

Effect of drought and carbon dioxide on nutrient uptake and levels of nutrient-uptake proteins in roots of barley

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PREMISE: Atmospheric carbon dioxide (CO₂) concentration is increasing, as is the frequency and duration of drought in some regions. Elevated CO₂ can decrease the effects of drought by further decreasing stomatal opening and, hence, water loss from leaves. Both elevated CO₂ and drought typically decrease plant nutrient concentration, but their interactive effects on nutrient status and uptake are little studied. We investigated whether elevated CO₂ helps negate the decrease in plant nutrient status during drought by upregulating nutrient-uptake proteins in roots.

METHODS: Barley (*Hordeum vulgare*) was subjected to current vs. elevated CO₂ (400 or 700 ppm) and drought vs. well-watered conditions, after which we measured biomass, tissue nitrogen (N) and phosphorus (P) concentrations (%N and %P), N- and P-uptake rates, and the concentration of the major N- and P-uptake proteins in roots.

RESULTS: Elevated CO₂ decreased the impact of drought on biomass. In contrast, both drought and elevated CO₂ decreased %N and %P in most cases, and their effects were additive for shoots. Root N- and P-uptake rates were strongly decreased by drought, but were not significantly affected by CO₂. Averaged across treatments, both drought and high CO₂ resulted in upregulation of NRT1 (NO₃⁻ transporter) and AMT1 (NH₄⁺ transporter) per unit total root protein, while only drought increased PHT1 (P transporter).

CONCLUSIONS: Elevated CO₂ exacerbated decreases in %N and %P, and hence food quality, during drought, despite increases in the concentration of nutrient-uptake proteins in roots, indicating other limitations to nutrient uptake.

KEY WORDS carbon dioxide; climate change; drought; Gramineae; nutrient uptake; nutrient-uptake protein; Poaceae; roots.

The atmospheric concentration of greenhouse gases is increasing due to human activity, including carbon dioxide (CO₂), which has increased by >40% since pre-industrial times and may reach 500–900 ppm by the end of this century (IPCC, 2014). As atmospheric CO₂ concentration increases, the Earth's surface temperature will increase, which will result in increases in the frequency and duration of drought in some regions of the world (Gutschick and BassiriRad, 2003; Pereira et al., 2006; IPCC, 2014). This increase in drought will negatively impact humanity, in part, by decreasing crop productivity at a time when human population is increasing (MEA, 2005).

Increases in CO₂ increase plant growth and reproduction by enhancing photosynthesis via decreases in photorespiration (C₃ plants) and/or by decreasing water use via decreases in stomatal opening (C₃ and C₄ plants) (Bowes, 1991; Drake et al., 1997; Makino and Mae, 1999; Poorter and Pérez-Soba, 2001; Lambers et al., 2008;

Leakey et al., 2009; DaMatta et al., 2010; Prior et al., 2011). However, elevated CO₂ levels compared to present levels cause a decrease in tissue nutrient concentration (% dry mass) in most plant species, especially for nitrogen (N) in C₃ non-legume species (Lüscher et al., 2000; Jablonski et al., 2002; Taub and Wang, 2008; Bloom et al., 2010; Myers et al., 2014). Multiple mechanisms likely cause the decrease in plant %N with elevated CO₂, including a dilution effect driven by increased carbohydrate production at high CO₂ (Taub and Wang, 2008) and an inhibition of shoot nitrate assimilation at high CO₂ (Bloom et al., 2002, 2010, 2014). Since elevated CO₂ decreases stomatal opening and water loss, it is anticipated that elevated CO₂ might mitigate the effects of drought on plants by decreasing stomatal conductance below that caused by drought alone, while increasing photosynthesis in drought-stressed plants.

Plants respond to decreases in water availability by decreasing stomatal conductance to slow the rate of water loss, which causes a

decrease in photosynthesis (Bradford and Hsiao, 1982; Christophe et al., 2011). The impact of a drought depends on its intensity and duration and when it occurs during the life cycle of the plant (Bradford and Hsiao, 1982). In most crops, drought has its most pronounced effects on yield when it occurs during the flowering or grain-filling stages (Meckel et al., 1984; Araus et al., 2002; Thomas et al., 2004). Drought also has a strong impact on plant nutrient relations. A recent meta-analysis demonstrated that drought stress typically decreases the concentration of N and P in plant tissues (He and Dijkstra, 2014), and several studies have shown that drought can decrease nutrient uptake from soil (Cramer et al., 2009; Waraich et al., 2011; Ge et al., 2012; Sardans and Peñuelas, 2012). Decreases in nutrient uptake during drought may occur for several reasons, including a reduction in nutrient supply by decreasing mineralization (Fierer and Schimel, 2002; Schimel et al., 2007; Sanaullah et al., 2012) and by reducing diffusion and mass flow of nutrients in the soil (Lambers et al., 2008).

Many studies have shown that elevated CO₂ can minimize or delay the effects of drought on plants, including on photosynthesis. Elevated CO₂ decreases stomatal opening (conductance) and water loss from leaves (transpiration), and increases photosynthesis in C₃ plants; hence, elevated CO₂ increases plant water-use efficiency (WUE) (Bunce, 1998; Curtis and Wang, 1998; Burkart et al., 2004; Ainsworth and Rogers, 2007; Robredo et al., 2007; Leakey et al., 2009; Manderscheid et al., 2014). Elevated CO₂ might also increase tolerance to drought by lowering plant osmotic potential, thereby maintaining higher pressure potential (Tyree and Alexander, 1993). In terms of nutrient relations, few studies have investigated the interaction between elevated CO₂ and water stress. For example, Robredo et al. (2011) observed that elevated CO₂ reduced the effects of water stress on nitrate reduction, ammonium assimilation, and protein content.

