Pak. J. Bot., 52(5): 1589-1594, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-5(15)

CADMIUM DECREASED SUPEROXIDE ANION DERIVED FROM NADPH OXIDASE THROUGH OVERLOAD OF CALCIUM IN WHEAT SEEDLING

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Abstract

Cadmium (Cd) pollution is an important environmental problem in society. Cd is released by industrial processes and phosphate fertilizers, permeates into the food chain. It inhibits plant growth and changes photosynthesis, and its pytotoxicity damages the plant. Over-production of reactive oxygen species (ROS) is believed to be one of the key factors responsible for Cd toxicity. Our objective was to clarify the roles of ROS and cytoplasm free calcium ($[Ca^{2+}]_{cyt}$) in wheat seedlings containing cadmium (Cd). Wheat grains were treated by a series of doses of cadmium chloride for six days. Results showed that wheat grains germination and seedling growth inhibition were increased with increasing Cd concentration. Additionally, low Cd concentration (0.05 mM) decreased NADPH oxidase (NOX) activity, and superoxide anion ($O_2^{\cdot-}$) and malondialdehyde (MDA) contents. Conversely, $[Ca^{2+}]_{cyt}$ was significantly increased at 0.05 mM Cd. To obtain deeper insights into the redox balance of wheat seedling response to Cd stress, $O_2^{\cdot-}$, H_2O_2 and MDA content *In vivo* were measured. The results showed that exogenous $O_2^{\cdot-}$markedly promoted seedling growth, increased $O_2^{\cdot-}$ content and NOX activity, and impaired $[Ca^{2+}]_{cyt}$ overload by Cd stress. Therefore, it was concluded that Cd inhibited early growth of seedlings mainly through inducing $[Ca^{2+}]_{cyt}$ overload which was attributed to the decrease of NOX-dependent $O_2^{\cdot-}$ production.

Key words: Cadmium, ROS, NADPH oxidase, Cytosolic free calcium.

Introduction

Currently, cadmium (Cd) pollution is one of the important environmental health problems in society. Cd is released into the environment as a industrial waste and phosphate fertilizers, and then transmitted to the food chain. Cd is an element that inhibits plant root and shoot growth (Baliardini *et al.*, 2015; Li *et al.*, 2017; Sager *et al.*, 2020), changes photosynthetic rate (Heyno *et al.*, 2008; Per *et al.*, 2016), and even causes plant mortality (Mohammadi-Bardbori & Rannug, 2014). Studies have revealed that Cd is strongly phytotoxic and causes various damages to plants. Over-production of reactive oxygen species (ROS) is believed to be one of the key factors responsible for Cd toxicity (Cuypers *et al.*, 2010).

ROS mainly includes superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). ROS activation by Cd attacks biomacromolecules and causes damage in wheat (Wang *et al.*, 2017), *Arabidopsis* (Baliardini *et al.*, 2015), and cucumber seedlings (Jakubowska *et al.*, 2015). Furthermore, as a non-redox-active metal, Cd activates NADPH oxidase (NOX) in membranes and gives rise to oxidative bursts (Cuypers *et al.*, 2010; Jiménez-Quesada *et al.*, 2016; Jedelská *et al.*, 2019). NOX utilizes cytoplasmic NADPH as the electron source to produce extracellular O_2 into $O_2^{\cdot-}$ (Jiménez-Quesada *et al.*, 2016), which is then transformed to H_2O_2 and other ROS.

Recently, a new role of ROS derived from NADPH oxidase (NOX) has been reported, that ROS regulates root growth (Foreman *et al.*, 2003; Monshausen *et al.*, 2007; Wilkins *et al.*, 2016). Tip-localized ROS produced from NOX is required to maintain normal growth rate of root hairs (Kanaoka & Torii, 2010; Jiménez-Quesada *et al.*, 2016). Oracz *et al.*, (2009) also reported the beneficial effect of HCN on germination of dormant embryos, related to an increase of O_2^- and H_2O_2 generation in the embryonic axes. Moreover, ROS plays a vital role in the regulation of germination of sunflower seed (Oracz *et al.*, 2007, 2009), and exogenous H_2O_2 increases the seed germination of warm-season prairie grasses (Sarath *et al.*, 2007). Overall, these studies indicate that ROS has positive roles in seed germination and plant growth.

