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Effects of exogenous zinc on the photosynthesis and carbonic anhydrase activity of millet (*Setaria italica* L.)

M.L. CAO*, Y.X. LI*, and H.L. DU**,+

*College of Agricultural Science, Shanxi Agricultural University, Taigu, 030801 Shanxi, China**
*College of Arts and Sciences, Shanxi Agricultural University, Taigu, 030801 Shanxi, China***

Abstract

This study aimed to evaluate the effects of Zn on the growth safety and activity of carbonic anhydrase (CA) in foxtail millet (*Setaria italica* L.). The photosynthetic characteristics, CA activity, and relative gene expression of different varieties of millet at the seedling stage were studied by spraying Zn solution under pot experiment and indoor culture conditions. Results showed that spraying low-concentration Zn solution (20, 40, and 60 mg L^{-1}) reduced malondialdehyde content and intercellular CO_2 concentration (C_i) but increased antioxidant enzyme activity, pigment content, and photosynthetic gas-exchange parameters (net photosynthetic rate, stomatal conductance, transpiration rate, except for C_i); meanwhile, spraying high Zn concentration (80 and 100 mg L^{-1}) exerted opposite effects. The optimal growth of millet was achieved when the Zn concentration was 40 mg L^{-1}. At this concentration, CA activity increased and β-CA family expression was upregulated, which exerted little or no effect on other CA families. Compared to Zhangzagu 10 (zinc-resistant variety), Jingu 21 (zinc-sensitive variety) showed a more significant change. This study may serve as a reference for further research on the function of CA and physiological processes, such as photosynthesis in millet, and a theoretical basis for the effective use of Zn fertilizer in millet.

Additional key words: carbonic anhydrase gene family; peroxidase; photosynthetic pigment; superoxide dismutase.

Introduction

Zinc is an essential trace element for plant growth and development. As the only metal element present in six enzymes, Zn plays an important regulatory role in plant photosynthesis, protein and nucleic acid metabolism, auxin metabolism, biofilm stability, and cell division (Prasad and Hagemeyer 1999, Kabata-Pendias and Pendias 2001). Appropriate amounts of Zn can increase the chlorophyll (Chl) content of crops, thereby increasing photosynthetic rate and raising yield (Gartler *et al.* 2013). High concentrations of Zn reduce pigment production (Chl, carotenoids, and phycobilisomes), affect PSII activity, and inhibit energy transfer from phycobilisomes to PSII centers, thereby inhibiting the photosynthesis and growth of millet plants (*Setaria italica* L.) (Zeng *et al.* 2009, Okmen *et al.* 2011, Xu *et al.* 2013). Zinc deficiency leads to the inactivation of certain enzyme cofactors or activators in plants, which negatively affects the physiological processes, such as photosynthesis and stress resistance, of plants (Shrotri *et al.* 1978, 1979, 1981).

Carbonic anhydrase (CA) is a Zn metalloenzyme widely found in animals, plants, bacteria, and fungi. Its active center contains Zn, which serves important biological functions, such as catalyzing the reversible hydration reaction between CO_2 and HCO_3^-, converting and diffusing CO_2 in respiration, and participating in pH regulation/adjustment, ion exchange, carboxylation and decarboxylation, and respiration (Khalifah 1971, Atkins *et al.* 1972, Graham *et al*. 1984, Moroney *et al.* 2001). The CA protein family can be divided into six subfamilies, such as α-, β-, γ-, δ-, ε-, and ζ-CA, according to the amino acid sequence homology and crystal structure similarity (Kaul *et al.* 2001). Higher plants mainly contain three subfamilies, such as α-, β-, and γ-CA, all of which are Zn-containing metalloenzymes. Recent studies have found that CA is closely related to photosynthesis, indicating its great significance in plant growth and development.

Many studies on CA have used rice (Qiao *et al.* 2014), rape (Deng *et al.* 2009a), and soybean (Ohki 1978) as test materials to explore the CA catalytic activity and related functions of leaf tissue. However, the CA activity and

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+Corresponding author; e-mail: duhuiling66@163.com
Abbreviations: CA – carbonic anhydrase; CAT – catalase; cDNA – complementary DNA; C_i – intercellular CO_2 concentration; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; MDA – malondialdehyde; PCR – polymerase chain reaction; P_N – net photosynthetic rate; POD – peroxidase; qPCR – real-time quantitative PCR detecting system; RNA – ribonucleic acid; SOD – superoxide dismutase.
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relative expression of millet are poorly understood. This study aims to clarify the relationship among CA, Zn, and photosynthesis, to provide valuable clues for further studies on CA function, and to serve as an important reference for improving the photosynthetic performance and quality of millet.

