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AQUATIC WEEDS

Response of the floating aquatic fern *Azolla filiculoides* to elevated CO_2, temperature, and phosphorus levels

Weiguo Cheng · Hidemitsu Sakai · Miwa Matsushima · Kazuyuki Yagi · Toshihiro Hasegawa

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Abstract *Azolla filiculoides* is a floating aquatic fern growing in tropical and temperate freshwater ecosystems. As *A. filiculoides* has symbiotic nitrogen-fixing cyanobacteria (*Anabaena azollae*) within its leaf cavities, it is cultivated in rice paddies to improve N availability and suppress other wetland weeds. To understand how C assimilation and N accumulation in *A. filiculoides* respond to elevated atmospheric carbon dioxide concentration (CO_2) in combination with P addition and higher temperatures, we conducted pot experiments during the summer of 2007 and 2008. In 2007, we grew *A. filiculoides* in pots at two treatment levels of added P fertilizer and at two levels of [CO_2] (380 ppm for ambient and 680 ppm for elevated [CO_2]) in controlled-environment chambers. In 2008, we grew *A. filiculoides* in four controlled-environment chambers at two [CO_2] levels and two temperature levels (34/26°C (day/night) and 29/21°C). We found that biomass and C assimilation by *A. filiculoides* were significantly increased by elevated [CO_2], temperature, and P level (all $P < 0.01$), with a significant interaction between elevated [CO_2] and added P ($P < 0.01$). Tissue N content was decreased by elevated [CO_2] and increased by higher temperature and P level (all $P < 0.01$). The acetylene reduction assay showed that the N-fixation activity of *A. filiculoides* was not significantly different under ambient and elevated [CO_2] but was significantly stimulated by P addition. N-fixation activity decreased at higher temperatures (34/26°C), indicating that 29/21°C was more suitable for *A. azollae* growth. Therefore, we conclude that the N accumulation potential of *A. filiculoides* under future climate warming depends primarily on the temperature change and P availability, and C assimilation should be increased by elevated [CO_2].

Keywords *Azolla filiculoides* · Carbon assimilation · Elevated atmospheric carbon dioxide · High temperature · Nitrogen fixation · Phosphorus nutrient

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Introduction

Azolla is a floating aquatic fern that grows in tropical and temperate freshwater ecosystems. As *Azolla* has symbiotic N-fixing cyanobacteria (*Anabaena azollae*) within its leaf cavities, it has been cultivated for many centuries in rice paddies in southern China and northern Vietnam as "green manure" to improve rice N availability (Watanabe & Liu, 1992; Wagner, 1997). Even though chemical N fertilizers have been substituted for *Azolla* as an N source, *Azolla* is still cultivated by organic farmers, especially in rice-fish-*Azolla* or rice-duck-*Azolla* multiple eco-production systems in China and Japan (Watanabe, 2006). In addition to providing N, *Azolla* is known to modify the physical, chemical, and biological properties of soil and the soil–water interface in rice fields and for mobilizing fixed phosphates, retarding the NH_3 volatilization that accompanies the application of chemical N fertilizer, and suppressing aquatic weeds in flooded rice fields (Mandal et al., 1999; Biswas et al., 2005).