Most plants obtain most of their mineral nutrients from soil using nutrient-uptake proteins located in the cellular membranes of roots. For example, in most plants, most N is taken up as inorganic nitrate (NO₃⁻) or ammonium (NH₄⁺), with lesser amounts taken up as amino acids or urea (Lambers et al., 2008; Nacry et al., 2013). Nitrate is taken up by members of the NRT1 and NRT2 families of transporters (NRT1: low-affinity NO₃⁻ transporters at high N levels; NRT2: high-affinity NO₃⁻ transporters at low N levels), and NH₄⁺ is taken up by the high-affinity AMT1 group (Nacry et al., 2013). Most phosphorus (P) is taken up by roots via the activity of PHT1-type transport proteins (Nussaume et al., 2011). Both drought and elevated CO₂ can alter nutrient uptake by affecting the kinetics of nutrient uptake by roots (BassiriRad, 2000). For example, studies have reported that whole-plant nutrient uptake is increased for many species under elevated CO₂ (Fangmeier et al., 2002; Shimono and Bunce, 2009); however, the effects of elevated CO₂ on the rate of nutrient uptake per unit root is variable (BassiriRad, 2000). In contrast, most previous studies have found that drought decreases the rate of nutrient uptake per unit root (Bradford and Hsiao, 1982; Christophe et al., 2011; Roupheal et al., 2012). For both CO₂ and drought, little is known about how they affect expression of nutrient-uptake proteins and how this relates to nutrient uptake (e.g., Bista et al., 2018, Jayawardena et al., 2017, and references therein).

In contrast to elevated CO₂ and drought singly, very little is known regarding the interactive effects of elevated CO₂ and drought on plant nutrient uptake (BassiriRad, 2000). To improve our basic understanding of plant responses to CO₂ and drought in combination, we investigated the effects of CO₂ and drought on the

concentration of nutrient-uptake proteins in roots, and how this relates to effects on nutrient-uptake rate and tissue nutrient concentration, using barley (*Hordeum vulgare* L., Poaceae) as a model. We then conducted a follow-up experiment with summer squash (*Cucubita pepo* L., Cucurbitaceae) to confirm our findings in barley, regarding drought × CO₂ effects on nutrient concentration. We tested two main hypotheses: (1) elevated CO₂ will mitigate the negative impacts of drought on the concentration of N and P in plant tissue, and (2) the effects of elevated CO₂ and drought, singly and in combination, on %N and %P will be correlated with the rates of N and P uptake by roots, which will be correlated with the concentrations of the major N- and P-uptake proteins in roots.

MATERIALS AND METHODS

Plant material, growth conditions, and harvesting

Hordeum vulgare L. (barley) was used in this study, because it is an agriculturally important drought-sensitive C₃ grass that has been extensively studied in terms of nutrient relations. Seeds were germinated in the greenhouse in trays containing calcined clay and were watered daily. After reaching the two-leaf stage, seedlings were transplanted to large pots (10-cm diameter × 50-cm depth, with mesh bottoms) containing calcined clay, with pots placed in individual shallow trays (15 × 15 × 3 cm). Thereafter, plants were fertilized with quarter-strength nutrient solution [full-strength = 4.5 mM KNO₃, 0.5 mM NH₄NO₃, 2.5 mM Ca(NO₃)₂, 1 mM MgSO₄, 0.5 mM KH₂PO₄, 100 μM diethylenetriaminepentaacetic acid, 20 μM H₃BO₃, 5 μM MnCl₂, 2.5 μM ZnSO₄, 1 μM CuSO₄, 0.1 μM Na₂MoO₄; pH 6.0]. The temperature of the greenhouse was maintained at 30°/23° ± 2°C (day/night), and light levels ranged from 200 to 1800 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR), depending on cloud cover, with a 15-h photoperiod. When light intensity went below 300 μmol m⁻² s⁻¹ PAR, supplemental lighting (ca. 200 μmol m⁻² s⁻¹ PAR) was automatically provided from lamps (250 W high-pressure sodium; Lucalox, General Electric, Boston, MA, USA) and 400 W metal-halide (Metalarc, Wilmington, MA, USA).

Five days after transplanting into pots, plants were moved to growth chambers (one per each of the four treatments) and allowed to acclimate for 24 h to the following chamber conditions: 500 μmol m⁻² s⁻¹ PAR, 14-h (0600–2000 hours) photoperiod, 28°/23°C (day/night) temperatures, and ambient CO₂ (400 ppm). The CO₂ concentration of the high-CO₂ chambers was increased to 700 ppm after 24 h, at which time plants were provided nutrient solution (full strength, as above) in sufficient volume to completely fill the soil pore space. Drought was imposed by withholding water, and the rate and severity of drought stress were controlled by daily monitoring of plant + soil + pot mass and stomatal conductance, and then replacing only 75% of the water that was lost the previous day. Imposing drought with this method (as done by Heckathorn et al., 2014; Bista et al., 2018) allowed plants to be exposed to a gradually increasing drought stress of duration similar to that in a field situation, wherein plants have the opportunity to acclimate. With this technique, we were able to extend the drought treatment to ca. 2 weeks (mimicking a natural drought) and in such a way that stomatal conductance was reduced by ca. 50% after 1 week. Before beginning the drought treatment, four randomly selected plants were harvested to serve as “time-zero” controls. Plants were harvested after 16 d of drought treatment. Plants were rotated within their

chambers every 2 days to avoid position effects and were switched between the two low-/high- CO_2 chambers every 7 d to avoid chamber effects. At harvest, plants ($n = 5\text{--}6$ per treatment combination) were separated into shoots and roots, and roots were rinsed with deionized water. The root system was divided longitudinally into halves, and one half was weighed (to determine fresh mass) and then oven-dried for nutrient analysis, while the other half was used for protein analysis. Dry mass of shoots and roots was determined after oven drying for 72 h at 65°C . Fresh root tissue was immediately frozen in liquid N_2 and stored at -80°C ; dry mass of this tissue was estimated from fresh-to-dry mass ratio of the other half of the root system.

To determine whether effects of drought and CO_2 on %N observed in barley were unique, we conducted a follow-up experiment with squash (*Cucurbita pepo*). In this experiment, plants were grown and treated as above, except for using a 50:50 mix of top soil and calcined clay and growing plants in 13×13 cm plastic pots. Then, the concentration of total protein in shoots was determined, rather than %N, since protein concentration is more relevant to human nutrition.

