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Exotic C₄ Grasses Have Increased Tolerance to Glyphosate under Elevated Carbon Dioxide

A. Manea, M. R. Leishman, and P. O. Downey*

The increase in atmospheric CO₂ levels can influence the growth of many invasive exotic plant species. However, it is not well-documented, especially for C₄ plants, how these growth responses will alter the effectiveness of the world's most widely used herbicide for weed control, glyphosate. We aimed to address this question by carrying out a series of glasshouse experiments to determine if tolerance to glyphosate is increased in four C₄ invasive exotic grasses grown under elevated CO₂ in nonlimiting water conditions. In addition, traits including specific leaf area, leaf weight ratio, leaf area ratio, root : shoot ratio, total leaf area, and total biomass were measured in order to assess their contribution to glyphosate response under ambient and elevated CO₂ levels. Three of the four mature grass species that were treated with the recommended concentration of glyphosate displayed increased tolerance to glyphosate under elevated CO₂. This was due to increased biomass production resulting in a dilution effect on the glyphosate within the plant. From this study, we can conclude that as atmospheric CO₂ levels increase, application rates of glyphosate might need to be increased to counteract the growth stimulation of invasive exotic plants.

Nomenclature: Glyphosate.

Key words: Alien plants, biomass, climate change, herbicide, photosynthetic pathway.

The nonselective herbicide glyphosate has been utilized extensively on a global basis since 1974 (Perez and Kogan 2003). Its ability to control a broad range of weeds has made it the world's most important herbicide (Baylis 2000; Powles 2008; Woodburn 2000). Glyphosate works by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme in the shikimic acid pathway. The inhibition of this enzyme prevents the biosynthesis of aromatic amino acid, which causes a halt in the formation of proteins and secondary compounds (Bradshaw et al. 1997).

With the emergence of climate change as a global issue, studying the response of plants to different climate change components and the resulting effects on the efficacy of glyphosate should be a priority. One critical component of climate change is the rising concentration of atmospheric CO₂. Over the last two decades, the amount of CO₂ available to plants has increased significantly (IPCC 2007). This is due mainly to the burning of fossil fuels and changes in land use that have caused an increase in the amount of atmospheric CO₂ from a preindustrial era concentration of 280 parts per million (ppm) to 379 ppm in 2005 (IPCC 2007). This trend is set to continue with atmospheric CO₂ predicted to reach 700 ppm by 2100 (IPCC 2007). An increase in atmospheric CO₂ could result in anatomical, morphological, and physiological changes in many plants that could influence uptake rates, transport, and the overall effectiveness of glyphosate (Bradshaw et al. 1997; Ziska and Teasdale 2000).

Plants that utilize the C₃ photosynthetic pathway are predicted to be favored under increased atmospheric CO₂ because the current CO₂ concentration is suboptimal for C₃ photosynthesis (Leegood 2002). Additional CO₂ causes a "fertilization effect," resulting in higher growth rates (Belote et al. 2003; Dukes 2002; Erickson et al. 2007; Poorter and Navas 2003; Sasek and Strain 1988, 1991; Smith et al. 2000;

Wray and Strain 1987; Ziska 2002; Ziska et al. 2004, 2005, 2007) and greater total leaf area (Sasek and Strain 1991; Wray and Strain 1987; Ziska et al. 2007). These changes might have a dilution effect on the glyphosate rendering it less effective than under current atmospheric CO₂ concentrations. In addition, C₃ plants display decreased stomatal conductance and stomatal number and increased leaf thickness that may further compromise glyphosate's effectiveness by restricting its foliar uptake (Ainsworth and Long 2005; Nowak et al. 2004; Ziska and Teasdale 2000).

Similar to C₃ plants, stomatal conductance in C₄ plants consistently is reduced under elevated CO₂ (Ainsworth and Long 2005; Wand et al. 1999). However, for C₄ plants, CO₂ fixation is saturated at 360 ppm, so these plants are less likely to show a positive response to additional CO₂ availability (Leegood 2002). This makes predicting the response of C₄ plants to increased atmospheric CO₂ more difficult (Dukes 2000). This unpredictability is demonstrated by studies that show the growth of C₄ plants can be stimulated (Bazzaz et al. 1989; Owensby et al. 1993, 1999), not affected (Erickson et al. 2007; Wray and Strain 1987), or inhibited (Belote et al. 2003) under elevated CO₂. These variable responses could result from variation in environmental conditions such as water availability (Owensby et al. 1993, 1999), making accurate predictions about the effectiveness of glyphosate on C₄ plants under elevated CO₂ more difficult in comparison to C₃ plants. For this reason we chose to focus on the efficacy of glyphosate under elevated CO₂ in C₄ plants.

We are aware of only three studies that have looked at changes in tolerance of plant species to glyphosate under elevated CO₂. Ziska et al. (1999) studied two weedy species, *Amaranthus retroflexus* L. and *Chenopodium album* L., which are C₄ and C₃, respectively. Irrespective of CO₂ level, *A. retroflexus* displayed a reduction in growth and subsequent elimination as a result of glyphosate application. In contrast, the growth of *C. album* was not affected by glyphosate application under elevated CO₂. From this, the authors concluded that C₃ plants have an increased tolerance to glyphosate under elevated CO₂. These results were reinforced by Ziska and Teasdale (2000) and Ziska et al. (2004) who studied the C₃ perennial weeds *Elytrigia repens* (L.) Desv. ex

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B.D. Jackson and *Cirsium arvense* (L.) Scop., respectively. The reason for this increased tolerance to glyphosate under elevated CO₂ is unclear, but it has been suggested that an increase in the root : shoot ratio might play a role (Ziska et al. 2004). Thus, it is apparent that more research is required on two fronts: (1) the tolerance of plants, especially C₄ plants, to glyphosate under elevated CO₂; and (2) the underlying mechanism responsible for changes in tolerance.

