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Elevated CO_2 plus chronic warming reduce nitrogen uptake and levels or activities of nitrogen-uptake and -assimilatory proteins in tomato roots

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Atmospheric CO_2 enrichment is expected to often benefit plant growth, despite causing global warming and nitrogen (N) dilution in plants. Most plants primarily procure N as inorganic nitrate (NO_3^-) or ammonium (NH_4^+), using membrane-localized transport proteins in roots, which are key targets for improving N use. Although interactive effects of elevated CO_2, chronic warming and N form on N relations are expected, these have not been studied. In this study, tomato (*Solanum lycopersicum*) plants were grown at two levels of CO_2 (400 or 700 ppm) and two temperature regimes (30 or 37°C), with NO_3^- or NH_4^+ as the N source. Elevated CO_2 plus chronic warming severely inhibited plant growth, regardless of N form, while individually they had smaller effects on growth. Although %N in roots was similar among all treatments, elevated CO_2 plus warming decreased (1) N-uptake rate by roots, (2) total protein concentration in roots, indicating an inhibition of N assimilation and (3) shoot %N, indicating a potential inhibition of N translocation from roots to shoots. Under elevated CO_2 plus warming, reduced NO_3^--uptake rate per g root was correlated with a decrease in the concentration of NO_3^--uptake proteins per g root, reduced NH_4^+ uptake was correlated with decreased activity of NH_4^+-uptake proteins and reduced N assimilation was correlated with decreased concentration of N-assimilatory proteins. These results indicate that elevated CO_2 and chronic warming can act synergistically to decrease plant N uptake and assimilation; hence, future global warming may decrease both plant growth and food quality (%N).

Introduction

Atmospheric CO_2 concentration has increased by >40% since pre-industrial times and may reach approximately 420–935 ppm by the end of this century, depending on future CO_2 emission scenarios (IPCC 2014). In general, CO_2 enrichment is expected to increase plant growth and productivity by enhancing photosynthesis, via decreases in photorespiration (in C_3 plants), and/or decreasing water use (C_3 and C_4 plants), via decreases in stomatal opening (Leakey et al. 2009, DaMatta et al. 2010). Although elevated CO_2 may often benefit plant growth, it is also a potent greenhouse gas that is the main cause of current global warming, and global warming

Abbreviations – DTPA, diethylene-triamine-penta-acetic acid; EB, extraction buffer; EDTA, ethylene-diamine-tetra-acetic acid; G_s, stomatal conductance; GOGAT, glutamine oxo-glutarate amino transferase; GS, glutamine synthetase; NR, nitrate reductase; PAR, photosynthetically active radiation; SDS, sodium dodecyl sulfate.

will often increase plant heat stress. For example, over the past three decades, the northern hemisphere has experienced the warmest 30-year period of the last 1400 years (IPCC 2014). Owing to global warming, the mean surface temperature of Earth is expected to rise by 1.4–5.8°C by the end of this century, increasing both chronic and acute heat stress (IPCC 2007). Models of agricultural systems in the current main crop-producing regions of the Earth predict large negative impacts from global climate change on food production and food security (IPCC 2014).

Most non-leguminous plants (primarily C_3) show a decline in nitrogen (N) status (i.e. the concentration of N in their tissues, or %N) when grown under atmospheric CO_2 enrichment (Taub and Wang 2008, Bloom et al. 2010); on the other hand, leguminous plants show relatively smaller changes in %N in response to elevated CO_2 (Rogers et al. 2009). Decreases in plant N status with elevated CO_2 are because of several factors, including high CO_2 growth dilution (Taub and Wang 2008) and inhibition of shoot N assimilation (Bloom et al. 2002, 2010, 2014). Importantly, declines in plant N status with elevated CO_2 will decrease the concentration of protein in food, and hence food quality, which will increase malnutrition and hunger for many humans (Taub et al. 2008, Bloom 2009, DaMatta et al. 2010). Although increased N fertilization could compensate for N deficiency to some extent in plants grown under elevated CO_2 (Taub et al. 2008, Bloom 2009), it is not the best solution to this global issue, given the high costs associated with N fertilizer production and that only 30–50% of N fertilizers applied are taken up by plants (Schroeder et al. 2013). Alternatively, improvements in crop N-uptake capacity or efficiency may be possible, using conventional breeding or transgenic approaches (Schroeder et al. 2013).

Most plants procure the majority of their N in one of the two inorganic forms, nitrate (NO_3^-) or ammonium (NH_4^+), using membrane-localized transport proteins specific to each N form. The main N-uptake proteins in roots are NRT1 (low-affinity NO_3^- transporter), NRT2 (high-affinity NO_3^- transporter) and AMT1 (high-affinity NH_4^+ transporter), with each type comprised of multiple members encoded by gene families (Nacry et al. 2013). NRT1 (low-affinity transport system) operates when the external [NO_3^-] is relatively high, while NRT2 (high-affinity transport system) operates when the external [NO_3^-] is relatively low (e.g. <100 μ*M*). The main NRT1 (NRT1.1) is both a HAT and LAT transporter. Once NO_3^- is taken up by plant roots via NRT1 and/or NRT2, it faces three fates: (1) reduction into NO_2^- by nitrate reductase (NR) in the cytosol and further reduction into NH_4^+ by nitrite reductase in plastids, (2) storage in the vacuole or (3) transport to shoots for storage or assimilation (Crawford and Forde 2002). Most of the NH_4^+ taken up by AMT1 or resulting from NO_3^- reduction enters the glutamine synthetase/glutamine oxo-glutarate amino transferase (GS/GOGAT) cycle, where it is fixed to a glutamate molecule by GS to form glutamine, with subsequent transfer of NH_2 from glutamine to oxo-glutarate by GOGAT (Masclaux-Daubresse et al. 2010). The main N-uptake proteins in roots, as well as the main N-assimilatory proteins, are key targets for improving N-use efficiency and nutritional quality of crops (Masclaux-Daubresse et al. 2010, Nacry et al. 2013, Schroeder et al. 2013). Given the differences in their assimilatory pathways, NO_3^- and NH_4^+ influence plant growth and nutrient status differently. For example, elevated CO_2 inhibits shoot NO_3^-, but not NH_4^+, assimilation (Bloom et al. 2002). Consequently, in some ways, NO_3^- and NH_4^+ should be treated as two separate nutrients, and research from plants treated with NO_3^- or NH_4^+ separately is often more informative than when they are provided as a mixture (Carlisle et al. 2012).

