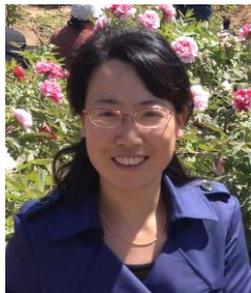


Hydrogen Peroxide Involved in Tip Growth of Wheat (*TriticumAestivum*) Root Hairs by High Concentration Chlorogenic Acid

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ABSTRACT

In order to clarify effects and mechanism of chlorogenic acid (CA) on the early development of six-day wheat seedlings, root growth were detected in the paper. Results have showed that moderate CA promoted the growth of root whereas high concentration CA inhibited the growth of root. In order to elaborate the mechanism of suppressive growth of root by high concentration CA, contents of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) in roots of wheat seedlings were measured. CA both decreased the contents of H_2O_2 and $O_2^{\cdot-}$. In addition, exogenous H_2O_2 could recover the repressive growth of primary root. Furthermore, CA markedly increased the activity of NADPH oxidases and significantly promoted Peroxidase (POD) activity to increase the scavenging ability of H_2O_2 . Therefore, inhibitory growth of primary roots and root hairs by CA potentially attributed to decrease of H_2O_2 which was eliminated by POD at root tips of wheat seedlings.

Keywords: Chlorogenic acid, H_2O_2 , NADPH oxidase, Root hairs, Tip growth.

Abbreviations: CA- chlorogenic acid, H_2O_2 - hydrogen peroxide, $O_2^{\cdot-}$ -superoxide anion, POD- peroxidase, SOD- superoxide dismutase.

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1. INTRODUCTION

Reactive oxygen species (ROSs) are by-products of many metabolic processes in higher plants, even under optimal conditions. Excess ROS can deoxidize nearby biological macromolecules including DNA, proteins and lipids [1-5]. In order to protect them against oxidative damage, higher plant cells have evolved enzymatic and non-enzymatic anti-oxidative systems. In the enzymatic system, superoxide dismutase (SODs) catalyzed the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and O_2 . The product H_2O_2 is catalyzed by a variety of peroxidases (PODs) which decompose H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (reduced ascorbic acid and glutathione) [6]. Accordingly, SOD and POD play vital roles in scavenging ROS.

More and more researches have showed that moderate ROS, such as O_2^- and H_2O_2 , have key roles in physiological and pathological process [7, 8]. Besides the organelles of mitochondria, chloroplast and peroxisome, the plasma membrane NADPH oxidases are the major resources of ROS in plant cells [5, 8, 9]. ROS derived from NADPH oxidases play vital roles in tip-growth of roots [10-12] and pollen tube [12-14]. The first NADPH oxidase gene was cloned in various alleles of *root hair defective 2* mutant (*rhd2*) of *Arabidopsis*, they were found to reside in a gene encoding the respiratory burst oxidase homolog C (AtRBOHC) [10] a homolog of the gp91^{phox} catalytic subunit of the mammalian NADPH oxidase responsible for ROS production during the phagocytic oxidative burst. These NADPH oxidases are integral plasma membrane enzymes that transfer of an electron from an NADPH substrate to FAD, then haem and finally to molecular oxygen across the membrane to generate O_2^- at the apoplast. O_2^- changed to H_2O_2 which can be passed through plasma membrane into the cell as a cellular signal due to the activity of aquaporin [15].

Chlorogenic acid (CA) is the first phenolic product from the phenylpropanoid biosynthetic pathway in high plants when the plants are subjects to biotic and abiotic stresses [16]. In addition, more and more researches have proved that CA participates in the development and growth of root, shoot and root hair in *Hypericum perforatum* and lettuce seedlings [17-19]. Besides that CA also involved in the development of cell wall in *Arabidopsis* and *Coffea* [20, 21]. The reason for that is that CA may work as a scavenger of ROS, in cooperation with ascorbic acid, at the apoplast [17, 22, 23].