In the current study, we examined four C₄ grass species to determine: (1) the efficacy of glyphosate on different age cohorts (seedling, juvenile, mature) of the plants under two CO₂ concentrations (ambient and elevated), (2) regrowth responses after glyphosate application under ambient and elevated CO₂, and (3) growth and allocation responses that might be responsible for altering the effectiveness of glyphosate under elevated CO₂. The grasses selected for this study all have been introduced to Australia and are invasive along the east coast from southeastern Queensland to northeastern Victoria (Harden 1993). The experiment was carried out in nonlimiting water conditions. As stomatal conductance is decreased in C₄ plants under elevated CO₂ (Ainsworth and Long 2005; Wand et al. 1999), nonlimiting water conditions should not affect the relative success of the grasses under elevated CO₂.

Materials and Methods

Species Selection. The four grass species selected were *Chloris gayana* Kunth, *Eragrostis curvula* (Schrad.) Nees, *Paspalum dilatatum* Poir., and *Sporobolus indicus* (L.) R. Br. Species selection was based on two criteria: (1) they had to be invasive exotics within Australia, and (2) they are chemically controlled with glyphosate for land management. All species are C₄ grasses and have a fairly rapid development so that mature developmental stages could be incorporated in the study.

Experimental Design. Each species was grown in a series of glasshouse experiments under ambient (380 to 420 ppm) and elevated (675 to 715 ppm) CO₂ conditions. These CO₂ concentration ([CO₂]) ranges were maintained by a CO₂ dosing and monitoring system.¹ The lower concentrations of these ranges tended to occur at nighttime, and the higher concentrations tended to occur during the daytime. The ambient CO₂ treatment represents the atmospheric CO₂ concentration during the turn of the 21st century (IPCC 2007). The elevated CO₂ treatment represents the potential atmospheric CO₂ concentration by 2100 (IPCC 2007). For each CO₂ treatment, individual plants were planted at three intervals (1, 2, and 3 mo) prior to glyphosate application. These planting intervals represent the postemergent developmental stages (seedling, juvenile, mature) of the grasses. Two glyphosate application rates or concentrations ([glyphosate]) were applied to each [CO₂] by cohort treatment: recommended (10 ml L⁻¹) and double recommended (20 ml L⁻¹). There were eight replicates for each of the 12 treatments (two [CO₂] by three cohorts by two [glyphosate] = 12 treatments). In addition, eight extra control plants per grass species were grown for each [CO₂] by cohort treatment to be harvested prior to glyphosate application. This design resulted in 576 plants (four grass species by two [CO₂] by three cohorts by two [glyphosate] by eight replicates + four grass species by two [CO₂] by three cohorts by eight replicates). In total, four

Table 1. Results from the log rank tests of overall survival rates for each cohort and glyphosate treatment or concentration ([glyphosate]) combination. Values shown are P values and significant differences are shown in bold. Letter in last column indicates under which CO₂ level (A = Ambient, E = Elevated) the grass species had a significantly (P = 0.05) higher survival rate.

[Glyphosate]	Cohort	df	χ^2	P value
Recommended	Young	1	0.509	0.476
Double	Young	1	0.509	0.476
Recommended	Juvenile	1	0.220	0.639
Double	Juvenile	1	0.220	0.639
Recommended	Mature	1	39.290	< 0.001 E
Double	Mature	1	2.392	0.122

glasshouses were used in this study, two for each CO₂ level. The pots within a CO₂ level were evenly split between the two glasshouses so each glasshouse contained the same number of replicates for each cohort by [glyphosate] treatment. The reason for doing this was twofold: (1) to act as insurance in case one glasshouse malfunctioned during the experiment, and (2) to average out the potentially differing conditions between the glasshouses. The temperature of the glasshouses was set for a maximum of 25 C and a minimum of 19 C. This temperature range was maintained with a reverse cycle inverter-ducted air conditioning unit. To avoid variation in growing conditions within each glasshouse, pots within each glasshouse were randomly moved to new positions on a fortnightly basis.

Seed Collection and Germination. Seeds for each of the four grass species were collected from a range of individual plants from sites in the Hawkesbury region of western Sydney. Once collected, the seeds for each of the grass species were germinated on moist filter paper within petri dishes.

Planting and Growth Conditions. Planting occurred 3, 2, and 1 mo prior to glyphosate application in order to produce three age cohorts of individuals: emergent seedlings, juveniles, and mature plants. For each cohort, seedlings were transplanted into pots in the glasshouses at the stage of cotyledon emergence. Multiple seedlings were transplanted into each pot to act as insurance against seedling mortality. After 3 d, any excess seedlings were removed from the pot. The seedlings were transplanted into pots 175 mm diam and 195 mm deep. The soil mixture used was obtained from a commercial supplier² and consisted of soil, double washed sand, composted sawdust, and graded ash in a ratio of 5 : 2 : 2 : 1. Each pot contained 2.4 L of this mixture. The pots were lined with newspaper to prevent soil loss through the drainage holes in the pots. The grasses were mist watered for 2 min three times daily. To counteract the nutrient loss resulting from this daily watering, 6.5 ± 0.2 g of slow release native plant fertilizer³ (23N : 2P : 17K) was added to each pot.