Materials and methods

Pot experiment was conducted in the greenhouse of the College of Resources and Environment of Shanxi Agricultural University (May–October 2017). The test soil was collected from Shenfeng Village, Taigu County, Shanxi Province. The soil was calcareous soil with pH 8.49, organic matter content of 19.61 g kg^{-1}, alkali nitrogen content of 55.38 mg kg^{-1}, available phosphorus content of 17.54 mg kg^{-1}, available potassium content of 113.85 mg kg^{-1}, total N content of 1.180 g kg^{-1}, total P content of 1.261 g kg^{-1}, and total K content of 20.18 g kg^{-1}. The millet varieties tested were Zhangzagu 10 (resistant, hybrid) and Jingu 21 (conventional). The Zn fertilizer tested was $ZnSO_4 \cdot 7H_2O$.

The pot used in the test pot had an inner diameter of 30 cm and a height of 25 cm, and was filled in May 2017. Exactly 10 kg of air-dried soil was filtered on a 6-mm sieve. Part of the soil was placed into the pot and then water was added before sowing. Seeds with full grain and uniform size were sown, and then the remaining soil was evenly covered over the millet until the thickness reached about 3 cm. In consideration of the moisture required for millet growth, water was poured once every 5 d (tap water). Different concentrations of Zn solution (0, 20, 40, 60, 80, and 100 mg L^{-1}) were sprayed at the seedling stage of millet and recorded as CK, Zn1, Zn2, Zn3, Zn4, and Zn5, respectively (35 d after planting). Each treatment was repeated three times. The material was taken on the 7th day after the treatment, and the physiological and photosynthetic indicators were measured.

Laboratory experiment: The experiment was conducted in the laboratory of College of Arts and Sciences, Shanxi Agricultural University (September–November 2017). Two millet varieties of Zhangzagu 10 and Jingu 21 were selected. Plump and well-sized seeds were selected, disinfected with 0.1% $HgCl_2$, and washed repeatedly with distilled water. The water on the seeds was absorbed by filter paper, dried in the shade, and then sown in a Petri dish with a diameter of 90 mm with 100 capsules per dish. The seeding substrate was distilled water, with 5 mL per dish, and the culture dish was placed in a *ZZSW-ZPO2-II*-type light culture rack (*Shanghai Zhizhong Laboratory Equipment*, *Ltd.*). The culture temperature was 25–28°C, the illumination was 12 h per day, and light intensity was 72–126 μmol(photon) m^{-2} s^{-1}. After germination, the seeds were watered three times a day, with 3 mL each time. After the plants were sown for 30 d, leaves were treated. The Zn solution was sprayed at the seedling stage at concentrations of 0 and 40 mg L^{-1}, which were recorded as CK and Zn2, respectively. Each treatment was repeated three times. On the 7th day after the treatment, the CA activity and the relative gene expression were measured.

Antioxidant enzyme activity and malondialdehyde (MDA) content: Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the nitroblue tetrazolium photochemical reduction inhibition method (Gao 2006), peroxidase (POD, EC 1.11.1.7) activity by the guaiacol method (Gao 2006), catalase (CAT, EC 1.11.1.6) activity by ultraviolet spectrophotometry (Gao 2006), and malondialdehyde (MDA) content by thiobarbituric acid spectrophotometry (Gao 2006). The pigment contents of the leaves were determined using acetone extraction (Gao 2006). Ultraviolet-visible spectrophotometer (*UV-5100H*, *Shanghai Yuanxi Laboratory Equipment Co*., *Ltd*., China) was used in the test. The activities were presented as the number of units per gram of fresh mass.

Photosynthetic characteristics: Net photosynthetic rate (P_N), transpiration rate (E), intercellular CO_2 concentration (C_i), and stomatal conductance (g_s) were measured using a *CI-340* handheld photosynthesis measurement system (*CID Bio-Science*, *Inc*., USA). Three samples of millet seedlings were randomly selected from 9:00–11:00 h on the 7th day after spraying the Zn solution, and the gas-exchange parameters were determined. The measurement was carried out with a fixed red-blue light source; the light intensity was 900 ± 50 μmol(photon) m^{-2} s^{-1}, the air temperature was 23 ± 2°C, and the CO_2 concentration was 400 ± 50 μmol mol^{-1}.

CA (EC 4.2.1.1) activity: The test was carried out using the plant CA enzyme-linked immunosorbent assay kit of *Shanghai Bio-Jining Industrial Co.*, *Ltd.* (China). The activity was presented as the number of units per gram of fresh mass.

Gene expression

Total RNA extraction: Fresh leaves (35 mg) were weighed from each sample and extracted using the total RNA extraction kit of *Tiangen Biotechnology Co.*, *Ltd.* (China). The purity and integrity of the RNA were determined by micro-spectrophotometry (*UV-8000*, *Shanghai Yuanxi Laboratory Equipment Co*., *Ltd*., China) and agarose gel electrophoresis (*JY-SPCT*, *Beijing Junyi Laboratory Equipment Co*., *Ltd*., China), respectively, after extraction.

