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Ameliorative Effects of Brassinosteroid on Excess Manganese-Induced Oxidative Stress in *Zea mays* L. Leaves

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Abstract

Manganese (Mn) is becoming an important factor limiting crop growth and yields especially on acid soils. The present study was designed to explore the hypothesis that brassinosteroid application can enhance the tolerance of maize (*Zea mays* L.) to Mn stress and if so, whether or not the mechanism underlying involves regulation of antioxidative metabolism in leaves. The effects of 24-epibrassinosteroid (EBR) on the growth, photosynthesis, water status, lipid peroxidation, accumulation of reactive oxygen species, and activities or contents of antioxidant defense system in maize plants under Mn stress were investigated by a pot experiment. At supplemented Mn concentrations of 150-750 mg kg^{-1} soil, the growth of plants was inhibited in a concentration-dependent manner. The semi-lethal concentration was 550 mg Mn kg^{-1} soil. Foliage application with 0.1 mg L^{-1} EBR significantly reduced the decrease in dry mass, chlorophyll content, photosynthetic rate, leaf water content, and water potential of plants grown in the soil spiked with 550 mg kg^{-1} Mn. The oxidative stress caused by excess Mn, as reflected by the increase in malondialdehyde (MDA) content and lipoxygenase (LOX, EC 1.13.11.12) activity, accumulation of superoxide radical and H_2O_2, was greatly decreased by EBR treatment. Further investigations revealed that EBR application enhanced the activities of superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2), and the contents of reduced ascorbate and glutathione, compared with the plants without EBR treatment. It is concluded that the ameliorative effects of EBR on Mn toxicity are due to the up-regulation of antioxidative capacity in maize under Mn stress.

Key words: excess manganese, brassinosteroid, oxidative stress, antioxidant defense system, maize (*Zea mays* L.)

INTRODUCTION

Manganese (Mn) is a trace element essential for growth and development of plants. However, excess Mn can be detrimental to plants, and represents an important factor limiting growth and crop yields particularly on acid and insufficiently drained soils with low redox potential (Schlichting and Sparrow 1988). Excess Mn inhibits chlorophyll biosynthesis, decreases photosynthetic rate, and induces deficiency of Fe, Mg, and Ca (Subrahmanyam and Rathore 2000; Hauck *et al*. 2003; Shi *et al*. 2006). Also, it results in imbalance of antioxidant defense system and accumulation of reactive oxygen species (ROS) in plants (González *et al*. 1998; Shi *et al*. 2006; Lei *et al*. 2007).

The accumulation of ROS, like $^1O_2$ (singlet oxygen), $O_2^{\cdot-}$ (superoxide radical), $^{\cdot}OH$ (hydroxyl radical), and H_2O_2, often takes place in tissues of plants under metal stresses. ROS are toxic to living cells, causing lipid peroxidation, loss of membrane integrity, and damage to DNA and proteins. To efficiently detoxify ROS

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thus protecting cells from oxidative damage, plants have developed a well-organized antioxidant defense machinery involving both enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), and ascorbate peroxidase (APX), and non-enzymatic substances, acting as free radical scavengers such as ascorbic acid, glutathione, cysteine and carotenoids (Noctor and Foyer 1998; Asada 1999). These antioxidants operate coordinately to keep a low level in ROS within cells. Plants with high levels of antioxidants, either constitutive or induced, have greater resistance to environmental stress-induced oxidative damage (Allen 1995; Gomes-Junior *et al*. 2006; Shi *et al*. 2006). Some investigations indicate that excess Mn induces higher capacity of antioxidants and lower oxidative stress in tolerant species or varieties than in sensitive one(s), suggesting the disturbance of antioxidant system and the occurrence of oxidative damage may at partly explain for Mn toxicity in plants, and the elevated activities or levels in antioxidants may contribute to Mn tolerance of plants in response to excess Mn (González *et al*. 1998; Fecht-Christoffers *et al*. 2003).

Brassinosteroids (BRs) are a new class of plant hormones, which play an essential role in plant growth and development (Clouse and Sasse 1998). Moreover, they confer resistance to plants against various abiotic/biotic stresses, including those caused by salt, chilling, heat, drought, and pathogens (Sasse 2003). Recently, it has been reported that BRs can alleviate the toxicity of heavy metal, for instance, Cd and Ni in plants (Hayat *et al*. 2007; Ali *et al*. 2008b). Several lines of study have shown that the protective effect of BRs against abiotic stresses is closely associated with the activation of antioxidant defense system and photosynthesis process in plants (Li *et al*. 1998; Núñez *et al*. 2003; Hayat *et al*. 2007; Arora *et al*. 2008; Hasan *et al*. 2008; Ali *et al*. 2008a). 24-epibrassinosteroid (EBR), a synthetic BR with a high and stable activity, has been shown to increase thermotolerance and pathogen resistance in plants (Krishna 2003). Recently, Dong *et al*. (2008) reported exogenous EBR can improve photosynthetic characteristics in soybean under Al stress. However, whether application of EBR and BRs in other forms can confer Mn tolerance in plants, and whether BRs-regulated antioxidative response is involved have not yet been known.