Although CA has vital roles in development of root, shoot and root hairs [17-19] the mechanism of CA on root development and growth was still unclear. In this manuscript, we shown that high concentration CA inhibited the number and length of root hairs and decreased the content of H_2O_2 , which possibly derived from NADPH oxidases.

2. MATERIALS AND METHODS

2.1. Plant Material

“Zhengmai9023”, from Henan Academy of Agriculture, China, is the wheat (*Triticum aestivum*) cultivar in the paper. The cultivar is selected for germination and seedlings experiments in the paper owe to the short dormancy and the most extensive planting area among high quality wheat in China.

2.2. CA Treatments

Wheat seeds were surface-sterilized for 15 min with 0.5% NaClO and washed extensively with deionized water. Seeds were put on 2 layer of filter paper in 9 cm Petri dish (35 seeds per dish) with embryo side facing up and then were placed in temperature-controlled incubator with 12h light and 12h darkness at 25°C for germination and early growth. During the whole process, the seeds or seedling were treated by different concentration of CA or in

combination with 0.1 mM H₂O₂ every day. Length of root, shoot, coleoptiles, number of root hairs, fresh weight and physiological indexes of six-day-seedlings were determined.

2.3. Determination of O₂^{-•} Content

Spectrophotometer assay for O₂^{-•} was determined by means of the method developed by [Elstner and Heupel \[24\]](#). In-situ detection and quantification for O₂^{-•} was detected by NBT staining of roots according to the method described by [Dunand, et al. \[25\]](#). Whole germinated seeds were put into the solution with roots totally immersed in it and stained for 30 min. The solution contains 0.75 mM NBT in 50 mM sodium phosphate buffer pH7.8. The reaction was stopped by transferring the seeds to distilled water. The roots were observed with a stereomicroscope and pictures were taken from 20 mm top of the roots. Settings were identical for all the pictures in the same experiment.

2.4. Determination of H₂O₂ Content

Spectrophotometer assay for H₂O₂ were ground wheat seedlings (0.2g FW) in 1.4 ml of cold 5% trichloroacetic acid (TCA) [\[26\]](#). The extracts were centrifuged at 12,000 g for 15 min and the supernatants were used to detect H₂O₂ content using H₂O₂ colorimetric detection kits (Nanjing Jiancheng Company of biological technology, China) according to the instructions of manufacturer [\[27\]](#).

In-situ detection and quantification for H₂O₂ was detected by DAB staining of roots according to the method described by [Tewari, et al. \[28\]](#).

2.5. Determination of NADPH Oxidase Activity

Protein extracts of six-day-seedling (1.0 g FW) were ground in a mortar and pestle with 3 ml cold (4°C) Na-phosphate buffer (pH 6.0, 10 mM), the homogenate were transferred to 10 ml tubes, kept on ice, and sonicated using a microtip for 15 s. The extracts were centrifuged at 13,400 rpm for 15 min, and the resulting supernatants were the crude germinated seed homogenates. Protein content was detected by coomassie brilliant blue [\[29\]](#). Crude germinated seed homogenates (0.2 ml) were precipitated with acetone (9:1 acetone: homogenate) at -20 °C for 15 min. Precipitated proteins were recovered by centrifugation at 14,000 g for 10 min at 4°C. Protein pellets were resuspended in buffer (50 mM Tris-HCl, 0.1 mM MgCl₂, 0.25 M Suc, 0.1% Triton-X-100, pH 8.0) and were used for photometry assay of NADPH oxidase activity.

Photometry assay for NOX activity was determined by nitrobluetetrazolium (NBT). The reaction contained 0.5 ml protein solution and 0.5 ml 730 μM NBT, after the addition of 1ml 100 μM NADPH to initiate reaction, NBT reduction was determined at 530 nm and an extinction coefficient of 12.8 mM⁻¹ cm⁻¹ was used for calculation of the oxidase activities. In-gel assay for NOX activity was performed according to the procedures described in reference [\[30\]](#).