Plant roots are often more sensitive to heat stress than shoots, which negatively impacts plant growth and productivity by reducing root growth and function, including nutrient uptake (Huang et al. 2012, Heckathorn et al. 2014). For example, Mainali et al. (2014) observed decreases in N uptake by roots during abrupt heat stress (35 and 40°C vs 30°C) in the warm-season C_4 grass, *Andropogon geradii* Vitman. In addition, abrupt heat stress (42°C vs 25°C) also decreased the uptake of N and other nutrients, as well as levels of nutrient-uptake and -assimilation proteins in tomato (*Solanum lycopersicum*) (Giri 2013, Heckathorn et al. 2014). Bassirirad (2000) showed that plant preference for NO_3^- or NH_4^+ depended on the soil or root temperature in many species, and the NH_4^+:NO_3^- uptake ratio decreased as temperature increased from suboptimal to optimal (sufficient data for supraoptimal temperatures were lacking). Rachmilevitch et al. (2006) found low NO_3^--assimilation rates at stressfully high soil temperature (37°C) in two *Agrostis* species differing in thermotolerance, and Hungria and Kaschuk (2014) observed differential effects of heat stress on NO_3^- and NH_4^+ assimilation in *Phaseolus vulgaris*. Cruz et al. (1993) found higher NH_4^+-uptake rates in *Ceratonia siliqua* under supraoptimal temperatures, which eventually caused toxicity to the plants from excess NH_4^+ accumulation. Notably, most previous studies which investigated the effects of temperature on N uptake focused on temperature rises from suboptimal to optimal temperatures (Clarkson and Alison 1979, Atkin and Cummins 1994, Bassirirad 2000), have subjected only roots (in intact plants) or root pieces to heating (Heckathorn et al. 2014)

and, in the case of NH_4^+, often used unrealistically high levels of NH_4^+ (Ganmore-Neumann and Kafkafi 1980, 1985). In addition, we are aware of no previous studies which have examined the effects of chronic warming or how high temperature stress interacts with N form (NO_3^- vs NH_4^+) to impact the levels of nutrient-uptake proteins in roots.

A limited number of previous studies have investigated CO_2 and N form interactions, showing that elevated CO_2 inhibits shoot NO_3^- but not NH_4^+ assimilation (Table S1, Supporting information). Several other previous studies have also investigated CO_2 and heat interactions (especially on photosynthesis, such as showing that effects of elevated CO_2 on photosynthetic thermotolerance depend on photosynthetic pathway) (Wang et al. 2012). Furthermore, a small number of studies have examined $CO_2 \times$ heat $\times$ N interactions, showing that effects of elevated CO_2 on photosynthetic thermotolerance depend on the photosynthetic pathway and N availability (Bassow et al. 1994, Wang et al. 2014). However, although it is expected that there will be interactive effects of elevated CO_2, heat stress and N form on root nutrient relations, this has not been studied (Bassirirad 2000, Heckathorn et al. 2014). Therefore, the objective of this study was to determine the interactive effects of elevated CO_2 and chronic warming in the presence of two forms of inorganic N (NO_3^- and NH_4^+) on growth, %N, N-uptake rates, concentrations and activities of major N-uptake proteins (NRT1 and AMT1) and concentrations of major N-assimilatory proteins (NR, GS and GOGAT), using tomato as a model. Based on the knowledge gaps discussed above, information from this study will help us better understand how climate change may impact plant productivity and food quality and how we might enhance plant N-use efficiency in a world with a changing climate.

Materials and methods

Chemicals

Ethylene-diamine-tetra-acetic acid (EDTA), Fe-diethylene-triamine-penta-acetic acid (DTPA) and NH_4Cl were purchased from Alfa Aesar, Ward Hill, MA; Becker Underwood Inc., Ames, IA and Reagent World Inc., Irvine, CA, respectively, and the rest of the chemicals were purchased from Thermo Fisher Scientific Inc., Fair Lawn, NJ, unless otherwise stated.

Plant material, growth conditions and treatments

Tomato (*S. lycopersicum* cv. Big Boy) was used as the model system, because this species is a heat-tolerant warm-season C_3 crop, and because this cultivar's growth and nutrient responses to abrupt heat stress (Giri 2013) and photosynthetic responses to abrupt heat stress and elevated CO_2 (Wang et al. 2008) have been previously studied. Previously, we observed the optimal growth temperature for this cultivar to be 30–32°C, when receiving high levels of N (e.g. 5–6 m*M*) provided primarily as NO_3^-; additionally, growth and photosynthesis were unaffected until temperatures exceeded 35–36°C (Giri 2013, Wang et al. 2008, Giri et al. unpublished data). In subsequent preliminary experiments where plants were grown with NO_3^- vs NH_4^+, we found that while tomato plants grown at 30°C grew better with solely NO_3^- than solely NH_4^+, we could achieve similar-sized plants when grown at 1.5 m*M* N (NO_3^- or NH_4^+); therefore, 1.5 m*M* N was selected for use in subsequent experiments.

Tomato seeds were germinated in calcined clay in a greenhouse, watered daily and provided quarter-strength complete Hoagland's nutrient solution [nutrient concentrations of full-strength solution: 2 m*M* $MgSO_4$, 1 m*M* KH_2PO_4, 1 m*M* K_2HPO_4, 2 m*M* $CaCl_2$, 6.2 m*M* KNO_3, 71 µ*M* Fe-DTPA, 10 µ*M* $MnCl_2$, 50 µ*M* H_3BO_3, 6 µ*M* $CuSO_4$, 6 µ*M* $ZnSO_4$ and 1 µ*M* Na_2MoO_4; pH = 6.0] twice (at 19 and 25 days after sowing). In the greenhouse, plants were exposed to approximately 25–30°C day/night temperatures and received ambient light levels plus day-extension lighting [<15% of full sunlight, provided by 250 W high-pressure sodium (GE Lighting Inc., Cleveland, OH) and 400 W metal-halide (Osram Sylvania products Inc., Manchester, NH) lamps] to provide a 15-h photoperiod. After 4 weeks (based on preliminary experiments), uniform seedlings with their first adult leaves were transplanted into 40 cylindrical pots [10 cm diameter × 40 cm length polyvinyl chloride pipes; one plant per pot] filled with coarse perlite (supported by mesh at the bottom to hold the perlite). The pots were placed in individual shallow trays that retained excess water and nutrient solution. Each transplanted seedling was treated twice (at 2 and 4 days after transplant) with 250 ml of quarter-strength nutrient solution (as described above) before they were transferred into growth chambers after reaching the two-adult leaf stage (5 days after transplant), at which point, CO_2, temperature and N treatments were initiated.