Glyphosate Application and Harvesting. One d prior to glyphosate⁴ application, the additional control plants from each cohort by [CO₂] treatment were harvested. These plants were separated into the following components: three fully expanded leaves, remaining leaf biomass, remaining above-ground biomass, and belowground biomass. The plant components were then washed free of soil before being oven-dried at 80 C for 48 h and weighed using a Mettler Toledo B-S electronic balance.

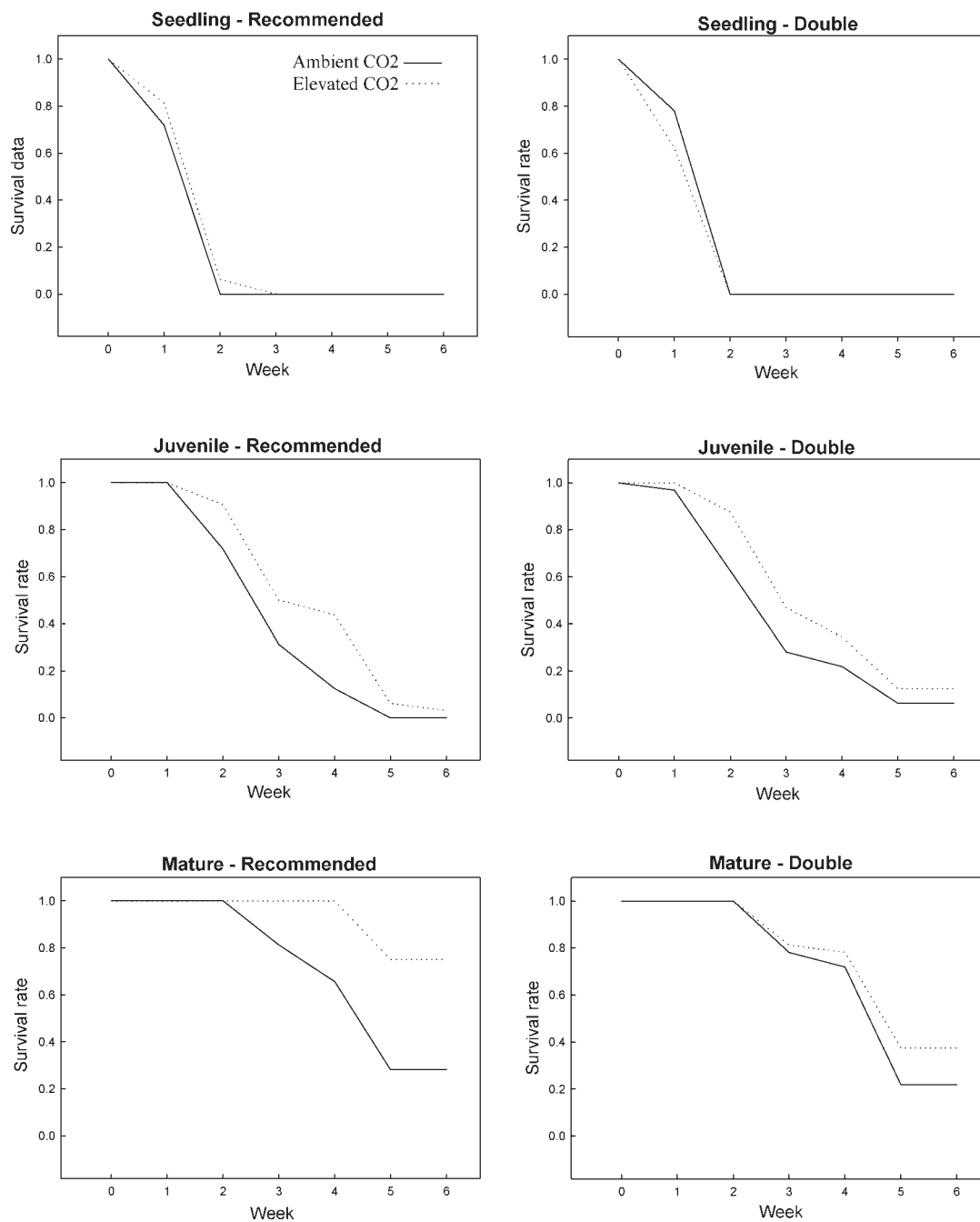


Figure 1. Survival rates of all the age cohort by glyphosate combinations grown under ambient and elevated CO₂ glasshouse conditions for 6 wk. The lines indicate the mean of eight plants grown under each CO₂ treatment (solid = ambient, dashed = elevated) for the four grass species combined.

On the day following the initial harvest, the glyphosate treatment (10 ml per plant) was applied to all remaining plants. After 42 d, the surviving plants were harvested and oven-dried as previously described but were not split into their components.

During the 42-d period after glyphosate application, the mortality of the grasses was recorded on a weekly basis. This involved classifying each individual grass as alive or dead. A grass was classified as dead if all its leaves became discolored and died. A plant was classified as alive if it showed signs of regeneration by the end of the 42 d. If a grass was classified as dead it remained in the experiment for the entire 42-d period and continued to be watered. Grasses that were classified as dead still were harvested after the 42-d period to ensure there was no living root biomass. There were no instances where

living root biomass was found in the pots of the grasses classified as dead, so we can assume this classification system was reliable.