2.6. Determination of SOD and POD Activities

The total activity of SOD was assayed according to [Meloni, et al. \[6\]](#) with some modifications. One gram of wheat seedling was ground with liquid nitrogen. For SOD, the enzyme was extracted with 5mL 50mM of phosphate buffer (pH 7.8) containing 1mM EDTA and 50 mg polyvinylpyrrolidone (PVP-10). Extracts were filtered through two layers of cheesecloth and centrifuged at 12,000×g for 15 min. SOD activity was determined spectrophotometrically at 560 nm with reduction of nitrobluetetrazolium (NBT). One unit of SOD activity was defined as the amount of sample to reach an inhibition of 50% of the NBT reduction rate.

POD activity was measured by use of guaiacol [27]. 0.5 gram of wheat seedling was ground with liquid nitrogen. For POD, the enzyme was extracted with 5mL of 0.1M Tris-HCL buffer (pH8.5) and centrifuged at 12,000×g for 15 min. POD activity was measured spectrophotometrically at 470 nm and recorded the value of change in 5 min after admixing of 0.8 mL extracts and the reaction solution. Reaction solution contained 50 mL 0.2 M phosphate buffer (pH6.0), 28 µL H₂O₂ and 19 µL guaiacol. One unit of POD activity was defined as the amount of sample to change 0.01 per min.

2.7. Statistical Analysis

All assays were routinely done in triplicate and each experiment repeated at least thrice. Each value was presented as means ± standard errors of the mean of triplicate (SE), with a minimum of three replicates. Statistical analysis was carried out by one-way ANOVA using the *t* test to evaluate whether the means were significantly different, taking $P < 0.05$ (*) and $P < 0.01$ (**) as significant and very significant.

3. RESULTS

3.1. High Concentration of CA Inhibited the Root and Shoot Growth of Wheat Seedlings

Table-1. Effects of different concentration CA on growth of wheat seedling.

CA (µM)	Root length (mm)	Shoot length (mm)	Coleoptile length (mm)	Fresh weight (mg)
0	84.95±4.71 ^c	108.71±1.86 ^b	22.88±1.38 ^a	0.16±0.01 ^a
1	88.05±3.28 ^b	108.05±1.08 ^b	22.9±1.19 ^a	0.17±0.01 ^a
10	93.75±4.23 ^a	112.5±2.92 ^a	23.05±0.94 ^a	0.18±0.02 ^a
50	84.38±4.56 ^c	103.0±4.72 ^c	22.24±0.75 ^a	0.17±0.02 ^a
100	87.10±5.31 ^b	109.9±1.02 ^{ab}	22.52±1.25 ^a	0.18±0.02 ^a
1000	58.19±1.80 ^d	67.19±4.63 ^d	20.57±0.93 ^b	0.13±0.02 ^b

Different small letters were significantly different at the 0.05 level.

From the Table 1, we have concluded that low concentration of CA significantly promoted the growth of wheat seedling whereas high concentration of CA significantly inhibited the growth of wheat seedling, such as shoot and root growth. For example, root length of seedling treated by 10 µM CA is longer 10.45 % than those of control. This finding is in agreement with previous paper reporting that 10⁻⁵ M CA promoted the growth of secondary root in lettuce seedlings [17]. In contrast, root and shoot length of seedling treated by 1 mM CA dropped to 33.2% and 56.2%, respectively.

3.2. High Concentration of CA Decreased of O₂⁻ and H₂O₂ Content In Root Tips

In order to detect the reason of inhibitory growth of root by high concentration CA, we detected H₂O₂ and O₂⁻ contents in roots of the sixth-day seedlings by means of hybridization in situ. From figure 1, 1mM CA prominently reduced contents of H₂O₂ and O₂⁻ in root tips, especially in meristem and elongation zone.

