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Future climate alleviates stress impact on grassland productivity through altered antioxidant capacity

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ABSTRACT

Predicting future ecosystem functioning requires a mechanistic understanding of how plants cope with different stressors under future climate conditions with elevated CO_2 concentrations and warmer temperatures. Nonetheless, studies of stress responses under combined elevated CO_2 and warming remain scarce.

We assembled grassland communities in sunlit, climate-controlled greenhouses and subjected these to three stressors (drought, zinc toxicity, nitrogen limitation) and their combinations. Half of the communities were exposed to ambient climate conditions (current climate) and the other half were continuously kept at 3 °C above ambient temperatures and at 620 ppm CO_2 (future climate).

Across all stressors and their combinations, future climate-grown plants coped better with stress, i.e. above-ground biomass production was reduced less in future than in current climate. Among several tested potential biochemical and ecophysiological stress-relief mechanisms, we found three mutually non-exclusive mechanisms underpinning an improved stress protection under future climate conditions: (i) altered sugar metabolism; (ii) up-regulated levels of total antioxidant capacity and polyphenols; and (iii) more efficient use of ascorbate and glutathione as antioxidants.

1. Introduction

Wherever they grow, plants are frequently subjected to a large variety of environmental stressors. Nitrogen (N) and water are considered the most important limiting factors of plant productivity in the vast majority of terrestrial ecosystems (Schulze et al., 1987; Vitousek and Howarth, 1991). In addition to resource limitations, plant communities have to deal with anthropogenic stressors such as acid rain, or organic and inorganic pollutants like pesticides or heavy metals. Indeed, metal toxicity limits plant growth in many areas affected by mining or smelting activities, sewage sludge deposition or traffic (Kabata-Pendias and Pendias, 2001; Arienzo et al., 2004).

Predicting future plant responses to the ongoing increase in frequency and intensity of droughts (IPCC, 2007) requires a mechanistic understanding of how plants cope with drought stress under conditions of elevated atmospheric CO_2 concentrations and warmer temperatures. Besides climate related stressors, this also holds for future responses to natural stressors like nutrient limitation or anthropogenic stressors like heavy metal pollution, which is why multifactorial experiments are often called for (Norby and Luo, 2004; Mittler, 2006). Nonetheless, field studies of stress responses under combined elevated CO_2 and warming remain very scarce (Casella et al., 1996; Dukes et al., 2005; Mikkelsen et al., 2008; Bloor et al., 2010).

Single factor studies, on the other hand, have been frequently reported, especially the effects of elevated CO_2. The two

Abbreviations: ANOVA, analysis of variance; ASC, ascorbate; D, drought stress; DOY, day of year; ET, evapotranspiration; GSH, glutathione; IS, insoluble sugars; MDA, malondialdehyde; N, nitrogen stress; PAR, photosynthetically active radiation; ROS, reactive oxygen species; SWC, soil water content; SD, standard deviation; SE, standard error; TAC, total antioxidant capacity; T_{air}, air temperature; U, unstressed; Z, zinc toxicity.

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fundamental responses of plants to rising atmospheric CO_2 concentrations are enhanced photosynthesis and reduced stomatal conductance. All other effects of elevated CO_2 on plants and ecosystems are derived from these changes (Long et al., 2004). Also the effect of single warming or elevated CO_2 on one environmental stressor has been reported in the literature. For example, elevated CO_2 can moderate water shortages during drought through its effect on stomatal conductance (Rogers et al., 1984), while warming can cause reduced water availability due to increased evapotranspiration (Loik et al., 2000). Future climate conditions may also affect the availability of N. Increased carbon (C) input under elevated CO_2 is found to decrease soil N availability through enhanced microbial immobilization (Luo et al., 2004; Hu et al., 2006), eventually leading to N limitation (Oren et al., 2001; Hungate et al., 2003; Luo et al., 2004). However, increasing soil temperatures could reverse this reduction in N availability, by enhancing net N mineralization (Loiseau and Soussana, 2000; Hovenden et al., 2008). Enhanced mineralization could also alter heavy metal availability if the metals are bound to soil organic matter (Antoniadis and Alloway, 2001). Hence, it is clear that a future climate can change the intensity of stress factors by decreasing or increasing the availabilities of resources and/or pollutants.

Regardless of a possible impact on the intensity of a stressor, a changing climate could also affect the plant's protective capacity and, as a consequence, their growth responses to stress. As a result of stress-induced perturbations in plant metabolism, levels of reactive oxygen species (ROS) generally increase (Mittler, 2002). The production of ROS during stress results from imbalances in pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. In addition, environmental stress has been shown to trigger the active production of ROS by NADPH oxidases (Knight and Knight, 2001). Reactive oxygen species can act as signals for the activation of stress response and defence pathways, but they can also be harmful for biological structures and processes, and can lead to DNA, amino acid and protein oxidation and lipid peroxidation (Asada, 1999). To circumvent the deleterious effects of ROS, plants have evolved robust antioxidant defensive systems to minimize free radicals damage (Mittler et al., 2004). Interestingly, oxidative stress and antioxidant systems may be altered in a future climate. Yet, the effect of combined elevated CO_2 and warming on stress-relief mechanisms has received little attention (Aranjuelo et al., 2008). Single elevated CO_2 can diminish intrinsic oxidative stress through decreased ROS formation, resulting from an enhanced use of reductant for assimilation in photosynthesis and a reduced photorespiration (Halliwell and Gutteridge, 1989; Schwanz and Polle, 1998). Consequently, a down-regulation of the protective mechanisms by elevated CO_2 has been reported in several studies (Schwanz and Polle, 1998; Vurro et al., 2009). Another observed effect of elevated CO_2 occurs prior to any photooxidative process and operates through an enhanced thermal dissipation of excessive energy (nonphotochemical quenching), resulting in an improved photoprotection (Aranjuelo et al., 2008). Furthermore, also moderate warming can contribute to alleviating oxidative stress through an enhancement of metabolic reactions (Han et al., 2009).