This study utilized a 2 × 2 × 2 factorial design (ambient or elevated $CO_2 \times$ near-optimal or high temperature × nitrate or ammonium). Treatments [ambient CO_2 (400 ppm)/near-optimal temperature (30/25°C; day/night), ambient CO_2/high temperature (37/32°C; day/night), elevated CO_2 (700 ppm)/near-optimal temperature and elevated CO_2/high temperature] were imposed in four growth chambers (model E36HO,

Percival Scientific Inc., Perry, IA), with N form nested within each chamber; consequently, plants were rotated every 4 days within chambers to prevent position effects. Prior to the start of treatments, plants were kept inside the growth chambers (10 plants in each) for 24 h under 500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR, supplied by 55 W Osram Dulux luminous lamps; Osram GmbH, Augsburg, Germany) with a 14-h (06:00–20:00 h) photoperiod, optimum temperature of 30/25°C (day/night) and ambient CO_2 (400 ppm) and humidity, to acclimatize to the new environment. Temperatures of the high temperature chambers were increased gradually over 3 days from 30/25 to 34/29 to 37/32°C (day/night), to avoid potential heat shock. Once final treatment temperatures were reached, a subset of plants (n = 4) was harvested and elevated CO_2 and N-form treatments were initiated (referred to hereafter as day 0). Plants were provided full-strength nutrient solution (as described above), except with N (1.5 m*M*) provided as either NO_3^- (KNO_3) or NH_4^+ (NH_4Cl) (the latter +1.5 m*M* KCl to balance K^+). Plants were irrigated with NO_3^- or NH_4^+ nutrient solution (250 ml for 9 days and then 750 ml for 9 days) every other day. When adding new nutrient solution, previously accumulated nutrient solution in the base of trays was discarded. Plants were misted with deionized water when needed, but water was not added to pots. Plants (n = 4) from each treatment combination were harvested 18 days after the start of treatments.

In a second independent experiment, to confirm the interactive effects of elevated CO_2 and warming on biomass, we grew and treated a second set of tomato plants (n = 8) as described above, except for providing plants with 500 ml of nutrient solution (NO_3^- as the sole N source) for all 18 days (vs 250 ml for 9 days and then 750 ml for 9 days, as described above). Prior to harvesting plants, we measured stomatal conductance (G_s) to water vapor in recently fully expanded leaves receiving steady-state irradiance, using a portable gas-exchange system [model 6400, LiCOR, Lincoln, NE; as described in the study of Wang et al. (2008)]. G_s was measured at 500 μmol m^{-2} s^{-1} PAR and the same CO_2 and temperatures as in experimental treatments, and measurements were made within 1 min, to ensure that G_s reflected chamber conditions; preliminary experiments indicated that measurement light levels were not photoinhibitory.

Plant nutrient relations measurements

Harvested plants were separated into shoots (leaves + stems) and roots. Shoots were immediately dried, while each root system was divided longitudinally. Half of the root system was immediately weighed, frozen in liquid N_2 and stored at −80°C, while the other half was dried for the determination of dry mass (both fresh and dry mass were determined on a subsample to allow determination of fresh-to-dry mass ratio). For dry mass, tissue was oven dried at 70°C for 72 h, weighed and ground to a fine powder in a mortar and pestle. The concentration (% dry mass) of C and N in roots and shoots was determined using the combustion-MS technique [as described in the study of Mishra et al. (2009)]. Total plant N content (mg) was determined from the product of %N and biomass, and N-uptake rate was determined by the following equation: N-uptake rate (mg g^{-1} dry root day^{-1}) = (total plant N content on day 18 − total plant N content on day 0)/root dry mass on day 18/18 days.

Total root proteins were extracted according to Mishra et al. (2012). A known amount (approximately 1 g) of fresh root tissue was ground in liquid N_2 to a fine powder and then ground with a known volume (4 ml per 1 g of root tissue) of extraction buffer [EB: 0.2 *M* Tris–HCl buffer, pH = 8.0; 5 m*M* EDTA, pH = 7.5; 0.7 *M* sucrose; 1% sodium dodecyl sulfate (SDS); 1 m*M* phenylmethylsulfonyl fluoride; 1 m*M* leupeptin and 2% *β*-mercaptoethanol]. The resulting mixture was transferred to a 15 ml tube, an equal volume of saturated phenol was added and it was centrifuged (10 000 *g* for 10 min at 4°C). Next, an equal volume of EB was added to the recovered phenol phase and centrifuged again. The phenol phase was recovered again and five volumes of chilled 0.1 *M* ammonium acetate were added. After overnight incubation at −20°C, root proteins were pelleted by centrifuging (as described above). The resulting pellet was washed several times with ammonium acetate and acetone. Finally, the pellet was dried under room temperature and re-solubilized with 1.2 ml of re-solubilizing buffer (62.5 m*M* Tris–HCl, pH = 6.8; 0.5% SDS and 20% glycerol) to dissolve the pelleted proteins. Total root protein concentration per g of fresh root was determined using a colorimetric assay (DC protein assay; Bio-Rad Laboratories Inc., Hercules, CA); bovine serum albumin was used as the protein standard.