Ca^{2+} is an important signaling molecule in cytosol, however, it is also toxic in plants when cytosolic free calcium ($[Ca^{2+}]_{cyt}$) is overloaded (Chen & Li, 2001; Wilkins *et al.*, 2016). Therefore, regulation of $[Ca^{2+}]_{cyt}$ is quite important in plant cells (Laohavisit *et al.*, 2009). Yeh *et al.*, (2007) has observed that Cd^{2+} induces ROS and $[Ca^{2+}]_{cyt}$ overload in rice roots. The increase of $[Ca^{2+}]_{cyt}$ by Cd in plants might also be one of the explanations for cell damage and growth inhibition (Yeh *et al.*, 2007; Rodriguez-Hernandez *et al.*, 2015). But the mechanisms for this are not clear. It is noteworthy that Cd exposure increases transcription of TPC1 which releases Ca^{2+} to the cytosol from vacuoles in roots of the aquatic plant *Typha latifolia* (Rodriguez-Hernandez *et al.*, 2015).

ROS and $[Ca^{2+}]_{cyt}$ are commonly believed to be associated with Cd toxicity due to their important role in seed germination and plant growth (Yeh *et al.*, 2007; Rodriguez-Hernandez *et al.*, 2015). But the exact mechanism under Cd stress is not clear. To identify the mechanism, the $O_2^{\cdot-}$ and H_2O_2 production *In vivo*, NOX activity, and malondialdehyde (MDA) content in wheat seedlings were determined under Cd stress. Additionally, to verify the regulatory relationship between $O_2^{\cdot-}$ and $[Ca^{2+}]_{cyt}$ in Cd toxicity, the effects of exogenous $O_2^{\cdot-}$ on $[Ca^{2+}]_{cyt}$ in seedling roots were also determined.

Plant material and treatment: Wheat (*Triticum aestivum*) grains "Zhengmai 9023" were obtained from Henan Academy of Agriculture, China. The grains were disinfected in 0.5% $NaClO_3$ for 10 min and washed

extensively with deionized water and incubated in 10 cm Petri dish (80 grains per dish) containing 2 layers of filter paper soaked with 20 ml deionized water or treatment solutions. A series of doses of $CdCl_2$ (0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 1mM) were treated wheat, respectively. Based on the experiment, 0.2 mM $CdCl_2$ was applied to the next experiment. 3 groups of treatment included: 0.2 mM $CdCl_2$, 0.2 mM $CdCl_2$ plus $O_2^{\cdot-}$ production system, 0.2 mM $CdCl_2$ plus 0.01% H_2O_2. The $O_2^{\cdot-}$ production system (methionine-riboflavin system) included: 26 mM L-methionine, 4 µM riboflavin and 20 µM EDTA-Na_2. This system produced $O_2^{\cdot-}$ when methionine and riboflavin irradiated (El-Zahaby *et al.*, 2004). Plants were cultivated at 25℃ and the alternative light system (1 h light and 1 h darkness, light intensity 120 $\mu E \cdot m^{-2} \cdot s^{-1}$) which was used for $O_2^{\cdot-}$ production to avoid the possible damage caused by continuous action (12 h light and 12 h darkness) of $O_2^{\cdot-}$. Germination percentage was noted daily for 3 days under a photoperiod (14 h light/10 h dark). The lengths of root, shoot, coleoptile, and other physiological indexes were determined by using 6-day-old seedlings. All solutions were refreshed every 2 days.

Determination of NOX activity: NOX activity was assayed based on the method developed by Sarath *et al.*, (2007). 6-day-old seedlings were pestled in a mortar with 10 mM sodium-phosphate buffer. The extracts were sonicated using a microtip in tubes on ice. The supernatants which were centrifuged were the crude seedling homogenates. The protein contents were tested by coomassie brilliant blue. The seedling homogenates were precipitated with acetone at -20°C for 15 min and then were centrifuged. The protein pellets were resuspended in 50 mM Tris–HCl buffer. The suspension liquid was used to detect NOX activity by photometry.