Mn bioavailability is strongly affected by soil pH, Mn reserve, and the availability of electrons. As soil pH decreases, highly available free Mn ions increase in the soil solution (Schlichting and Sparrow 1988). With the rapid development of industry, acid deposition has been an increasingly serious environmental problem, which results in serious acidification of soil and higher availability of Mn in soil (Blake and Goulding 2002), especially in tropical and subtropical region. In addition, application of acidic and physiologically acidic fertilizers in agriculture aggravates the process of soil acidification (Petrie and Jackson 1984). Xiangtan City, Hunan Province in the south of China, has a famous manganese mine. Waste water, gases, and residue discharged from mining and smelting process have led to serious contamination of local air, water, and soil. Moreover, acid deposit takes place frequently in this region. Nowadays, Mn contamination issues are becoming increasingly common in Xiangtan City and its vicinities. Maize is a crop with relatively higher tolerance to Mn (Kamprath and Foy 1985). Therefore, the present study aims to test whether the application of EBR will ameliorate the Mn toxicity of maize, and to monitor the EBR-induced alterations in the levels of antioxidant activities or contents, thus giving implications for crop productivity in artificially-contaminated lands, and providing evidence of the involvement of EBR in alleviating Mn-induced oxidative stress.

MATERIALS AND METHODS

Soil

A red, silty clay soil was collected from the botanical garden of Hunan University of Science and Technology, China. Selected physical and chemical properties of the soil are presented below: pH 4.8; organic matter 17.6 g kg^{-1}; CEC 9.7 cmol kg^{-1}; available Mn 76.2 mg kg^{-1}. After air-drying, pounding, and passing through a 2-mm sieve, the soil was mixed with composite fertilizer at ratio of 0.15 N:0.10 P_2O_5: 0.15 K_2O as a base fertilizer. Then, the soil was spiked with $MnSO_4$ at rates of 150, 250, 350, 450, 550, 650, and 750 mg Mn kg^{-1} soil. The soil without Mn addi-

tion was used as the control. After 4-week incubation, the resulted artificially-contaminated soil was ready for investigations.

Plant materials and treatments

Seeds of maize (*Zea mays* L., Luyu 13) were sterilized with 0.1% $HgCl_2$ for 10 min and washed extensively with distilled water. After 12 h of imbibition in distilled water, the seeds were sown in trays of the control soil. When the second leaf was fully expanded, 6 uniform seedlings were transplanted to each plastic pot containing 4 kg artificially-contaminated soil. The pot experiment was conducted in a greenhouse with day/night cycle of 14/10 h, 24-26°C/18-20°C, respectively, photosynthetically active radiation of 100 μmol photons m^{-2} s^{-1}, and relative humidity between 65 and 75%. The plants were watered daily. After 10 d of growth, the plants were sprayed with various concentrations (0.01, 0.05, 0.1, 0.2, and 0.3 mg L^{-1}) of EBR (Sigma, USA) containing 0.01% (v/v) Tween-20 till the whole plants were fully wet, and sprayed again 2 d later for a strengthening treatment. The plants sprayed with 0.01% (v/v) Tween-20 served as controls. At 3, 6, 9, and 12 d after the strengthening treatment (DAT), the second leaves (from upper to lower) were sampled for analyses.

Plant growth and Mn content

The harvested plants were washed with deionized water three times and divided into roots and shoots. The samples were oven-dried at 70°C for 72 h to a constant weight and weighted. Dried plant material was powdered and digested in a mixture of HNO_3/$HClO_4$ (2/1, v/v). Mn content was determined by inductively coupled plasma spectrometry method (ICP-7510, Shimadzu, Japan).

Chlorophyll content, net photosynthetic rate (Pn, CO_2 exchange), and chlorophyll fluorescence

Chlorophyll was extracted by acetone and measured according to Arnon (1949). Pn was measured with a portable photosynthesis system (Li 6400, LI-COR, Lincoln, NE, USA). Chlorophyll fluorescence was determined with a pulse amplitude modulated fluorometer (PAM-2000, Heinz Walz, Effeltrich, Germany). Fv/Fm, reflecting the maximal quantum yield of PSII photochemistry, was calculated.

Relative water content (RWC), water potential, and free proline content

RWC was determined according to the method of Turner (1981). Water potential was measured hygrometrically on six leaf discs (10 mm diameter, excluding midrib) cut from three leaves taken from the randomly selected plants, using a Wescor (Logan, UT) microvoltmeter (model HR 33T) and C-52 leaf chambers. Proline extraction and its concentration determination were carried out by the method of Bates *et al*. (1973).

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) according to the thiobarbituric acid reaction (Heath and Packer 1968). MDA concentration was calculated by its extinction coefficient of 155 mmol L^{-1} cm^{-1}.

Measurement of $O_2^{\cdot-}$ generating rate and H_2O_2 content

$O_2^{\cdot-}$ generation was measured by monitoring the reduction of sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate at 470 nm in the presence of $O_2^{\cdot-}$ (Able *et al*. 1998). Corrections were made for the background absorbance in the presence of 50 units SOD. $O_2^{\cdot-}$ generation rate was calculated using an extinction coefficient of 21.6 mmol L^{-1} cm^{-1}.

H_2O_2 content was measured as described by Brennan and Frenkel (1977) and, calculated from the standardized H_2O_2 curve.

Assay of lipoxygenase (LOX)

Leaves were homogenized in ice-cold 50 mmol L^{-1} potassium phosphate buffer (pH 7.5) containing 0.1% (w/v) CHAPSO with a dilution fold of 5-10 (v/m), and then centrifuged at 4°C for 15 min at 12 000 × g. The ex-

tract was ready for use. LOX activity was assayed in borate buffer (0.2 mmol L^{-1}, pH 9.0) by monitoring the increase in absorption at 234 nm due to formation of conjugated linoleate hydroperoxides, using linoleic acid as a substrate (Surrey 1964).