Measuring Plant and Allocation Traits. In order to determine if there was any relationship between plant traits and tolerance to glyphosate, the following range of traits were measured or calculated for each grass species: Specific leaf area (SLA) was measured as the leaf area per unit leaf mass for three randomly selected outer canopy leaves. Leaf area was measured using a LI-3100C Area Meter⁵ prior to oven-drying the leaves for weighing. Leaf weight ratio (LWR) was calculated as total plant leaf mass divided by total plant mass.

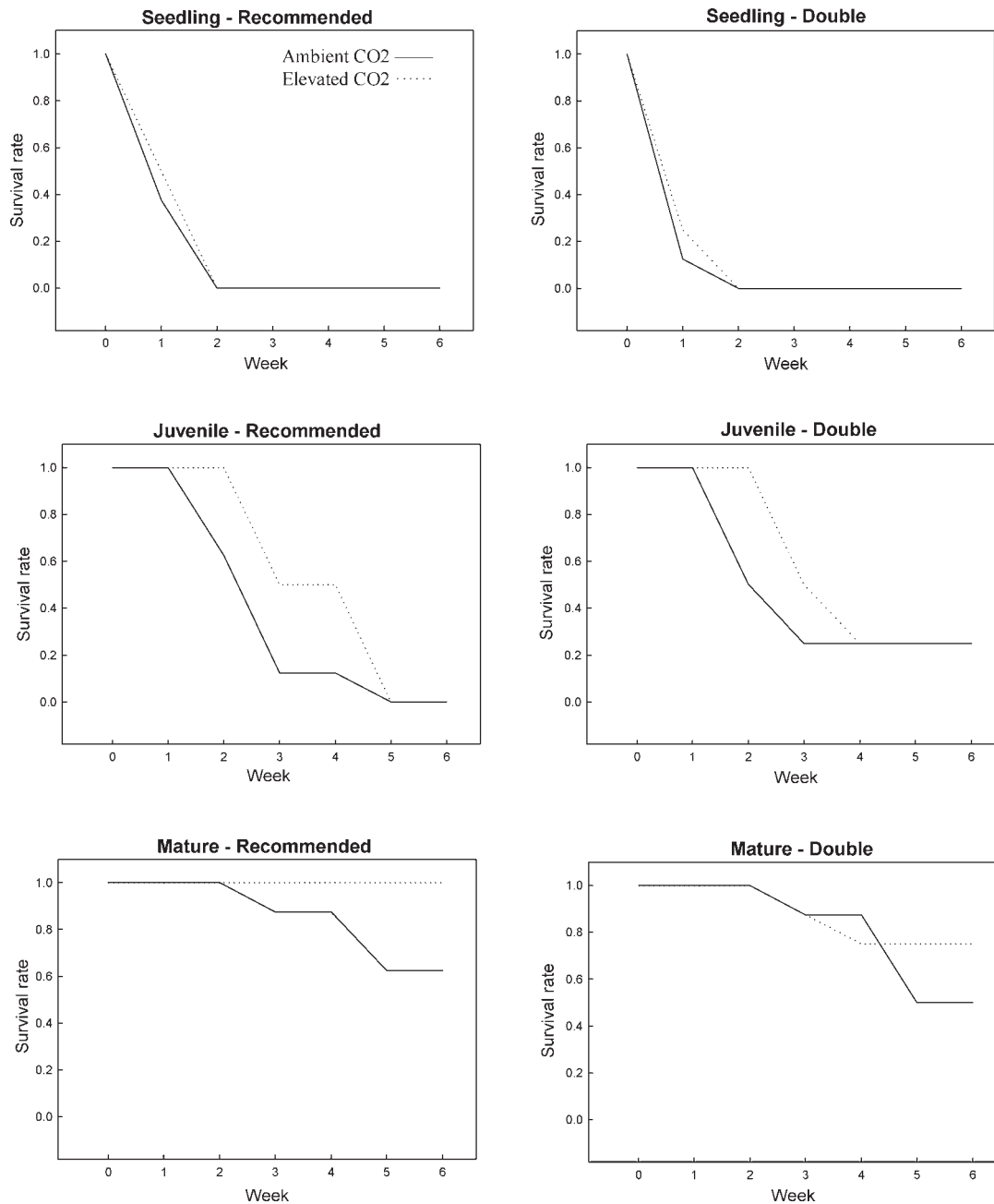


Figure 2. Survival rates of all the age cohort by glyphosate combinations grown under ambient and elevated CO₂ glasshouse conditions for 6 wk. The lines indicate the mean of eight plants grown under each CO₂ treatment (solid = ambient, dashed = elevated) for *C. gayana*.

Leaf area ratio (LAR) was calculated as SLA times LWR. Root-to-shoot ratio (R : S) was calculated as total root biomass divided by total shoot biomass. Total leaf area (L_T) was measured as the sum of the leaf area of all leaves on a plant. Total biomass (B_T) was calculated as the sum of the dry weight of all of the harvested components.

Across-Species Data Analysis. To determine the survival function of all the grass species in response to CO₂ level, Kaplan-Meier survival curves were generated. The survival distributions for each of the grass species under ambient and elevated CO₂ were compared using a log rank test, with significance determined at $P < 0.05$. These analyses were carried out for each cohort by glyphosate treatment across all grass species.

Species-Level Data Analysis. To determine the survival function of each grass species in response to CO₂ level, Kaplan-Meier survival curves were generated. The survival distributions for each of the grass species under ambient and elevated CO₂ were compared using a log rank test, with significance determined at $P < 0.05$. These analyses were carried out for each cohort by glyphosate treatment within each grass species.