Reverse transcription: Total RNA (5 μL) was used as a reverse transcription template. Reverse transcription was performed by *QuantScript RT* of *Tiangen Biotechnology Co.*, *Ltd.*, and first-strand cDNA was synthesized by oligodT, a reverse transcription primer. The reverse transcription system (20 μL) was as follows:

Composition	Dosage [μL]
Total RNA	5.0
OligodT primer (10μM)	1.0
5× RT MasterMix	4.0
RNase-free H_2O to final volume	20.0

Primer design: The primers required for the essay were designed according to the special requirements of the primer design by real-time quantitative PCR. The primer sequences are shown in the text table, *actin* is the reference gene in millet.

Gene family	Primer	Sequences [5'→3']	NCBI retrieval
α-CA	SiCA1-F	CGCAATGCGCGAGAGGGACG	XM-004975507
	SiCA1-R	GCGTACAGCGCGGCCAGCTT	
	SiCA2-F	GCCGCACTATTGTATGTTTGC	XM-004954909
	SiCA2-R	CTCCGAGTATAATTCAGCCAG	
	SiCA3-F	CGCCACTGTCGGGGCGCTC	XM-004973687
	SiCA3-R	CGCTCGATGGCGGGCTGCAG	
	SiCA4-F	GTACGCGGCGGCGGCCCTGG	XM-004965848
	SiCA4-R	CCAGCCTGCGTATGGTCCGA	
	SiCA5-F	GCATATGGGTCGTCCAGCTC	XM-004973604
	SiCA5-R	GTAGAGGACGCCGATCACCGC	
β-CA	SiCA6-F	CGAGGTCCTTGGGATCAGCGG	XM-012844113
	SiCA6-R	CAGCAAACTCTAGTGCAGCAC	
γ-CA	SiCA7-F	GCAACTACTTCTTCCACGAG	XM-004967603
	SiCA7-R	CTATTTCATCATCAGTGAGC	
	SiCA8-F	CTCGGATCCACCCTCCAGG	XM-004958441
	SiCA8-R	TGTGCGCATACTTCTTCCTC	
Actin	Siactin-F	TGTGACAATGGTACTGGAATG	XM-004970638
	Siactin-R	CAAGGTCCAATCGAAGAATAG	

Real-time PCR: The SYBR Green-qPCR method was used for amplification using a *QuantStudio 6* real-time quantitative PCR instrument (*MA-1620Q*, *Shandong Boke Laboratory Equipment Co*., *Ltd*., China). The amplification conditions were as follows: predenaturation at 95°C for 2 min; denaturation at 95°C for 10 s; annealing at 60°C for 30 s; extension at 72°C for 1 min, 40 cycles. The reaction system was as follows:

Composition	Dosage [μL]
Primer F [10μM]	1
Primer R [10μM]	1
5× Pfu buffer	5
dNTPs [2.5Mm]	2
Pfu polymerase [2.5 U·μL^{-1}]	0.5
cDNA template	2
SYBR Green I	5
ddH_2O to final volume	25

Statistical analysis: The data were plotted using *Microsoft Excel* software, analyzed by analysis of variance (*ANOVA*) with *SPSS 17.0*, and tested by *Duncan*'s new complex range method for significance. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Results

Antioxidant enzyme activity: After spraying Zn on millet, the antioxidant enzyme activities of the two varieties initially increased and then decreased, and these changes were different between varieties (Fig. 1). The antioxidant enzyme activities reached the highest levels after Zn2 treatment in each variety and were significantly different from those under CK. However, the antioxidant enzyme activities under Zn4 were lower than those under CK, and the effect of Zn5 treatment was more obvious. Compared with CK, Zn1, Zn2, and Zn3 treatment increased the SOD activity of Jingu 21 by 12.0, 19.6, and 8.9%, respectively, while those of Zhangzagu 10 by 2.6, 7.8, and 3.0%, respectively. Compared with CK, Zn4 and Zn5 treatment decreased the SOD activity of Jingu 21 by 2.9 and 7.1%, respectively, and those of Zhangzagu 10 by 2.5 and 5.3%, respectively. Compared with CK, Zn1, Zn2, and Zn3 treatment increased the POD activity of Jingu 21 by 12.7, 26.1, and 7.3%, respectively, while those of Zhangzagu 10 by 6.1, 10.4, and 4.4%, respectively. Compared with CK, Zn4 and Zn5 treatment decreased the POD activity of Jingu 21 by 8.6 and 17.0%, respectively, and those of Zhangzagu 10 by 1.7 and 7.6%, respectively. Compared with CK, Zn1, Zn2 and Zn3 treatment increased the CAT activity of Jingu 21 by 12.6, 24.3, and 9.5%, respectively, while those of Zhangzagu 10 by 2.1, 4.2, and 1.7%, respectively. Compared with CK, Zn4 and Zn5 treatment decreased the CAT activity of Jingu 21 by 8.0 and 10.8%, respectively, and those of Zhangzagu 10 by 2.1 and 4.4%, respectively. The change degree of Jingu 21 was higher than that of Zhangzagu 10, and POD activity changed the most among the three antioxidant enzymes.