Relative levels of the major N-uptake proteins (NRT1 and AMT1) and N-assimilatory proteins (GS, GOGAT and NR) per unit total root protein were determined by enzyme-linked immunosorbent assay, using protein-specific antibodies generated by the Heckathorn laboratory (NRT1 and AMT1) or purchased commercially (GS, GOGAT and NR; Agrisera, Vännäs, Sweden). For NRT1 and AMT1, rabbit polyclonal antiserum was generated using oligopeptide antigens of conserved domains of each N-uptake protein. Protein sequences of NRT1 and AMT1 families were collected using open-access databases (e.g. NCBI and TAIR), aligned

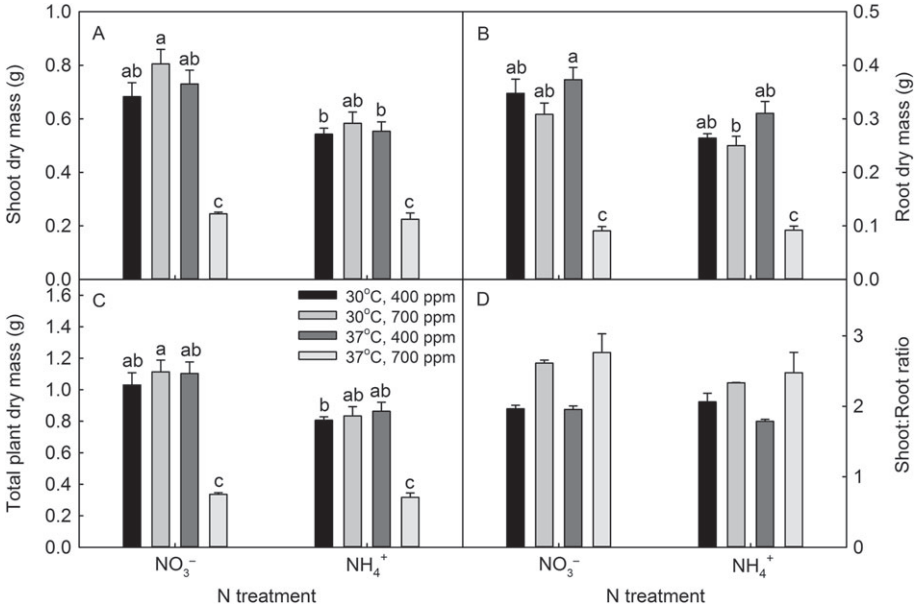

Fig. 1. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on (A) shoot dry mass, (B) root dry mass, (C) total plant dry mass and (D) shoot:root ratios of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- or 1.5 m*M* NH_4^+ for 18 days. Each bar represents mean (n = 4) + 1 SEM of each treatment combination. Within each panel, bars without a common letter are significantly different ($P < 0.05$, Tukey's test). Note: scales are different among panels.

with CLUSTAL W software (Mac Vector Inc., Cary, NC) and further analyzed for conserved domains using DNASTAR software (MegAlign function; DNASTAR Inc., Madison, WI). Potential hydrophobicity of the selected peptide sequences of each N-uptake protein was analyzed with the ProSite database (http://prosite.expasy.org). The specificity of antisera was confirmed by western blotting, comparing results for pre-immune vs immune sera, antigen-purified vs crude sera and tomato vs *Arabidopsis thaliana* samples. The relative quantity of N transporters, both per unit total root protein and per g dry root, was determined by relativizing samples to a standard root protein extract from tomato, following subtraction of background owing to non-specific binding using pre-immune serum. The relative activity per uptake protein was then calculated as specific uptake rate per g of root divided by the relative amount of transporter per g of root.

Statistical analysis

Data were analyzed using two-way (two levels of CO_2 × two levels of temperature) and/or three-way (two levels of CO_2 × two levels of temperature × two forms of N) ANOVA, with CO_2, temperature and N form as fixed factors, using JMP 12 (SW) software (SAS Institute Inc., Cary, NC). Tukey's post hoc test was used for multiple comparisons, only if ANOVA results were significant; results were considered significant if $P < 0.05$. Model assumptions of independence, normality and equal variance were checked with residual vs fitted, normal Q-Q and S-L plots, respectively, using R version 3.1.2. [R Core Team (2013), Vienna, Austria] If results required transformation to meet model assumptions (only necessary for biomass, total root proteins and N-uptake protein activity), log transformation proved to be optimal and sufficient. Figures were generated using SIGMAPLOT 12.5 software (Systat Software Inc., Chicago, IL). Results presented in figures are untransformed means and SEM.

Results

Shoot, root and total plant biomass (dry mass) were each significantly affected individually by CO_2, temperature and N form, but there were significant interactive effects only for CO_2 × temperature on these variables (ANOVA, $P < 0.005$; Table S2). At 30°C, elevated CO_2 did not have a statistically significant influence on plant biomass (although elevated CO_2 plants tended to have higher shoot biomass and lower root biomass than ambient CO_2 plants for both N treatments); however, at 37°C, plant biomass (shoot, root and total) significantly decreased at elevated CO_2, in both NO_3^-- and NH_4^+-treated plants (Fig. 1A–C). The chronic warming treatment (37°C vs 30°C) did not significantly influence plant biomass under ambient CO_2 in either NO_3^-- or NH_4^+-treated plants,

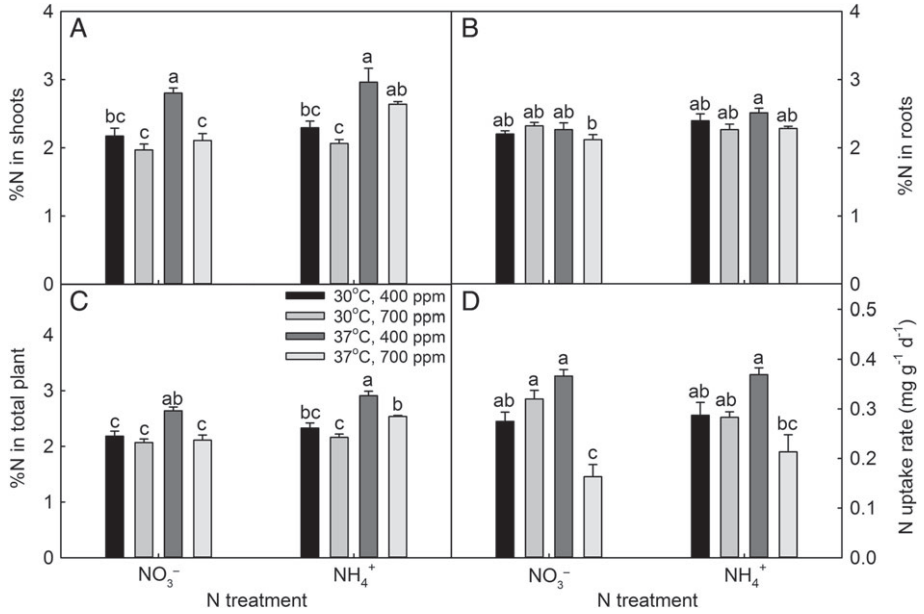

Fig. 2. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on (A) %N in shoots, (B) %N in roots, (C) %N in total plant and (D) N-uptake rate per g of dry root mass per day of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- or 1.5 m*M* NH_4^+ for 18 days. Each bar represents mean (n = 4) + 1 SEM of each treatment combination. Within each panel, bars without a common letter are significantly different ($P < 0.05$, Tukey's test). Note: scales are different among panels.