Species that survived glyphosate treatment had total leaf senescence followed by resprouting. To assess this regrowth response for each grass species under ambient and elevated CO₂, the regrowth biomass data was analysed using two sample t-tests. This analysis was carried out for both the recommended and double glyphosate treatments in each cohort for each of the four grass species.

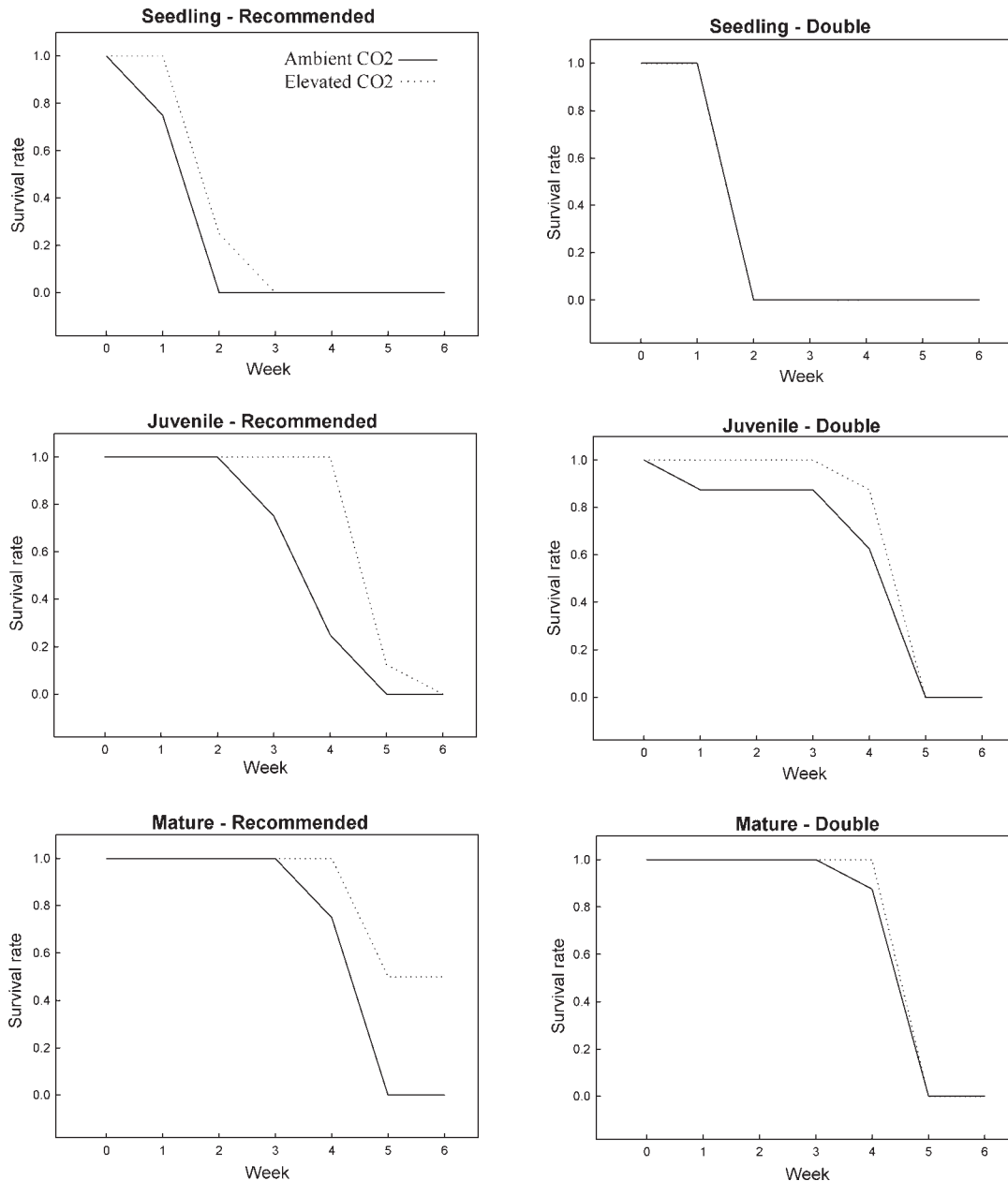


Figure 3. Survival rates of all the age cohort by glyphosate combinations grown under ambient and elevated CO₂ glasshouse conditions for 6 wk. The lines indicate the mean of eight plants grown under each CO₂ treatment (solid = ambient, dashed = elevated) for *E. curvula*.

To determine if the plant growth and allocation traits were influenced by CO₂ level, the trait data was analysed using two sample *t*-tests. This analysis was carried out for all three cohorts for each of the four grass species.

For both the overall and species-level data analyses, the significance level was set at 0.05. The regrowth biomass and trait data analyses were carried out using Minitab 15 Statistical Software (Minitab, Inc. 2007). The survival analysis was carried out using SAS 9.2 (SAS Institute, Inc. 2008).