Malondialdehyde content: The trend of MDA content was opposite that of the antioxidant enzymes. As shown in Fig. 1*D*, the MDA content initially decreased and then increased with the increasing Zn concentration. The MDA

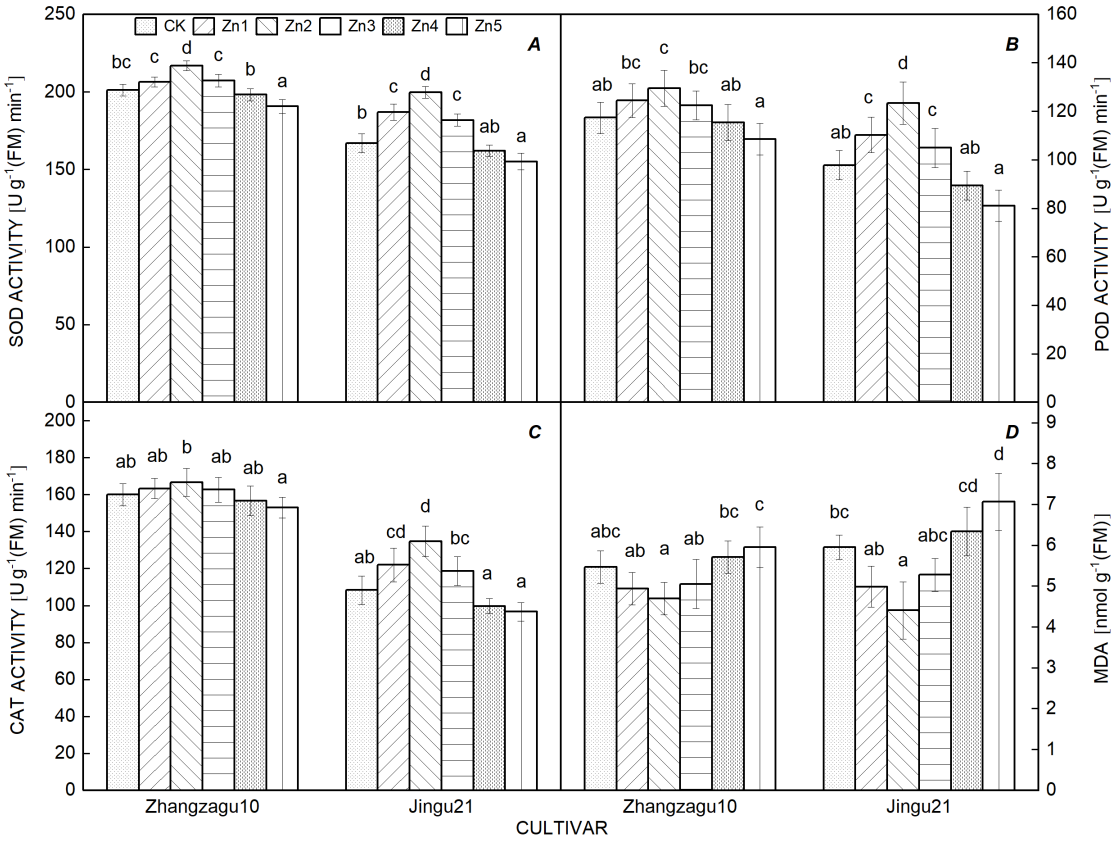

Fig. 1. Effects of different exogenous zinc concentrations on the superoxide dismutase (SOD) (*A*), peroxidase (POD) (*B*), catalase (CAT) (*C*) activities, and malondialdehyde content (*D*) of two millet varieties. The leaves of two millet cultivars were determined at the seedling stage. *Different letters* per treatment indicate statistical significant differences (p<0.05) showed by *Tukey*'s test. Different concentrations of Zn solution (0, 20, 40, 60, 80, and 100 mg L^{-1}) were sprayed at the seedling stage of millet and recorded as CK, Zn1, Zn2, Zn3, Zn4, and Zn5, respectively.

content reached a minimum value and then gradually increased under Zn2, and it was higher under Zn4 than that in CK. Compared with CK, Zn1, Zn2, and Zn3 treatment for 7 d decreased the MDA contents of Jingu 21 by 16.3, 26.0, and 11.4%, respectively, while those of Zhangzagu 10 by 9.7, 14.2, and 7.6%, respectively. Compared with CK, Zn4 and Zn5 treatment for 7 d increased the MDA contents of Jingu 21 by 6.5 and 18.7%, respectively, and those of Zhangzagu 10 by 4.4 and 8.8%, respectively.