Grasslands cover 15% of the European land area and are an important food source for livestock (Ciais et al., 2010). An understanding of potential stress-induced reductions in biomass production is thus relevant, both now and under changing climate conditions. In the present study we have determined how grassland communities respond to a variety of stressors in a future climate at both the cellular and the community level. Hence, this study responds to the need for research that relates molecular information to whole plant processes (Chaves et al., 2003). The experimental set-up contained realistically assembled grassland communities that were grown under a current and a projected future climate, and subjected to drought, N limitation, Zn toxicity and their combinations. We hypothesize that combined elevated CO_2 and warming ameliorates plant protection against stress and that this increased protection mitigates the decline in grassland productivity in response to stress.

2. Materials and methods

2.1. Experimental set-up

The study was performed on assembled grassland communities at the Drie Eiken Campus, University of Antwerp, Wilrijk, Belgium (51°09′ N, 04°24′ E, 10 asl). Average annual precipitation at this location is 776 mm, average annual air temperature is around 10.8 °C. The experimental set-up consisted of six sunlit, south facing climate-controlled chambers. The interior surface area was 1.5 m × 1.5 m, the height at the north side 1.5 m and at the south side 1.2 m. The top of the chambers consisted of a colourless polycarbonate plate (4 mm thick), whereas the sides were made of polyethylene film (200 μm thick), both UV transparent. Three of the six chambers tracked the current climate with current air temperature (T_{air}) and CO_2 concentration, while the other three chambers were exposed to a future climate scenario with 3 °C warming and a target CO_2 concentration of 620. We further refer to these climate scenarios as 'current' and 'future climate', respectively, although it is recognized that future climate conditions as predicted by the IPCC also include other aspects such as changes in precipitation and wind patterns, more frequent extreme events and increased emissions of aerosols, methane and nitrous oxide (IPCC, 2007). The CO_2 concentration was measured and regulated with a CO_2 control group with an infrared analyser (WMA-4, PPSystems, Hitchin, UK). In the current climate chambers the concentration was 394 ± 34 ppm (SD) while in the future climate chambers it was 625 ± 53 ppm (SD). Every half hour, T_{air} was monitored with a temperature sensor (Siemens, type QFA66, Germany) and photosynthetically active radiation was measured with a quantum sensor (SDEC, type JYP1000, France). During the experiment monthly average T_{air} was 17.1, 16.4 and 18.4 °C in May, June and July 2008, respectively. In the current climate chambers T_{air} was on average 0.6 ± 1.6 °C (SD) lower than outside and the future climate chambers were 2.5 ± 1.6 °C (SD) warmer than outside. Total monthly irrigation equalled 61.5, 64.4 and 85.1 mm in May, June and July, respectively. Irrigation was calculated from the monthly rainfall over the period 1995–2005 and corrected for differences in evapotranspiration (ET) inside and outside the chambers. To this end, De Boeck et al. (2006) calculated ET inside current climate chambers from changes in soil water content (SWC) and the amount of administered water, and the outside ET with Hamon's equation (Haith and Shoemaker, 1987) based on day length, vapour pressure and T_{air}.

2.2. Plant communities

This study was part of a larger experiment that consisted of 30 randomly placed grassland communities per chamber. Each community was composed of six species, selected from three functional groups which were equally represented: two grass species (*Poa pratensis* L. and *Lolium perenne* L.), two N-fixing dicots (*Medicago lupulina* L. and *Lotus corniculatus* L.), and two non-N-fixing dicots (*Rumex acetosa* L. and *Plantago lanceolata* L.). The communities contained 18 individuals (three per species) planted in a hexagonal grid with a 4.5 cm interspace, with interspecific interactions maximized by avoiding clumping. Communities were established early May 2008 (day of year (DOY) 134–137) by transplanting 5-week-old seedlings to PVC containers (tubes, 24 cm inner diameter and 40 cm height, closed with a lid at the bottom) filled with sandy soil

(93.2% sand, 4.6% silt, 2.2% clay; pH 7.6; 1.0% total C, total Kjeldahl-N 0.42 g kg^{-1}; CEC 3.9 meq 100 g^{-1} fresh weight; field capacity 0.13 m^3 m^{-3}; wilting point 0.069 m^3 m^{-3}; background Zn concentration 21.4 mg kg^{-1} dry soil). The containers were buried in the soil to avoid unnatural soil temperatures.

2.3. Imposed stressors

For the present study, a subset of 8 communities per chamber was used. In each chamber one community was given no stress (U: "unstressed") and seven communities were subjected to drought stress (D), nitrogen limitation (N), zinc toxicity (Z) or a combination of these stressors (DN, DZ, NZ or DNZ). Drought stress was applied by withholding water for a period of 14 days in the first half of July 2008 (DOY 182–196). Soil water content was measured with a PR2 soil moisture sensor (Delta-T Devices Ltd., UK) two times a week during the drought treatment and once a week throughout the rest of the experiment.