Daily measurements of leaf stomatal conductance to water vapor (G_s) and net photosynthesis (P_n ; net CO_2 assimilation) were used to monitor progress of the drought and the eventual extent to which drought reduced G_s and P_n during the experiment. At the same time of day (1200–1300 hours), G_s and P_n were measured on 3–5 random plants using a portable photosynthesis system with infrared gas analyzer equipped with a 6-cm^2 leaf chamber and CO_2 , light, and temperature control (Model 6400, LiCOR Biosciences, Lincoln, NE, USA). Gas-exchange measurements were made within 1 min of enclosing leaves within the cuvette and after gas-exchange rates stabilized. For P_n , recently expanded, illuminated attached leaves were measured while maintaining CO_2 at 400 ppm and light intensity at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. Immediately before harvest, plant water status, measured as xylem pressure potential (ψ_{leaf}) was determined with a pressure chamber.

Nutrient and protein analysis

The concentration of C and N in homogenized plant tissue (% dry mass) was determined by mass spectrometry (MS), while P was measured by inductively coupled plasma–optical emission spectroscopy (ICP-OES), as explained by Mishra et al. (2009). Nutrient concentrations were then used to determine total nutrient amounts in the plants (Total amount in plant = Tissue concentration \times Tissue mass, for shoot + root). Nutrient-uptake rates per gram of root dry mass were determined from the increase in total plant nutrient content from one harvest to the next, divided by grams of root dry mass at the later harvest, divided by days between harvests. For example, N uptake rate per gram of dry root on day 16 harvest was determined as N uptake rate (mg g^{-1} dry root day^{-1}) = [(Total plant N content on day 16 – Total plant N content on day 0) / Root dry mass on day 16] / 16 days.

For protein analysis, total root protein was extracted by grinding fresh tissue (0.5–1 g) in liquid N_2 in a mortar and pestle and then in 2 to 4 mL of extraction buffer (0.2 M Tris pH 8, 1% v/v SDS, 0.7 M sucrose, 5 mM EDTA, 2% β -mercaptoethanol, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride), as per Mishra et al. (2012). The homogenate was then mixed with an equal volume of phenol and incubated for 15 min at room temperature on ice, with occasional vortexing. Samples were then centrifuged at $10,000 \times g$ for

10 min at 4°C to separate the aqueous and organic phases. The upper phenol phase was recovered, washed with an equal volume of extraction buffer, and again centrifuged as above. The supernatant was then stored overnight at -20°C in 5 volumes of chilled 0.1 M ammonium acetate, after which, root proteins were pelleted by centrifugation. The pellets were washed twice with ammonium acetate, once with 80% acetone, and then twice with 100% acetone. Finally, the pellets were dried under room temperature and re-solubilized 62.5 mM Tris pH 6.8, 0.5% v/v SDS, and 20% v/v glycerol. Total root protein concentration per gram of fresh root was determined using a colorimetric assay (DC protein assay, Bio-Rad, Hercules, CA, USA), using BSA to generate standard curves.

Using protein-specific antibodies (see detailed description below) and enzyme-linked immunosorbent assay (ELISA), we determined the relative concentrations (per unit total root protein) of specific nutrient-uptake proteins (NRT1, AMT1, and PHT1). For ELISA, equal total root protein was loaded per well, background from nonspecific binding was subtracted using pre-immune serum, and all samples were relativized to a standard root-protein extract. Primary antibodies were detected colorimetrically, using a secondary antibody conjugated to alkaline phosphatase. Using root fresh-to-dry mass ratio, the relative concentration of specific nutrient-uptake proteins per unit total root protein was converted to concentration per gram of root dry mass. Then, the apparent relative uptake rate per transporter was determined from nutrient uptake rate per gram of root and the relative concentration of transporter per gram of root (i.e., Nutrient uptake rate per gram of root = Concentration of nutrient-uptake protein per gram of root \times Rate per transporter). Uptake rate per transporter was estimated only for PHT1, since PHT1, a phosphate transporter, is the main P-uptake protein in roots, that roots take up P primarily as phosphate (Lambers et al., 2008), and P was provided only as phosphate

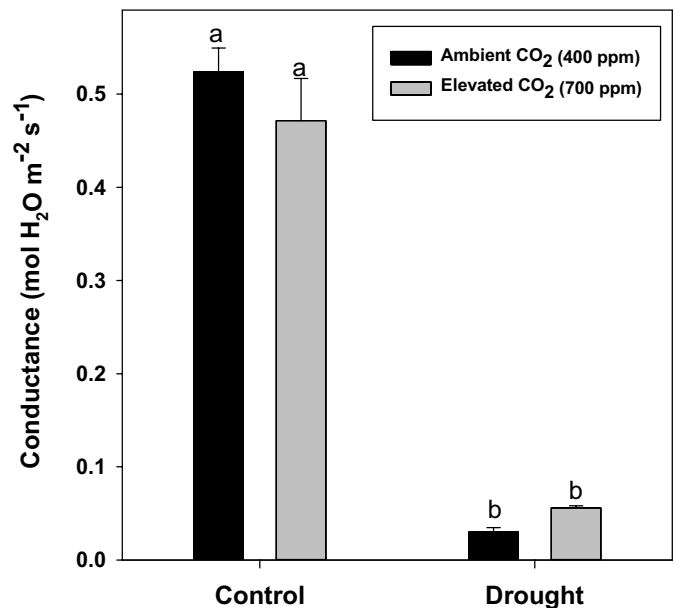
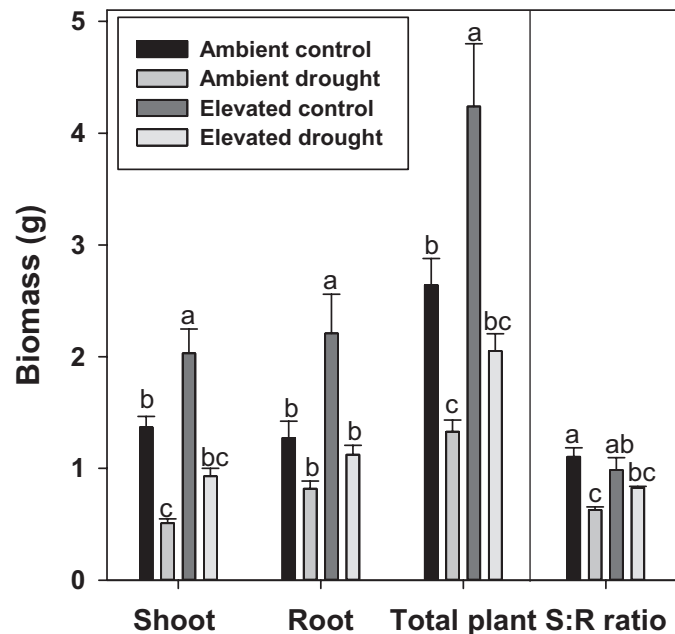