Pigment content: The effect of Zn on the pigment content of millet leaves is shown in Table 1. The pigment content in the leaves of millet increased with the increasing Zn concentration until Zn2. However, the pigment content under both Zn4 and Zn5 treatments decreased, and the content was even lower than that under CK. Although the two varieties showed similar trends, they differed in the degree of increase or decrease in their pigment content, and the change degree in Jingu 21 was significantly higher than that in Zhangzagu 10.

Compared with CK, Zn1 treatment for 7 d significantly increased the Chl *a*, Chl *b*, total Chl, and carotenoid contents of Jingu 21 by 9.0, 9.3, 9.0, and 5.5%, respectively, and those of Zhangzagu 10 by 4.9, 8.1, 5.8, and 4.9%, respectively. Zn2 treatment for 7 d significantly increased the Chl *a*, Chl *b*, total Chl, and carotenoid contents of Jingu 21 by 13.7, 24.0, 16.2, and 7.9%, respectively, and those of Zhangzagu 10 by 7.6, 11.9, 8.8, and 5.3%, respectively. Zn3 treatment for 7 d significantly increased Chl *a*, Chl *b*, total Chl, and carotenoid contents of Jingu 21 by 6.8, 7.3, 6.9, and 2.4%, respectively, and those of Zhangzagu 10 by 3.4, 5.5, 4.0, and 1.9%, respectively. Compared with CK, Zn4 treatment for 7 d decreased Chl *a*, Chl *b*, total Chl, and carotenoid contents of Jingu 21 by 4.5, 9.1, 5.7, and 3.5%, respectively, and those of Zhangzagu 10 by 3.2, 7.9, 4.5, and 2.3%, respectively. Zn5 treatment for 7 d decreased Chl *a*, Chl *b*, total Chl, and carotenoid contents of Jingu 21 by 9.5, 18.2, 11.6, and 4.1%, respectively, and those of Zhangzagu 10 by 5.4, 13.3, 7.6, and 3.4%, respectively, which reached significant levels except for carotenoids. Chl *b* content changed the most among the four pigment indicators.

Photosynthetic gas-exchange parameters: After Zn treatment, the P_N, g_s, and E of the two varieties initially increased and then decreased with increasing Zn concentration. Changes in these indicators differed between varieties (Table 2). The three indicators reached their

maximum levels in Zn2 and then gradually decreased. These indicators were lower under Zn4 than under CK and reached a significant level between treatments. Compared with CK, Zn2 treatment for 7 d significantly increased the P_N, g_s, and E of Jingu 21 by 11.4, 5.3, and 6.0%, respectively, and those of Zhangzagu 10 by 7.7, 3.6, and 4.2%, respectively. Zn2 treatment for 7 d significantly increased the P_N, g_s, and E of Jingu 21 by 22.2, 8.8, and 9.8%, respectively, and those of Zhangzagu 10 by 16.0, 6.0, and 7.0%, respectively. Zn3 treatment for 7 d significantly increased the P_N, g_s, and E of Jingu 21 by 8.1, 4.0, and 5.1%, respectively, and those of Zhangzagu 10 by 6.1, 2.7, and 3.1%, respectively. Compared with CK, Zn4 treatment for 7 d decreased the P_N, g_s, and E of Jingu 21 by 11.2, 5.0, and 5.4%, respectively, and those of Zhangzagu 10 by 6.4, 2.2, and 3.1%, respectively. Zn5 treatment for 7 d decreased the P_N, g_s, and E of Jingu 21 by 16.1, 8.5, and 10.1%, respectively, and those of Zhangzagu 10 by 12.9, 4.7, and 6.0%, respectively (Table 2). P_N changed the most among the three indicators and the change degree in Jingu 21 was significantly higher than that in Zhangzagu 10.