Depending on population growth and energy use scenarios, atmospheric CO_2 concentration (CO_2) is expected to rise from its current level of 380 ppm to between 485 and 1000 ppm by 2100 (Intergovernmental Panel on Climate Change (IPCC), 2007). Increases of CO_2 and other greenhouse gases (methane and nitrous oxide) are predicted to cause an average global warming of 1.1–6.4°C by 2100 (IPCC, 2007). The stimulative effect of atmospheric CO_2 enrichment on plant growth and development has been predicted to increase vegetative productivity, with large variations between species (Kimball et al., 2002; Ainsworth & Long, 2005). The variations in growth and photosynthetic enhancements under elevated [CO_2] may be associated with the differential responses of species to other limiting factors, such as temperature, nutrients, light, and water stress (Kimball et al., 2002). As nitrogen (N) already limits productivity in most ecosystems and because tissue N content is a major determinant of photosynthesis, the CO_2 fertilization effect often decreases with increasing exposure to elevated [CO_2] as a result of down-regulation of photosynthetic capacity under elevated [CO_2] (Luo et al., 2004; Reich et al., 2006). In contrast, N-fixing plant species often show a larger growth response to elevated [CO_2] than nonfixing species if other nutrients are not deficient (Lee et al., 2003). After N, phosphorus (P) is the other most frequently limiting nutrient for terrestrial and aquatic plant growth (Kobayashi et al., 2008), especially for N-fixing plants (Singh & Singh, 1988; Vitousek et al., 2002; Černá et al., 2009). Our objective was to understand how the floating aquatic fern *Azolla* responds to elevated [CO_2] in combination with P addition and higher temperatures; and how these changes in climate parameters and P levels affected the N-fixation activity of *Azolla filiculoides*. Since *A. azollae* symbiotically fixes atmospheric N and supplies fixed N to *Azolla*, we hypothesize that *Azolla* growth would be increased by elevated [CO_2], and the stimulatory effect of elevated [CO_2] would be enhanced by P addition and increased temperature. We tested this hypothesis by examining growth of the biomass, C assimilation, and N accumulation by two experiments using controlled-environment chambers in the summers of 2007 and 2008.

Materials and methods

Experimental design and controlled-environment chambers

We conducted two separate pot experiments during the summer seasons in 2007 and 2008 at the National Institute for Agro-Environmental Sciences, Tsukuba, Japan (36°01′N, 140°07′E). We used four controlled-environment chambers (Climatron; Shimadzu, Kyoto, Japan) to maintain the two [CO_2] and two temperature treatments. Each chamber was 4 m × 2 m × 2 m (L × W × H) and could hold 72 pots. The pots were used to grow rice (*Oryza sativa* L.), *Azolla filiculoides*, and some aquatic weeds, included *Monochoria vaginalis* and Barnyardgrass (*Echinochloa crus-galli*) during 2007 and 2008 summer season.

We have used these chambers since 1996 to carry out elevated [CO_2] experiments with rice, and the chambers have performed well in controlling atmospheric CO_2 concentration and temperature (Cheng et al., 2001, 2006; Sakai et al., 2001, 2006). During the 2007 season, we used only two chambers to study how elevated [CO_2] and P nutrient levels affected the growth of *A. filiculoides*. During the 2008 season, all four chambers were used to study interaction effects of elevated [CO_2] and high temperature on growth of *A. filiculoides*. Details of the controlled-environment

chamber systems have been described by Sakai et al. (2001). *Azolla filiculoides* inocula (IRRI code FI 1001) were provided by Dr. Y. Kishida of Okayama University, Japan.

Experiment 1: Effect of elevated [CO_2] and P on *A. filiculoides* growth

Azolla filiculoides was grown in 12 plastic pots (19.5 cm inside diameter, 27.0 cm height, 0.2 cm thickness) filled with 5.0 kg gray sandy soil, which had been collected from the plow layer (about 15 cm of the top layer) of a typical rice field in Kujukuri, Chiba Prefecture, Japan. This plow layer contained 8.1 ± 0.2 g kg^{-1} organic C and 0.9 ± 0.01 g kg^{-1} total N on a dry weight basis, and the average pH was 6.3 (Cheng et al., 2007). Six pots were set up as P-enrichment treatments by the application of superphosphate of lime at 1 g P_2O_5 per pot. Superphosphate of lime was mixed with the soil 1 day before the start of the experiment. All pots were flooded and maintained with tap water at a depth of about 15 cm on the soil surface. A removable cross-shaped plastic bar was floated on the water in each pot to divide the area into four blocks for four replicate samplings (Fig. 1).