Assay of antioxidative enzymes

0.5 g leaves were homogenized in 5 mL of ice-cold 50 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 1 mmol L^{-1} EDTA and 1% polyvinyl-pyrrolidone, with the addition of 1 mmol L^{-1} reduced ascorbate (ASC) in the case of APX and dehydroascorbate reductase (DHAR) assay in a chilled pestle and mortar. The homogenates were centrifuged at 4°C for 20 min at 12 000 × g and the resulting supernatants were immediately used for the following enzyme assays. Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The absorbance was recorded at 560 nm, and one unit of SOD was defined as the amount of enzyme required to cause a 50% inhibition of the reduction of NBT. CAT activity was measured as the decline in absorbance at 240 nm due to the decomposition of H_2O_2, as described by Dhindsa *et al*. (1981). The assay of POD activity was based on the method as described by Braber (1980). Activity was determined by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation. APX activity was assayed by monitoring the decrease in absorbance at 290 nm as ASC was oxidized, according to Nakano and Asada (1981). The assay of DHAR activity was carried out by measuring the increase in absorbance at 265 nm due to ASC formation (Nakano and Asada 1981). Glutathione reductase (GR) activity was measured by following the decrease in the absorbance of NADPH at 340 nm as oxidized glutathione (GSSG)-dependent oxidation of NADPH, as described by Foyer and Halliwell (1976). Protein content was determined according to the method of Bradford (1976) with BSA as standard.

Determination of nonenzymatic antioxidants

0.5 g leaves were homogenized with 10 mL of 5% ice-cold trichloroacetic acid and 5% sulfosalicylic acid. The homogenates were centrifuged at 4°C for 20 min at 12 000 × g and the resulting supernatants were immediately used for assay. ASC and total ascorbate [ASC plus DHA (dehydroascorbate)] were measured according to Hodges *et al*. (1996). Total glutathione [GSH (reduced glutathione) plus GSSG (oxidized glutathione)] was determined by a method of enzymatic cycling assay according to Griffiths (1980). GSSG was determined after the removal of GSH by 2-vinylpyridine derivatization. GSH was calculated by the subtraction of GSSG from total glutathione.

Statistics

All results presented are the mean of three independent replicates (n = 6). The data were analyzed by analysis of variance (ANOVA) using SPSS for Windows ver. 11.5 (SPSS Inc, Chicago, USA). Differences between treatments were compared by the least significant difference (LSD) test at a 0.05 level.

RESULTS

Growth, photosynthesis, and leaf water status

Maize plants grown in 150-750 mg Mn kg^{-1} soil showed growth inhibition with the increase of Mn concentrations. Plant dry mass dropped to about the half of the control at 550 mg Mn kg^{-1}, and this was regarded as the semi-lethal concentration used for following experiments (Fig.1-A). To determine the point where EBR showed the most significant effect, a preliminary experiment with EBR at 0, 0.01, 0.05, 0.1, 0.2, and 0.3 mg L^{-1} was carried out. As shown in Fig.1-B, spraying with EBR at 0.1 mg L^{-1} exerted the greatest effect on the Mn-caused inhibition of growth, with an increase of 35.0% in the dry mass, relative to the treatment of 550 mg Mn kg^{-1} alone. However, a further increase in EBR concentration up to 0.2 mg L^{-1} EBR had a detrimental effect on the plant growth, indicating higher concentrations of EBR might be toxic to maize plants.

Chlorosis, curling of leaf apices and subsequent slight wilting were observed in the leaves. A significant decrease in chlorophyll content was recorded in maize plants exposed to 550 mg Mn kg^{-1} (Table 1). Also, the Mn

stress caused an evident decrease in Pn and Fv/Fm, indicating a great impairment on the photosynthesis of plants. Moreover, the decreased water potential and RWC, increased free proline content (Fig.2), and visible leaf wilting in the Mn-stressed plants suggested malfunction of water uptake. EBR alone had a non-significant influence on these parameters (Fig.2). However, the plants supplied excess Mn along with 0.1 mg L^{-1} EBR, exhibited a significant improvement in their phenotypic appearance, chlorophyll contents, photosynthetic rate and in leaf water status (Table 1).

Mn content

As expected, Mn contents in both shoots and roots dramatically increased with exposure days in the presence of Mn excess (Table 2). This was particularly pronounced in shoots, where Mn content increased dramatically by nearly 20 folds at 12 DAT as compared to the control. However, no evident changes in Mn content and distribution between shoots and roots following EBR application, regardless of exposure to Mn excess.

Lipid peroxidation

Mn stress resulted in a significant increase in LOX activity as compared with the control (Table 3). EBR treatment greatly decreased the increase in LOX activity of the Mn-stressed plants, suggest-