The communities were fertilized with 15 g N m^{-2} NH_4NO_3, 7.5 g m^{-2} P_2O_5, 15 g m^{-2} K_2O and micro-elements (Fe, Mn, Zn, Cu, B, Mo), with the exception of the communities that were subjected to N limitation. These received all nutrients in the same dose except for nitrogen. All nutrients were given dissolved in water, half on DOY 146 and the other half on DOY 171. Nitrogen concentrations were measured with a CN element analyser (NC-2100, Carlo Erba Instruments, Milan, Italy) after grinding the samples. Total community N stock (above-ground + roots) was determined on each community by multiplying N concentrations with biomass, separately for the above-ground and the root compartment.

Zinc was mixed with the soil 40 days before the seedlings were transplanted to the containers, so that the soil could stabilize. Per container 280 ml of a 50 g Zn L^{-1} $ZnCl_2$ solution was administered. Concentrations of Zn in roots and shoots were measured after digestion by warming with HNO_3 and H_2O_2, using mass spectrometry (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany).

2.4. Biomass harvest

Above-ground (shoot – above 3.5 cm – and stubble) and root biomass were harvested immediately after the imposed drought period (DOY 196–200). Above-ground biomass was subdivided by species. In each chamber one community was harvested per treatment, yielding three replicates (chambers) per climate. Per species a subsample was taken from the shoot biomass and its fresh weight was determined. The remaining shoot was weighed fresh and immediately frozen in liquid nitrogen (see further). The total shoot dry weight per species was calculated from the total shoot fresh weight and the dry weight/fresh weight ratio of the subsample. Dry weights were determined after drying the biomass for 48 h at 70 °C. The shoot dry weight of the community was calculated as the sum of the shoot dry weights of the six composing species. Root biomass was estimated from 12 soil cores (2 cm diameter) per community. To adequately represent the total root biomass in the soil, six cores were taken directly below the plants (one per species) and six cores in the middle of a triangle between three plant positions. Root samples were washed until they were free of soil. Total biomass included the sum of above-ground and root biomass.

2.5. Biochemical parameters

To detect the underlying metabolic processes that determine the effect of climate on the stress response of the communities, we analyzed several biochemical parameters: insoluble sugar (IS) concentrations, lipid peroxidation, total antioxidant capacity (TAC), polyphenol concentrations, tocopherol concentrations and, ascorbate (ASC) and glutathione (GSH) concentrations and redox status.

2.5.1. Insoluble sugar concentration

Insoluble sugar (IS) concentrations were determined according to Leyva et al. (2008). Fifty mg frozen shoot tissue was homogenized in 2 ml of pure acetone to extract and remove interfering pigments and soluble sugars, centrifuged at 9.5 g for 20 min, and the supernatant decanted. Insoluble sugars were extracted by adding 1.1% HCl to the residues, heating for 30 min at 100 °C, cooling and centrifugation (9.5 g, 20 min). The supernatant was diluted with water to 5 ml. Freshly prepared anthrone reagent (150 μl) was combined with 50 μl of the sample dilutions in a microplate, and absorbance was measured at 620 nm (Multiskan RC plate reader type 351, Labsystems, Colorado, USA). Glucose was used for the standard curve.

2.5.2. Lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid–malondialdehyde (TBA–MDA) assay (Hodges et al., 1999). Five hundred microliter of plant shoot extract (see above) was combined with 500 μl of MDA reagent [20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluene], with or without 0.65% (w/v) TBA. The samples were mixed, incubated at 95 °C for 30 min, cooled and re-centrifuged. Absorbance was measured (Milton Roy Spectronic 301, New York, USA) at 440 nm, 532 nm and 600 nm. MDA equivalents were calculated as described by Hodges et al. (1999).

2.5.3. Total antioxidant capacity

A modified ferric ion reducing antioxidant power (FRAP) assay was used to estimate the total antioxidant capacity (TAC) of plant extracts (Benzie and Strain, 1996). Two hundred mg of frozen shoot tissue was extracted in 2 ml of 80% (v/v) ethanol using by mortar and pestle, followed by centrifugation (3000 × *g* for 15 min, 4 °C). The samples were then mixed with the FRAP reagent, and the absorption was measured at 600 nm after 3 and 20 min (Multiskan RC plate reader type 351). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard.

2.5.4. Polyphenol concentration

The concentration of polyphenols in the 80% (v/v) ethanol plant extracts was determined spectrophotometrically using the Folin–Ciocalteu reagent, following the method of Galvez et al. (2005). Briefly, 100 μl of extract were diluted with 2 ml water, 200 μl Folin–Ciocalteu phenol reagent, and 1 ml of 15% (w/v) Na_2CO_3, mixed and allowed to stand for 2 h. The absorbance was measured at 765 nm (Milton Roy Spectronic 301). Gallic acid was used to make a standard curve and the polyphenol concentration is expressed as mg ml^{-1} gallic acid equivalents (GAE).