On 29 July 2007, the first day of the experiment, the surfaces of the 48 blocks in the 12 pots were inoculated with equal fresh weights (0.2 g) of *A. filiculoides*. Half of the 12 pots (three control pots and three P-enriched pots) were placed in a controlled-environment chamber under ambient [CO_2], and the other six pots were placed in a chamber under elevated [CO_2]. The temperatures in both chambers were the same (32/22°C, day/night). The four treatments were designated as follows:

(1) "EP," elevated [CO_2] (680 ppm) with added P;
(2) "AP," ambient [CO_2] (380 ppm) with added P;
(3) "EC," elevated [CO_2] without added P (control); and
(4) "AC," ambient [CO_2] without added P (control).

Samples of the fern mats from each block in all pots were collected 7, 14, 21, and 28 days after the inoculation by a stainless mesh screen, washed with tap water, and then oven dried at 80°C for 3 days and weighed. Dried fern for the final samples were ground, and the C and N content of the tissues were determined using an elemental analyzer (EA1108; Fisons, Italy).

Experiment 2: Effect of elevated [CO_2] and temperature on *A. filiculoides*

The second experiment was carried out during the 2008 summer season. As in experiment 1, 12 plastic pots filled with 5.0 kg gray sandy soils were used to grow *A. filiculoides*. Superphosphate of lime was applied to all pots at the rate of 1 g P_2O_5 per pot before the start of the experiment. Flooding with water and the 4-block design in the pots were the same as in experiment 1 (Fig. 1).

On 2 July 2008, the surfaces of the 48 blocks in the 12 pots were inoculated with equal fresh weights (0.2 g) of *A. filiculoides*. Three pots were put into each of four controlled-environment chambers for exposure to the combinations of two [CO_2] levels and two temperature treatments. The four treatments were designated as follows:

(1) "EH," elevated [CO_2] (680 ppm) and high temperature (34/26°C, day/night);
(2) "AH," ambient [CO_2] (380 ppm) and high temperature (34/26°C);
(3) "EL," elevated [CO_2] and low temperature (29/21°C, day/night); and
(4) "AL," ambient [CO_2] and low temperature (29/21°C).

Owing to the schedule for chamber use, we shortened the growth period in this experiment. After

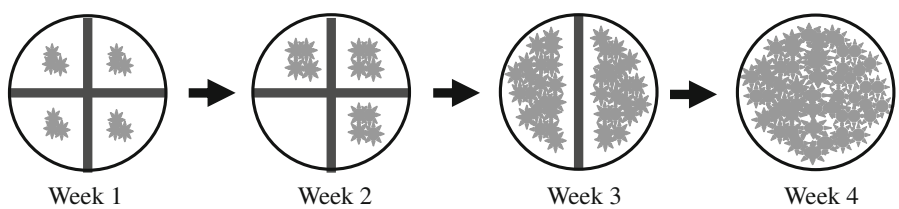

Fig. 1 Experimental sampling design and schedule for experiment 1. A removable cross was used to divide the plants in each pot for four sampling times. See text for details

7, 12, and 16 days, samples of the fern mats were collected from each block in all pots using a stainless mesh screen, washed with tap water, oven dried at 80°C for 3 days, and weighed. C and N content of *A. filiculoides* tissues for the final sampling were determined using an elemental analyzer, as described for experiment 1.

Measurement of biological N-fixation activity

On the last day of each experiment, part of fresh pieces of *A. filiculoides* fern were collected from the different treatments to measure biological N-fixation activity, using the acetylene reduction method (Yoshida & Ancajas, 1971; Cheng et al., 2001). To apply this method, fresh fern samples (equivalent to about 50 mg dry wt) were placed in 68-ml serum bottles that were capped with a butyl rubber stopper. Ten percent by volume of the bottle headspace air was replaced with pure acetylene gas, using a syringe. After the bottles were incubated at 30°C for 48 h, ethylene production was measured by a gas chromatograph (Shimadzu GC-7A, Kyoto, Japan) with a flame ionization detector (Cheng et al., 2001). The dry weight of *A. filiculoides* in the bottles was determined after oven drying at 80°C for 3 days. The biological N-fixation activity was calculated as μmol C_2H_4 per g dry wt of *A. filiculoides* per day (μmol C_2H_4 g^{-1} day^{-1}).