FIGURE 1. Effects of control vs. drought treatments and ambient vs. elevated CO_2 on leaf stomatal conductance to water vapor (G_s) in barley (*Hordeum vulgare*) at the final harvest (i.e., end of drought treatment). Each bar represents mean ± 1 SE. Means not sharing the same letters differed significantly.

TABLE 1. Results (*P*- and *F*-values) from ANOVA statistical analysis of data from barley.

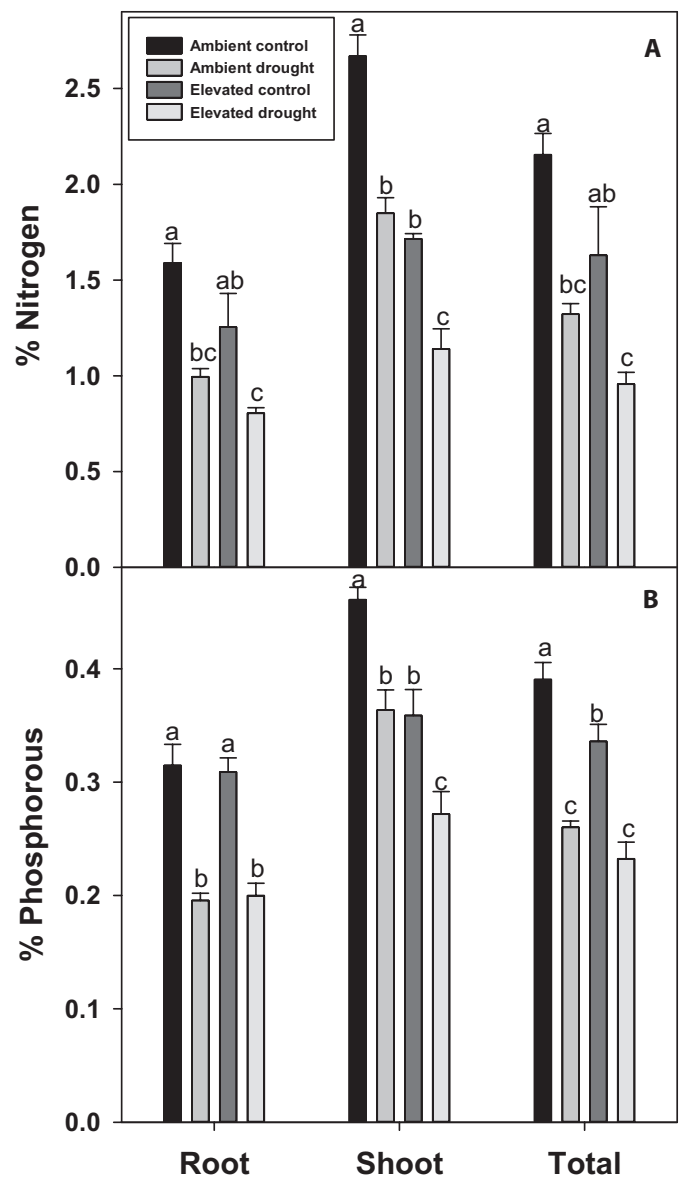
Variable	<i>P</i> -values			<i>F</i> -values
	Drought	CO ₂	D × CO ₂	
Conductance	<0.0001	0.5777	0.1125	$F_{3,15} = 131.60$
Shoot dry mass	<0.0001	0.0002	0.3098	$F_{3,18} = 30.84$
Root dry mass	0.0005	0.0030	0.0986	$F_{3,18} = 10.61$
Total plant dry mass	<0.0001	0.0009	0.1530	$F_{3,18} = 17.53$
Shoot-to-root mass	<0.0001	0.5450	0.0228	$F_{3,18} = 10.54$
Root %N	<0.0001	0.0134	0.4563	$F_{3,18} = 12.48$
Shoot %N	<0.0001	0.0003	0.9036	$F_{3,18} = 16.23$
Total plant %N	<0.0001	0.0035	0.5561	$F_{3,18} = 14.48$
Root %P	<0.0001	0.9497	0.6871	$F_{3,18} = 28.77$
Shoot %P	0.0001	<0.0001	0.7868	$F_{3,18} = 17.16$
Total plant %P	<0.0001	0.0050	0.3118	$F_{3,18} = 31.03$
N-uptake rate	<0.0001	0.7617	0.0539	$F_{3,18} = 58.51$
P-uptake rate	<0.0001	0.9127	0.0049	$F_{3,18} = 92.85$
Total root protein	<0.0001	0.0250	0.1462	$F_{3,18} = 18.86$
NRT1/unit root protein	0.0024	0.0166	0.7494	$F_{3,13} = 8.34$
NRT1/g dry mass	0.8079	0.0013	0.8006	$F_{3,9} = 7.15$
AMT1/unit root protein	0.0094	0.0144	0.5210	$F_{3,13} = 6.39$
AMT1/g dry mass	0.0732	0.4047	0.8514	$F_{3,13} = 1.39$
PHT1/unit root protein	0.0049	0.7871	0.2733	$F_{3,13} = 4.48$
PHT1/g dry mass	0.0014	0.0021	0.0014	$F_{3,14} = 12.70$
PHT1 activity	<0.0001	0.0668	0.8825	$F_{3,13} = 24.31$

**FIGURE 2.** Effects of control vs. drought treatments and ambient vs. elevated CO₂ on shoot, root, and total plant dry mass, and shoot to root (S:R) mass ratio in barley. Each bar represents mean + 1 SE. Means not sharing the same letters differed significantly.

in this study. Since the plants in this study were provided N in both NO₃⁻ and NO₄⁺ forms, and the amount or proportion of N taken up as NO₃⁻ vs. NO₄⁺ is unknown, we cannot estimate the relative uptake rate per unit protein for NRT1 or AMT1.