but decreased plant mass at elevated CO_2. The ratio of shoot-to-root biomass was affected only by CO_2, increasing in elevated compared with ambient CO_2 ($P < 0.0001$) (Fig. 1D).

Shoot and total plant %N were significantly affected by CO_2, temperature and N form, while root %N was significantly affected only by N form, and there were significant interactive effects of CO_2 × temperature and N form × temperature on total plant %N (ANOVA, $P < 0.05$; Table S2). Across both temperatures and N forms, CO_2 enrichment caused a decline in shoot and total plant %N, and this decline was especially pronounced for NO_3^--treated plants at 37°C (Fig. 2A, C). Chronic warming (37°C vs 30°C) caused significant increases in shoot and total plant %N for NO_3^-- and NH_4^+-treated plants at both CO_2 levels, except for plants treated with NO_3^- and grown under elevated CO_2 (Fig. 2A, C). Interestingly, temperature and CO_2 did not have significant influences on %N of roots in either NO_3^-- or NH_4^+-treated plants (Fig. 2B).

Nitrogen-uptake rate was significantly affected by CO_2 and the interactive effect of CO_2 × temperature (ANOVA, $P < 0.05$; Table S2). Elevated CO_2 significantly reduced the N-uptake rates of both NO_3^-- and NH_4^+-treated plants at 37°C. Furthermore, there was a trend for chronic warming treatment (37°C vs 30°C) to increase plant N-uptake rate at ambient CO_2, although the effect was non-significant, while warming decreased it (significantly in NO_3^-, non-significantly in NH_4^+) at elevated CO_2 (Fig. 2D).

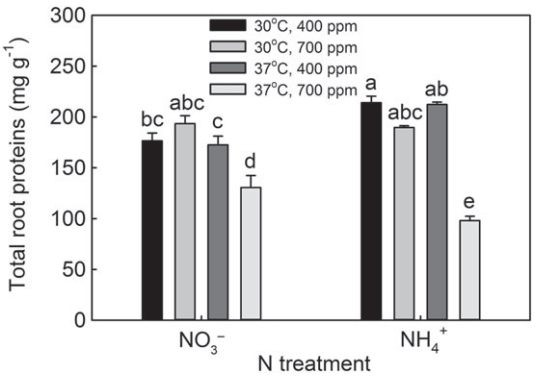

Fig. 3. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on total root proteins per g of dry root mass of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- or 1.5 m*M* NH_4^+ for 18 days. Each bar represents mean (n = 4) + 1 SEM of each treatment combination. Bars without a common letter are significantly different ($P < 0.05$, Tukey's test).

Total root protein concentration was significantly affected by the interaction of CO_2 × temperature × N form (ANOVA, $P < 0.05$; Table S2). Elevated CO_2 significantly reduced total root protein concentrations of both NO_3^-- and NH_4^+-treated plants only at 37°C, and this reduction was more pronounced in NH_4^+- than in NO_3^--treated plants. Interestingly, chronic warming

treatment (37°C vs 30°C) did not show a negative influence on total root proteins at ambient CO_2 (Fig. 3).

The concentration of NRT1 per g dry mass was significantly affected by both temperature and the interaction of CO_2 × temperature (ANOVA, $P < 0.05$; Table S2). Elevated CO_2 significantly reduced NRT1 concentration per g dry mass at 37°C, but not at 30°C, and was significantly reduced by warming treatment at elevated CO_2, but not at ambient CO_2 (Fig. 4B). Although the effects were non-significant, the combination of elevated CO_2 and warming tended to decrease concentration of NRT1 per unit total protein relative to the other treatments. The concentrations of AMT1 in roots, both per unit total protein and per g dry mass, were significantly affected only by the interactive effect of CO_2 × temperature (ANOVA, $P < 0.05$; Table S2). Elevated CO_2 tended to decrease both AMT1 concentrations at 37°C, but increase them at 30°C, while warming treatment tended to decrease both AMT1 concentrations at elevated CO_2, but increase them at ambient CO_2 (Fig. 4A, B). The relative activity per NO_3^- transporter (NRT1) was not significantly affected by warming or elevated CO_2, while activity per NH_4^+ transporter (AMT1) was affected by the interaction of CO_2 × temperature (ANOVA, $P < 0.05$; Table S2), with activity highest at 30°C and ambient CO_2 (Fig. 4C).

The concentration of NR per unit total root protein was significantly affected only by CO_2, while NR per g dry mass was significantly affected by temperature, CO_2 and their interaction (ANOVA, $P < 0.05$; Table S2). Overall, elevated CO_2 decreased NR concentration per unit total root protein, while NR concentration per g root mass was reduced by the combination of elevated CO_2 and warming (Fig. 5A, B). The concentrations of GS per unit root protein and per g root were both significantly affected by CO_2 and N form. In addition, GS concentration per g root dry mass was significantly affected by the interactions of temperature × CO_2 and temperature × N form (ANOVA, $P < 0.05$; Table S2). Elevated CO_2 tended to decrease the concentration of GS (both per unit root protein and per g root) regardless of the temperature and N form, and this decline was especially pronounced per g dry mass of NH_4^+-treated plants at 37°C. Although the warming treatment tended to show a positive influence on both concentrations of GS at ambient CO_2 regardless of the N form, it reduced GS concentration per g dry mass at elevated CO_2 regardless of the N form (significantly so only in NH_4^+-treated plants) (Fig. 5C, D). The concentrations of GOGAT per unit root protein and per g root were both significantly affected by CO_2 and interaction of CO_2 × temperature. In addition, GOGAT concentration per g dry mass was significantly affected by temperature and the interaction of CO_2 × N form (ANOVA, $P < 0.05$; Table S2). Notably, GOGAT concentration per