2.5.5. Tocopherol concentration

Tocopherols were extracted with hexane from fresh shoot material (MagNA Lyser, Roche, Vilvoorde, Belgium, 1 min, 7000 rpm). The dried extract (CentriVap concentrator, Labconco, Kansas, USA) was resuspended in 100 μl hexane, and tocopherols were separated by HPLC (HPLC system, Shimadzu, 's Hertogenbosch, The Netherlands) (normal phase conditions, Particil Pac 5 μm colum material, length 250 mm, i.d. 4.6 mm). Elution was isocratic with 8% tetrahydrofuran in hexane (column temperature at 40 °C). A fluorescence detector (RF-10A, Shimadzu, ex = 295 nm, em = 330 nm) and a diode array detector (SPD-M10AVP, Shimadzu) were used in line, for optimal sensitivity and to check peak purity. Dimethyl tocol (DMT) (Matreya LLC, Pennsylvania, USA) was used as internal standard (5 ppm). Data were analyzed with Shimadzu Class VP software 6.14.

2.5.6. Ascorbate and (homo)glutathione concentration and redox status

Ascorbate (ASC), glutathione (GSH) and homoglutathione (hGSH) levels were determined by HPLC analysis (Shimadzu) (Potters et al., 2002). Two hundred mg of frozen tissue were extracted with a mortar and pestle with 1 ml of ice-cold 6% (w/v) meta-phosphoric acid. After centrifugation (16,000 × g, 4 °C, 10 min), antioxidants were separated on a reversed phase column (100 × 4.6 mm Polaris C18-A, 3 μm particle size; 40 °C; Varian, CA, USA) with an isocratic flow rate of 1 ml min^{-1} of the elution buffer (2 mM KCl, pH 2.5 adjusted with o-phosphoric acid). The components were quantified using a custom-made electrochemical detector with a glassy carbon electrode and a Schott Pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks were confirmed using a diode array detector (SPD-M10AVP, Shimadzu), in line with the electrochemical detector. Chromatogram analysis was performed using the Shimadzu Class VP software 6.14. Total antioxidant concentrations (reduced + oxidized) were determined after reducing 100 μl sample with DTT (50 μl, 200 mM DTT in 400 mM Tris), for 10 min at room temperature and protected from light. The redox statuses were determined by dividing the reduced form of the antioxidant by the total concentration. In the N-fixing species (*M. lupulina*, *L. corniculatus*), GSH is largely replaced by hGSH, and we used the added concentrations (hGSH + GSH).

2.6. Calculations and statistical analysis

All data are analyzed at community level rather than at species level since analyses of the species responses would ignore the relative share of each species in the community. Furthermore, this approach is more relevant when predicting agricultural productivity losses or stress impacts on the C cycle. To determine the contribution of each species to the overall community for the biochemical parameters, individual species values were multiplied with the corresponding dry weight biomass proportion of the species in the community. Biochemical parameters are expressed on a fresh weight basis.

To allow separation of a climate effect on the response to stress from a climate effect on the unstressed communities, we calculated the stress responses (impact) for each climate. We therefore subtracted the values of the unstressed communities from the values of the stressed communities of the corresponding climate (further referred to as 'stress impact'). The analysis of both the absolute values of the stressed communities and the stress impact, indirectly gave us information on the climate effect in unstressed communities. For example, if there was a significant effect of climate on the absolute values of the stressed communities, and no effect on stress impact, we concluded that the observed climate effect was present in the unstressed communities as well, as they served as the reference to calculate the stress impact. To visualize the stress impact results, the stress impact in future climate was plotted against the stress impact in current climate for all different parameters (see for example Fig. 1a). The 1:1 line in these graphs represents a stress impact that is not altered by climate; values above the 1:1 line represent an increased positive or a reduced negative impact of stress in future climate; values below the 1:1 line represent a reduced positive or an increased negative impact of stress in future climate.

Hence, per parameter two statistical analyses were performed: one on the absolute values of the parameter in the stressed communities and a second on the stress impact values. ANOVA's were performed in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) using the mixed procedure (Littell et al., 1996) with climate and stress (= all stressors and their combinations) as fixed factors, and chamber as a random factor nested within climate. Effects were considered significant at $P < 0.05$. Non-significant terms were excluded from the model. We did not perform a multifactorial analysis with the three stressors as fixed factors because the scope of this paper is to study the effect of climate on stress impact rather than the interactions between stressors. Combining the stressors and their combinations in one fixed factor increased the statistical power to detect an effect of climate on the stress impact.

3. Results

3.1. Intensity of the stressors

Application of stressors effectively deteriorated growth conditions for the plant communities. Nitrogen limited communities (N, NZ, DN, DNZ) had a lower total N stock than unstressed communities ($F_{4,22} = 12.58$, $P < 0.001$; a posteriori comparison of unstressed with stressed communities, $P < 0.05$ in all cases; Figure S1). Zn concentrations were higher in communities with added Zn (Z, NZ, DZ, DNZ) than in unstressed communities, for shoots as well as for roots ($F_{4,24} = 5.61$, $P = 0.025$ and $F_{4,24} = 14.09$, $P < 0.001$, a posteriori comparison of unstressed with stressed communities, $P < 0.05$ in all cases; Figure S2). Logically, SWC decreased in the drought stressed communities (D, DN, DZ, DNZ) after withholding water (DOY 182, Figure S3).

The N stock of the communities did not differ among the climates for the unstressed nor for the N limited communities ($F_{1,4} = 0.01$, $P = 0.941$ and $F_{1,17} = 3.41$, $P = 0.082$, respectively; Figure S1). Likewise, the shoot and root Zn concentrations did not differ among the climates in both the unstressed and the Zn-polluted communities (shoots: $F_{1,4} = 0.01$, $P = 0.948$ and $F_{1,22} = 0.02$, $P = 0.895$; roots: $F_{1,4} = 0.13$, $P = 0.735$ and $F_{1,22} = 1.14$, $P = 0.297$, respectively; Figure S2). In contrast, SWC in both the unstressed and stressed communities did differ among the climates ($F_{1,4} = 7.88$, $P = 0.048$ and $F_{1,4} = 34.26$, $P = 0.0043$, respectively; Figure S3). On average drought stressed communities (D, DN, DZ, DNZ) reached wilting point at DOY 189 and 186 in current and future climate, respectively (Figure S3).