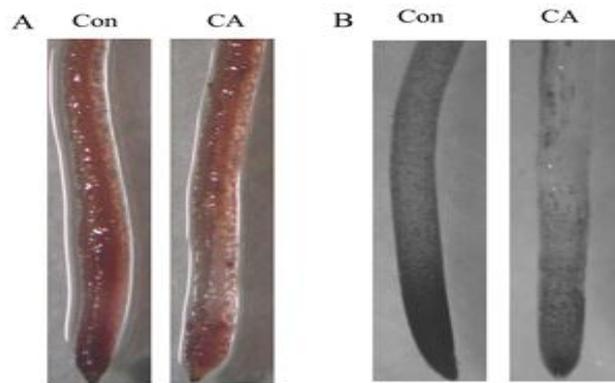


Fig-1. Effects of CA on H₂O₂ and O₂⁻ contents in root tips of wheat seedlings. Compared with control, wheat seedlings were treated by 1 mM CA. H₂O₂ and O₂⁻ contents were showed by DAB (A) and NBT (B) dye in situ in root tip of wheat seedling, respectively. H₂O₂ and O₂⁻ contents in root meristematic and elongation zone were significantly decreased by 1 mM CA.

3.3. Exogenous H₂O₂ Resumed The Suppressive Growth Of Primary Roots And Root Hairs By CA

Table-2. Exogenous H₂O₂ relieved the inhibitory growth of wheat seedlings by CA.

Treatments	Root length (mm)	Shoot length (mm)	Coleoptile length (mm)	Fresh weight (g)
Con	65.2±2.5 ^a	81.0±4.9 ^a	22.8±0.6 ^a	0.14±0.02 ^a
CA	54.5±2.3 ^b	67.9±2.0 ^b	22.7±0.6 ^a	0.13±0.01 ^b
CA+H ₂ O ₂	65.1±2.1 ^a	84.5±3.2 ^a	22.7±0.5 ^a	0.15±0.01 ^a

Different small letters were significantly different at the 0.05 level.

Based on the results of mentioned above, we applied exogenous H₂O₂ and O₂⁻ separately to alleviate the inhibitory growth of root and shoot. Exogenous H₂O₂ notably relieved the root and shoot growth of seedlings treated by 1 mM CA (Table 2). For example, the root length and shoot length of seedlings treated by CA could be recovered to 19.45% and 24.45% respectively by exogenous H₂O₂ (Table 2). On the contrary, Exogenous O₂⁻ slightly aggravated the inhibitory growth of root and shoot (data not shown). Therefore, exogenous H₂O₂, not O₂⁻, could promote the suppressive growth of shoot and primary root of seedlings treated by 1 mM CA.

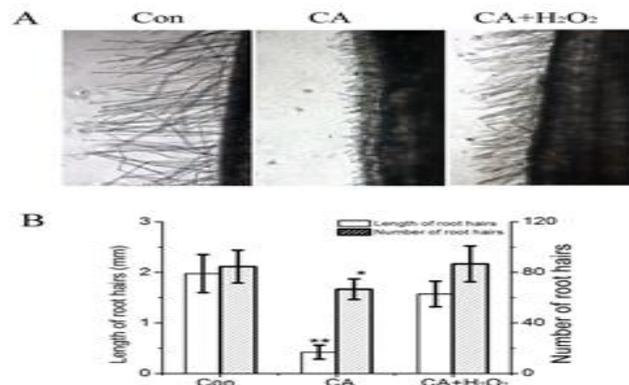


Fig-2. Effects of CA only and combined with exogenous hydrogen peroxide on growth of root hairs. Image of root hairs (A) treated by CA only and combined with exogenous hydrogen peroxide were recorded by Nikon DSLR camera under optical microscope. Length and number of root hairs (B) were measured by means of Photoshop. CA markedly decreased length and number of root hairs and exogenous hydrogen peroxide could recover the inhibitory growth of root hairs by CA.