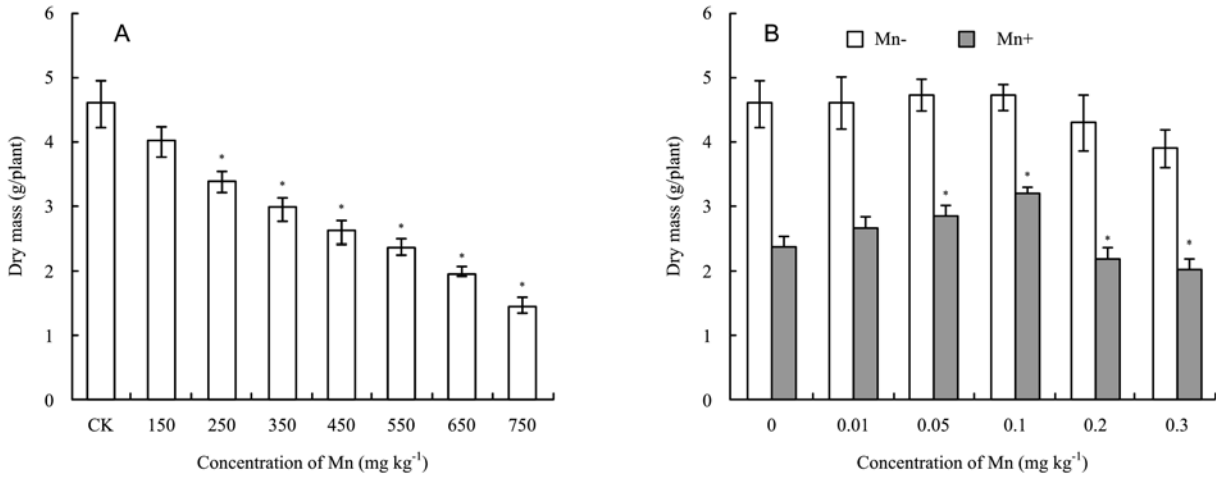

Fig. 1 Effect of Mn and EBR on the dry mass of maize plants at 12 d after EBR treatment. Plants were exposed to Mn at the indicated concentrations (A) or at 550 mg kg^{-1} (B) for 10 d and then applied with EBR. Vertical bars represent the SE of means (n=6); the same as below. Asterisks indicate that values are significantly different between Mn treatments and control (A), or between treatments of Mn + EBR and Mn alone (B) ($P<0.05$); the same as below.

Table 1 Effects of EBR (0.1 mg L^{-1}) on the chlorophyll content, net photosynthetic rate (Pn) and chlorophyll fluorescence (Fv/Fm) of maize plants exposed to 550 mg Mn kg^{-1} at 3, 6, 9 and 12 d after EBR treatment (DAT)[1)]

Parameters	DAT	Control	EBR	Mn	Mn+EBR
Chlorophyll (mg g^{-1} FW)	3	2.74±0.13 ab	2.83±0.17 a	2.31±0.09 c	2.60±0.06b
	6	2.67±0.17 ab	2.81±0.14 a	1.96±0.07 c	2.35±0.12 b
	9	2.52±0.08 a	2.64±0.09 a	2.10±0.06 b	2.45±0.11 a
	12	2.43±0.11 ab	2.52±0.13 a	1.71±0.16 c	2.14±0.10 b
Pn (μmol m^{-2} s^{-1})	3	38.6±1.3 a	39.2±2.6 a	31.1±1.9 b	36.5±1.7 a
	6	41.2±3.7 ab	43.0±2.1 a	29.7±2.4 c	35.0±2.0 b
	9	44.0±2.7 a	45.3±2.2 a	28.2±1.8 c	39.2±1.6 b
	12	43.1±1.9 a	45.6±3.3 a	28.5±1.4 c	36.7±2.2 b
Fv/Fm	3	0.837±0.010 a	0.849±0.020 a	0.783±0.015 c	0.815±0.012 b
	6	0.823±0.021 a	0.825±0.017 a	0.757±0.023 b	0.808±0.020 a
	9	0.831±0.012 a	0.824±0.016 a	0.763±0.019 b	0.819±0.014 a
	12	0.820±0.017 a	0.827±0.021 a	0.746±0.018 b	0.811±0.022 a

[1)] Data represent the means± SE (n=6). Values with different letters in the same row are significantly different ($P<0.05$). The same as beow.

ing it may lessen lipid peroxidation induced by Mn stress via decreasing the activity of this enzyme.

The Mn stressed-plants showed an evident increase in MDA content (Table 3), indicating pronounced lipid peroxidation occurred under the stressful Mn condition. However, the Mn-induced increase in MDA level was greatly reduced by EBR application.

ROS levels

As depicted in Table 3, excess Mn led to a remarkable enhancement in $O_2^{\cdot-}$ production rate and H_2O_2 content, demonstrating an evident oxidative stress occurred in the Mn-stressed plants. EBR alone had no obvious effects on $O_2^{\cdot-}$ production rate and H_2O_2 content, but significantly reduced the increase in their levels when plants were exposed to excess Mn, indicating a great alleviation of the Mn-induced oxidative stress by EBR application.

Response of antioxidant defense system

Antioxidant enzyme activities changed in a time-dependent manner after excess Mn and/or EBR treatments. Upon excess Mn, SOD activity revealed an average increase of 53.9% during 3-9 DAT, when compared to the control (Fig.3-A). Similarly, Mn stress led to a significant enhancement of POD activity (Fig.3-B). In contrast, activities of CAT, APX, DHAR and GR (Fig.3-C, F) were inhibited by excess Mn. Single EBR application had no effects on POD activity, whereas promoted activities of the other antioxidant enzymes. Moreover, activities of all the antioxidative enzymes tested were up-regulated in the EBR plus Mn stressed-plants, with an average increase of 16.8-79.2% during 3-9 DAT, as compared with those of excess Mn treatment alone.

The control plants maintained a stable ASC level of around 1.27 nmol g^{-1} FW in leaves during 3-9 DAT (Fig.4-A). Application with EBR alone resulted in a slight but not significant increase in ASC content. When

Table 2 Effects of EBR (0.1 mg L^{-1}) on Mn content of maize plants exposed to 550 mg Mn kg^{-1} at 3, 6, 9 and 12 d after EBR treatment (DAT)