3.2. Plant community biomass

The above-ground biomass of the stressed communities was consistently higher in the future than in the current climate, independent of the stress treatment (Fig. 1a, Table 1). The stress impact plot for above-ground biomass (Fig. 1b) shows all observations above the 1:1 line and in the lower left quadrant (negative values), demonstrating a smaller stress-induced decrease of biomass production in the future climate (Table 1). This observation indicates a mitigated impact of a variety of stressors on above-ground biomass in the future relative to the current climate. In contrast to above-ground biomass, roots responded differently to climate and stress (Figure S4 and Section S5). To help unravel the molecular basis for the mitigated stress impact on above-ground biomass in future climate, we investigated changes in the sugar content and in plant oxidative stress responses.

3.3. Insoluble sugar concentration and lipid peroxidation

The insoluble sugar concentration reflects the levels of structural carbohydrates and starch, and is an indicator of sugar storage and cell wall formation activities. The IS levels of the stressed communities did not differ among the climates (Fig. 2a, Table 1). The stress impact on IS, on the other hand, was smaller in future (near zero) than in current climate conditions (positive values) (Fig. 2b; below 1:1 line, Table 1). Hence, relative to unstressed plants, plants under stress allocated less C towards IS in the future than in the current climate.

Malondialdehyde (MDA) levels are commonly used as an indicator of ROS-induced membrane damage. The absolute and stress

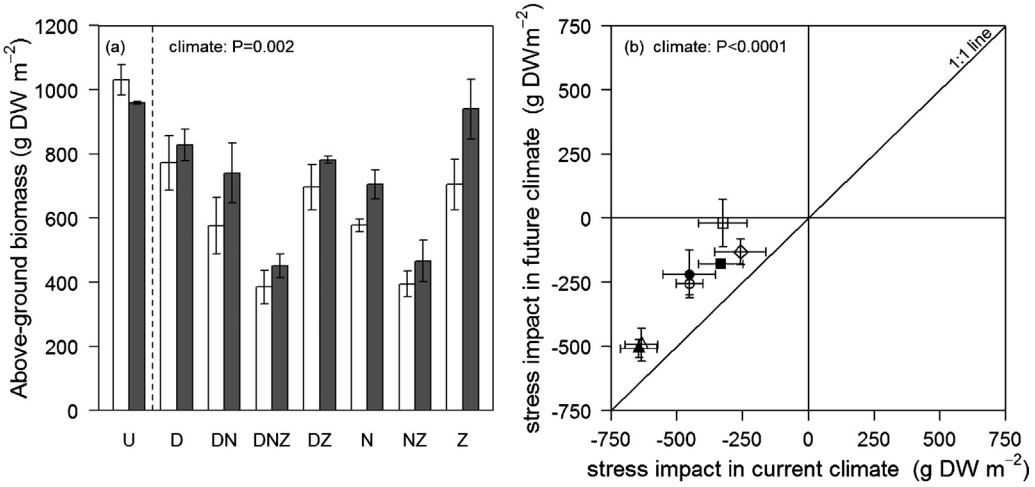

Fig. 1. Above-ground biomass of experimental grassland communities in current and future climate (elevated CO_2 and temperature), with and without exposure to three stressors and their combinations. Panel (a): absolute values in current (white bars) or future (grey bars) climate, means ± SE (n = 3). P-value of climate effect in ANOVA on stressed communities (bars to the right of the dashed line). Panel (b): differences between stressed and unstressed communities (stress impact) in future climate plotted against stress impact in current climate, means ± SE (n = 3). P-value of climate effect in ANOVA. Stressors: drought: D, open diamond; N limitation: N, open circle; Zn toxicity: Z, open square; and their combinations: DN, filled circle; DZ, filled square; NZ, open triangle; DNZ, closed triangle; U = unstressed communities.

Table 1
Analyses of variance (ANOVA) of measured parameters of experimental grassland communities exposed to three stressors (drought, N limitation and Zn addition) and their combinations in two climates (current climate and future climate with elevated CO_2 and air temperatures). ANOVA's were performed on the absolute values of the stressed communities and on the difference between stressed and unstressed communities (stress impact). Degrees of freedom were 1, 34 for climate, 6, 34 for stress and 6, 28 for C × S. Significance levels (P-values) are presented in bold when significant (<0.05). Abbreviations: insoluble sugars (IS), total antioxidant capacity (TAC), ascorbate (ASC), glutathione (GSH).