For each nutrient-uptake protein, rabbit polyclonal antiserum was generated using oligopeptide antigens of conserved domains that were

designed using bioinformatics, as described in detail by Jayawardena et al. (2017). The oligopeptide antigens were TGGLKSSVSGFGSDQFDES for NRT1, KLLRISAEDMAGMDLTRH for AMT1, and GDYPLSATIMSEYANKKTRG for PHT1. These peptide sequences are highly conserved across species, including in barley, and within a species, they are found in most isoforms of each uptake-protein type, especially the most abundant and important isoforms in roots (i.e., NRT1.1 and NRT 1.2, AMT1.1 and AMT1.2, and PHT1.1-1.4) (Lauter et al., 1996; Von Wiren et al., 2000; Nussaume et al., 2011; Nacry et al., 2013). The specificity of antisera was confirmed by western blotting, comparing results for pre-immune vs. immune sera, antigen-purified vs. crude sera, and against tomato and *Arabidopsis*

**FIGURE 3.** Effects of control vs. drought treatments and ambient vs. elevated CO₂ on (A) %N and (B) %P per dry mass in root, shoot, and total plant tissue in barley. Each bar represents mean + 1 SE. Within each panel, means not sharing the same letters differed significantly.

thaliana samples (species for whom the molecular masses of the above nutrient-uptake proteins are known).

Statistical analyses

Data were analyzed using two-way (two levels of CO₂ × two levels of drought) ANOVA, with CO₂ and drought levels as fixed factors ($n = 5-6$). Tukey's post-hoc test was used for multiple comparisons if ANOVA results were significant for at least one factor or their interaction; unless otherwise indicated, results were considered significant if $P < 0.05$. Analyses were conducted using JMP 12 software (SAS Institute, Cary, NC, USA).

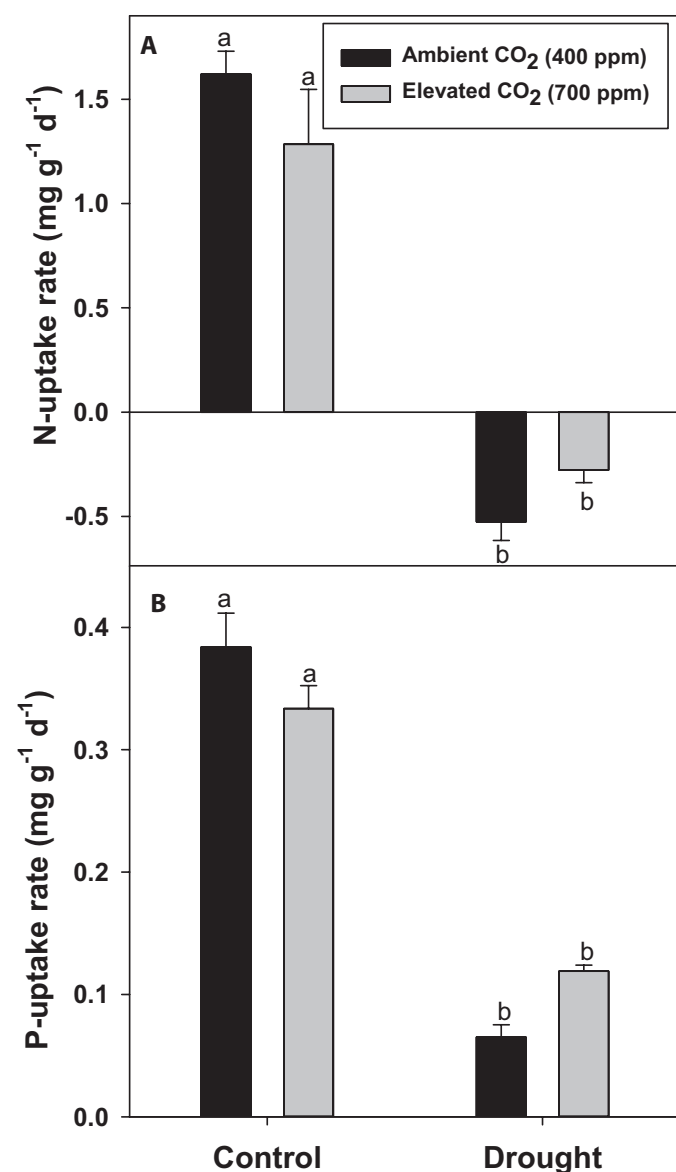


FIGURE 4. Effects of control vs. drought treatments and ambient vs. elevated CO₂ on uptake rate per dry mass of roots for (A) N and (B) P in barley. Each bar represents mean + 1 SE. Within each panel, means not sharing the same letters differed significantly.

RESULTS

After 16 days of drought, stomatal conductance was reduced by 94% and 89% for ambient and high CO₂ treatments, respectively (Fig. 1, Table 1). Leaf water potential (MPa, mean + 1 SE) at the end of drought was $-0.43 + 0.02$ and $-0.34 + 0.02$ for control treatments (400 and 700 ppm CO₂, respectively) and $-1.68 + 0.03$ and $-1.28 + 0.05$ for drought treatments (400 and 700 ppm CO₂, respectively) (data not shown). Both shoot and root dry biomass, and, hence, total plant biomass were affected by drought and CO₂, but there were no significant interactive effects (Fig. 2, Table 1). In general, drought decreased biomass and elevated CO₂ increased biomass, including during drought. As a consequence of large reductions in shoot mass, but smaller reductions in root mass, drought decreased the shoot-to-root biomass ratio, especially in plants exposed to 400 ppm CO₂ (Fig. 2, Table 1).