As the concentration of Zn increased, C_i initially decreased and then increased, which was in contrary to the trend of P_N, and the reduction was the most obvious under Zn2 treatment (Table 2). Compared with CK, Zn1, Zn2 and Zn3 treatment for 7 d significantly decreased the C_i of Jingu 21 by 11.3, 16.9, and 6.2%, respectively, while those of Zhangzagu 10 by 8.0, 11.2, and 4.0%, respectively. Compared with CK, Zn4 and Zn5 treatment for 7 d increased the C_i of Jingu 21 by 4.5 and 7.2%, respectively, and those of Zhangzagu 10 by 2.4 and 5.4%, respectively. The change trend of C_i in Jingu 21 in the different Zn concentrations was as follows: Zn5 > Zn4 > CK > Zn3 > Zn2 > Zn1. Zhangzagu 10 exhibited a similar trend. Although Zhangzagu 10 and Jingu 21 showed similar

Table 1. Effect of exogenous zinc on the pigment content of millet leaves. Values are means ± SE (n = 3). *Different letters* in the same column indicate significant difference at the p<0.05 level by *Duncan*'s new multiple range test. Different concentrations of Zn solution (0, 20, 40, 60, 80, and 100 mg L^{-1}) were sprayed at the seedling stage of millet and recorded as CK, Zn1, Zn2, Zn3, Zn4, and Zn5, respectively.

Cultivar	Treatment	Chl *a* [mg g^{-1}(FM)]	Chl *b* [mg g^{-1}(FM)]	Chl (*a*+*b*) [mg g^{-1}(FM)]	Carotenoid [mg g^{-1}(FM)]
Zhangzagu 10	CK	9.66 ± 0.18^{c}	3.77 ± 0.17^{b}	13.43 ± 0.11^{c}	1.40 ± 0.04^{ab}
	Zn1	10.13 ± 0.05^{d}	4.07 ± 0.12^{cd}	14.21 ± 0.17^{e}	1.47 ± 0.04^{c}
	Zn2	10.40 ± 0.09^{e}	4.21 ± 0.03^{d}	14.62 ± 0.10^{f}	1.47 ± 0.03^{c}
	Zn3	9.99 ± 0.07^{d}	3.97 ± 0.11^{c}	13.96 ± 0.04^{d}	1.42 ± 0.01^{bc}
	Zn4	9.35 ± 0.10^{b}	3.47 ± 0.09^{a}	12.82 ± 0.19^{b}	1.36 ± 0.05^{ab}
	Zn5	9.14 ± 0.10^{a}	3.27 ± 0.11^{a}	12.41 ± 0.10^{a}	1.35 ± 0.02^{a}
Jingu 21	CK	7.36 ± 0.11^{c}	2.38 ± 0.16^{bc}	9.74 ± 0.16^{c}	1.31 ± 0.09^{ab}
	Zn1	8.02 ± 0.12^{d}	2.60 ± 0.32^{c}	10.62 ± 0.25^{d}	1.38 ± 0.13^{ab}
	Zn2	8.36 ± 0.09^{e}	2.95 ± 0.13^{d}	11.32 ± 0.12^{e}	1.42 ± 0.03^{b}
	Zn3	7.86 ± 0.05^{d}	2.56 ± 0.14^{c}	10.41 ± 0.19^{d}	1.34 ± 0.05^{ab}
	Zn4	7.02 ± 0.09^{b}	2.17 ± 0.07^{ab}	9.19 ± 0.16^{b}	1.27 ± 0.01^{a}
	Zn5	6.66 ± 0.09^{a}	1.95 ± 0.17^{a}	8.61 ± 0.09^{a}	1.26 ± 0.08^{a}

Table 2. Effect of exogenous zinc on photosynthetic gas-exchange parameters of millet. Values are means ± SE (n = 3). *Different letters* in the same column indicate significant difference at the p<0.05 level by *Duncan*'s new multiple range test. Different concentrations of Zn solution (0, 20, 40, 60, 80, and 100 mg L^{-1}) were sprayed at the seedling stage of millet and recorded as CK, Zn1, Zn2, Zn3, Zn4, and Zn5, respectively. P_N – net photosynthetic rate; g_s – stomatal conductance; E – transpiration rate; C_i – intercellular CO_2 concentration.