	Absolute values of stressed communities			Stress impact		
	Climate	Stress	C × S	Climate	Stress	C × S
Above-ground biomass	**0.002**	**<0.001**	0.792	**<0.001**	**<0.001**	0.885
Root biomass	0.404	0.443	0.228	**0.003**	0.599	0.414
IS	0.966	**0.007**	0.424	**0.003**	0.073	0.736
Lipid peroxidation	0.087	**0.047**	0.717	0.489	**0.007**	0.457
α-Tocopherol	0.363	**0.002**	0.245	0.330	**0.006**	0.355
TAC	**0.025**	**0.038**	0.933	0.932	0.120	0.970
Polyphenols	**0.047**	0.082	0.845	0.685	0.077	0.838
ASC concentration	0.689	**0.048**	0.888	**<0.001**	0.117	0.935
ASC redox status	0.903	0.301	0.789	**<0.001**	0.558	0.917
GSH concentration	0.804	0.069	0.886	**<0.001**	0.401	0.981
GSH redox status	0.626	0.159	0.883	0.955	0.255	0.926

impact values of MDA did not differ among the climates (Fig. 2c and d; scattered around the 1:1 line, Table 1). Hence, stress-induced damage to cell membranes was probably limited, and did not differ among the climates, neither in unstressed nor in stressed communities.

3.4. Changes in antioxidant metabolism

One of the most common defence responses of plants against oxidative stress involves changes in ROS scavenging antioxidant metabolites. The overall TAC was higher in the future than in the current climate for stressed communities, whereas the stress impact was not affected by climate (Fig. 2e and f; scattered around the 1:1 line, Table 1). From this we conclude that the future climate led to an up-regulation of small molecular weight antioxidants, which contributes to the TAC in both unstressed and stressed conditions.

The TAC of plant tissues is determined by a large variety of antioxidant molecules, including polyphenols, tocopherols, ASC and GSH. We measured changes in the levels of each of these groups of metabolites. The polyphenol levels followed the same pattern as for the TAC; i.e. in the future climate an up-regulation in both stressed and unstressed conditions was observed (Table 1, Fig. 2g and h). The finding that polyphenol levels correlated with the TAC suggests that the TAC, for the largest part, was determined by polyphenolic constituents. The levels of α-tocopherol, a membrane-embedded lipophilic antioxidant, were not altered by climate neither for the absolute nor for the stress impact values (Table 1, Figure S6a and b). This result is consistent with the lipid peroxidation data, supporting the idea of limited cell membrane damage and, consequently, minimal changes in the biosynthesis of lipophilic-antioxidant compounds.

Ascorbate and glutathione are two essential plant molecular antioxidants interconnected in the so-called ASC-GSH cycle (Mittler et al., 2004). Both the absolute levels and the redox status of these molecules are considered important oxidative stress parameters. Absolute ASC levels and ASC redox status of the stressed communities did not differ between the climates. Stress impact values, on the other hand, were 'zero to negative' in the future climate compared to positive in the current climate, for both parameters (Fig. 2i and j (ASC) and Figure S6c and d (redox status); below the 1:1 line, Table 1). This indicates that in the future climate there was an up-regulation of ASC and an increase of the ASC redox status in unstressed communities, which was no longer detectable under stress conditions. This result is explained by an increase of ASC and ASC redox status under stress in the current climate, and a