The activity of NOX was tested by nitroblue tetrazolium (NBT) reduction. The protein solution and 730 µM NBT were mixed as the reaction solution. And 100 µM NADPH was added to start the reaction. Finally, the products of NBT reduction were measured at 530 nm and in order to calculate of the oxidase activities, 12.8 mM cm^{-1} was used for the extinction coefficient.

Determination of $O_2^{\cdot-}$ content: $O_2^{\cdot-}$ content was assayed as described by Jing *et al.*, (2012). The 6-day-old seedlings were ground in mortar at 4°C with 50 mM of sodium phosphate buffer. The homogenate was centrifuged. The supernatant was first bathed for 30 min at room temperature with 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffers. Then, the mixture was bathed with 17 mM sulfanilamide and 7 mM 2-naphtylamine. The above mixture was centrifuged at 13, 000 g for 15 min and the absorbance was detected at 530 nm. Sodium nitrite was used to establish a calibration curve.

Determination of H_2O_2 content: The content of H_2O_2 was assayed as described by Zhang (2009). The 6-day-old seedlings were ground in mortar with 1.5 ml of 5% trichloroacetic acid (Patterson *et al.*, 1984). The homogenate was centrifuged at 12 000 g for 20 min. The H_2O_2 content was used to measure by means of H_2O_2 colorimetric detection kits (Nanjing Jiancheng, China).

Detection and quantification of $O_2^{\cdot-}$ and H_2O_2 in-situ: The $O_2^{\cdot-}$ in situ in roots of wheat seedlings was assayed by NBT staining as described by Dunand *et al.*, (2007). The roots of 6-day-old seedling were incubated in 0.75 mM NBT for 30 min. The pH 7.2 phosphate buffer was used to stop the reaction. The stereomicroscope foots were observed under and photos were taken by Nikon motor. Content of H_2O_2 was measured by diaminobenzidine (DAB) staining of roots following that method of by Tewari *et al.*, (2008).

Assay of MDA content: The content of MDA was measured by thiobarbituric acid (TBA). The 6-day-old seedlings were ground in mortar with 50 mM sodium phosphate buffer. The extracts were centrifuged and the supernatant was incubated with 20% TCA with 0.5% TBA. The mixture solution was warmed at 90℃ for 25 min, and then was centrifuged after cooling. The absorbance of the mixture was recorded at 450nm, 532 nm and 600 nm, respectively. In order to calculate the MDA content, the extinction coefficient was 155 $mM \cdot cm^{-1}$.

Detection of $[Ca^{2+}]_{cyt}$ in wheat seedling roots: Oregon Green 488 BAPTA-1 as the Ca^{2+}-sensitive fluorescent staining (Invitrogen, USA) were used to localize the $[Ca^{2+}]_{cyt}$ in roots of 4-old-day seedlings (Yeh *et al.*, 2007). The intact roots were incubated in solution contained 10 mM Oregon Green 488 BAPTA-1 for 2 h at 4°C in the darkness (Zhang *et al.*, 1998). Then the roots were bathed in 0.2 mM $CaCl_2$ solution for 1 or 2 h at 20°C in the darkness. A Nikon TE2000-U fluorescent inverted microscope equipped with a green fluorescent protein filter (excitation 450–490 nm, emission 500–530 nm) was used for taking fluorescence photos. Exposure timings were same to all samples. At the exposure time, no auto fluorescence was observed in unstained control. The Photoshop (Adobe Systems Inc., San Jose, CA, USA) histogram function was used to evaluate the mean fluorescent intensity of the root (0, white; 255, black) (Dunand *et al.*, 2007).