Moreover, the growth of root hairs of wheat seedlings at six days was demonstrated by image and data statistics. Results have shown that root hairs of control were denser and longer than those of treated by 1mM CA (Table 1 should be Figure 1A). Length of root hairs was shortened to 78.67% and number of root hairs was reduced to 78.85 % by 1mM CA treatment (Fig.2B). Fortunately, length and number of root hairs were recovered to 79.58% and 104.87% respectively by exogenous hydrogen peroxide (Fig.2). Therefore, CA markedly inhibited the growth of primary root and root hairs whereas exogenous hydrogen peroxide had ability of counteracting the inhibitory effects.

3.4. Exogenous H₂O₂ also Increased H₂O₂ Production in Root

In order to make further elaborate mechanism of exogenous H₂O₂ promote the growth of the root, the O₂⁻ and H₂O₂ production in roots of the sixth-day seedlings were tested by means of spectrophotometry and hybridization in situ. Exogenous H₂O₂ had no effect on the decrease of O₂⁻ content in root cells by 1mM CA (Fig.3).

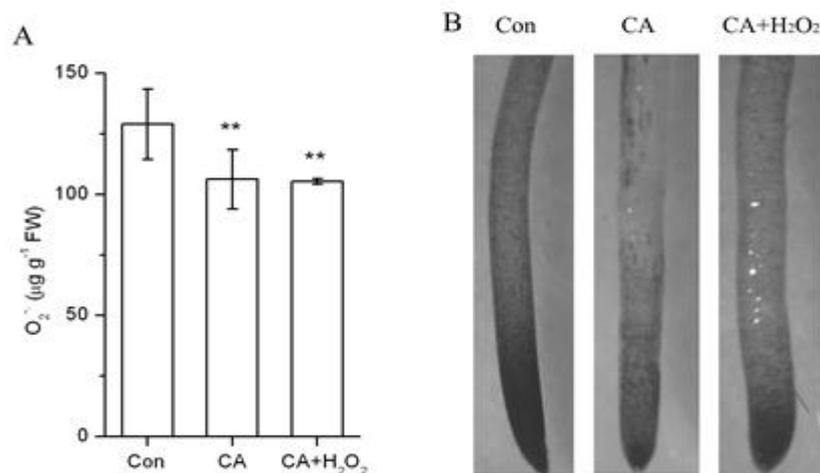


Fig-3. Effects of CA only and combined with exogenous hydrogen peroxide on hydrogen peroxide content in root of wheat seedlings. Hydrogen peroxide content in root of wheat seedlings were detected by spectrophotometer (A) and dye in situ (B). Decrease of superoxide anion content by exogenous CA (1 mM) could not be released by exogenous hydrogen peroxide.

That is to say exogenous H₂O₂ could not improve the content of O₂⁻ content in root cells which was decreased by 1mM CA. The reason is obvious that O₂⁻ could be changed to H₂O₂ freely or dismutation by superoxide dismutase (SODs), but not vice versa. Exogenous H₂O₂ significantly added the decrease of H₂O₂ product by 1mM CA in root tip cells, especially in root tip of meristematic area and elongation area by hybridization in situ (Fig.4B). On the contrary, the content of H₂O₂ product measured by spectrophotometry had showed that 1 mM CA did not change H₂O₂ content and exogenous H₂O₂ increased markedly H₂O₂ content (Fig. 4A). The difference of H₂O₂ content between two methods probably attributed to the materials used from different root segment. The whole roots was used to detect the H₂O₂ content by spectrophotometry whereas H₂O₂ content just in root tips was showed by hybridization in situ. Therefore CA prominently decreased the H₂O₂ and O₂⁻ production in meristematic and elongation zone of root tips and just exogenous H₂O₂ alleviated decrease of H₂O₂ production.