Parameters	DAT	Control	EBR	Mn	Mn+EBR
Shoot Mn (mg g^{-1} DW)	3	0.097±0.007 b	0.113±0.005 b	2.187±0.167 a	1.946±0.110 a
	6	0.163±0.012 b	0.170±0.015 b	3.233±0.261 a	3.190±0.224 a
	9	0.193±0.013 b	0.198±0.008 b	3.513±0.159 a	3.375±0.163 a
	12	0.210±0.009 b	0.205±0.006 b	3.677±0.213 a	3.861±0.270 a
Root Mn (mg g^{-1} DW)	3	0.046±0.008 b	0.051±0.007 b	0.873±0.055 a	0.795±0.039 a
	6	0.093±0.005 b	0.088±0.006 b	1.072±0.057a	1.110±0.098 a
	9	0.120±0.007 b	0.103±0.009 b	1.142±0.032 a	1.229±0.064 a
	12	0.132±0.011 b	0.145±0.012 b	1.204±0.070 a	1.213±0.110 a

Table 3 Effects of EBR (0.1 mg L^{-1}) on the accumulation of H_2O_2 (μmol g^{-1} FW) and $O_2^{\cdot-}$ (nmol min^{-1} g^{-1} FW), MDA content (nmol g^{-1} FW), and LOX activity (nmol min^{-1} g^{-1} FW) in maize plants exposed to 550 mg Mn kg^{-1} at 3, 6, 9, and 12 d after EBR treatment (DAT)

Parameters	DAT	Control	EBR	Mn	Mn+EBR
H_2O_2 content	3	2.8±0.2 c	3.1±0.4 c	5.3±0.3 a	4.0±0.1 b
	6	2.6±0.1 c	2.8±0.2 bc	4.1±0.1 a	3.2±0.2 b
	9	2.3±0.3 c	2.5±0.1 c	4.7±0.2 a	3.9±0.3 b
	12	3.2±0.2 c	3.0±0.2 c	4.5±0.4 a	3.6±0.3 b
$O_2^{\cdot-}$ generating rate	3	9.1±0.5 c	8.7±0.3 c	19.6±1.4 a	13.8±0.9 b
	6	10.7±0.9 c	11.2±0.7 c	18.4±1.1 a	15.1±0.6 b
	9	12.3±0.7 c	11.4±0.4 c	17.5±0.8 a	13.7±0.5 b
	12	11.6±0.6 c	10.4±0.5 c	20.1±1.0 a	15.8±1.2 b
MDA content	3	7.2±0.6 c	7.0±0.4 c	13.1±1.1 a	9.8±0.8 b
	6	8.3±0.6 c	7.8±0.3 c	11.4±0.4 a	9.5±0.4 b
	9	8.9±0.3 c	8.4±0.6 c	14.6±0.9 a	11.3±1.0 b
	12	9.2±0.7 bc	8.9±0.5 c	12.7±0.6 a	10.0±0.3 b
LOX activity	3	0.48±0.02 c	0.52±0.04 c	0.77±0.03 a	0.61±0.01 b
	6	0.62±0.03 c	0.58±0.05 c	0.93±0.06 a	0.75±0.02 b
	9	0.74±0.06 c	0.70±0.03 c	1.18±0.08 a	0.86±0.05 b
	12	0.66±0.02 c	0.61±0.04 c	0.97±0.05 a	0.80±0.04 b

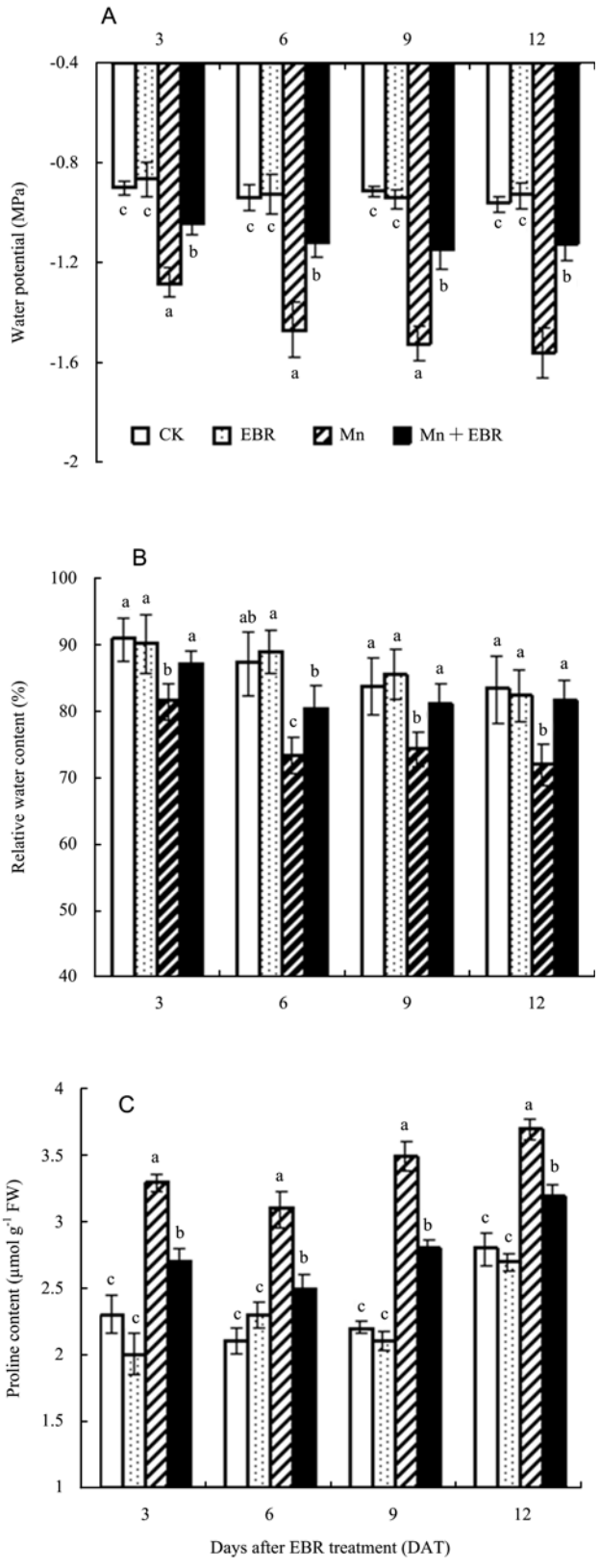

Fig. 2 Effect of EBR (0.1 mg L^{-1}) on the water potential (A), relative water content (B), and proline content (C) of maize plants exposed to 550 mg Mn kg^{-1}. Different letters indicate that values are significantly different ($P<0.05$).