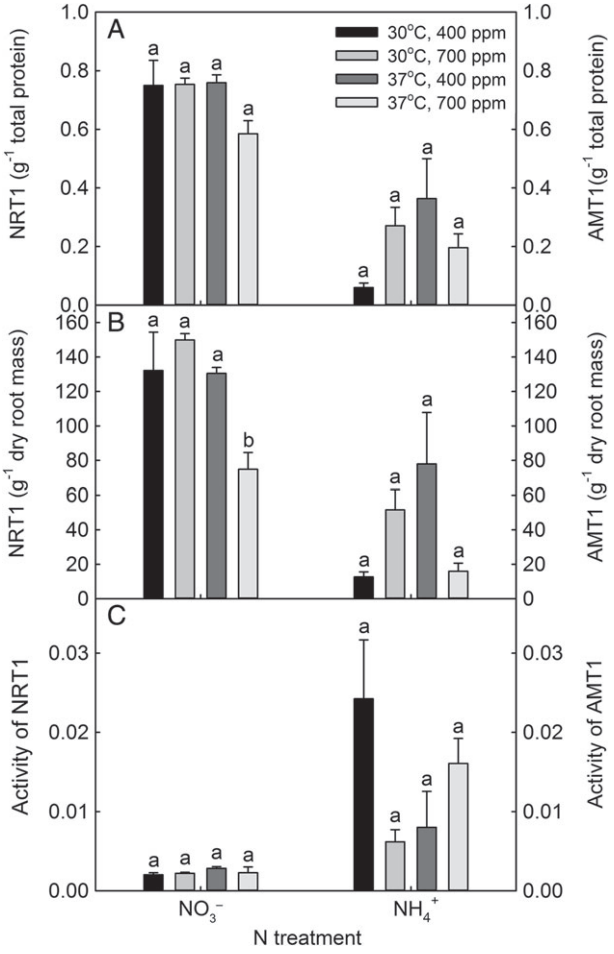

Fig. 4. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on the relative concentrations of the major NO_3^--uptake protein, NRT1, and NH_4^+-uptake protein, AMT1, (A) per unit total root protein, (B) per gram of dry root mass and (C) activities of NRT1 and AMT1 of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- or 1.5 m*M* NH_4^+ for 18 days. Each bar represents mean (n = 4) + 1 SEM of each treatment combination. Within each panel and N form, bars without a common letter are significantly different ($P < 0.05$, Tukey's test). Note: scales are different among panels.

g dry mass of both NO_3^-- and NH_4^+-treated plants was significantly reduced by elevated CO_2 at 37°C, but not at 30°C, and was significantly reduced by warming treatment at elevated CO_2, but not at ambient CO_2 (Fig. 5E, F).

In the second confirmatory experiment, the combination of warming and elevated CO_2 again significantly decreased total plant biomass, relative to warming or elevated CO_2 alone (Fig. 6A). In this second experiment, elevated CO_2 alone slightly (but not significantly) increased biomass, as in the first experiment, while warming alone slightly (but not significantly) decreased biomass. In contrast to biomass, G_s was unaffected by

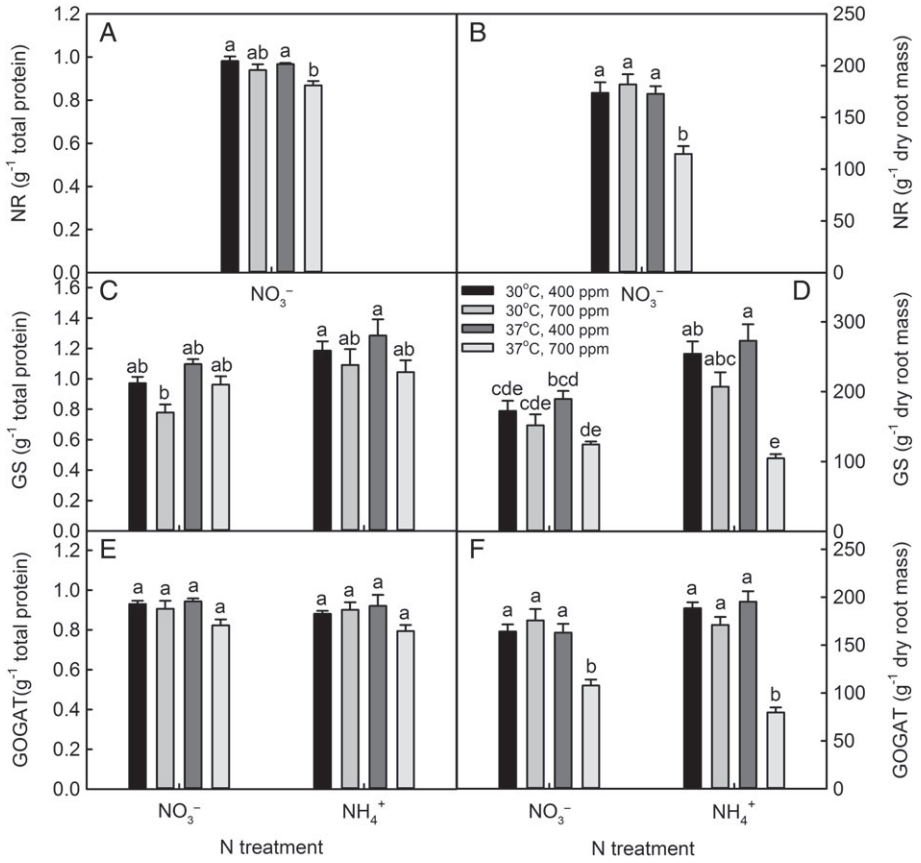

Fig. 5. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on the relative concentrations of (A) NR per unit total root protein, (B) NR per g of dry root mass, (C) GS per unit total root protein, (D) GS per g of dry root mass, (E) GOGAT per unit total root protein and (F) GOGAT per g of dry root mass of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- or 1.5 m*M* NH_4^+ for 18 days. Each bar represents mean (n = 4) + 1 SEM of each treatment combination. Within each panel, bars without a common letter are significantly different ($P < 0.05$, Tukey's test). Note: scales are different among panels.

elevated CO_2 or warming, singly or in combination (Fig. 6B).