Cultivar	Treatment	P_N [μmol m^{-2} s^{-1}]	g_s [mmol m^{-2} s^{-1}]	E [mmol m^{-2} s^{-1}]	C_i [μmol mol^{-1}]
Zhangzagu 10	CK	6.95 ± 0.13^{c}	54.92 ± 0.56^{c}	2.388 ± 0.051^{c}	212.05 ± 1.05^{d}
	Zn1	7.50 ± 0.21^{d}	56.90 ± 0.34^{e}	2.489 ± 0.019^{e}	195.18 ± 1.28^{b}
	Zn2	8.07 ± 0.15^{e}	58.24 ± 0.27^{f}	2.555 ± 0.020^{f}	188.22 ± 2.54^{a}
	Zn3	7.38 ± 0.11^{d}	56.42 ± 0.44^{d}	2.463 ± 0.023^{d}	203.63 ± 4.39^{c}
	Zn4	6.51 ± 0.13^{b}	53.71 ± 0.29^{b}	2.314 ± 0.016^{b}	217.23 ± 3.26^{e}
	Zn5	6.06 ± 0.22^{a}	52.35 ± 0.27^{a}	2.243 ± 0.014^{a}	223.44 ± 2.49^{f}
Jingu 21	CK	5.76 ± 0.13^{c}	50.56 ± 0.84^{c}	2.154 ± 0.071^{c}	249.58 ± 1.33^{d}
	Zn1	6.42 ± 0.12^{e}	53.23 ± 0.39^{e}	2.284 ± 0.029^{d}	221.47 ± 0.82^{b}
	Zn2	7.04 ± 0.13^{f}	55.02 ± 0.20^{f}	2.365 ± 0.011^{e}	207.34 ± 1.73^{a}
	Zn3	6.23 ± 0.14^{d}	52.61 ± 0.34^{d}	2.265 ± 0.013^{d}	234.20 ± 4.38^{c}
	Zn4	5.11 ± 0.15^{b}	48.05 ± 0.12^{b}	2.037 ± 0.021^{b}	260.70 ± 3.12^{e}
	Zn5	4.83 ± 0.18^{a}	46.24 ± 0.33^{a}	1.937 ± 0.036^{a}	267.47 ± 2.31^{f}

trends at different concentrations, the increase or decrease in C_i was not the same, and the change in Jingu 21 was significantly higher than that in Zhangzagu 10.

CA activity and relative gene expression: CA activity increased after Zn2 treatment, the CA activities of Jingu 21 and Zhangzagu 10 increased by 45.2 and 26.6%, respectively (Fig. 2). Compared to Zhangzagu 10, Jingu 21 showed a greater change, but both reached significant levels.

Table 3 shows that after Zn treatment, *SiCA1*, *SiCA2*, and *SiCA7* genes from the CA gene family were not expressed in the two varieties, and the expression levels of *SiCA3*, *SiCA4*, *SiCA5*, and *SiCA8* were lower. However, the degree of change of Jingu 21 was greater than that of Zhangzagu 10. A higher expression level was obtained only in *SiCA6*, indicating that Zn induces the expression of the β-CA family but exerts little or no effect on the α and γ families. Compared with CK, Zn2 treatment for 7 d significantly increased the relative expression of the *SiCA6* gene of Jingu 21 and Zhangzagu 10 by 160.7 and 253.9%, respectively.

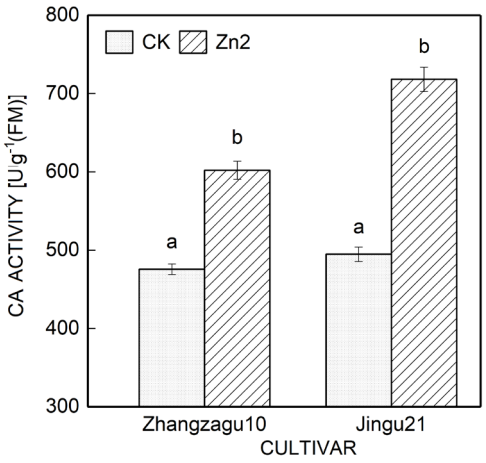

Fig. 2. Effect of exogenous zinc on carbonic anhydrase (CA) activity of millet leaves. Leaves of two millet cultivars were determined at the seedling stage. *Different letters* per treatment indicate statistical significant differences (p<0.05) showed by *Tukey*'s test. The Zn solution was sprayed at the seedling stage at concentrations of 0 and 40 mg L^{-1}, which were recorded as CK and Zn2, respectively.

Discussion

Zinc is an essential trace element in plant metabolism (Rengel 1999, Kabata-Pendias and Pendias 2001) and moderate amounts of Zn can promote plant growth. However, excessive amounts can cause plant toxicity (Chaney 1993), which also exerts a specific impact on the Calvin cycle and optical system activities (Van Assche and Clijsters 1986). Zinc can activate several antioxidant enzymes, such as SOD, POD, and CAT (Jat *et al.* 2007, Saeed *et al.* 2013). Fernàndez-Martínez *et al.* (2014) showed that plant photoprotection and antioxidant capacity increase with increasing Zn concentration; however, high concentrations of Zn produce high toxicity, which can reduce crop biomass, decrease photosynthetic pigment content, and damage the photochemical processes and photosynthesis. Mateos-Naranjo *et al.* (2014) showed that a suitable amount of Zn (30 mmol L^{-1}) can increase the total biomass, relative growth rate, and photosynthetic pigment content of *Juncus acutus*, but high concentrations of Zn (> 60 mmol L^{-1}) inhibit plant growth, reduce its growth rate and photosynthetic pigment content, and affect the chlorophyll fluorescence characteristics. This study shows

Table 3. Effect of exogenous zinc on the relative expression of carbonic anhydrase (CA) genes. The Zn solution was sprayed at the seedling stage at concentrations of 0 and 40 mg L^{1}, which were recorded as CK and Zn2, respectively.