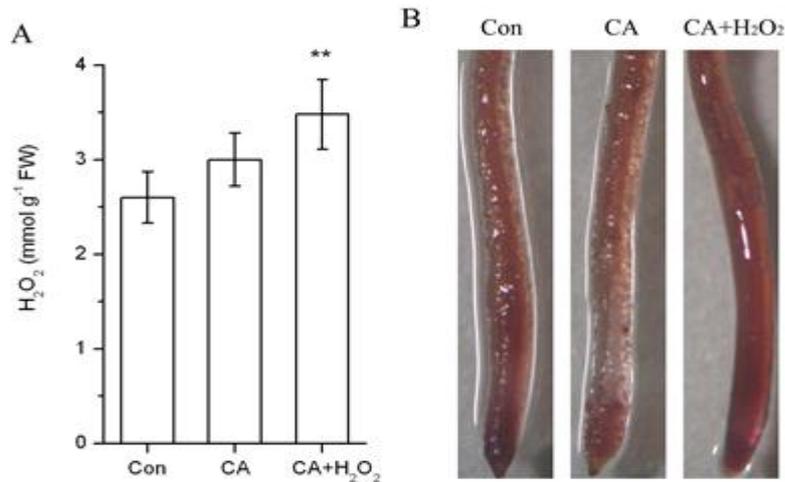


Fig-4. Effects of CA only and combined with exogenous hydrogen peroxide on superoxide anion content in root of wheat seedlings. Hydrogen peroxide content in root of wheat seedlings were detected by spectrophotometer (A) and dye in situ (B). CA only has little change hydrogen peroxide content and CA combined with H₂O₂ increased hydrogen peroxide content in whole root of wheat seedlings (A). Whereas decrease of hydrogen peroxide content by exogenous CA (1 mM) could be released partially by exogenous hydrogen peroxide in root meristematic and elongation zone (B).

3.5. High Concentration of CA Increased POD Activity in Roots

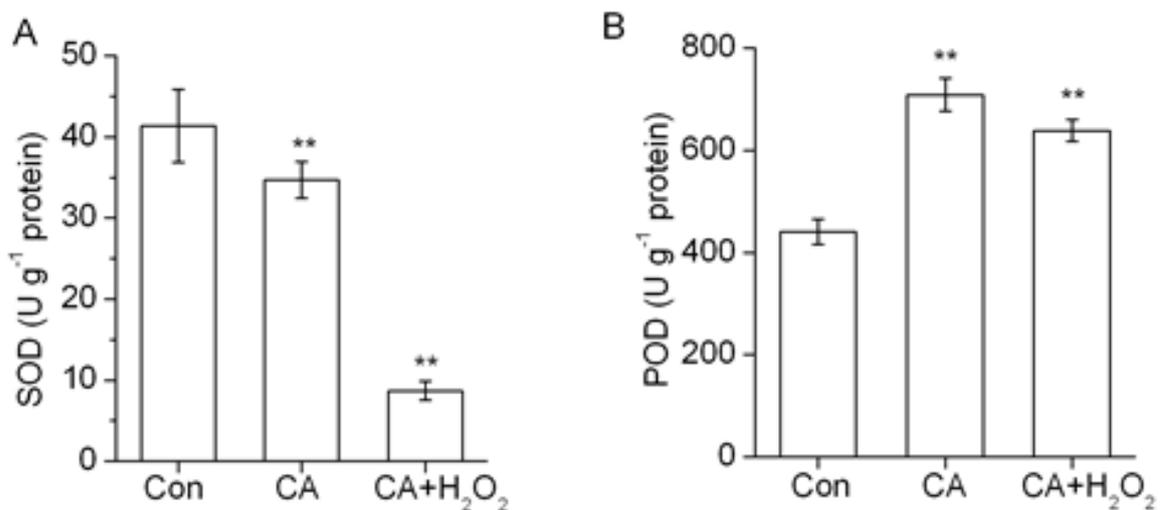


Fig-5. Effects of CA and hydrogen peroxide on the activities of SOD and POD. CA decreases the SOD activity (A) whereas POD activity was activated by exogenous CA. Exogenous H₂O₂ released further strengthen CA on activities POD and SOD.