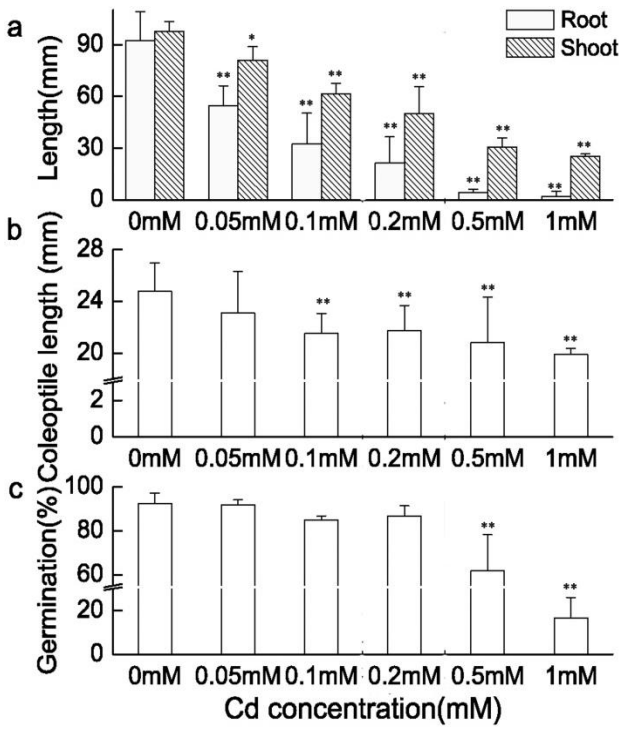
Statistical analysis

Statistical analysis was done by one-way ANOVA using the *t* test. $p<0.05$ (*) and $p<0.01$ (**) represented significant or very significant, respectively. Each value in the paper was presented as "means ± standard errors" (n=3).

Results

Cd inhibition of seed germination and early growth of wheat seedlings: With increased concentrations of Cd^{2+} from 0.05 mM to 1mM, root and shoot length were all very significantly reduced gradually (Fig. 1a). Seedling shoot length was markedly inhibited by low Cd (0.05 mM Cd^{2+}, Fig. 1a). However, low Cd didn't affect coleoptile length, but was reduced by 0.1 mM Cd^{2+} or more (Fig. 1b). Germination of seeds was not noticeably affected by moderate Cd^{2+} until concentrations reached 0.5 mM (Fig. 1c). We suggest that wheat seedling growth is more sensitive to Cd toxicity than germination. A concentration of 0.2 mM Cd^{2+} decreased shoot, root and coleoptile

length by c. 50%, 80% and 14%, respectively (p<0.01, Fig. 1a, b). Conversely, 0.2 mM Cd^{2+} had little effect on the germination of wheat seeds (Fig. 1c). Therefore, to study the mechanism of Cd on wheat seedling growth, this concentration was chosen for further Cd stress experiments.



Cd decreased $O_2^{\cdot-}$ content and NOX activity in wheat seedlings: To obtain deeper insights into the redox balance of wheat seedling response to Cd stress, $O_2^{\cdot-}$, H_2O_2 and MDA content *In vivo* were measured. Results showed that Cd very significantly reduced $O_2^{\cdot-}$ content in whole wheat seedlings (p<0.01, Fig. 2a). Furthermore, $O_2^{\cdot-}$ in root meristem and elongation zones of Cd-treated seedlings was much less than in controls (Fig. 2c). Moreover, H_2O_2 was distributed in the whole root, especially in the meristem zone and elongation zones (Fig. 2d). Cd didn't change whole seedling H_2O_2 content (Fig. 2b), although Cd marginally decreased H_2O_2 content in the meristem zone (Fig. 2d). Additionally, Cd decreased activity of NOX in the apoplast (Fig. 3a), and in the MDA content (Fig. 3b). Therefore, Cd inhibited early growth of wheat seedlings by decreasing NOX activity, $O_2^{\cdot-}$ and MDA content.

Exogenous $O_2^{\cdot-}$ improved early growth and $O_2^{\cdot-}$ content under Cd stress: To investigate if the decrease of $O_2^{\cdot-}$ was the major reason for Cd toxicity, exogenous $O_2^{\cdot-}$ was added to seedlings under Cd stress. The results showed that pulse stimulation of exogenous $O_2^{\cdot-}$ significantly promoted the growth of Cd-treated seedlings whereas continuous stimulation had no recovery effect (data not shown). Compared with Cd-stressed seedlings, shoot, root, and coleoptile length in the pulse stimulation group was increased by 83%, 93% and 10%, respectively (Fig. 4). However, the exogenous $O_2^{\cdot-}$ treated seedlings was relatively spindly compared to the control. These results indicated that exogenous $O_2^{\cdot-}$ effectively activated Cd growth inhibition. Conversely, root and coleoptile lengths were not affected by exogenous H_2O_2 (Fig. 4). However, exogenous H_2O_2 increased seedling shoot length by 110.4% (p<0.01, Fig. 4). Therefore, re-supply of $O_2^{\cdot-}$ but not H_2O_2 reversed seedling growth inhibition under Cd treatment.

