730	0.36
Flower	0.211	0.552	0.240
Total	0.77	0.48	0.007*
Root:shoot	0.005*	0.84	0.029*
P_n	0.001*	0.149	0.042*
G_s	0.330	0.816	0.525
C_i	<0.0001*	0.914	0.962
Chlorophyll:			
Total	0.100	0.007*	0.078
Chl a/b	0.036*	0.016*	0.167
Sugar			
Leaf	<.0001*	0.046*	0.55
Root	0.0005*	0.166	0.526
Leaf [B]	0.0001*	<.0001*	0.00006*
Root [B]	0.082	<.0001*	0.112
B-uptake rate	<.0001*	0.0079*	0.0043*
BOR1	0.25	0.7	0.69

* Indicates significant differences among treatments at $P<0.05$

that were affected by B or for which there were significant B *x* CO_2 interactions in either leaves or roots (Fig. 4a–d). However, as in many past studies, elevated CO_2 affected the concentration of nutrients in most instances here (all but P in roots and shoots, Mg and S in roots, and Fe and Zn in shoots; not shown). B affected [P] in leaves (decreasing [P] at 45 μM B; $P=0.0078$), and both [Fe] (highest [Fe] at 450 μM B; $P=0.0279$) and [Zn] in roots (highest at 450 μM B in ambient CO_2 only; $P=0.0188$). Interactive effects of B and CO_2 were evident only for Cu in roots ($P=0.0100$); marginally-significant effects were observed for Zn in roots ($P=0.0655$).

As with geranium, we observed B *x* CO_2 effects on biomass in barley. While elevated CO_2 increased shoot, root, and whole-plant biomass on average in barley, when comparing elevated vs. ambient CO_2 within each B level, growth was stimulated significantly by high CO_2 only at 30 μM B for shoots, roots, and whole-plant biomass (Fig. 5a–c). In addition, while shoot, root, and whole-plant biomass was greatest at 30 μM B in elevated CO_2, no decline in biomass was evident at 0 vs 30 μM B at ambient CO_2, and declines in biomass at 1,000 vs. 30 μM B were not significant. A significant B *x* CO_2 interaction was also observed for root:shoot biomass in barley, wherein B had no effect on root:shoot mass at ambient CO_2, but root:shoot mass increased with increasing B at elevated CO_2 (Fig. 5d).

We also observed a significant interactive effect of B*x*CO_2 on dry mass of *Azolla caroliniana* (Fig. 6), and these effects were similar to those in geranium and barley. Total plant mass in *Azolla* was decreased by both low and high, relative to medium, B, and elevated CO_2 increased mass. When comparing mass between elevated and ambient CO_2 within each B level, biomass was enhanced both at 0 and 10 μM B, but not at 1,000 μM B.

Discussion

The present study found that (1) growth of plants in elevated, relative to current, atmospheric CO_2 affected B relations, (2) CO_2 and B have interactive effects on growth and function, and (3) elevated CO_2 exacerbated effects of low B as predicted, but did not minimize effects of high B as expected; instead, high CO_2 increased B stress at high levels of B. Regarding effects of elevated CO_2 on B relations, high CO_2 decreased the concentration of B in plant tissues (especially leaves), as well as the rate of B uptake by roots, especially at high B (450 μM). Though not statistically significant, elevated CO_2 also tended to increase the levels of the B transport protein, BOR1, at low and medium B (4.5 and 45 μM).