Results and Discussion

In this study we tested whether the efficacy of glyphosate on invasive exotic grass species is affected under elevated CO₂ conditions. Our results showed that the mature invasive exotic grasses that were sprayed with the recommended concentra-

tion of glyphosate had a significantly higher survival rate under elevated CO₂ compared with ambient CO₂ ($\chi^2 = 39.290$, $P < 0.001$; Table 1; Figure 1). To determine if particular species were driving this result, the data were analyzed at the species level. This analysis showed that *C. gayana* ($\chi^2 = 8.641$, $P = 0.003$; Figure 2), *E. curvula* ($\chi^2 = 7.752$, $P = 0.005$; Figure 3), and *P. dilatatum* ($\chi^2 = 31.200$, $P < 0.001$; Figure 4) had significantly higher survival rates under elevated CO₂ conditions, whereas *S. indicus* ($\chi^2 = 0.830$, $P = 0.362$; Figure 5) showed no difference in response between CO₂ treatments. Sufficient individuals survived of only two species to enable analysis of this regrowth response (the mature cohort of *C. gayana* and *S. indicus* at both glyphosate treatments). The regrowth responses of *C. gayana* ($t_6 = -0.240$, $P_{\text{Recommended}} = 0.818$; $t_5 = 0.752$, $P_{\text{Double}} = 0.486$) and *S. indicus* ($t_7 = 0.096$, $P_{\text{Recommended}} = 0.926$;

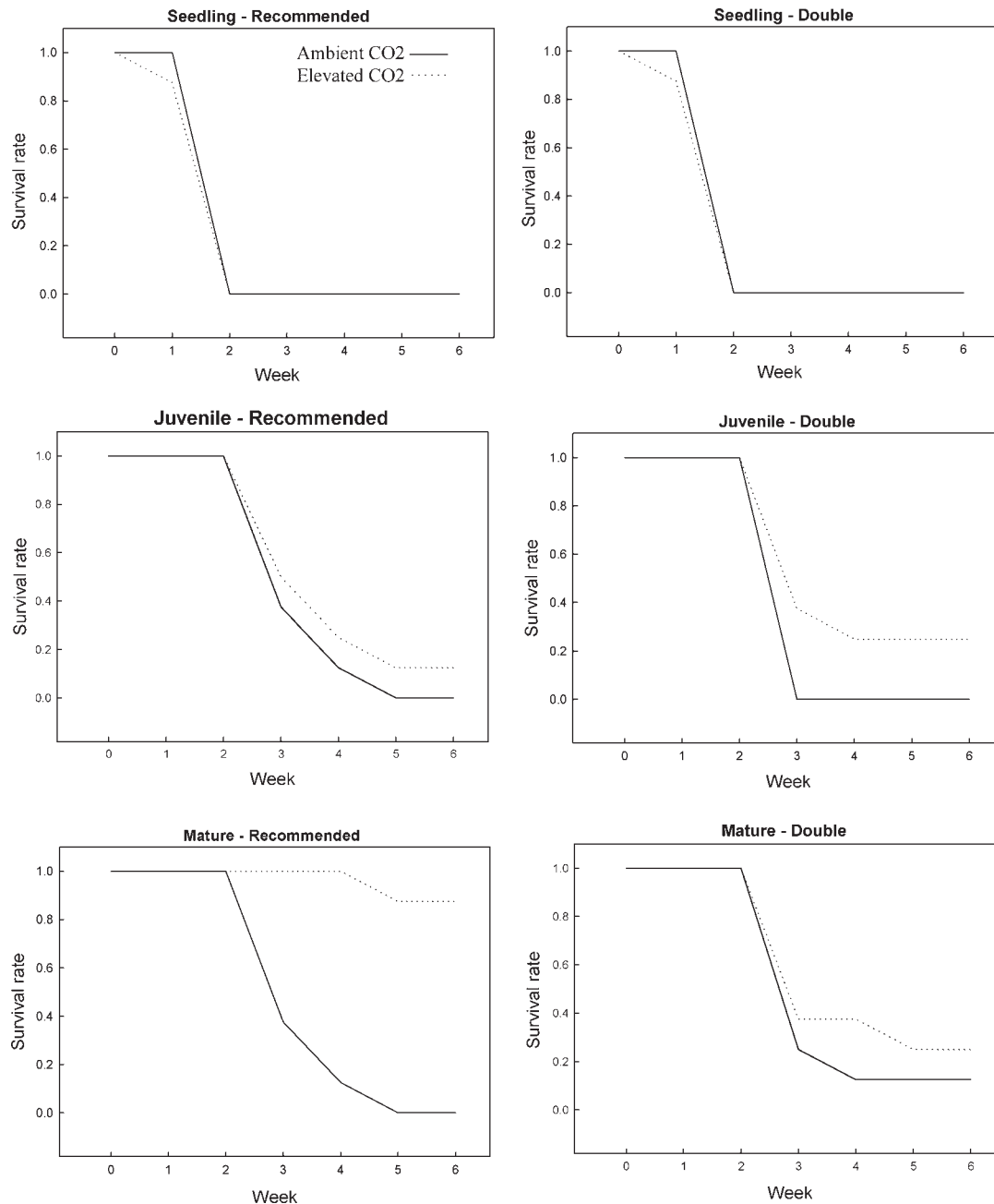


Figure 4. Survival rates of all the age cohort by glyphosate combinations grown under ambient and elevated CO₂ glasshouse conditions for 6 wk. The lines indicate the mean of eight plants grown under each CO₂ treatment (solid = ambient, dashed = elevated) for *P. dilatum*.

$t_2 = 1.709$, $P_{\text{Double}} = 0.229$) did not differ significantly between ambient and elevated CO₂ for either of the glyphosate treatments.