Statistical analysis

We conducted two-way ANOVA for all measured parameters at the last sampling, with $[CO_2]$, P nutrient levels and the $[CO_2]$ × P interaction effects in experiment 1, and $[CO_2]$, temperature and $[CO_2]$ × temperature interaction effects in experiment 2. The analysis was carried out using the SPSS 14 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Effect of elevated $[CO_2]$ and P on *A. filiculoides*

We found significant exponential relationships for the changes in *A. filiculoides* biomass over the 28-day growth period under four combinations of $[CO_2]$ and P nutrient levels (Fig. 2). These relationships were described by:

$$y = C \cdot \exp(kx), \quad (1)$$

where y is the *A. filiculoides* biomass (mg) on incubation day x (days); C is the *A. filiculoides*

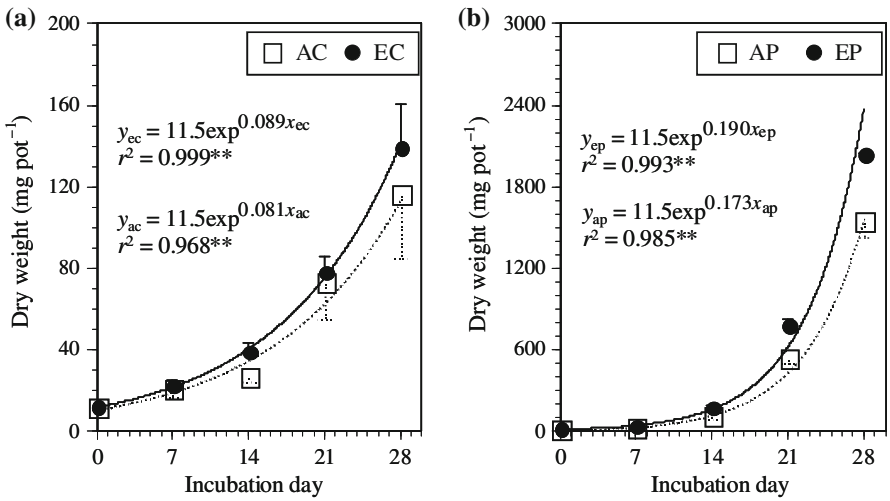

Fig. 2 Changes in dry weight of *A. filiculoides* in pots without (**a**) and with (**b**) P application under both ambient (380 ppm) and elevated (680 ppm) $[CO_2]$ levels during 28-day incubations in controlled-environment chambers (experiment 1). *Bars* indicate standard deviations ($n = 3$). *AC* ambient $[CO_2]$ without added P fertilizer, *EC* elevated $[CO_2]$ without added P, *AP* ambient $[CO_2]$ with P added, and *EP* elevated $[CO_2]$ with P added. All growth curves show significant fits to exponential models (** $P < 0.01$)

Table 1 Effects of elevated [CO_2] and P on *A. filiculoides* growth after 28 days incubation

P level	CO_2 level (abbrev.)	Dry weight biomass (mg/pot)	C content (%)	N content (%)	C/N (wt/wt)	C assimilation (mg C/pot)	N accumulation (mg N/pot)
Control	Elevated (EC)	139	39.9	1.76	22.7	55.5	2.5
	Ambient (AC)	116	40.7	2.01	20.3	47.1	2.3
	% Change by e[CO_2]	19.6	−2.0	−12.2	11.7	17.8	4.9
P addition	Elevated (EP)	2030	40.3	1.92	21.0	818.1	38.9
	Ambient (AP)	1544	40.6	2.42	16.9	627.4	37.4
	% Change by e[CO_2]	31.5	−0.8	−20.6	24.8	30.4	4.1
% Change by P addition		1300	0.4	15.1	−11.9	1309	1491
ANOVA results							
P		**	ns	**	**	**	**
CO_2		**	ns	**	**	**	ns
P × CO_2		**	ns	*	ns	**	ns