In general, or averaged across treatments, both drought and elevated CO₂ decreased shoot, root, and total plant %N, and %N was lowest in the drought + high-CO₂ treatment (Fig. 3A, Table 1); there were no interactive effects on %N. Similarly, both drought and elevated CO₂ decreased shoot and total plant %P, while only drought affected root %P, and %P was lowest in shoots in the drought + high-CO₂ treatment (Fig. 3B, Table 1). The uptake rates of N and P by roots were decreased strongly by drought (and were negative during drought, indicating a net loss of N from plants). Elevated CO₂ did not significantly affect N and P uptake, though high CO₂ tended to decrease uptake in well-watered plants and increase uptake in drought-stressed plants (Fig. 4A and B, Table 1).

Drought (averaged across CO₂ treatments), and to a lesser extent CO₂ (averaged across drought treatments), significantly decreased the concentration of total protein in roots (Fig. 5, Table 1). In contrast to total root protein concentrations, the concentrations of NRT1, AMT1, and PHT1 per unit total root protein were significantly

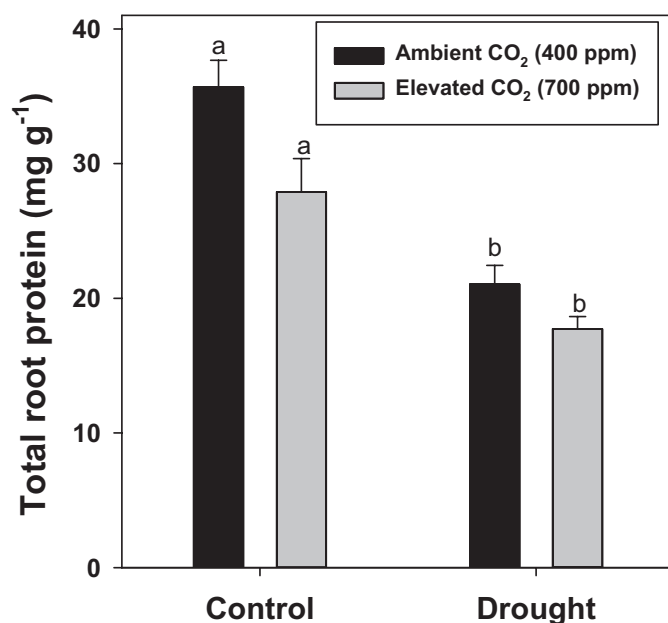


FIGURE 5. Effects of control vs. drought treatments and ambient vs. elevated CO₂ on the concentration of total protein per dry mass of roots in barley. Each bar represents mean + 1 SE. Means not sharing the same letters differed significantly.

upregulated by drought and high CO₂, excluding PHT1 with high CO₂ (Fig. 6, Table 1). Consequently, the concentration of AMT1 per gram root mass did not change significantly with drought or CO₂, NRT1 per gram root increased modestly with high CO₂ (with no drought effect), and PHT1 per gram root decreased with high CO₂ in well-watered plants only (Fig. 6, Table 1). In contrast, the relative activity of PHT1 decreased significantly with drought only, though PHT1 activity increased modestly with high CO₂ (marginally significant) (Fig. 7, Table 1).

In the follow-up study with squash, drought significantly decreased total plant fresh mass, while elevated CO₂ increased it slightly in both control and drought conditions (not shown). As with %N in barley, in squash, drought and elevated CO₂ both decreased the concentration of total protein in shoots ($P = 0.09$ and 0.10 , respectively), and protein concentration was lowest in the drought \times high-CO₂ treatment (Fig. 8).

DISCUSSION

As greenhouse gases, mainly CO₂, cause further climate warming, many parts of the globe are expected to experience increases in evapotranspiration or decreases in precipitation, leading to increased drought (IPCC, 2014). Both drought and elevated CO₂ are known to decrease the concentration of N and P in plant tissues (Taub and Wang, 2008; He and Dijkstra, 2014; Myers et al., 2014). In the present study, we tested two main hypotheses: (1) elevated CO₂ will mitigate the negative impacts of drought on the concentration of N and P in plant tissue, and (2) the effects of elevated CO₂ and drought, singly and in combination, on %N and %P will be correlated with rates of N and P uptake by roots, which will be correlated with concentrations of the major N- and P-uptake proteins in roots. To our knowledge, this is the first study to quantify the effects of drought on the levels of nutrient-uptake proteins in roots during drought and elevated CO₂.

In barley in this study, drought significantly decreased shoot and root biomass, and decreased shoot-to-root mass ratio at ambient CO₂. Meanwhile, elevated CO₂ increased biomass similarly in both well-watered controls and drought-stressed plants. Hence, elevated CO₂ partially ameliorated the negative impact of drought on plant growth. In contrast, both drought and elevated CO₂ decreased %N and %P (barley) or total protein concentration (squash), and there were additive negative effects of drought and elevated CO₂ in barley and squash. Further, while drought strongly decreased N- and P-uptake rates of roots, elevated CO₂ slightly increased these rates, and elevated CO₂ tended to increase the concentration of the major N-uptake proteins (NRT1, AMT1) and the activity of the major P-uptake protein, PHT1. Hence, elevated CO₂ did not ameliorate the impact of drought on %N and %P, and the impacts of drought and elevated CO₂ on %N and %P were not often correlated with nutrient-uptake rates or concentrations of nutrient-uptake proteins in roots. These results indicate that nutrient uptake was limited during drought by something other than levels of nutrient-uptake proteins.