We examined growth and allocation traits of the four species before glyphosate application in order to aid interpretation of the survival data (Table 2). The three grass species (*C. gayana*, *E. curvula*, and *P. dilatum*) that had a significantly higher survival rate under elevated CO₂ also produced more biomass (39, 83, and 59% increase, respectively) and leaf area (40, 67, and 24% increase, respectively) under these conditions. These trait differences between ambient and elevated CO₂ were significant for the biomass production of *E. curvula* ($t_{14} = 3.475$, $P = 0.005$) and *P. dilatum* ($t_{14} = 4.134$, $P = 0.001$), and total leaf area of *E. curvula* ($t_{14} = 5.309$, $P < 0.001$). In contrast, the biomass (5% decrease) and total leaf area (34% decrease)

produced by *S. indicus*, the only species not to have a significantly higher survival rate under elevated CO₂, decreased between the two CO₂ levels. This suggests that the effectiveness of glyphosate might be proportional to the amount of plant tissue upon which it has to act. That is, a larger amount of biomass might dilute the glyphosate within the plant, rendering it less effective. In the future, if invasive exotic plant growth is stimulated under elevated CO₂ conditions, it might be necessary to increase the concentration at which glyphosate is applied to these plants to counteract the increase in biomass production.

The results of this study are consistent with the few previous studies that have found an increase in tolerance of invasive exotic C₃ plant species to glyphosate under elevated CO₂ levels compared to ambient CO₂ levels (Ziska et al. 1999, 2004; Ziska and Teasdale 2000). Our study has

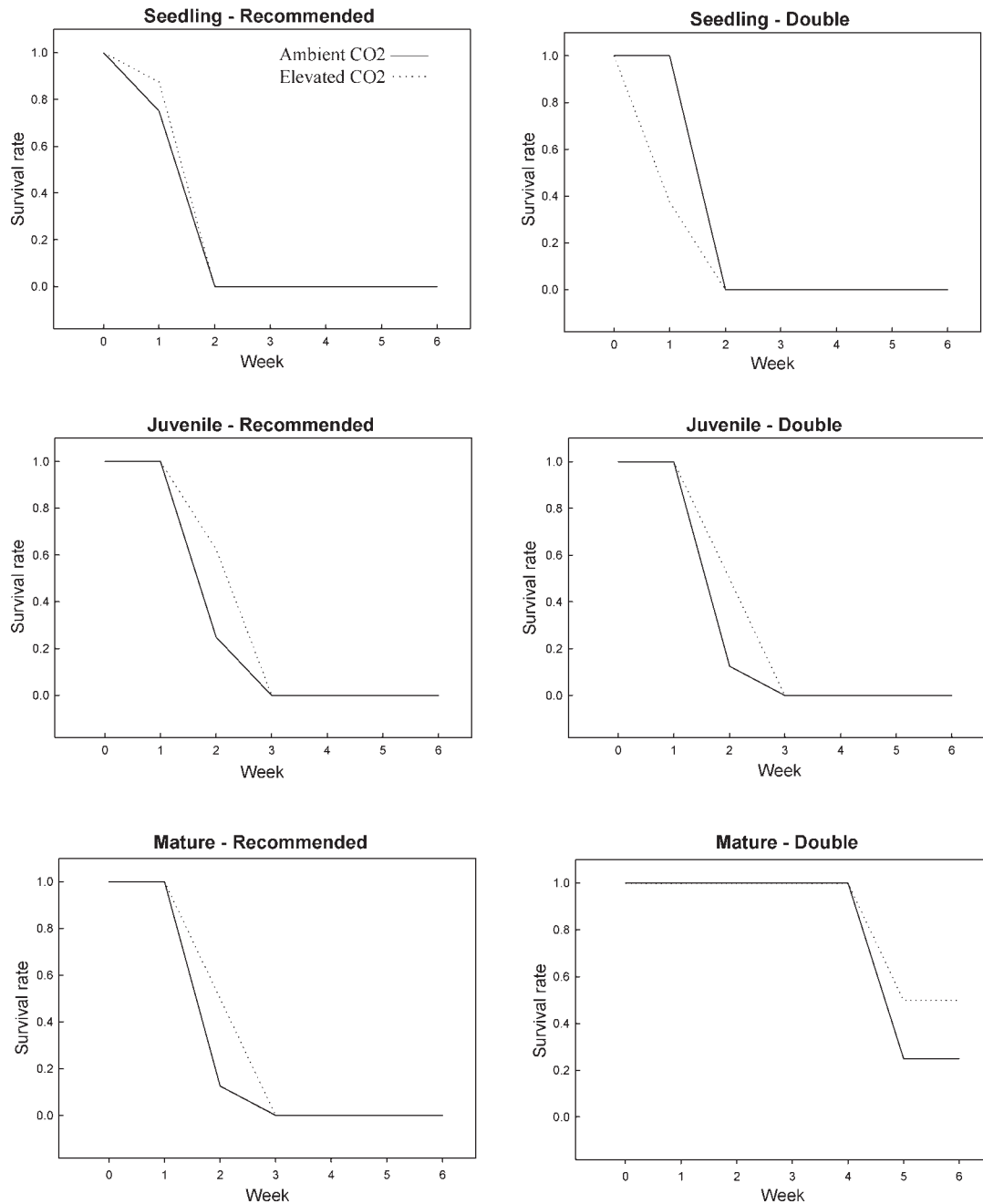


Figure 5. Survival rates of all the age cohort by glyphosate combinations grown under ambient and elevated CO₂ glasshouse conditions for 6 wk. The lines indicate the mean of eight plants grown under each CO₂ treatment (solid = ambient, dashed = elevated) for *S. indicus*.

expanded this knowledge base to include a larger number of C₄ plant species. Ziska et al. (1999) is the only study of which we are aware that has tested the efficacy of glyphosate on a C₄ plant under ambient and elevated CO₂ levels. They found that glyphosate tolerance of the C₄ weedy species *A. retroflexus* was not affected by CO₂ concentration. Thus of the five C₄ species studied so far, three have shown increased tolerance of glyphosate under elevated CO₂ and two have shown no difference in tolerance.