grown in the soil added with 550 mg kg^{-1} Mn, plants exhibited a significant decline in ASC content. However, ASC increased sharply in the EBR plus Mn stressed-plants in relative to those of Mn stress alone. Similar changes in GSH content were observed except for the plants exposed to excess Mn alone for 3 DAT, when GSH content was stimulated to increase over the control value (Fig.4-B).

DISCUSSION

It has been well documented that excess Mn affects some physiological and metabolism processes involved in plant growth and development (Quartin *et al*. 1988; Subrahmanyam and Rathore 2000; Hauck *et al*. 2003; Shi *et al*. 2006). The present study has revealed that excess Mn at 550 mg kg^{-1} inhibited the growth of maize plants, as reflected by a considerable decrease in the dry matter accumulations (Fig.1-A). Reduction in biomass accumulation is often a reliable indication of the plants' sensitivity to various stresses, as it represents the cumulative influences of damaged or inhibited physiological functions. On the other hand, the photosynthetic pigment contents, Fv/Fm and Pn also exhibited a significant decrease under the influence of excess Mn (Table 1). Moreover, excess Mn induced water deficit in leaves, as indicated by decrease in water potential and RWC (Fig.2-A, B) in the young foliage of plants, and also by curling of leaf apices and subsequent wilting. The present study also presents evidence of the beneficial effect of EBR on the growth, photosynthesis and water status of maize under Mn stress. Similar results have been obtained in *Brassica juncea* under Cd stress (Hayat *et al*. 2007) and Ni stress (Ali *et al*. 2008b), and in soybean under Al stress (Ali *et al*. 2008a). The EBR-induced enhancement in Pn of Mn stressed-maize may be due to the increased chlorophyll content and Fv/Fm (Table 1), thus promoting the efficiency of light harvest and transfer, and/or to the increased stomatal conductance, carbonic anhydrase activity, concentration and activity of RuBPCase, thus enhancing CO_2 transportation and fixation (Hayat *et al*. 2007; Ali *et al*. 2008a; Dong *et al*. 2008). A further investigation showed no evident difference in Mn contents at the organ levels following EBR treatments (Table 2), suggesting lowering Mn uptake, or retention of Mn in roots