Cultivar	Gene family	Gene name	Treatment	
			CK	Zn2
Zhangzagu 10	α-CA	*SiCA1*	0.0010 ± 0.0001	0.0036 ± 0.0002
		SiCA2	0.0012 ± 0.0001	0.0043 ± 0.0002
		SiCA3	0.2436 ± 0.0180	0.3645 ± 0.0118
		SiCA4	0.1882 ± 0.0013	0.2194 ± 0.0137
		SiCA5	0.2362 ± 0.0142	0.3585 ± 0.0097
	β-CA	*SiCA6*	0.8222 ± 0.0370	2.1437 ± 0.0258
	γ-CA	*SiCA7*	0.0013 ± 0.0001	0.0156 ± 0.0008
		SiCA8	0.2719 ± 0.0124	0.3718 ± 0.0273
Jingu 21	α-CA	*SiCA1*	0.0630 ± 0.0065	0.2386 ± 0.0338
		SiCA2	0.0641 ± 0.0003	0.2579 ± 0.0153
		SiCA3	0.2521 ± 0.0153	0.5312 ± 0.0202
		SiCA4	0.2763 ± 0.0115	0.6215 ± 0.0202
		SiCA5	0.2711 ± 0.0029	0.6043 ± 0.0064
	β-CA	*SiCA6*	0.9463 ± 0.0235	3.3486 ± 0.0750
	γ-CA	*SiCA7*	0.0698 ± 0.0093	0.2854 ± 0.0122
		SiCA8	0.3511 ± 0.0049	0.9182 ± 0.0195

that low concentrations of Zn (20, 40, and 60 mg L^{-1}) can activate the production of antioxidant enzymes, reduce the content of harmful substances of MDA, and promote photosynthesis, but the high concentrations of Zn (80 and 100 mg L^{-1}) can inhibit the production of protective enzymes, increase MDA content, and affect photosynthesis, thus influencing crop growth. The results are consistent with those reported above. Zinc plays an important role in the anti-lipid peroxidation of millet. The suitable Zn concentration can alleviate the peroxidation of membrane lipids and improve the antioxidant activity and delay the rapid senescence of foxtail millet (Kakade *et al*. 2009, Maurya and Kumar 2014). Therefore, the application of a suitable concentration of Zn to foxtail millet could help improve its resistance to stress.

CA is the first Zn enzyme discovered in 1940 and is one of the most important Zn enzymes in photosynthetic CO_2 fixation (Bird *et al.* 1980, Rengel 1995). CA activity and its relative gene expression are linearly positively correlated with Zn concentration in a certain Zn concentration range of plant leaf (Pandey and Sharma 2000, Pandey *et al.* 2002, Qiao *et al.* 2014). When Zn is deficient, CA activity decreases (Sasaki *et al.* 1998), but excessive Zn also decreases CA activity (Han *et al.* 2003). The present study shows that 40 mg L^{-1} concentration of Zn increased the CA activity in millet and induced the expression of the β-CA family but exerted little or no effect on the α and γ families. These changes varied in the different cultivars. This result is consistent with the above findings and some papers (Wu *et al*. 2006, Deng *et al*. 2009b) also reported it. The difference in optimal growth concentrations may be due to the different species and application periods. CA is a Zn metalloenzyme that is activated by zinc treatment, and the activity increase may be due to the increased degree of activation after the application of Zn. CA can increase carbon dioxide concentration at carbon dioxide fixation sites in chloroplasts, thus affecting photosynthesis. Whereas β-CA is mostly found in chloroplasts of higher plants, α-CA and γ-CA are mostly found in cytoplasm of animals or algae, which may be the reason why zinc treatment only induces the expression of β-CA gene family. Given the limited test conditions, this experiment only analyzed the effect of spraying Zn fertilizer at the seedling stage of the two millet varieties. Therefore, the test results only represent the effect of Zn fertilizer on millet during this period. This study provided a theoretical basis for the rational application of zinc and the relationship between CA and photosynthesis in foxtail millet.

Conclusion: Zinc exhibits a 'low promotion and high inhibition' growth phenomenon in millet and it exhibits the best effects on growth at the concentration of 40 mg L^{-1}. At 40 mg L^{-1}, zinc can enhance the photosynthesis of plants, and induces the expression of the β-CA family, but the high concentration of Zn can damage crops and inhibit the photosynthesis of plants. The order of the effects of each concentration on millet is as follows: Zn2 > Zn1 > Zn3 > CK > Zn4 > Zn5. Studying the relationship among Zn, photosynthesis, and CA provides a theoretical support for the rational application of Zn fertilizer on millet.

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