The results also showed that exogenous $O_2^{\cdot-}$ increased $O_2^{\cdot-}$ content (Fig. 2a, 2c), NOX activity (Fig. 3a) and MDA content (Fig. 3b), but didn't change H_2O_2 content (Fig. 2b, 2d). Conversely, exogenous H_2O_2 didn't alter the $O_2^{\cdot-}$ content (Fig. 2a, 2c), NOX activity (Fig. 3a) and MDA content (Fig. 3b) although H_2O_2 content was markedly increased by exogenous H_2O_2 (Fig. 2b, 2d). It was concluded that exogenous $O_2^{\cdot-}$, not H_2O_2, retrieved $O_2^{\cdot-}$ production and NOX activity.

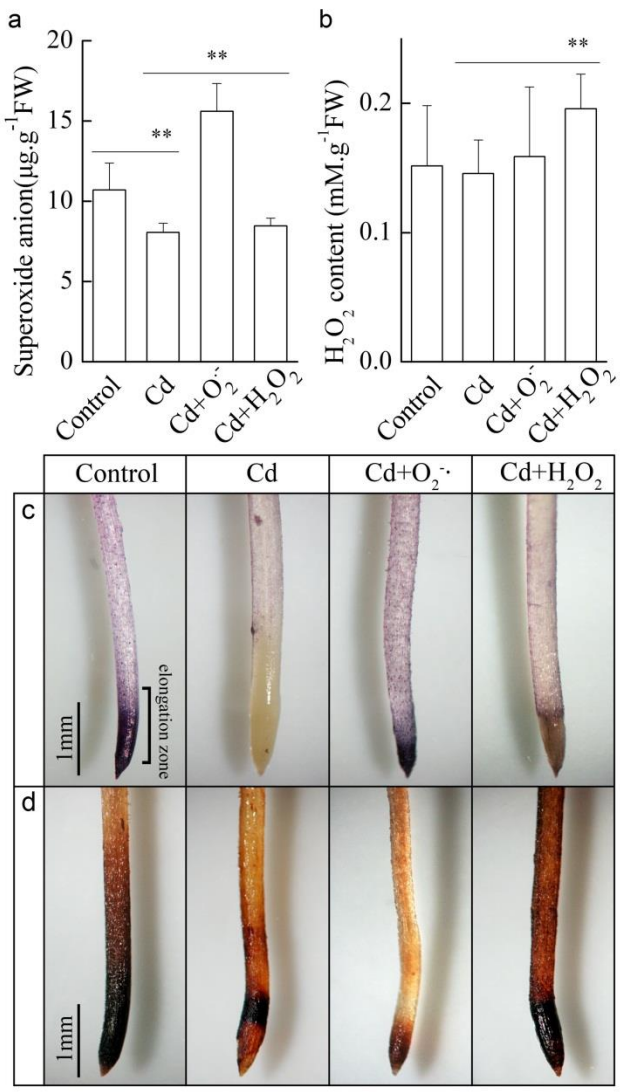


Fig. 2. Exogenous $O_2^{\cdot-}$ and H_2O_2 changed $O_2^{\cdot-}$ and H_2O_2 contents in wheat seedlings under Cd treatment. a-b, $O_2^{\cdot-}$ and H_2O_2 contents were detected by spectrophotometer using the whole seedlings (n=3). c, $O_2^{\cdot-}$ content in roots was stained by NBT. d, H_2O_2 content in roots was stained by DAB. Differences were significant at p<0.05 (*) and very significantly at p<0.01 (**) according to t-test. Control: deionized water; Cd: 0.2mM $CdCl_2$; Cd+$O_2^{\cdot-}$: 0.2mM $CdCl_2$ plus $O_2^{\cdot-}$ production system. Cd+H_2O_2: 0.2mM $CdCl_2$ plus 0.01% H_2O_2. FW: fresh weight.