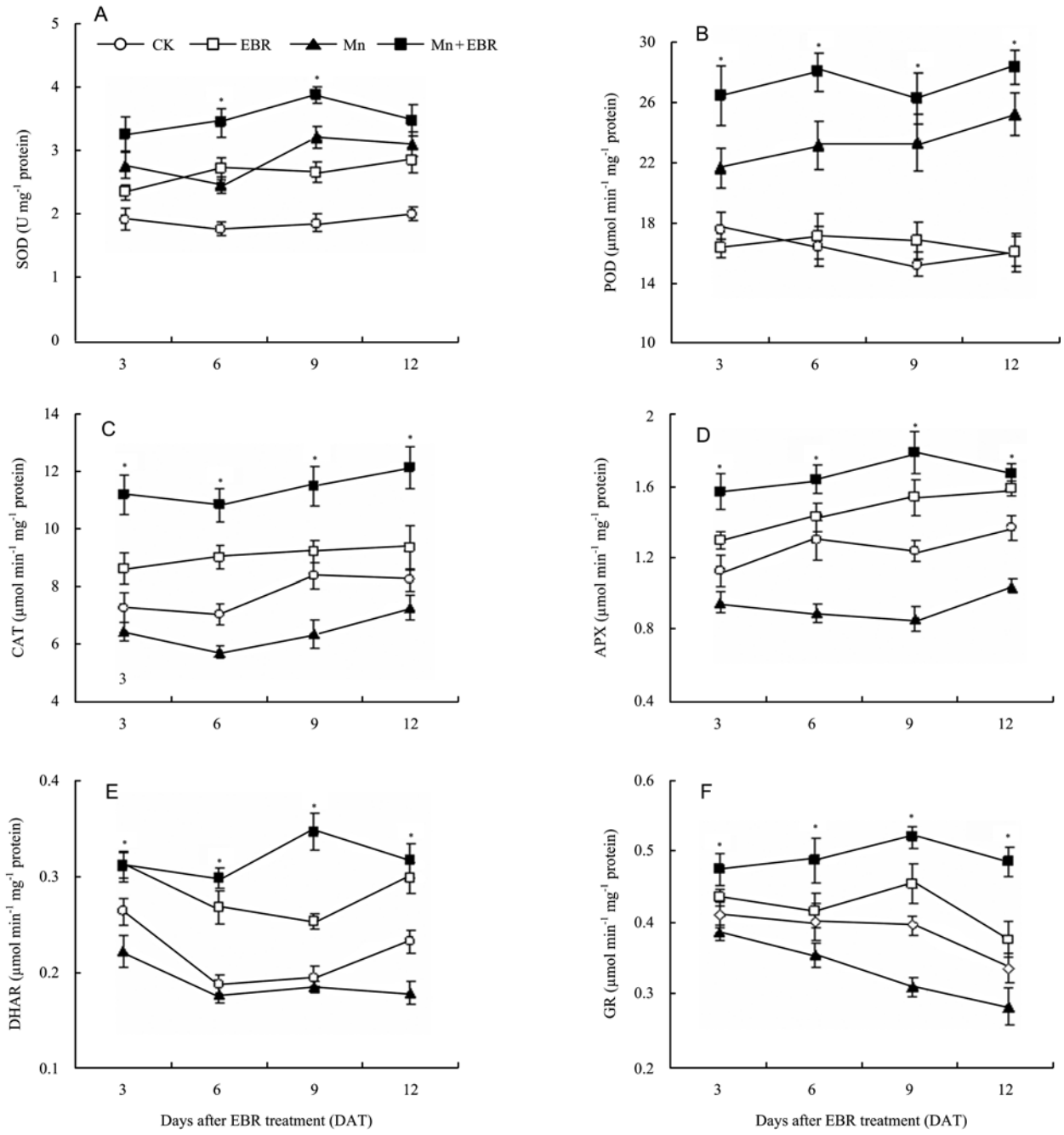

Fig. 3 Effect of EBR (0.1 mg L^{-1}) on the activities of SOD (A), POD (B), CAT (C), APX (D), DHAR (E), and GR (F) in maize plants exposed to 550 mg Mn kg^{-1}.

may have not contributed to the alleviation of Mn toxicity by EBR.

Water deficit can lead to inhibition of photosynthesis by stomatal or nonstomatal mechanisms (Ni and Pallardy 1992), and also induce oxidative stress in plant cells (Smirnoff 1993). Therefore, the induction of water deficit by Mn stress might have contributed to the declined photosynthetic rate (Table 1) and oxidative damage (Table 3) thereby aggravating Mn-toxicity. However, the physiological drought generated by excess Mn, in the present study, was greatly alleviated by EBR treatment, reflecting a partial relief from Mn-toxicity. This notion is further supported by the fact that the increase in proline content under Mn stress

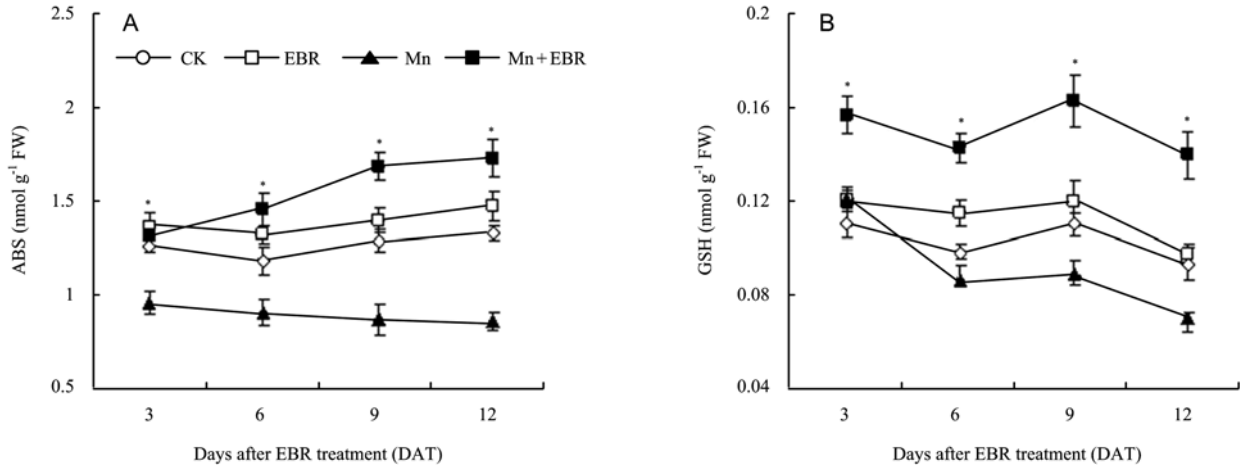

Fig. 4 Effect of EBR (0.1 mg L^{-1}) on the contents of ASC (A) and GSH (B) in maize plants exposed to 550 mg Mn kg^{-1}.

was greatly counteracted by EBR application (Fig.2-C), since there is evidence that proline accumulation is partly related to a heavy metal-induced water deficit and may be considered as a suitable indicator of heavy metal stresses (Schat *et al*. 1997; Metwally *et al*. 2003).

Accumulation of ROS often results in oxidative damage directly or indirectly in plants subjected to a variety of stresses and may cause significant damages to cellular constituents, especially membrane lipids. LOX is considered as an indicator of oxidative stress, which catalyzes oxygenation of polyunsaturated fatty acids into lipidhydroperoxides during responses to various environmental stresses. Enhanced $O_2^{\cdot-}$ and H_2O_2 accumulation, MDA content, and LOX activity (Table 3) in plants exposed to excess Mn were evident here, indicating the metal led to the appearance of oxidative damage, which is in agreement with the other studies carried out in common bean and cucumber (González *et al*. 1998; Shi *et al*. 2006). By contrast, during exposure to excess Mn, the plants treated with EBR exhibited lower ROS generation, MDA content and LOX activity, suggesting the beneficial effect of EBR on plant growth is, at least partly, due to the decline in the level of oxidative damage. This view is supported by the earlier findings registered in water-stressed (Li *et al*. 1998) and salt-stressed maize (Arora *et al*. 2008).