ns not significant; * $P < 0.05$; ** $P < 0.01$

biomass (mg) at the beginning of the experiment; and k is the growth rate constant (days^{-1}) for the exponential model. For the four treatments in experiment 1, the value for the initial dry weight (C) was 11.5 mg. The rate constant k increased under elevated [CO_2] by 10.2% without added P and by 9.4% with added P. On average, k increased by 112.9% from the application of P fertilizer (Fig. 2). At the final sampling after 28 days of growth, the biomass of *A. filiculoides* was significantly increased by both elevated [CO_2] and P application (both $P < 0.01$), with a significant interaction of [CO_2] and P application ($P < 0.01$). The biomass of *A. filiculoides* increased on average by a factor of 13 by the application of P (Table 1). The C content of *A. filiculoides* tissues at final sampling was similar (about 40%) among the four treatments, so the rates of C assimilation by *A. filiculoides* followed the same pattern as those of the dry weights (Table 1).

The N content of *A. filiculoides* tissues was significantly lower under elevated [CO_2] and higher with added P (both $P < 0.01$), with a significant interaction of [CO_2] and P ($P < 0.05$). The accumulation of N in *A. filiculoides* tissues was significantly greater by a factor of about 15 following P application but was not affected by elevated [CO_2] (Table 1). As a consequence, the C/N ratios in *A. filiculoides* tissues were significantly increased by elevated [CO_2] and decreased by P application (both $P < 0.01$; Table 1).

Effect of elevated [CO_2] and temperature on *A. filiculoides* growth

As we found in experiment 1, the changes in *A. filiculoides* biomass during the 16-day growth period under four treatment combinations of two [CO_2] levels and two temperature levels showed significant fits to the exponential model (Eq. 1, Fig. 3). The value of C for these models was 10.5 mg, the dry weight of the initial *A. filiculoides* inoculation. In experiment 2, the growth rate constant k increased under elevated [CO_2] conditions by 5.7% at higher temperatures (34/26°C) and by 5.3% at lower (29/21°C) temperatures. On average k increased by 4.5% with a temperature increase of 5°C (Fig. 3). At the final sampling after 16 days of growth, the biomass of *A. filiculoides* was significantly higher under conditions of elevated [CO_2] and higher temperature (both $P < 0.01$). The interaction of [CO_2] and temperature was not significant ($P = 0.11$; Table 2). The C content of *A. filiculoides* tissues at final sampling was around 40% for all four treatments, similar to those in experiment 1 (Table 2).

The N content of *A. filiculoides* tissues was significantly lower under elevated [CO_2] and higher under higher temperature conditions (both $P < 0.01$). There was no detectable interaction of [CO_2] and high temperature for tissue N content ($P = 0.37$; Table 2). Accumulation of N in *A. filiculoides* tissues