Exogenous $O_2^{\cdot-}$ decreased $[Ca^{2+}]_{cyt}$ overload by Cd in wheat seedling roots: To clarify if protection of exogenous $O_2^{\cdot-}$ on Cd-treated seedlings was related to $[Ca^{2+}]_{cyt}$ in wheat seedlings, measurement of $[Ca^{2+}]_{cyt}$ with Oregon green 488 BAPTA-1 was carried out. As shown in Fig. 5, green fluorescence was markedly activated in Cd-treated seedling root tips, and decreased by exogenous $O_2^{\cdot-}$. The results indicated that Cd induced $[Ca^{2+}]_{cyt}$ while exogenous $O_2^{\cdot-}$ recovered it.

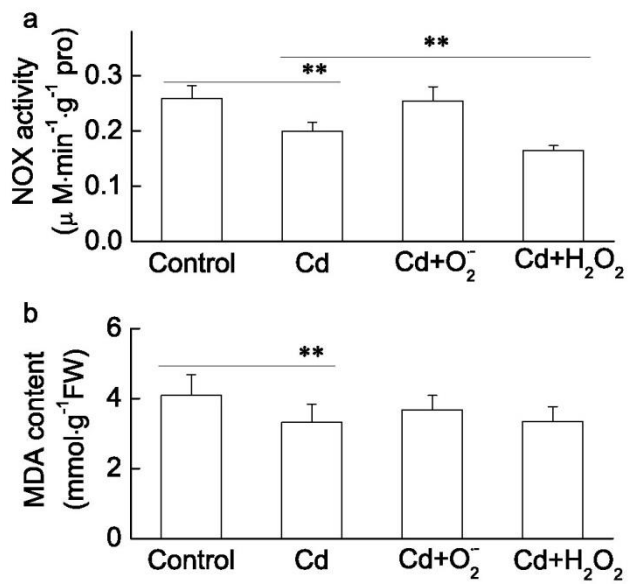


Fig. 3. NOX activity (a) and MDA content (b) in wheat seedlings were changed by exogenous $O_2^{\cdot-}$ (n=3). Differences were significant at $p<0.05$ (*) and very significantly at $p<0.01$ (**) according to *t*-test. The treatment was same to Fig.2. FW: fresh weight.

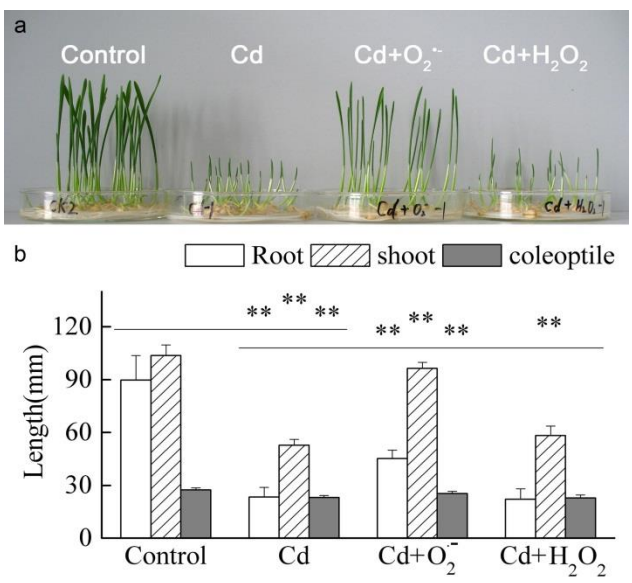


Fig. 4. Exogenous $O_2^{\cdot-}$ but not H_2O_2 recovered the growth of Cd-treated wheat seedlings. a, Photographs of the 6-day-old seedlings were taken. b, The root, shoot and coleoptile lengths of wheat seedlings with different treatments were calculated (n=23). Differences were significant at $p<0.05$ (*) and very significantly at $p<0.01$ (**) according to *t*-test. The treatment was same to Fig.2. FW: fresh weight.