Drought can decrease the rate of nutrient uptake by roots, such as by decreasing soil nutrient availability or decreasing the mass flow of nutrients to roots, and it has been hypothesized that drought can decrease nutrient uptake due to decreases in the activity or expression of nutrient-uptake proteins in roots (Bradford and Hsiao, 1982; Schimel et al., 2007; Christophe et al., 2011; Roupahel et al., 2012; Sanuallah et al., 2012). In this study, as in others, drought decreased N- and P-uptake rates per gram of root. Meanwhile, drought upregulated NRT1,

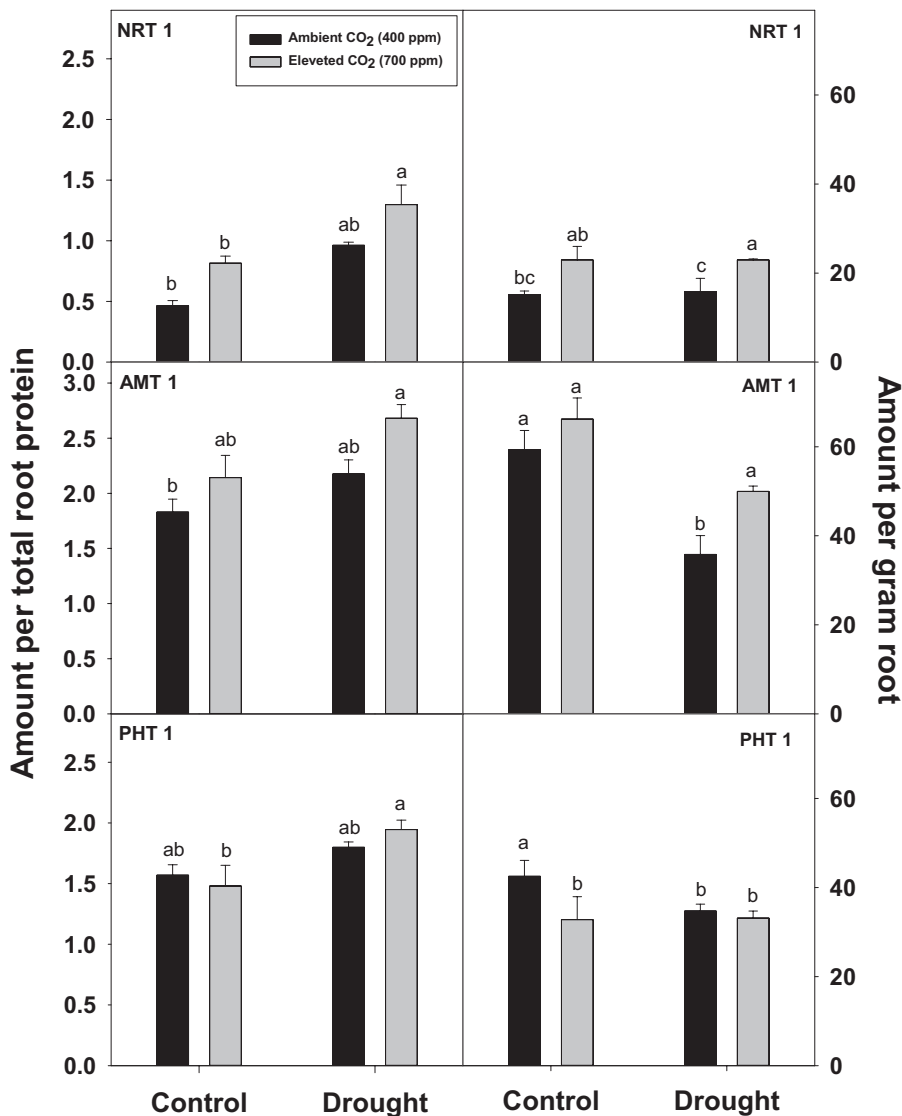


FIGURE 6. Effects of control vs. drought treatments and ambient vs. elevated CO₂ on the relative concentration of the nitrate-uptake protein, NRT1, the ammonium-uptake protein, AMT1, and the phosphate-uptake protein, PHT1, of roots in barley. Each bar represents mean + 1 SE. Within each panel, means not sharing the same letters differed significantly.

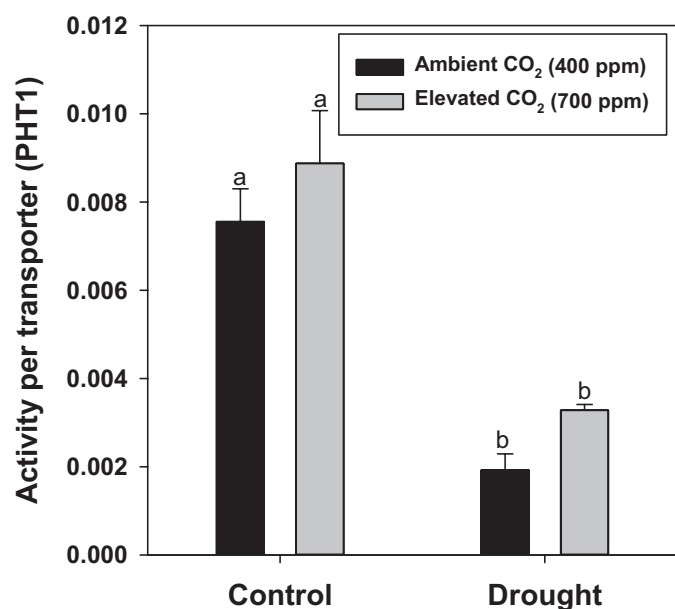


FIGURE 7. Effect of control vs. drought treatments and ambient vs. elevated CO₂ on the relative activity of the phosphate-uptake protein, PHT1, of roots in barley. Each bar represents mean + 1 SE. Means not sharing the same letters differed significantly.