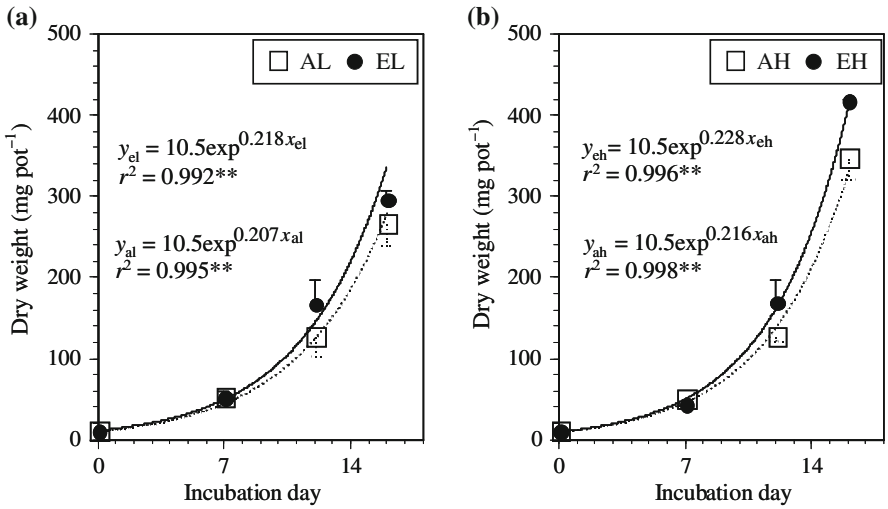

Fig. 3 Changes in dry weight of *A. filiculoides* in pots under four treatments in controlled-environment chambers during 16-day incubations (experiment 2). *Bars* indicate standard deviations ($n = 3$). **a** *AL* ambient [CO_2] and low temperature, *EL* elevated [CO_2] and low temperature. **b** *AH* ambient [CO_2] and high temperature, *EH* elevated [CO_2] and high temperature. All growth curves show significant fits to exponential models (** $P < 0.01$)

Table 2 Effects of elevated [CO_2] and temperature on *A. filiculoides* growth after 16 days incubation

Temperature (day/night)	CO_2 level (abbrev.)	Dry weight biomass (mg/pot)	C content (%)	N content (%)	C/N (wt/wt)	C assimilation (mg C/pot)	N accumulation (mg N/pot)
High (34/26°C)	Elevated (EH)	417	40.9	3.13	13.1	170.6	13.1
	Ambient (AH)	348	40.8	3.30	12.4	142.1	11.5
	% Change by e[CO_2]	19.8	0.2	−5.2	5.8	20.1	13.6
Low (29/21°C)	Elevated (EL)	295	40.1	2.56	15.7	118.5	7.6
	Ambient (AL)	267	39.7	2.84	14.0	105.9	7.6
	% Change by e[CO_2]	10.8	1.1	−9.8	12.0	12.0	−0.1
% Change by Inc. temperature		36.2	2.3	19.1	−14.3	39.4	62.4
ANOVA results							
Temperature		**	*	**	**	**	**
CO_2		**	ns	**	**	**	ns
Temperature × CO_2		ns	ns	ns	ns	ns	ns

ns not significant; * $P < 0.05$; ** $P < 0.01$

was significantly increased only by higher temperature (by 62.4%) and was not affected by elevated [CO_2] (Table 2). The C/N ratios in *A. filiculoides* tissues were significantly increased by elevated [CO_2] and significantly decreased by high temperature (both $P < 0.01$; Table 2).

Effect of elevated [CO_2], P addition, and temperature on biological N-fixation activity in *A. filiculoides*

We compared the biological N-fixation activities of *A. filiculoides* grown in experiments 1 and 2, as determined on the final day of sampling, by the acetylene reduction method (Fig. 4). Overall, the acetylene reduction activity of *A. filiculoides* was lower under elevated [CO_2] conditions than under ambient [CO_2] in both experiments 1 and 2, but the differences were not significant ($P = 0.15$ for experiment 1 and $P = 0.24$ for experiment 2). The acetylene reduction activity was significantly increased by application of P fertilizer in experiment 1 ($P < 0.05$, Fig. 4a) and was significantly decreased by the temperature increase from 29/21°C to 34/26°C in experiment 2 ($P < 0.05$, Fig. 4b). There were no significant interactions between [CO_2] and P addition ($P = 0.61$; Fig. 4a) or between [CO_2] and higher temperature ($P = 0.82$; Fig. 4b).