In geranium, we observed interactive effects of CO_2 and B on leaf, stem, and whole-plant dry mass (flower mass showed a similar non-significant pattern), root:shoot ratio, net photosynthesis, leaf [B], B-uptake rate, and root [Zn]. B *x* CO_2 effects were also observed for shoot, root, and whole-plant biomass in barley grown at 30 μM B and transferred to 0, 30, or 1,000 μM B, and for whole-plant biomass in *Azolla* grown at 10 μM B and transferred to at 0, 10 and 1,000 μM B. In both geranium and barley, statistically-significant stimulation of growth by elevated CO_2 was

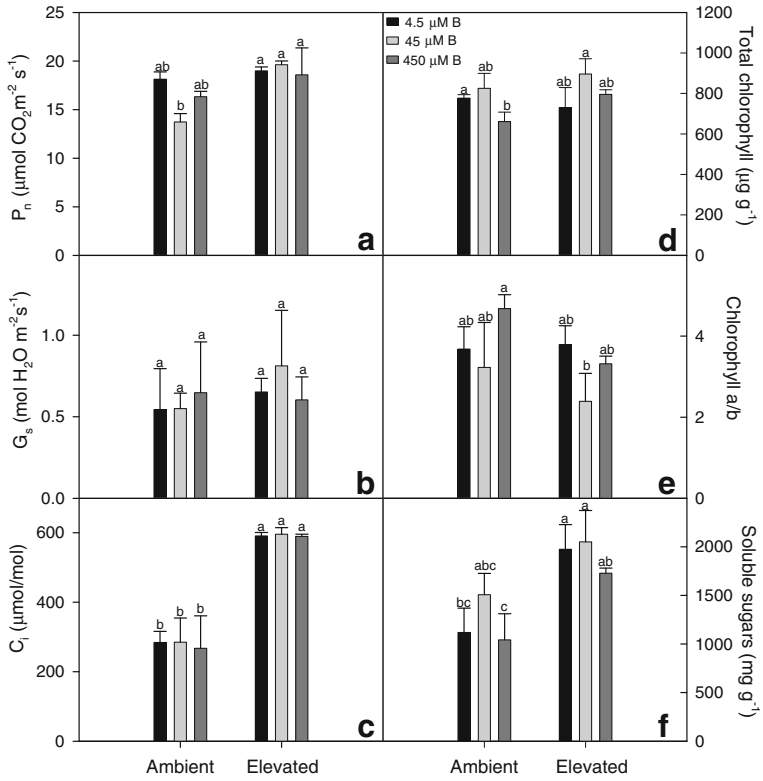

Fig. 2 Effect of B (4.5, 45, and 450 μM) and CO_2 (ambient=370 and elevated=700 ppm) on **a** net photosynthesis (P_n), **b** stomatal conductance to water vapor (G_s) **c** internal CO_2 concentration (C_i), **d** total chlorophyll content, **e** chlorophyll *a:b* ratio, and **f** soluble sugars of geranium roots. Each bar represents the mean (±1 SD) of three independent replicates. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

observed at medium B levels, but not at low or high B; in fact, in geranium at high B, elevated CO_2 decreased biomass relative to ambient CO_2 levels. In *Azolla*, elevated CO_2 stimulated growth and 0 and 10 μM B, but not at 1,000 μM B. Also, in geranium, peak biomass of leaves, stems, and whole-plants was