Discussion

To the best of our knowledge, this study is the first to investigate the interactive effects of CO_2, warming and N form (NO_3^- vs NH_4^+) on root N uptake and the concentration of N-uptake and N-assimilatory proteins in roots. Individually, elevated CO_2 and warming had small effects on plant growth and on root N relations, but the combination of elevated CO_2 and chronic warming acted synergistically to severely inhibit plant growth, the rate of N uptake by roots, the concentration of total protein per g root as well as N-assimilatory proteins (NR, GS and GOGAT) in roots in both NO_3^-- and NH_4^+-treated plants; for N-uptake proteins, elevated CO_2 plus warming decreased the concentration (but not activity) of NO_3^--uptake proteins (NRT1), but decreased the activity (but not concentration) of NH_4^+-uptake proteins (AMT1). This negative interaction of elevated CO_2 and temperature contrasts with positive interactions of elevated CO_2 and drought or salinity, wherein elevated CO_2 can increase plant N assimilation and N concentration by improving plant water status via reductions in G_s and transpiration (Robredo et al. 2011, Zaghdoud et al. 2016).

In both experiments, by itself, elevated CO_2 slightly increased shoot and total biomass of both NO_3^-- and NH_4^+-treated plants grown at 30°C, as occurs in most plants grown at optimal temperatures and with adequate supply of N (Leakey et al. 2009, Wang et al. 2012). In contrast, by itself, 37°C (chronic warming) slightly increased or decreased (depending on experiment) shoot, root and total biomass of both NO_3^-- and NH_4^+-treated plants at ambient CO_2; hence, 37°C (vs 30°C) was by itself, at worst, only modestly stressful at ambient CO_2 for plants treated with either NO_3^- or

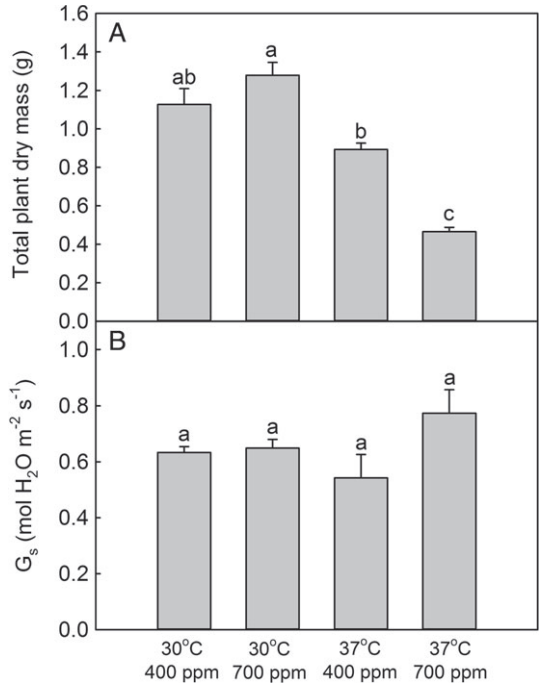

Fig. 6. Effects of ambient (400 ppm) vs elevated (700 ppm) CO_2 and 30°C (near optimal) vs 37°C (chronic warming) daytime temperatures on (A) total plant dry mass and (B) G_s of *Solanum lycopersicum* provided 1.5 m*M* NO_3^- for 18 days. Each bar represents the mean (n = 8 for total plant dry mass and n = 6 for G_s) + 1 SEM of each treatment combination. Within each panel, bars without a common letter are significantly different ($P < 0.05$, Tukey's test). Note: scales are different among panels; data are from the confirmatory experiment.

NH_4^+. However, the combination of elevated CO_2 and chronic warming severely inhibited the growth of both NO_3^-- and NH_4^+-treated plants (Figs 1A–C and 6A). The interactive negative effect of elevated CO_2 and chronic warming on growth was likely not because of the inhibition of shoot NO_3^- assimilation or NH_4^+ toxicity at 37°C vs 30°C, as similar effects were observed for both NO_3^-- and NH_4^+-treated plants. Interestingly, while we observed a negative effect of elevated CO_2 on growth during chronic warming, as did Wang et al. (2014) in barley plants grown at low N (on net photosynthesis), most previous studies have observed a positive effect of elevated CO_2 on growth during heat treatments, although the benefit of elevated CO_2 declined as the temperature of the heat treatments increased (reviewed in Wang et al. 2012). We are currently investigating the possibility that the combination of warming and elevated CO_2 affects leaf angle, and this reduces growth, relative to elevated CO_2 or warming alone.

Elevated CO_2 alone slightly decreased shoot and total plant %N in both NO_3^-- and NH_4^+-treated plants indicating that plant N uptake did not keep pace with increased growth at elevated CO_2, causing growth dilution of tissue N at elevated CO_2 (as explained in Taub and Wang 2008). In contrast, chronic warming significantly increased shoot and total %N in both NO_3^-- and NH_4^+-treated plants at ambient CO_2 (Fig. 2A, C). This is consistent with Shen et al. (2009), who observed a higher concentration of shoot N in warm-season bermudagrass (*Cynodon dactylon*) following 3 weeks of heat stress, although not in cool-season Kentucky bluegrass (*Poa pratensis*). Notably, the magnitude of the decline in %N in plants grown at elevated compared with ambient CO_2 was greater under chronic warming than at ambient temperature, although roots were able to maintain relatively similar levels of %N among all treatment combinations, indicating a possible negative effect on N translocation from roots to shoots, especially in NO_3^--treated plants where the decline in shoot %N was larger (Fig. 2A, B). In accordance with our results, Wang et al. (2012) observed decreases in leaf N concentration in both high CO_2 grown C_3 and C_4 plants, compared with those grown at ambient CO_2, and the magnitude of the decrease increased with moderate vs severe heat stress. Based on the greater positive effects of elevated CO_2 on plant function in legumes than on non-legumes, Wang et al. (2012) further suggested that a decline in leaf N concentration with elevated CO_2 might play a role in decreasing the benefits of elevated CO_2 during heat stress compared with optimal temperatures.

