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Sulfur dioxide derivatives alleviate cadmium toxicity by enhancing antioxidant defence and reducing Cd^{2+} uptake and translocation in foxtail millet seedlings

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ABSTRACT

Sulfur dioxide (SO_2) was recently proposed as a novel bio-regulator in mammals. However, the possible advantageous effects of SO_2 in plant adaptation to heavy metal-contaminated environments are largely unknown. In the present study, using Na_2SO_3/$NaHSO_3$ derivatives as SO_2 donors, we investigated the possible roles and regulation mechanisms of SO_2 in alleviating Cd^{2+} toxicity in foxtail millet seedlings. Exogenous SO_2 derivatives (0.5 mM) application significantly reduced the seedling growth inhibition caused by Cd^{2+} stress. Cd^{2+}-induced oxidative damage was also alleviated by SO_2 derivatives, which was supported by the decreased malondialdehyde (MDA) level in the leaves of seedlings pretreated with SO_2 derivatives. These responses were related to the enhanced activities of representative antioxidant enzymes, including catalase and superoxide dismutase, as well as the up-regulation of ascorbate-glutathione cycle, which contributed to the scavenging of Cd^{2+}-elicited $O_2^{\bullet -}$ and H_2O_2 within the leaves of foxtail millet seedlings. Also, SO_2 derivative application promoted sulfur assimilation and increased the content of glutathione and phytochelatins, which may help to enhance Cd^{2+} detoxification capacity in foxtail millet seedlings. Moreover, application of SO_2 derivatives caused down-regulation of the transcript expression levels of several genes involved in Cd^{2+} uptake and translocation, such as *NRAMP1*, *NRAMP6*, *IRT1*, *IRT2*, *HMA2*, and *HMA4*, thus resulting in reduced Cd^{2+} accumulation in the shoots and roots of Cd^{2+}-stressed seedlings. Collectively, these results suggest that exogenous SO_2 derivative application can alleviate oxidative damage and restrict Cd^{2+} buildup, thereby reducing Cd^{2+}-induced growth inhibition in foxtail millet seedlings upon Cd^{2+} exposure. This novel finding indicates that the usage of SO_2 derivatives may be an effective approach for enhancing Cd^{2+} tolerance in foxtail millet and other crops.

1. Introduction

Cadmium (Cd), one of the most toxic heavy metals, has become a widespread environmental contaminant mainly due to anthropogenic activities including metallurgic industries, waste incinerators, and application of phosphate fertilizers (Dalcorso et al., 2008). Excessive Cd^{2+} exerts adverse effects on a series of physiological processes such as photosynthesis, respiration, and nutrient metabolism, leading to growth inhibition and eventually plant death (Gill et al., 2012; Feng et al., 2017). One of the primary causes of Cd^{2+} toxicity within cells is oxidative damage due to a burst of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which can harmfully alter protein structure, degrade phospholipids, and even cause cell death (Chmielowska-Bąk et al., 2014; Han et al., 2016).

Sulfur dioxide (SO_2), a non-flammable gas with a penetrating odour, has traditionally been considered to be a common and harmful air pollutant. Prolonged exposure to excessive SO_2 can cause neurological disorders and respiratory diseases (e.g., asthma, emphysema, chronic bronchitis, etc.; Meng, 2003; Sang et al., 2010). Recently, it was discovered that SO_2 is produced endogenously and exhibits beneficial roles during a variety of physiological processes and disease responses in mammals, such as protecting against myocardium injury, improving pulmonary vascular structural remodelling, and enhancing myocardial antioxidant capacity (Liu et al., 2010; Liang et al., 2011), which has changed people's opinion of this toxic gas. In the food industry, SO_2 has become the most widespread fruit preservative, not only due to its antimicrobial properties but also because it induces plant biotic defence responses (Giraud et al., 2012). Our latest research revealed that SO_2 exposure can enhance disease resistance against *Botrytis cinerea* in *Arabidopsis* by promoting defence-related gene expression and enzyme activity (Xue and Yi, 2018).

SO_2 can be metabolized and used as sulfur source in plants, by

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feeding into sulfur (S) assimilation, to form cysteine (Cys) and other sulfur-containing compounds (Aghajanzadeh et al., 2015). S assimilation has been considered as an important step for plant tolerance to Cd^{2+} stress (Gill and Tuteja, 2011). The S assimilation pathway initiates with the uptake of sulfate from the soil which is facilitated by sulfate transporters. Once within cells, sulfate can be activated by ATP via ATP sulfurylase (ATPS). The product is reduced by 5′-adenylylsulfate reductase to sulfite, which is further reduced to sulfide by sulfite reductase. Cys is the final product of the S assimilation pathway and is supposed to be a precursor of glutathione (GSH), a non-protein thiol protecting the plants from Cd^{2+}-induced oxidative stress. GSH also acts as the substrate for biosynthesis of phytochelatins (PCs), which has a proven role in Cd^{2+} detoxification (Gill and Tuteja, 2011; Gill et al., 2012). In many previous studies, Cd^{2+} tolerance was found to be associated with enhanced Cys, GSH, and PCs biosynthesis in plants (Domínguez-Solís et al., 2004; Asgher et al., 2014; Liang et al., 2016).

In plants, SO_2 is taken up mainly through the stomata, followed by distribution within the intercellular space of leaf tissues. In the aqueous phase of the apoplast and/or cytoplasm, SO_2 dissociates into its sulfite derivatives (sulfite ions/bisulfite ions, 3:1 M/M; Shapiro, 1977). Thus, $Na_2SO_3/NaHSO_3$ (3:1, M/M), which can be made relatively safely and easily in comparison with SO_2 gas, is often used as a SO_2 donor to investigate the specific toxicological effects or physiological functions of SO_2 in animals and plants (Liang et al., 2011; Yi et al., 2012; Hu et al., 2014; Wei et al., 2015). More recently, it was reported that exogenously applied SO_2 derivatives can act as antioxidant agents to mitigate aluminium (Al^{3+}) and Cd^{2+} toxicity during wheat seed germination (Hu et al., 2015; Zhu et al., 2015). However, to the best of our knowledge, the possibility that SO_2 derivatives might be beneficial for seedling growth, especially in C_4 crops subjected to Cd^{2+} stress, has not been addressed.