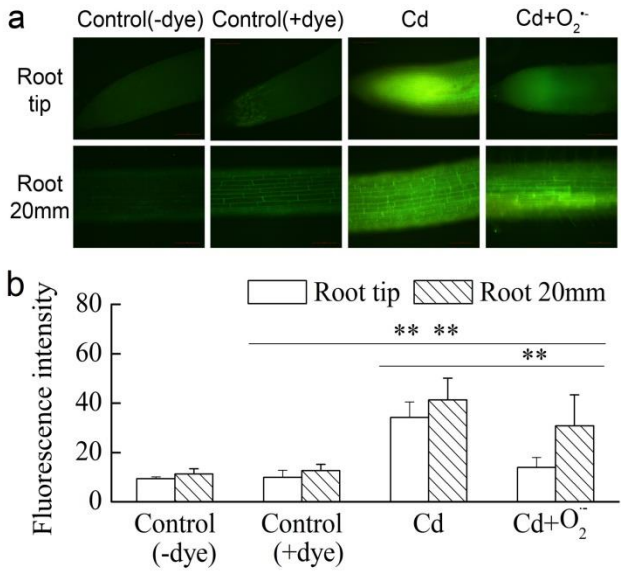


Fig. 5. Exogenous $O_2^{\cdot-}$ lowered $[Ca^{2+}]_{cyt}$ in roots of Cd-treated wheat seedlings. a, $[Ca^{2+}]_{cyt}$ in 20 mm from root tip of wheat seedlings was stained by Oregon Green 488 BAPTA-1. b, fluorescence intensity showed in figure 5a ($n \geqslant 7$) was calculated using photoshop's histogram function (0, white; 255, black). Differences were significant at $p<0.05$ (*) and very significantly at $p<0.01$ (**) according to *t*-test. Cd: 0.2mM $CdCl_2$, Cd+$O_2^{\cdot-}$: 0.2mM $CdCl_2$ plus $O_2^{\cdot-}$ production system.

Discussion

Cd inhibited growth of wheat seedlings by decreasing $O_2^{\cdot-}$: Cd decreases germination and early growth of wheat seedlings in many plants (Pena *et al.*, 2012; Groppa *et al.*, 2012; Jakubowska *et al.*, 2015; Wang *et al.*, 2017; Sager *et al.*, 2020). This is supported by our results, for example, Cd decreases wheat seed germination and shoot and root length (Fig. 1). Although Cd is a non-redox-active metal, many studies suggest that Cd causes oxidative stress in plants by increasing ROS production indirectly (Rodriguez-Serrano *et al.*, 2009a, b; Baliardini *et al.*, 2015; Nemmiche, 2016; Wang *et al.*, 2017). However, some research now demonstrates that Cd decreases ROS, for example, Cd^{2+} depresses production of $O_2^{\cdot-}$ in extracted soybean plasma membranes and intact roots of 3-4 day soybean or cucumber seedlings (Heyno *et al.*, 2008). $O_2^{\cdot-}$ generation diminishes in leaf discs of Cd treated sunflower (Groppa *et al.*, 2012), and Cd^{2+} decreases or doesn't change the $O_2^{\cdot-}$ production in wheat roots (Pena *et al.*, 2012; Jakubowska *et al.*, 2015). Our results supported these patterns, for example, $O_2^{\cdot-}$ content in Cd-treated seedlings was much lower than in controls *In vivo* (Fig. 2a), a finding supported by in situ staining of $O_2^{\cdot-}$ in root tips (Fig. 2c). Additionally, Cd slightly decreased H_2O_2 content in root tips (Fig. 2b) but didn't change H_2O_2 content in whole seedlings (Fig. 2d).