Surprisingly, elevated CO_2 and chronic warming acted synergistically to severely inhibit the rate of N uptake by roots, independent of the N treatment (Fig. 2D). Inhibition of N-uptake rate per g root at elevated CO_2 and warming was concomitant with a reduction of NRT1 concentration per g root in NO_3^--treated plants, but correlated with a reduction in activity of AMT1 in NH_4^+-treated plants (Fig. 4B, C). These results suggest that the combined effect of elevated CO_2 and chronic warming can inhibit root N-uptake rate, in part, by decreasing root N-uptake protein concentration or activity per transporter, depending on N form. Although the rate of N uptake can be influenced by the rate of plant transpiration, as it affects the rate of mass-flow transport of N to the root surface for subsequent uptake (Taub and Wang 2008), decreases in N uptake from warming plus elevated CO_2 in this study were not because of the decreases in root water uptake rate, as warming plus elevated CO_2 slightly increased G_s (and hence transpiration; Fig. 6). By itself, elevated CO_2 had no significant impact on N-uptake rate at 30°C, in contrast to Asensio et al. (2015), where they observed higher NO_3^- absorption rate (per g plant) in wheat plants grown at elevated CO_2. However, in general, there is no consistent effect of elevated CO_2 on NO_3^- or NH_4^+ uptake by roots (Bassirirad

2000). Also, by itself, chronic warming only slightly (and not significantly) accelerated the root N-uptake rates of both NO_3^-- and NH_4^+-treated plants at ambient CO_2. As few past studies have examined effects of either chronic or acute supraoptimal temperatures on nutrient uptake by roots in intact plants, it is not possible to generalize about effects of heat stress on nutrient uptake; however, it is clear that warming often increases nutrient-uptake rate if temperatures are not high enough to cause decreases in plant growth (i.e. if warming does not cause heat stress) (Bassirirad 2000, Heckathorn et al. 2014). As discussed by Bassirirad (2000), enhancement of nutrient uptake by warming might be because of the stimulation of root uptake kinetics.

Elevated CO_2 alone slightly increased the concentration of total proteins in roots of NO_3^--treated plants, while it decreased the concentration in NH_4^+-treated plants at 30°C. This coincides with our previous work (Heckathorn et al., unpublished data), where we saw the same trend in tomato plants treated with 2.5 m*M* NO_3^- or NH_4^+ and grown under elevated CO_2. Furthermore, these results are in conformity with Bloom et al. (2002), who saw the same trend in wheat. In contrast, chronic warming by itself did not have any positive or negative influences on the concentration of total proteins in roots of either NO_3^-- or NH_4^+-treated plants at ambient CO_2 in the current study, indicating a lack of heat-related damage to protein synthesis, which typically occurs in roots during severe heat stress (Huang et al. 2012). Notably, although %N in roots among all treatments was similar, the combination of elevated CO_2 and warming acted synergistically to severely reduce the concentration of total protein in roots of both NO_3^-- and NH_4^+-treated plants, indicating an inhibition of N assimilation in roots (Figs 2B and 3). Supporting the possibility of inhibition of N assimilation in roots, we saw a decline in the concentrations (mainly per g dry mass) of all N-assimilatory proteins at elevated CO_2 and chronic warming. The concentrations of NR and GOGAT, per g dry mass, were significantly reduced by elevated CO_2 and chronic warming in NO_3^--treated plants, while GS and GOGAT concentrations per g dry mass were significantly reduced by elevated CO_2 and warming in NH_4^+-treated plants (Fig. 5B, D, F). The reduction of total root protein concentration at high CO_2 and temperature was more pronounced in NH_4^+-treated than in NO_3^--treated plants, suggesting that inhibition of N assimilation was greater in NH_4^+-treated plants (Fig. 3). This mirrors the greater reductions of GS and GOGAT concentrations per g dry mass in NH_4^+-treated plants compared with NO_3^--treated plants (Fig. 5D, F). The effects of elevated CO_2 plus high temperature on plants were not related to tissue %C, which did not differ among treatments for either shoots or roots (data not shown). Taken together, our results suggest that inhibition of N assimilation under elevated CO_2 and chronic warming was mainly because of reduced concentrations of N-assimilation proteins, regardless of the N form supplied.

In summary, this study shows that elevated CO_2 and chronic warming (37°C vs 30°C) acted synergistically to severely inhibit plant growth, root N-uptake rate and the concentrations of total proteins, N uptake and N-assimilation proteins in the roots of tomato plants. The synergistic effect of elevated CO_2 and chronic warming negatively affected the root N-uptake rate of both NO_3^-- and NH_4^+-treated plants, which was associated with reduced concentration of root NO_3^--uptake proteins and activity of NH_4^+-uptake proteins, suggesting that reduction of N uptake is at least partly because of the reduction of N-uptake protein concentration or activity. The combined effect of elevated CO_2 and warming caused a potential negative effect on N translocation from roots to shoots in both N treatments, especially in NO_3^--treated plants, while the combined effect of elevated CO_2 and warming inhibited N assimilation, especially in NH_4^+-treated plants. As inhibition of N assimilation was associated with reduced concentration of N-assimilatory proteins, the inhibition of N assimilation is potentially because of the reduction of N-assimilatory proteins driven by the synergistic effect of elevated CO_2 and chronic warming. Furthermore, greater inhibition of N assimilation in NH_4^+-treated plants is potentially because of the greater reduction of GS and GOGAT concentrations (per g dry mass) under elevated CO_2 and chronic warming. Taken together, findings from this study indicate that individually, elevated CO_2 and chronic warming may not impact nutrient uptake and assimilation by plants, but in combination, they may hinder both nutrient uptake and assimilation, and thereby decrease the growth and food quality (protein and N concentration) of plants, including crops. Hence, future efforts to improve crop productivity and quality, either through conventional plant breeding or genetic engineering, should include a focus on minimizing detrimental effects of warming and elevated CO_2 on nutrient-uptake and -assimilatory proteins.

Author contributions

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

harvest plants. S. M., J. K. B. and C. R. K. provided valuable feedback.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Past studies on interactive effects of CO_2 and forms of N (NO_3^- vs NH_4^+) on different root parameters.

Table S2. Results from statistical ANOVA.

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