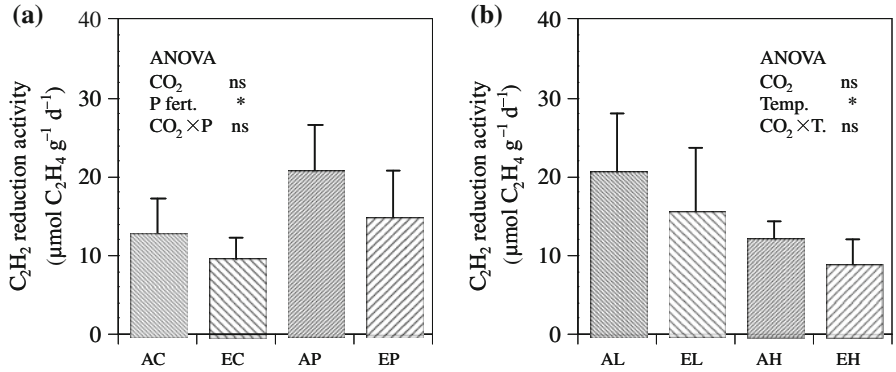

Fig. 4 Acetylene reduction activity of *A. filiculoides* tissues collected from the four treatments in experiment 1 (**a**) and experiment 2 (**b**). *Bars* indicate standard deviations ($n = 3$). *AC* ambient [CO_2] without added P fertilizer, *EC* elevated [CO_2] without added P, *AP* ambient [CO_2] with added P, *EP* elevated [CO_2] with added P. *AL* ambient [CO_2] and low temperature, *EL* elevated [CO_2] and low temperature, *AH* ambient [CO_2] and high temperature, and *EH* elevated [CO_2] and high temperature. *Insets* show ANOVA results: *ns* not significant; * $P < 0.05$

Discussion

Biomass and C assimilation

During the past three decades there have been numerous studies to understand the effects of elevated [CO_2] on various plant species in various ecosystems, such as crops in farmlands, woody plants in forests, herbaceous plants in grasslands, and emergent vegetation in wetlands, using chamber and free-air CO_2 enrichment (FACE) experiments (Kimball et al., 2002; Saarnio et al., 2003; Nowak et al., 2004; Ainsworth & Long, 2005; Kim & Kang, 2008). Although the stimulative effect of elevated [CO_2] on plant growth varies widely between species, the biomass and C assimilation of plants under elevated [CO_2] conditions are higher than under ambient conditions primarily because of enhanced plant photosynthesis under elevated [CO_2] (Kimball et al., 2002; Ainsworth & Long, 2005). Our results show that the C content of *A. filiculoides* tissues was similar, around 40%, in the eight treatment conditions in both experiments 1 and 2, although there was a significant but small increase (2.3%) under elevated temperature. Therefore, differences in biomass among all treatments are reflected in the C assimilation rates.

We found that the biomass of *A. filiculoides* was significantly increased by elevated [CO_2] and P application (both $P < 0.01$), with a significant interaction of [CO_2] and P ($P < 0.01$) in experiment 1, and was significantly increased by elevated [CO_2] and higher temperature (both $P < 0.01$), without any interaction between [CO_2] and high temperature in experiment 2. The increase in C assimilation rates resulting from elevated [CO_2] by the time of the final sampling ranged from 10.8 to 31.5% in both experiments, which is similar to observations for rice and others plants (Kimball et al., 2002; Kim et al., 2003; Sakai et al., 2006; Cheng et al., 2009). Idso et al. (1987) also reported that the responses of *Azolla* to elevated [CO_2] are similar to the responses of three terrestrial species (carrot, radish, and cotton) and another floating aquatic plant, the water hyacinth. In that study, *Azolla* was cultured in N-free nutrient solution. In our study, the changes in *A. filiculoides* biomass under all eight treatment conditions in both experiments significantly fit the exponential model, suggesting that the increases resulting from elevated [CO_2] could become greater if the plants were grown with sufficient nutrients for a longer time.