In order to explore the reason of decrease of H₂O₂ in roots by CA, the activities of SOD and POD were detected in wheat seedlings at six days. CA alone lowered SOD activity to 83.85%. CA and H₂O₂ made the SOD activity further decrease to 20.02%. On the control, POD activity by CA alone or both and exogenous H₂O₂ is higher 60.66% and 44.78% than one of control (Fig.5). So, CA increased the scavenging ability of ROS by increasing activity of POD.

3.6. High Concentration of CA Increased NOX Activity in Roots

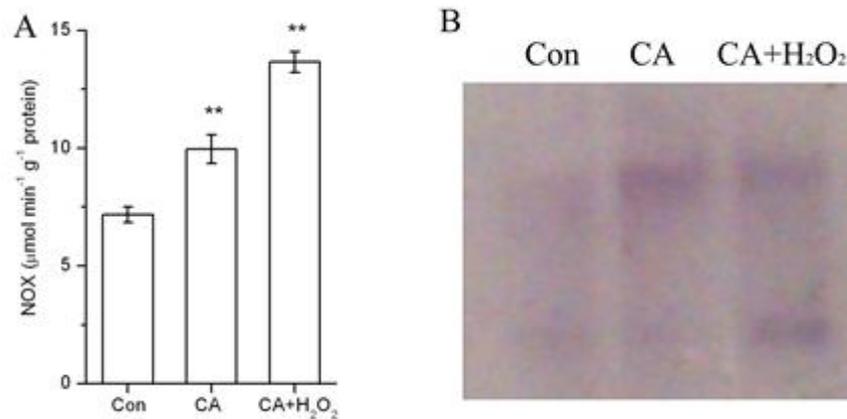


Fig-6. Effects of CA only and combined with hydrogen peroxide on the of NOX activity. NOX activity in root of wheat seedlings was detected by spectrophotometer (A) and polyacrylamide gel electrophoresis (B). NOX activity could be increased by CA only and be further promoted by exogenous hydrogen peroxide.

More and more researches have shown that ROS derived from NOX had vital roles in root tip growth. In order to reveal the change of ROS in seedlings roots was from NOX. NOX activity of seedling root was detected by spectrophotometry and polyacrylamide gel electrophoresis. NOX activity in seedlings roots was increased to 138.31 % by 1 mM CA and exogenous H₂O₂ further increased the NOX activity to 179.80 % (Fig. 6A). The same result was concluded from the figure 6B. Thus, CA enhanced production of H₂O₂ by improving NOX activity.

4. DISCUSSION

4.1. CA Had Dual Functions on Early Growth of Wheat Seedlings.

CA, as a common phenolic compound, could induce the development of root, shoot and root hairs from the callus when combined with NAA in *Hypericum perforatum* Franklin and Dias [19]. Narukawa, et al. [17] research has shown that 10⁻⁵ M CA was the optimal concentration for root hair formation in lettuce seedlings at pH4. Likewise, we also improved that 10 μM CA had ability of promoting the growth of primary roots (Table 1), in agreement with the conclusion above mentioned [17]. However, high concentration of CA (1mM) significantly suppressed the growth of primary roots and shoots (Table 1). Most important of all, we also found that CA not only decreased the number but also lowered the length of root hairs (Fig. 2). It is well known that root and root hairs is closely related to absorption of water and mineral nutrition and eventually decided to production of crop. So, the growth of root and root hairs is particular important and next we emphasized on elucidating the inhibitory growth of root by CA.

4.2. H₂O₂ Has Vital Roles in Tip Growth of Root Hairs

ROS is the byproducts of metabolic activity under normal conditions, biotic stress and abiotic stress conditions in aerobic plant. To date, more and more researches have showed that moderate ROS is an important modulator of plant development, such as tip growth of root hair and pollen tubes [14, 31, 32]. Growth of root mainly depended on the cell division in meristematic zone and cell expansion in elongation zone of root tips. Liszkay, et al. [33] have reported that H₂O₂ and O₂⁻ contents were demonstrated in the meristematic zone of the root hair. In the paper, we also found that H₂O₂ and O₂⁻ distributed along the root tip especially in the meristematic and elongation zone (Fig.1, 3 and 4). H₂O₂ participated in secondary root growth of *Pisumsativum* seedlings under dessication owe to redox receptor reversibly [34]. Moreover, H₂O₂ inhibited the growth of root tip due to controlling the expression

of cyclin, such as PCN1, CyclinB1, E2FA, in meristematic zone [35]. Therefore, hydrogen peroxide has vital roles in growth of primary root.