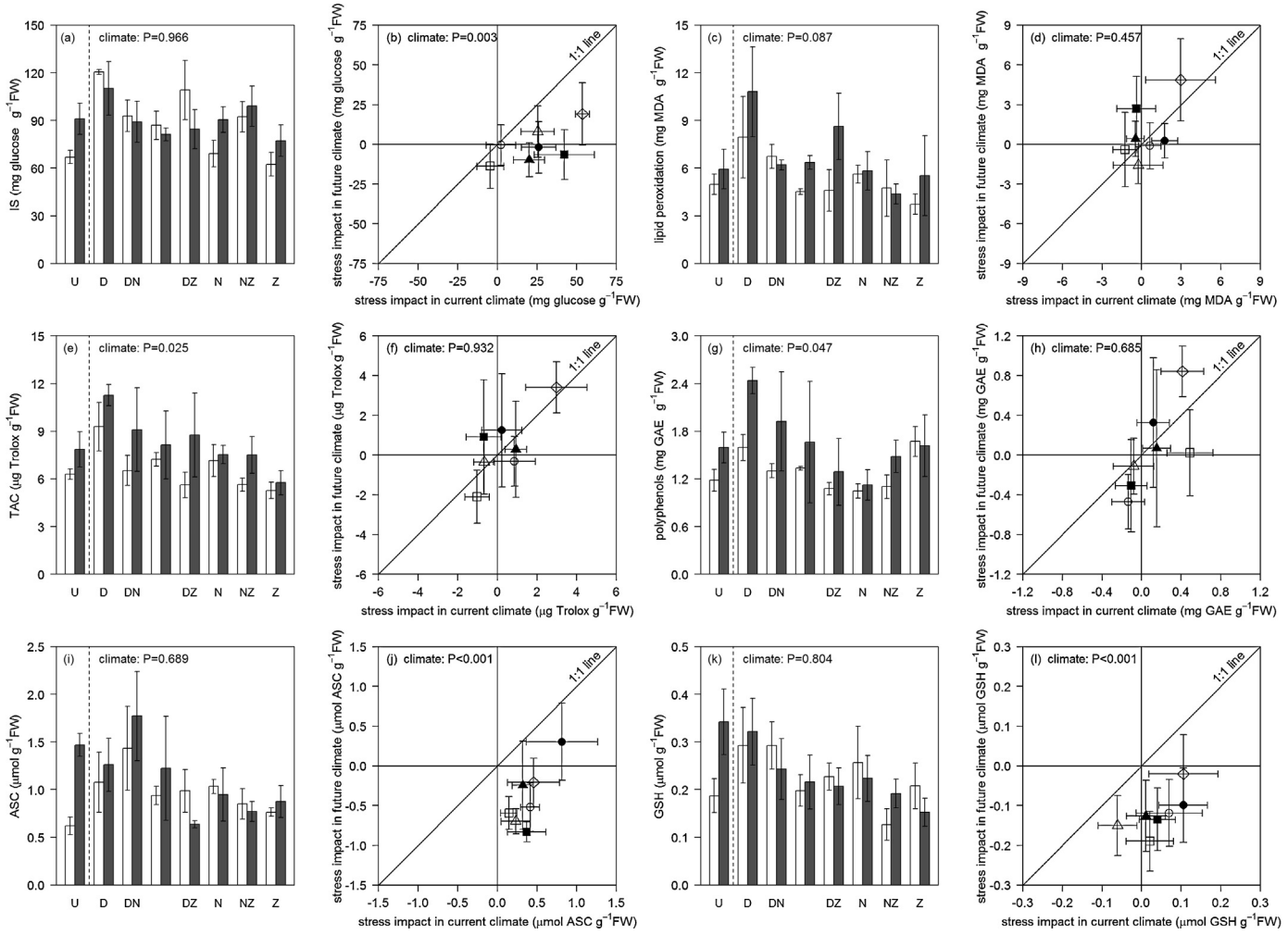

Fig. 2. Biochemical parameters of experimental grassland communities in current and future climate (elevated CO_2 and temperature), with and without exposure to three stressors and their combinations. Insoluble sugars (IS) (A, B); lipid peroxidation (C, D); total antioxidant capacity (TAC) (E, F); polyphenols (G, H); ascorbate concentration (ASC) (I, J) and glutathione concentration (GSH) (K, L), details see Fig. 1. The 1:1 line represents a stress impact that is not altered by climate, values above the 1:1 line represent an increased positive or a reduced negative impact of stress in future climate, values below the 1:1 line represent a reduced positive or an increased negative impact of stress in future climate.

decrease in the future climate (nearly all located in the lower right quadrant (Fig. 3j and Figure S6d)). The GSH levels showed the same pattern (Fig. 3k and l; Table 1) of responses as ASC and ASC redox status. However, the GSH redox status was not affected by climate neither in absolute nor in stress impact values (Figure S6e and f; scattered around the 1:1 line, Table 1). This indicated that there were no stress-induced changes in the GSH redox status in plants grown under either climate conditions.

4. Discussion

The present study unambiguously demonstrates that, across three different abiotic stressors, above-ground biomass production of grassland communities was reduced less by stress in a future than in a current climate. Despite some recent efforts (Aranjuelo et al., 2006; Bloor et al., 2010; Kongstad et al., 2012), very few studies have focused on the interactions between stress impact and climate change. For N limitation, similar results were found in an earlier manipulation experiment on identical grassland communities in which N limitation decreased biomass production under a current climate, but not under a future climate (Van den Berge et al., 2011b). However, in the same experimental set-up drought impact was not altered by a future climate (Naudts et al., 2011, 2013). A similar observation has been reported for *Lotus corniculatus* L. monocultures grown under laboratory conditions at a constant temperature (Carter et al., 1997).

What mechanisms could be responsible for the decreased impact of stress on above-ground productivity under future climate conditions? We have examined three possible explanations. Firstly, a future climate could affect drought intensity or the availability of N and Zn. Secondly, a future climate could alter the uptake of N, Zn and water by stimulating root production and thirdly, a future climate could affect the ability of plants to cope with stress through changes in plant metabolism.

The first mechanism affects exposure to stress through changes in the availability of N, Zn or water. Plant Zn concentrations were not altered by climate. Zinc concentrations found in plant material are a good indicator for metal bioavailability in the soil; hence it is safe to assume that Zn availability did not differ between climates in our study (Maiz et al., 2000; Van den Berge et al., 2011a). If water could explain the smaller stress impact on above-ground biomass in the future climate in drought stressed communities, we would expect higher water availability than in the current climate. This was not the case; we found that SWC was lower in the future climate throughout the entire experiment. The plant N stock in our study did not differ between climates, suggesting no change

in N availability in a future climate. However, the fact that the *P*-value of the interaction between climate and stress in the N limited communities was close to 0.05 (0.082) (see Section 3.1) suggests that we cannot completely rule out changes in N availability as an explanation for the altered biomass response to stress. Moreover, an earlier study by Van den Berge et al. (2011b) revealed a modified relation between added N and plant available N in a future climate, potentially contributing to an altered biomass response to N limitation.

The second potential mechanism also operates via changes in exposure to stress and hinges on altered root production. Stimulated root production in a future climate could change N, Zn and water uptake. This would moderate effects of N limitation and drought, but could intensify Zn toxicity. In previous studies, an increased root/shoot ratio under elevated CO_2 concentrations increased drought tolerance by improving water and nutrient exploration (Rogers et al., 1994; Erice et al., 2006). In the present study, a future climate did indeed enhance root production in unstressed communities, but this stimulation was lost under stress. Hence, an altered uptake of N, Zn or water due to an altered root production is unlikely to explain the above-ground biomass response.

Differences in exposure to stress due to altered availability or uptake of Zn and water were thus unlikely to explain the altered biomass response under future climate conditions. In the specific case of N limitation, an altered availability could not be excluded. However, this does not eliminate the possibility of a simultaneous increase in protection through changes in plant metabolism, which is the third mechanism we explored. Therefore, we analyzed C allocation to starch and cell wall material (IS), as well as plant antioxidant responses.