N accumulation and N-fixation activity

N is the element that most often limits plant growth, and progressive nitrogen limitation can affect plant responses to elevated [CO_2] (Luo et al., 2004). Our previous studies showed that rice growth and grain yield respond to elevated [CO_2] are dependent on plant N accumulation, and that enhancement of rice canopy C gain by elevated [CO_2] is sensitive to leaf

N content (Kobayashi et al., 2006; Sakai et al., 2006). The application of chemical N fertilizer is an option for improving rice production, but it is neither environmental friendly nor economically viable (Mandal et al., 1999). If N-fixation activity of the cyanobacteria *Anabaena*, which lives symbiotically in leaf cavities of *Azolla*, is enhanced by elevated [CO_2] and rising temperature, then rice growth and the fertility of paddy soils should be improved by adding *Azolla* to rice paddies in the future to help adjust to climate change.

The N accumulated by *A. filiculoides* floating on the water flooding rice paddies has two potential sources. One source is mineral N absorbed from the flooding water, originally from soil mineralization; the other source is via N-fixation by the symbiotic cyanobacteria *Anabaena*. In this study, we measured the tissue N content and also the N-fixation activity at the final sampling time in both experiments. Our results showed that the N content of *A. filiculoides* tissues was significantly reduced by elevated [CO_2], with decreases ranging from 5.2 to 20.6%, which is consistent with observations summarized by Cotrufo et al. (1998) for woody and nonwoody species and by Kimball et al. (2002) for agricultural crops. The lower N content under elevated [CO_2] can be explained by the dilution effect caused by increased biomass and C accumulation. However, our results also showed that the acetylene reduction activity of *A. filiculoides* on a dry weight basis was lower under elevated [CO_2] conditions in both experiments 1 and 2, although the differences are not significant. Even on a total biomass basis, the effect of elevated [CO_2] on acetylene reduction activity is still negative. On the basis of meta-analysis, Van Groenigen et al. (2006) reported that elevated [CO_2] has no effect on N-fixation in the absence of added non-N nutrients (e.g., P, molybdenum, and potassium), and had a stimulative effect on N-fixation if non-N nutrients were available. In this study, the application of P fertilizer significantly increased the N content of *A. filiculoides* tissues by 15.1%, the N accumulation rate by a factor of 15 and N-fixation activity by 59.7% on average (experiment 1), but the N accumulation rates and N-fixation activity stimulated by P addition did not show significant interactions with elevated [CO_2]. It is not clear why elevated [CO_2] did not significantly affect N-fixation activity either with or without P fertilizer application, but one possible explanation is that the availability of N in the flooding water supplied from soil mineralization was not limiting.

There was also no effect of elevated [CO_2] on N-fixation activity of *A. filiculoides* under the two temperature regimes in experiment 2. Raising the temperature from 29/21°C to 34/26°C significantly increased the N content of *A. filiculoides* tissues by 19.1% and the N accumulation rate by 62.4% and reduced N-fixation activity by 42.7% on average. The increased N accumulation under higher temperatures was presumably because the higher temperatures accelerated soil N mineralization and provided more mineral N for *A. filiculoides* growth. Higher temperatures enhance N mineralization in submerged rice paddies (Inubushi et al., 1985; Cheng et al., 2000). The decrease in N-fixation activity under the higher temperatures may be explained by the fact that the optimum temperature for growth of the microorganism *Anabaena azollae* is about 25°C (Wagner, 1997).

Our results provide convincing evidence that elevated [CO_2], the addition of P, and higher temperatures significantly increase *A. filiculoides* growth and C assimilation, with a significant interaction between elevated [CO_2] and P nutrient application. The N content of *A. filiculoides* tissues decreased under elevated [CO_2], whereas it increased with the addition of P and under higher temperatures. The acetylene reduction assay showed that the N-fixation activity of *A. filiculoides* was not affected by elevated [CO_2] but was significantly stimulated by P application and inhibited by increasing the temperatures from 29/21°C to 34/26°C. Therefore, we conclude that the N accumulation potential of *A. filiculoides* under future climate warming mainly depends on the temperature change and availability of P and that C assimilation should increase under the elevated [CO_2]. Note, however, that our results are based on two separated experiments in different years. Further studies on the interactive effects of 3 factors (elevated [CO_2], high temperature, and P application) should be carried out together to confirm our results under future climate warming scenarios.

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