AMT1, and PHT1 per unit total root protein, which is consistent with the results of (1) Wang et al. (2017), who observed increased mRNA levels of NRT1.2, NRT2.5, AMT1.1, and AMT1.3 in roots of corn during drought, and (2) Bista et al. (2018), who observed upregulation of NRT1, AMT1, and PHT1 per total root protein in barley, corn, and big bluestem (*Andropogon gerardii*) during drought. Perhaps upregulation of nutrient-uptake proteins

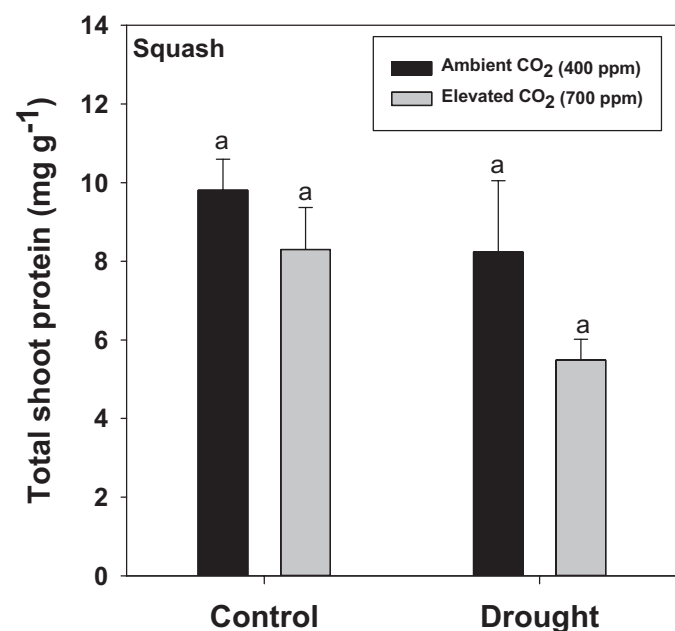


FIGURE 8. Effect of control vs. drought treatments and ambient vs. elevated CO₂ on the concentration of total protein (per gram fresh mass) in shoots of squash (*Cucubita pepo*). Each bar represents mean + 1 SE. Means not sharing the same letters differed significantly.

per unit total protein during drought is a response by the plant to try to increase nutrient-uptake rates. However, since drought reduced the concentration of total protein per gram of roots in our study, the concentration of NRT1 and AMT1 per gram of root did not increase with drought, while PHT1 even decreased. Drought also strongly decreased the relative activity (uptake rate) per PHT1 transporter. Although we could not calculate the effect of drought on the activity of NRT1 and AMT1 separately, since N-uptake rates per gram of root decreased with drought, but levels of NRT1 and AMT1 per gram of root did not change significantly, then it is likely that the activities of NRT1 and AMT1 decreased with drought, too. The cause of the decrease in activity of N- and P-uptake proteins with drought is unknown, but might include damage to the nutrient-uptake proteins or decreased concentration of N and P at the root surface due to drying soil.

Elevated CO₂ helps ameliorate the effect of drought on plants by decreasing plant water loss, thus delaying the onset of drought (Bunce, 1998; Curtis and Wang, 1998; Burkart et al., 2004; Ainsworth and Rogers, 2007; Robredo et al., 2007; Leakey et al., 2009; Oliver et al., 2009; Manderscheid et al., 2014). In this study, elevated CO₂ modestly increased (though usually not significantly) N- and P-uptake rates, the concentration of NRT1 and AMT1 per unit total root protein and per gram of root, and the relative activity of PHT1 during drought. Consistent with these results, past studies have observed similar effects of elevated CO₂ upregulating the expression of genes encoding nutrient-uptake proteins (Jin et al., 2009; Lekshmy et al., 2009; Vincente et al., 2016). In the case of NO₃⁻ uptake, elevated CO₂ increased mRNA levels of N-uptake proteins in wheat roots, especially at high vs. low NO₃⁻ (Lekshmy et al., 2009; Vincente et al., 2016). Elevated CO₂ also increased mRNA for several Fe-uptake proteins in tomato roots (Jin et al., 2009). At the protein level, elevated CO₂ had inconsistent effects on expression of nutrient-uptake proteins in tomato (Jayawardena et al., 2019; Vicente et al., 2016).

With climate change, the frequency, duration, and intensity of drought will increase in many regions of the earth. Elevated CO₂ will help combat drought to an extent, by lessening the negative impact of drought on plant growth, but this study indicates that elevated CO₂ can exacerbate the impact of drought on the nutritional quality of plant tissues (i.e., the concentration of N and P or protein decreased more with drought plus elevated CO₂ than with either factor alone in barley and squash). Worldwide, more than two billion people already suffer from iron and zinc deficiency, since most plant tissue has low concentrations of these and other nutrients, including the cereal grains that provide most of the calories for humanity (Schroeder et al., 2013; Myers et al., 2014; and references therein). In addition, increases in atmospheric CO₂ levels, drought, and heat stress all tend to decrease the uptake of mineral nutrients by plant roots and cause decreases in the concentration of most nutrients in plant tissues (including in seeds) (Jablonski et al., 2002; He and Dijkstra, 2014; Heckathorn et al., 2014; Myers et al., 2014). The proportion of humanity suffering from malnutrition, which is caused by insufficient quantity or quality of food (especially protein, vitamins, and mineral nutrients) (Myers et al., 2014), is likely to increase in the coming decades due to the aforementioned factors. Hence, efforts to improve the tolerance of crops to climate change should include adaptations which help plants procure more nutrients in a warmer, often drier, high-CO₂ world, which in the case of drought, and based on this study, may not necessarily include increasing expression of nutrient-uptake proteins.

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AUTHOR CONTRIBUTIONS

D.R.B., S.A.H., and J.K.B. were involved in the conceptual design of the study. D.R.B. performed the experiments, collected and analyzed data, generated figures, and wrote the draft manuscript. S.A.H. was the main faculty advisor of D.R.B. and was involved in data interpretation and manuscript revision. J.K.B. and D.M.J. provided valuable feedback on the manuscript. D.M.J. helped with harvesting and ELISA.

DATA AVAILABILITY

All data from this study are available on request, as are samples of antiserum produced by the Heckathorn lab and used in this work.

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