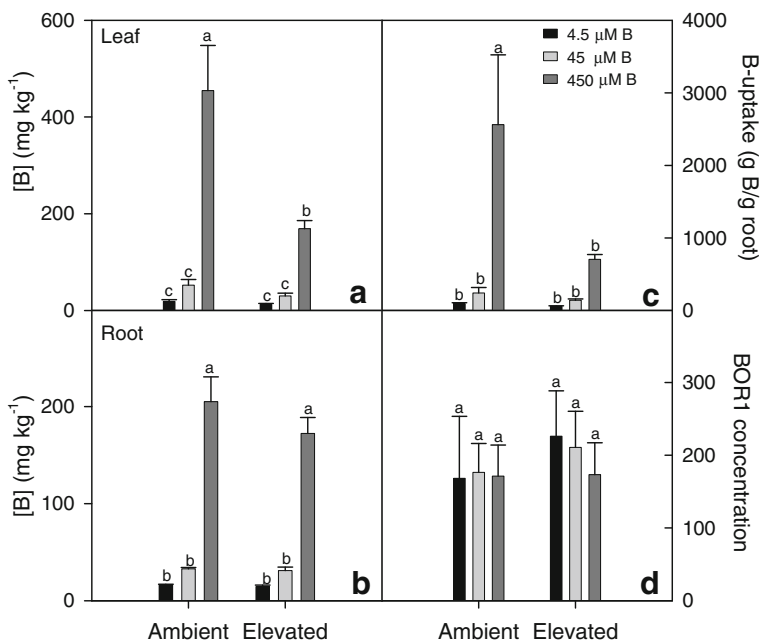

Fig. 3 Effect of B (4.5, 45, and 450 μM) and CO_2 (ambient=370 and elevated=700 ppm) in geranium on: **a**, **b** Boron concentration in leaf and roots, respectively (dry mass basis), **c** specific uptake rate of B (total g plant B/g dry roots), and **d** boron transporter protein (Bor1) concentration (per unit total protein, relative to a standard). Each bar represents the mean (± 1 SD) of three independent replicates. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

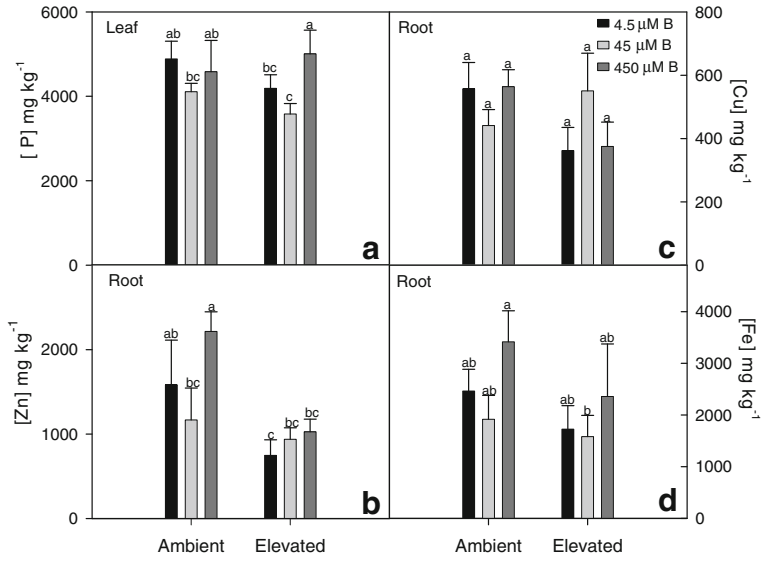

Fig. 4 Effect of B (4.5, 45, and 450 µM) and CO_2 (ambient=370 and elevated=700 ppm) on concentration (dry mass basis) of selected nutrients in shoots or roots of geranium. Shown are only those nutrients for which there were either significant B or B *x* CO_2 effects. Each bar represents the mean (± 1SD) of three independent replicates. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

observed at 450 µM at ambient CO_2, but at 45 µM B at elevated CO_2. In barley, peak mass was observed at 0 and 30 µM B in ambient CO_2, but at 30 µM B in elevated CO_2, while in *Azolla,* peak mass was observed at 10 µM B under both ambient and elevated CO_2.

In geranium, the pattern of B *x* CO_2 effects on biomass was not reflected in any other response variable measured, except for shoot:root biomass (i.e., the inverse pattern for root:shoot results shown), suggesting that the pattern of B *x* CO_2 effects on biomass was likely a result of effects on biomass allocation. Notably, the pattern of B *x* CO_2 effects on biomass was unrelated to tissue [B]. Hence, though low-B plants were smaller than medium B plants at high, but not low, CO_2, consistent with our *apriori* prediction that elevated CO_2 would increase the potential for B deficiency, this was not caused by simple effects on tissue [B]. In contrast, we predicted *apriori* that elevated CO_2 would decrease the potential for B toxicity at high B, but in fact, we observed the opposite, and elevated CO_2 caused a decrease in plant growth at high vs. medium B. Thus, in a future high-CO_2 world, B stress may become more prevalent, at