Foxtail millet (*Setaria italica* L. Beauv) is an important and popular cereal crop in southern Europe and Asia. It is a model plant system for studying C_4 grass biology and agronomic traits related to nutritional quality, photosynthetic efficiency and biomass potential (Pant et al., 2016). Furthermore, this crop was found to be resistant to multiple abiotic and biotic stresses such as drought (Lata et al., 2011), salinity (Ardie et al., 2015) and fungal diseases (Han et al., 2017). However, there is little information available regarding foxtail millet responses to Cd pollution. Based on the above mentioned studies, we hypothesized that the usage of SO_2 derivatives might be a potential approach to enhance Cd^{2+} tolerance in foxtail millet plants. The aim of this study was to investigate whether and how the SO_2 derivatives may function in alleviating Cd^{2+}-induced toxicity in foxtail millet. This study would extend our knowledge of the beneficial roles of SO_2 derivatives in cereal crops and provide novel strategies for improving plant tolerance against heavy metal pollutants.

2. Materials and methods

2.1. Plant cultivation

Seeds of foxtail millet (*Setaria italica*, ecotype Changnong42) were kindly provided by the Millet Research Institute of Shanxi Academy of Agricultural Science in China. Seeds were sterilized in 75% (v/v) ethanol for 30 s and in 10% (v/v) sodium hypochlorite solution for an additional 10 min. Sterilized seeds were then grown on Petri dishes with three layers of gauze at the bottom and 5 mL of water. After germination in the dark at 25 °C, the cultures were kept in a growth room with a temperature of 25 °C under a 12 h light/12 h dark photoperiod and 150 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation. Seedlings were watered with liquid MS (Murashige and Skoog) solution. The solution was replaced every 2 days.

2.2. Plant treatments

A mixture of sodium sulfite and sodium bisulfite (3:1 mM/mM) was used as exogenous SO_2 derivatives as described by Yi et al. (2012). Ten-day-old seedlings with uniform size were divided into three groups for various treatments according to the following: (1) seedlings were cultured in liquid MS solution supplemented with different concentrations of $CdCl_2$ (0, 125, 250, 375, 500, and 625 μM). After 72 h, root length, shoot fresh weight, and malondialdehyde (MDA) levels in shoots were determined. (2) Seedlings were pretreated with various concentrations of SO_2 derivatives (0, 0.25, 0.5, and 1.0 mM) for 12 h and then were cultivated in liquid MS solution containing different concentrations of $CdCl_2$ (0, 250, and 500 μM). Seedling samples were harvested at 72 h to measure shoot fresh weight and root length. (3) Seedlings were exposed to 0 or 0.5 mM SO_2 derivatives for 12 h and then treated with 0, 250, and 500 μM $CdCl_2$ for another 72 h. Leaves, roots, and shoots were harvested to analyse physiological indexes and gene expression.

2.3. Measurement of total chlorophyll, MDA, H_2O_2, and $O_2^{\bullet -}$ contents

Total chlorophyll content in leaves was measured by detecting absorbance at 663 and 645 nm in 80% acetone extracts. The content of MDA was analysed as described previously (Fang et al., 2014). The H_2O_2 and $O_2^{\bullet -}$ contents were assayed in leaf samples according to the methods reported by He et al. (2011).

2.4. Antioxidant enzymes activities assay

Total activities of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) were determined as described previously (Han et al., 2016). Total glutathione reductase (GR) activity was assayed using a GR assay kit A062 (Nanjing Jiancheng Bioengineering Institute, China). One unit of GR was defined as the amount of enzyme that catalysed the oxidation of 1 mol of NADPH per minute.

2.5. Determination of GSH, oxidized glutathione (GSSG), ascorbic acid (ASA), dehydroascorbic acid (DHA), Cys and PCs contents

GSH, GSSG, ASA, and DHA content was assayed as described previously (Fang et al., 2014). Cys content was determined using a Cys assay kit BC0180 (Beijing Solarbio Science & Technology Co., Ltd, China). PCs content was calculated according to the method of Liang et al. (2016) by subtracting the content of GSH from that of non-protein thiol (NPT).

2.6. Examination of Cd^{2+} content

Shoots and roots of seedlings were harvested separately and dried at 65 °C for 48 h. Dry samples (0.1 g) were digested with $HNO_3/HClO_4$ (85/15, v/v), and the Cd^{2+} content was measured using an inductively coupled plasma optical emission spectrometer (OPTIMA 2000; PerkinElmer, USA).

2.7. Quantitative real-time PCR analysis

Total RNA was isolated from foxtail millet roots using an RNeasy plant mini kit (Qiagen, Valencia, USA). Extracted RNA was treated with RNase-free DNase (Promega, Madison, USA). cDNA was synthesized from 1 μg of the total RNA using M-MLV reverse transcriptase (Promega, Madison, USA) according to the manufacturer's instructions. Expression levels of genes were assessed by quantitative real-time PCR using SYBR Green Master Mix in a Real-Time PCR System (Life Technologies Corp., Carlsbad, USA). Primer sequences are listed in Supplementary material Table S1. The housekeeping gene *Actin* was used as an internal control (Fang et al., 2014). The expression data of target genes were normalized to the *Actin* transcript level and analysed