The positive stress impact values of IS in a current climate indicate that plants responded to stress by incorporating more C into structural carbohydrates. In contrast, plants in the future climate did not exhibit such increased C allocation to storage or structural elements (stress impact values around 0) upon stress. Converting sugars to storage and structural materials may at first seem a beneficial response upon stress exposure, but retaining higher amounts of soluble sugars could enhance stress protection. Soluble sugars have been found to assist in osmotic adjustments (Tarczynski et al., 1993), membrane and protein stabilization (Amiard et al., 2003; Hincha et al., 2003), and can have a signalling function in regulating stress and defence responses (Gomez-Ariza et al., 2007). Moreover, an increasing number of studies indicate that sugar and sugar-like compounds can act as ROS scavengers, working in concert with vacuolar phenolic compounds and the 'classic' cytosolic antioxidant mechanisms (Nishizawa et al., 2008; Peshev et al., 2013). Our results suggest that future climate conditions affect the stress-induced changes in sugar metabolism, which could potentially explain an enhanced protective capacity. Further analysis of metabolite levels and sugar dynamics is necessary to understand these metabolic changes and to elucidate the importance of this response in stress protection under future climate conditions.

Increased levels of enzymatic and molecular antioxidants are often associated to stress tolerance (Mittler, 2002; Le Martret et al., 2011). A key explanation for the reduced stress impact on plant biomass production that we observed, was therefore found in the increased levels of several small molecular antioxidants in the future climate-grown plants. Increases in TAC and polyphenol levels were observed for unstressed and stressed communities, indicating that future climate improves the protective capacity of plant communities. Higher antioxidant levels probably protected plant metabolism against oxidative stress impact and contributed to the smaller reduction in above-ground biomass in the future climate treatment. The improved protective capacity could be explained by an increased capacity for detoxification and repair through an increased internal availability of C under elevated CO_2 (Carlson and Bazzaz, 1982).

Increases in other antioxidants, ASC and GSH, and in the ASC redox status were only apparent in the unstressed communities. The stress impact values for ASC and GSH levels and ASC redox status were positive in the current climate, but zero to negative in the future climate. These results are consistent with the idea that these antioxidants were 'more effectively consumed' by the plants grown in a future climate, supporting their role in protection and continued growth. The difference in response to stress conditions for ASC, GSH (not increased) and TAC (increased), is not a surprise, as ASC and GSH represent only a fraction of the TAC, and therefore not necessarily show the same pattern.

Comprehensive analyses of plant stress responses, at various levels of organization, under different climate conditions, provide crucial information to predict future ecosystem functioning. Since it is unfeasible to test all possible stressors in a realistic experimental set-up, a mechanistic understanding of stress responses in a future climate is highly important. Reports on multiple stress responses under combined elevated CO_2 and warming, containing biochemical as well as ecological response variables are very rare. Also, studies on the single effects of elevated CO_2 on antioxidant metabolism remain inconclusive (Tausz et al., 2007). A reduction in ROS formation under elevated CO_2 can lead to a down-regulation of the protective systems in plants (Schwanz and Polle, 1998; Vurro et al., 2009; Gillespie et al., 2011), but other studies have found an enhanced protective capacity (Schwanz and Polle, 2001; Geissler et al., 2010) or even an increase in oxidative stress (Qiu et al., 2008). One other experiment tackling the effect of climate on drought stress, did not show an increased protective effect of future climate at the antioxidant level, which was in line with the absence of an effect of future climate at the biomass level (Farfan-Vignolo and Asard, 2012). This discrepancy with our findings indicates that the protective effect of future climate – at the biochemical and therefore also at the biomass level – is highly dependent on the timing, intensity and duration of the stressor. In a study on the combined effects of elevated CO_2, warming and drought on regrowth of *Medicago sativa* L. 30 days after cutting, a smaller reduction in regrowth was found in future than in current climate (Erice et al., 2007). With regard to concentrations of antioxidant metabolites (ASC and GSH) there was no effect of climate in unstressed communities, while in stressed communities the future climate caused an up-regulation of GSH (Erice et al., 2007). In our study we found an up-regulation in both the ASC and GSH levels in a future climate in unstressed, but not in stressed communities. We hypothesize that the up-regulation of antioxidants in a future climate is explained by an increased availability of substrates for their synthesis. However, the mechanistic understanding of the effect of a future climate on antioxidant metabolism should be the subject of further research. We therefore suggest a more extensive study that includes the determination of additional biochemical parameters related to plant defence mechanisms including proline biosynthesis, sugar dynamics, changes in osmolyte levels, and the analysis of plant antioxidant enzymes.

In conclusion, we can confirm the hypothesis that a future climate ameliorates plant protection against stress. Under stress, plants grown in a future climate had a smaller reduction in above-ground biomass production than plants grown in current climate. Our results indicate three mutually non-exclusive explanations for this response: (i) TAC and polyphenol levels indicate a better protection against stress, (ii) ASC and GSH are more efficiently used as antioxidants, and (iii) changes in sugar metabolism provide an enhanced stress protection in future climate. Most notably about our findings is the striking consistency of the protective effect of future climate across seven stress treatments. There could be worldwide implications connected to the alleviation of the stress

impact on grassland productivity under future climate conditions. For instance, the enhanced protection against drought could mitigate anticipated productivity losses in regions where more frequent and more intense droughts are predicted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2013.11.003.

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