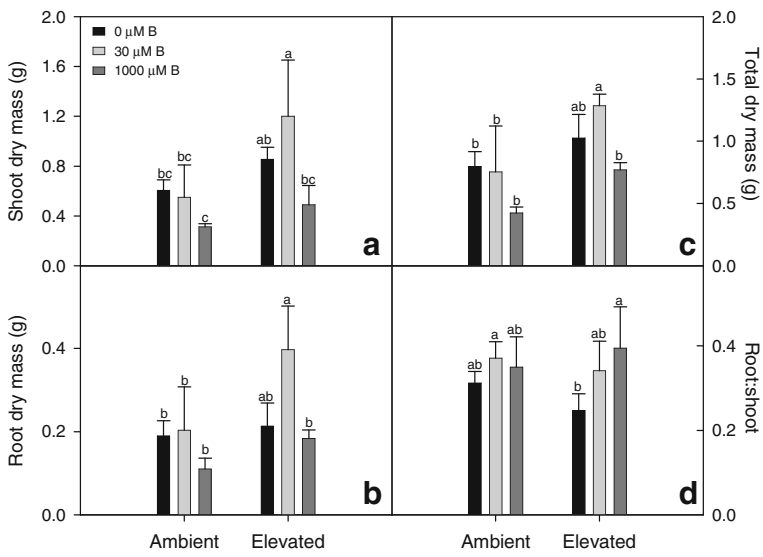

Fig. 5 Effect of B (0, 30, and 1,000 µM) and CO_2 (ambient=370 and elevated=700 ppm) on shoot and root mass of barley (*H. vulgare*). Two-week-old seedlings were grown hydroponically in complete nutrient solution with 30 µM B, and then subsets of plants were transferred to 0, 30, or 1,000 µM B. Each bar represents the mean (± 1 SD) of three independent replicates. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

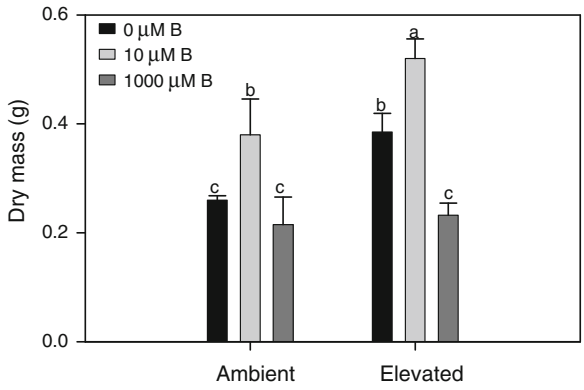

Fig. 6 Effect of B (0, 10, and 1,000 μM) and CO_2 (ambient= 370 and elevated=700 ppm) on total plant biomass of water fern (*Azolla caroliniana*). Plants were grown hydroponically for many generations in complete nutrient solution with 10 μM B, and then subsets of plants were transferred to 0, 10, or 1,000 μM B. Each bar represents the mean (± 1 SD) of four independent replicates. *P* values from ANOVA for treatments: *B*=<.0001, CO_2=<.0001 and B *x* CO_2=0.014. Within each variable, different letters above the bars indicate a significant difference among treatments ($P<0.05$)

both low-B and high-B stress, though the specific mechanism for this is not known.