It is well known that root hair initiation and tip growth is two different processes during development of root growth. More and more researches have shown that O_2^- determined the initiation of root hairs [12, 32]. However, tip growth of root hairs attributed to H_2O_2 [32]. Moreover, exogenous CA inhibited primary root and root hair growth (Table 1 and Fig 2) by decrease of hydrogen peroxide and superoxide anion in meristematic and elongation zone of primary roots (Fig. 1,3,4) and exogenous H_2O_2 markedly increased the length of primary root (Table 2) and root hairs (Fig. 2). Such results also confirmed that hydrogen peroxide has more important roles in root tip growth than in root initiation.

4.3. Exogenous CA Decreased Hydrogen Peroxide Maybe Derived from NOX

Early reports have shown that ROS derived from NADPH oxidase had vital roles in plant development [12, 14, 36]. ROS derived from RHD2/AtrbohC focused on the tip of root hairs and attributed to the formation of tip-high calcium gradient [12]. Expression of AtrBOHC often accumulates in the membrane at the site of root hair initiation [37]. However, once the root hairs transition to tip growth, the protein is most highly accumulated toward the apex of the elongating hair [12]. Therefore, ROS derived from AtrBOHC has vital roles in root initiation and tip growth. Besides in Arabidopsis, ROS derived from NOX control cell expansion in maize (*Zea mays*) roots [33]. Results have showed that 1mM CA increased the activity of NOX in roots of wheat seedlings (Fig. 6). Therefore, H_2O_2 derived from NOX would be raised whereas the H_2O_2 in roots of wheat seedlings was reduced by 1 mM CA.

It was well known that except the generation of H_2O_2 , the scavenging of ROS, such as POD and SOD, also has vital roles in controlling the concentration of H_2O_2 . SOD activity altered O_2^- to H_2O_2 and POD activity catalyzed H_2O_2 to H_2O by oxidation other reductants, such as glutathione and ascorbic acid. Accordingly, CA decreased the product of H_2O_2 by restraining activity of SOD and increased the reduction of H_2O_2 by increase of POD activity (Fig. 5). With respect to O_2^- content, maybe other scavenger enzymes and reductants participated in decrease of O_2^- in root tips.

In a word, exogenous CA has dual effects on the growth of shoot and root and high concentration of CA restrained the growth of primary root and root hairs. Then, we focused on elaborating mechanism of the inhibitory effect of primary root and root hairs by CA and found that CA depressed products of O_2^- and H_2O_2 in meristem and elongation zone of root tips. Otherwise, exogenous H_2O_2 , but not O_2^- , could restore the inhibitory growth of primary roots and root hairs. Although CA increased the activity of NOX but POD activity was dramatically promoted by CA. Therefore, H_2O_2 content was decreased in root tip of wheat seedlings.

5. CONCLUSION

In the paper, redox state was used to assess response to different concentration CA in wheat. Roots were analyzed for physiological changes occurring in response to CA. CA had dual effects on wheat seedlings growth depending on concentration of CA. H_2O_2 and O_2^- in roots were measured by histochemical stain and spectrophotometer. CA changed contents of H_2O_2 and O_2^- in roots and exogenous H_2O_2 could alleviate the inhibition root growth by high concentration CA. Further research have showed that H_2O_2 derived from NOX would be raised whereas the H_2O_2 in roots of wheat seedlings. These integrated data provide a perspective on cellular events regulated by CA in wheat seedlings root. These findings provide new insight into CA response in wheat roots that will be valuable in early development of wheat root growth.

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