The EBR-modulated changes in ROS accumulation in maize leaves would be associated with the alterations in the activities of antioxidant enzymes. H_2O_2 produced from $O_2^{\cdot-}$ catalyzed by SOD is degraded by CAT, POD, and APX (Asada 1999). Therefore, concurrent functioning of these enzymes is vital for quenching ROS to a low level. Our investigations show that SOD activity was significantly enhanced while the activities of CAT and APX were inhibited in the Mn-stressed plants (Fig. 3-C, D), indicating the cooperative functionality of antioxidative enzymes was disrupted. This change resulted in more H_2O_2 production (Table 3). However, EBR application led to a further increase in SOD and POD (Fig.3-B) activities, and also an increase in CAT, POD, and APX activities as compared to those under excess Mn alone. Consequently, the content of $O_2^{\cdot-}$ and H_2O_2 significantly decreased in the Mn-stressed maize following EBR treatment. The EBR-modulated increase in the activities of antioxidative enzymes, such as SOD, CAT, APX, and POD, may represent a defense mechanism against the Mn stress-induced oxidative damage, as also observed in salinity stressed-maize following EBR application (Arora *et al*. 2008).

In contrast with the other H_2O_2-detoxifying enzymes, POD in plants is quite distinguishable in physiological functions. It participates in metabolic processes such as lignin biosynthesis, indoleacetic acid decomposition, responses to biotic and abiotic stresses, including heavy metal stresses (Passardi *et al*. 2004). The remarkable enhancement of POD activity in maize upon either Mn stress or EBR treatment (Fig.3-B) suggests its important role in response to Mn stress. Whereas, its function in plants may be more complex when exposed to excess Mn. The occurrence of brown spots in older leaves, a visible symptom of Mn toxicity observed in a variety of plants (Horst 1988), represents local depos-

its of oxidized Mn and oxidized phenolic compounds in the cell wall, and is proposed to be mediated by a H_2O_2-consuming POD (Wissemeier and Horst 1992). Seemingly, the increase in POD activity by EBR under Mn stress is inconsistent with the alleviation of Mn toxicity in maize. However, Fecht-Christoffers *et al*. (2003) confirmed the development of Mn toxicity in pea is dependent on the spatial activation of POD, which is located in the apoplast. Presumably, apoplastic POD activity in maize was not be activated by EBR under Mn stress. Alternatively, EBR might cause a decline in the content of apoplastic H_2O_2, i.e., decreasing the substance concentration of apoplastic PODs thus limiting the formation of Mn toxicity symptoms.

Ascorbate-glutathione cycle is an important H_2O_2-detoxifying system, which comprises enzymes such as APX, DHAR and GR, and nonenzymatic antioxidants such as ascorbate and glutathione (Noctor and Foyer 1998; Asada 1999). In this cycle, APX decomposes H_2O_2 via oxidation of ASC, and then ASC is regenerated from DHA using GSH as an electron donor, followed by recycling of GSH by GR activity. Accumulating evidence reveals that the availability of antioxidants and the activities of antioxidant enzymes in this system are involved in tolerance to metal stresses (Ma *et al*. 2007). In this study, the activities of APX, DHAR and GR were down-regulated in response to excess Mn (Fig.3-D-F), whereas, substantially enhanced by EBR application, suggesting their important role in the ameliorative effects of EBR on Mn toxicity of maize. Surprisingly, the activities of the three enzyme, together with CAT, were even higher in the EBR plus Mn-treated plants than in the control or EBR-treated ones. This effect may be attributed to appropriate H_2O_2 accumulation in the EBR plus Mn-treated plants, which might function as a signal to trigger the activation of the antioxidative enzymes. In rice, H_2O_2-mediated induction of APX mRNA was observed when experiencing oxidative stress (Morita *et al*. 1999). However, the dual function of H_2O_2 in causing oxidative damage and signalling in the stimulation of defense response may be dependent on the extent of H_2O_2 accumulation in tissues. H_2O_2 content was rather higher in the plants subjected to Mn stress, and this could determined the type of role played by H_2O_2. Presumably, the signalling role of H_2O_2 in the activation of antioxidative enzymes might be limited in the Mn-stressed plants.

Being a major contributor to the cellular redox state, ASC is involved in oxidative defense in plants (Noctor and Foyer 1998). In common bean, genotypic Mn tolerance may be associated with the maintenance of higher ASC concentrations in leaf tissue under Mn stress (González *et al*. 1998). Moreover, it is an effective scavenger for phenoxy radicals, which may lead to oxidation of phenols, thus forming necrosis in plants (Sánchez *et al*. 1997). Excess Mn caused a significant decline in ASC content in maize plants (Fig.4-A). This may be resulted from the lowered activity of DHAR (Fig.4-E), which is responsible for generation of ASC from DHA, and increasing oxidation of ASC or prevention of ASC synthesis under Mn stress (Shi *et al*. 2006). Owing to its redox active thiol group, GSH is another prominent cellular antioxidant and has been considered to be closely associated with tolerance to a variety of heavy metal stresses (Hall 2002; Freeman *et al*. 2004). In the present study, the overall level of GSH was decreased under Mn stress (Fig.4-B). However, the decreased levels in ASC and GSH could be reversed by simultaneous application of EBR, suggesting they may play an important role in the beneficial effects of EBR on the growth of maize plants exposed to Mn stress.

In summary, EBR application exerts a significant ameliorative effect on the growth, photosynthesis and water status in maize plants subjected to Mn stress. Enhanced antioxidant defense system is involved in the alleviation of Mn toxixity in maize following EBR treatment, as revealed by the activation of antioxidative enzymes, such as SOD, POD, CAT, APX, DHAR, and GR, and the increase in ASC and GSH poll size. Probably, a "tolerance" rather than an "avoidance" mechanism may be involved in the positive influence on the Mn-stressed plants, since the total Mn concentrations in shoots and roots were not changed by EBR application. The alterations in the uptake and distribution of other mineral elements and their relation to the EBR-induced ameliorative effect need a further investigation.

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