The B *x* CO_2 effects on biomass allocation observed in this study are similar to those observed by Sicher (2005), where the same trend was seen in barley roots grown under different P levels and ambient vs. high CO_2. Exposure of plant canopies to high CO_2 concentration often stimulates the growth of both shoot and root, but the question remains whether elevated atmospheric CO_2 concentration will affect roots and shoots of crop plants proportionally. Since elevated CO_2 can induce changes in plant structure and function, there may be differences in allocation between root and shoot, at least under some conditions (Rogers et al. 1996). It is generally observed that root:shoot ratio responds to deficits in light (Boote 1976), water (Kramer and Boyer 1995), and major mineral nutrients (Cakmak et al. 1994; Gutschick 1993), with the root:shoot response to a given factor usually towards diverting dry weight to the plant part that is the most limiting to growth under prevailing environmental conditions (Wilson 1988). However, the effects of elevated atmospheric CO_2 on root-to-shoot are much less clear (Rogers et al. 1996). The response of root-to-shoot to elevated atmospheric CO_2 is highly variable among species. For example, there were significant increases in root-to-shoot for soybean (*Glycine max*; Rogers et al. 1992) and in *Quercus alba* L. seedlings (Norby et al. 1986) exposed to elevated CO_2, while in cotton *(Gossypium hirsutum)* grown under field conditions, root:shoot mass appeared to be unaffected by CO_2 concentration (Prior et al. 1994).

Though increases in photosynthesis and growth are typical under elevated vs. current CO_2 for most C_3 species, decreases in tissue nutrient concentrations often occur too, and not just for nitrogen (e.g., Cure and Acock 1986; Sicher and Bunce 1999; Vandermeiren et al. 2002; Norby et al. 1986; Roberntz and Stockfors 1998; Fangmeier et al. 1996; Luomala et al. 2005). The increase in biomass under elevated CO_2 is largely attributed to increases in net photosynthesis and nutrient limitation has generally been found to suppress this response (Conroy 1992; McKee and Woodward, 1994; Lloyd and Farquhar, 1996; Stitt and Krapp, 1999). For examples, when birch (*Betula pendula*; Pettersson et al. 1993; Silvola and Ahlholm 1995), loblolly pine (*Pinus taeda*; Gebauer et al. 1996), rice (*Oryza sativa*; Ziska et al. 1996), cotton (*Gossypium hirsutum*; Rogers et al. 1993), wheat (*Triticum aestivum*; Rogers et al. 1996), and tobacco (*Nicotiana tabacum*; Geiger et al. 1999) were grown at various N supplies, elevated CO_2 led to large increases of biomass at the highest N supply, small increases at a moderately limiting N supply, and no increase, or even a slight decrease, at the lowest N supply. Therefore, nutrient supply and, consequently, the nutrient status of plants should be a critical factor determining growth responses to the elevated CO_2.

In this study, growth at elevated CO_2 led to lower tissue B concentrations in geranium, though this was statistically significant only in leaves at the highest B level, and to decreases in B uptake rate. Decreases in [B] with growth under elevated CO_2 have also been observed in most (Peñuelas et al. 2001; Norby et al. 1986; O'Neill et al. 1987; Liu et al. 2007), but not all (Peñuelas et al. 1997; Luomala et al. 2005), previous studies wherein B was measured. Decreases in the uptake rate of B at high CO_2 in this study were unrelated to the presence or absence of high-CO_2-stimulation of growth, and so are unlikely to be linked to total plant demand for B. Further, B uptake rates decreased with elevated CO_2 despite that fact that B concentrations in geranium leaves decreased under high CO_2 to levels approaching B deficiency at 4.5 and 45 μM B (Blevins and

Lukaszewski 1998; El-Shintinawy 1999; Wimmer et al. 2005). High-CO_2-related decreases in B uptake rate were also unrelated to levels of expression of the B transport protein, BOR1, since BOR1 levels were unaffected by B and increased slightly at elevated CO_2 (4.5 and 45 μM B). In contrast, Jin et al. (2009) recently reported that several Fe transporter genes were up-regulated more under elevated than current CO_2 levels in tomato plants grown under iron deficiency conditions. Thus, the reason that B levels decreased at elevated CO_2 in this study remain unknown. However, we did not examine effects of B and CO_2 on levels of the other known major B transport protein, Nip5;1, and it is possible that this protein responds differently than BOR1.

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