Several reports suggest that the ROS is beneficial for seed germination and plant growth (Foreman *et al.*, 2003; Muller *et al.*, 2009; Oracz *et al.*, 2009; Jiménez-Quesada *et al.*, 2016; Kou *et al.*, 2018), for example, $O_2^{\cdot-}$ regulates growth of root hairs (Jiménez-Quesada *et al.*, 2016). It has been shown that $O_2^{\cdot-}$ plays vital role in early growth of wheat seedlings (Jing *et al.*, 2012), in our study, $O_2^{\cdot-}$

production was strongly reduced in Cd-treated roots (Fig. 2a, 2c). However, exogenous $O_2^{\cdot-}$ increased $O_2^{\cdot-}$ root content and distributed it evenly (Fig. 2c). Furthermore, exogenous $O_2^{\cdot-}$ activated growth inhibition by Cd (Fig. 4). On the contrary, Cd didn't decrease wheat seedling H_2O_2 content. Exogenous H_2O_2 increased H_2O_2 content in Cd-treated seedlings (Fig. 2b, 2d) but scarcely reversed growth (Fig. 4). In short Cd inhibited root and shoot growth by decreasing $O_2^{\cdot-}$, but not H_2O_2 production.

Decrease of $O_2^{\cdot-}$ by Cd originated from NOX: Besides the electron transport chains in mitochondria or chloroplasts, the primary origins of ROS in plants are NOX in the plasma membrane (Sandalio *et al.*, 2008; Zhang *et al.*, 2009; Jiménez-Quesada *et al.*, 2016). It has been suggested that NOX is associated with plant growth and development (Swanson & Gilroy 2010; Jiménez-Quesada *et al.*, 2016). To investigate the origin of $O_2^{\cdot-}$, we determined NOX activity, and Fig. 3 clearly showed that Cd strongly inhibited NOX activity. It had been suggested that the decrease of $O_2^{\cdot-}$ was mainly due to decreasing NOX activity. In our study, exogenous $O_2^{\cdot-}$ reversed $O_2^{\cdot-}$ production (Fig. 2a, 2c), NOX activity (Fig. 3a) and Cd growth inhibition (Fig. 4). Therefore, Cd inhibited seedling growth by inhibiting NOX-dependent $O_2^{\cdot-}$ production. These results broadly concur those of previous studies. The immediate (≤1h) consequence of exposure to Cd^{2+} *In vivo* is decreased ROS by inhibition of NOX activity (Heyno *et al.*, 2008). Cd diminishes $O_2^{\cdot-}$ generation in treated sunflower leaf discs through inhibition gene expression of NOX (Groppa *et al.*, 2012). Interestingly, exogenous $O_2^{\cdot-}$ activates the NOX (Fig. 3a) maybe through self-amplification of $O_2^{\cdot-}$ (Swanson & Gilroy, 2010).

Exogenous $O_2^{\cdot-}$ retrieved growth inhibition through impairing $[Ca^{2+}]_{cyt}$ overload by Cd: The Ca^{2+} signal is a core regulator of plant cell response to the environment (Dodd *et al.*, 2010; Wilkins *et al.*, 2016), and $[Ca^{2+}]_{cyt}$ activation by Cd has been observed in many plants. The increase of $[Ca^{2+}]_{cyt}$ is demonstrated in rice roots following 15 min of Cd exposure (Yeh *et al.*, 2007). In roots of the hydrophytic plant *Typha latifolia*, Cd induces $[Ca^{2+}]_{cyt}$ by increasing TPC1 transcription which releases Ca^{2+} from the vacuoles to the cytoplasm (Rodriguez-Hernandez *et al.*, 2015). Furthermore, $[Ca^{2+}]_{cyt}$ overload is one of cytotoxic response to Cd and causes growth inhibition (Rodriguez-Hernandez *et al.*, 2015). In our study, $[Ca^{2+}]_{cyt}$ was increased by Cd stress in wheat seedling roots (Fig. 5). However, exogenous $O_2^{\cdot-}$ decreased it. In conclusion, exogenous Cd inhibited seedling growth by causing $[Ca^{2+}]_{cyt}$ overload and exogenous $O_2^{\cdot-}$ protected seedlings from Cd toxicity by impairing it.

Acknowledgments

This work was supported by Natural Science Project of Henan Science and Technology Department (162300410175), National Engineering Laboratory for Wheat & Corn Further Processing, Henan University of Technology (NL2016011), Fundamental Research Funds for the Henan Provincial Colleges and Universities in Henan University of Technology (2016QNJH20) and Sichuan Province Key Technology Support Program (2015SZ0209). The work was also funded by National Key Laboratory of Wheat and Maize Crop Science in Henan.

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(Received for publication 6 December 2018)