This study suggests that the differing response in survival among species to glyphosate application between ambient and elevated CO₂ is related to their biomass production. The high mortality of seedlings and juvenile plants of all species under both glyphosate and CO₂ treatments is consistent with this.

However it should be stressed that there are other CO₂-induced effects that were not measured by this study which might have influenced our results. Stomatal number and cuticle thickness are consistently reduced under elevated CO₂, which might have limited the uptake of glyphosate into the plant tissue (Ainsworth and Long 2005; Wand et al. 1999). Protein content per gram of tissue also can be reduced under elevated CO₂, which might reduce the need for aromatic amino acids (Bowes 1996). Glyphosate inhibits the production of these aromatic amino acids, but if there is not a large demand for them, glyphosate will be less effective (Ziska et al. 1999).

This study focused on the response of invasive exotic C₄ grasses to glyphosate application under ambient and elevated CO₂ for plants grown as individual plants under nonresource-

Table 2. Species-level trait means and standard errors for the four species grown under ambient and elevated CO₂ and harvested at 3 mo after planting. Significant P values from *t*-tests are shown in bold.

Species	Trait	Ambient CO ₂		Elevated CO ₂		df	<i>t</i>	P
		Mean	SE	Mean	SE			
<i>Chloris gayana</i>	Total biomass	17.240	2.790	23.922	5.448	14	1.798	0.094
	Specific leaf area	16.373	1.786	20.662	0.502	14	5.253	< 0.001
	Leaf weight ratio	0.306	0.016	0.278	0.020	14	-1.232	0.246
	Leaf area ratio	5.054	0.852	5.732	0.627	14	1.367	0.199
	Root : shoot ratio	0.390	0.027	0.417	0.010	14	0.403	0.693
<i>Eragrostis curvula</i>	Total leaf area	872.411	175.187	1,222.533	138.408	14	2.155	0.052
	Total biomass	5.303	0.465	9.697	0.708	14	3.475	0.005
	Specific leaf area	16.838	1.969	18.224	2.324	14	1.109	0.289
	Leaf weight ratio	0.527	0.025	0.658	0.017	14	0.299	0.317
	Leaf area ratio	5.364	1.177	5.340	0.961	14	-0.048	0.962
<i>Paspalum dilatatum</i>	Root : shoot ratio	0.193	0.018	0.240	0.032	14	1.056	0.310
	Total leaf area	287.283	19.068	479.019	42.303	14	5.309	< 0.001
	Total biomass	30.335	1.379	48.128	0.900	14	4.134	0.001
	Specific leaf area	27.693	3.061	28.789	1.627	14	0.574	0.575
	Leaf weight ratio	0.283	0.017	0.202	0.019	14	-6.051	< 0.001
<i>Sporobolus indicus</i>	Leaf area ratio	7.899	1.414	5.825	0.240	14	-2.920	0.014
	Root : shoot ratio	0.308	0.024	0.375	0.046	14	1.870	0.098
	Total leaf area	1,990.130	97.082	2,463.355	90.617	14	1.562	0.142
	Total biomass	2.629	0.620	2.496	0.583	14	-0.156	0.878
	Specific leaf area	19.576	0.912	17.045	0.717	14	-2.182	0.048
	Leaf weight ratio	0.378	0.018	0.465	0.053	14	1.570	0.151
	Leaf area ratio	7.409	0.511	7.930	0.934	14	0.489	0.635
	Root : shoot ratio	0.327	0.034	0.176	0.045	14	-2.673	0.019
	Total leaf area	196.101	44.775	129.657	41.737	14	-1.086	0.296

limited glasshouse conditions. These conditions might not accurately represent those experienced in the grasses' natural environment where there is competition for resources such as soil moisture, nutrients, and light. Previous work has shown that plant response to elevated CO₂ is constrained by resource availability (Ören et al. 2001; Poorter et al. 1996; Reich et al. 2006). Nevertheless, our study has shown clearly that when plants are able to respond to elevated CO₂ with greater growth and biomass production, particularly leafy biomass, this will increase their tolerance of glyphosate application. This suggests that increasing atmospheric concentrations of CO₂ might require an increase in application rates of glyphosate, which could have significant economic and environmental consequences.

Sources of Materials

¹ CO₂ dosing and monitoring system, Canary Company Pty. Ltd., 1/163 Burns Bay Rd., Lane Cove, New South Wales 2066, Australia.

² Coarse river sand and organic garden mix, Australian Native Landscapes, Terrey Hills, New South Wales 2084, Australia.

³ Slow release low phosphorus fertilizer, J. R. Somplot Co., P.O. Box 27, Boise, ID 83707.

⁴ Glyphosate as isopropylamine salt, Monsanto Company, P.O. Box 1750, Columbus, OH 43216-1750.

⁵ LI-3100C Area Meter, Li-Cor Corporation, 4808 Progressive Avenue, Lincoln, NE 68504.

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