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Exogenous phytic acid inhibits early growth of wheat seedlings (*Triticum aestivum* L.) by decreasing superoxide anion derived from NADPH oxidase

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Abstract

Reactive oxygen species (ROS), especially H_2O_2 and $O_2^{-\cdot}$, are found to be a signal of regulating plant development in recent years. PA is a famous anti-nutrient factor and antioxidant. The aim of this study is to investigate the effect of exogenous phytic acid (PA, $C_6H_{18}O_{24}P_6$) on seed germination and early seedling growth in wheat, and to determine whether ROS are involved in it. Seeds of wheat were grown in PA and the production of H_2O_2 and $O_2^{-\cdot}$ were detected in leaves and roots of six-day-seedling. Results show that exogenous PA depresses the early wheat seedling growth (leaves expansion and root tip-growth) in a dose dependent manner and reduces $O_2^{-\cdot}$ production in leaves and roots whereas does not affect germination and H_2O_2 production. To verify whether the growth inhibition by PA results from the decrease in the production of $O_2^{-\cdot}$, exogenous $O_2^{-\cdot}$ combined with PA was used to treat wheat seeds, and the production of H_2O_2 and $O_2^{-\cdot}$, the activities of NADPH oxidase, POD and SOD were investigated. Results show that exogenous $O_2^{-\cdot}$ reverses markedly the inhibition of early seedling growth by PA and the production of $O_2^{-\cdot}$ rather than H_2O_2 was increased by exogenous $O_2^{-\cdot}$. PA alone decreases the activities of POD and NADPH oxidase in leaves and $O_2^{-\cdot}$ can recover them partially. Therefore, exogenous PA restrains early development of wheat seedlings by reduction of $O_2^{-\cdot}$ derived from NADPH oxidase and it also indicates that $O_2^{-\cdot}$ plays a vital role in leave expansion and root tip-growth of wheat seedlings.

Keywords: phytic acid; reactive oxygen species; $O_2^{-\cdot}$; NADPH oxidase; hydrogen peroxide.
Abbreviations: ROS_Reactive oxygen species; PA_Phytic acid; POD_Peroxidase; SOD_Superoxide dismutase; MDA_ Malonaldehyde; $O_2^{-\cdot}$_Superoxide anion; TBA_Thiobarbituric acid; NBT_Nitroblue tetrazolium; TCA_Trichloracetic acid.

Introduction

Reactive oxygen species (ROS) are by-products of many metabolic processes in higher plants, even under optimal conditions. Besides the organelles such as chloroplasts, mitochondria and peroxisomes (Edreva, 2005), ROS in plant cells also come from the plasma membrane NADPH oxidases, cell wall peroxidases, apoplastic oxalate oxidases and amine oxidases (Apel et al., 2004). ROS derived from NADPH oxidases play vital roles in tip-growth of roots (Foreman et al., 2003; Carol and Dolan, 2006) and pollen tube (Potocky et al., 2007). To date, many researches have shown that ROS, especially hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{-\cdot}$), act as signals in regulating plant germination. For example, transient burst of H_2O_2 and $O_2^{-\cdot}$ is found during the germination in *Pisum sativum* (Kranner et al., 2010) and sunflower (Oracz et al., 2009, 2007). Otherwise, Sarath et al. (2007) reported that exogenous H_2O_2 can increase the seed germination in warm-season prairie grasses, indicating the universal role of H_2O_2 in seed germination.

On the other hand, lots of ROS, such as H_2O_2, $O_2^{-\cdot}$ and hydroxyl radical ($OH^{\cdot}$), have ability of oxidizing nearby biomacromoles including DNA, proteins and lipids (Barja, 2004; Yin and Chen, 2005), and the oxidized biomacromoles can be fatal for the cell (Mittler, 2002). For example, short-lived $O_2^{-\cdot}$ (half-life of approximately 2-4 μs) not only decreases the activities of metal-containing enzymes through depressing the transition of Fe^{3+} and Cu^{2+} (Vranová et al., 2002), but also evokes membrane lipid peroxidation to form toxic product—malondialdehyde (MDA) (Breusegem et al., 2001). Accordingly, MDA is usually used as an index of oxidative stress (Hodges et al., 1999).

In order to protect them against oxidative damage, higher plant cells have evolved enzymic and non-enzymic antioxidative systems. In the enzymic system, superoxide dismutases (SODs) catalyse the dismutation of $O_2^{-\cdot}$ to H_2O_2 and O_2. Three kinds of isoforms exiting in plant cells have different subcellular locations, which contain different metals. Cu–Zn-SOD and Fe-SOD isoforms are mainly located at chloroplasts (Droillard and Paulin, 1990) whereas Mn-SOD exists primarily in mitochondria and peroxisomes (Rabinowitch and Fridovich, 1983; Sandilo et al., 1987). H_2O_2, generated from the dismutation of $O_2^{-\cdot}$, 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) (Meloni et al., 2003). Accordingly, SOD and POD play vital roles in scavenging ROS.
Myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, commonly called phytic acid (PA), is the primary storage form of phosphorus in cereal, legumes and oil seeds and accounts for one to several percent of the dry weight.
It is typically found in the outer layers (aleuron) of cereal grains and the endosperm of legumes and oil seeds. It is well known that PA is regarded as the anti-nutrient factor for monogastric animals (Lo¨nnerdal., 2003). Because it chelates effectively the positively charged cations and forms phytate

(also called phytin) in protein bodies or protein storage vacuoles (Raboy, 2002). In order to increase the bioavailability of macro and micro-nutrients, some *lpa* (low phytic acid) mutants have been constructed in recent years but seed vigor of the mutants is different from that of the wild type (Aluru et al., 2011; Campion et al., 2009; Kim et al., 2011). The germination rate is higher for maize mutant *lpa1-1* and bean mutant *lpa-280-10* than for their wild types under varying ageing or stress conditions (Aluru et al., 2011; Campion et al., 2009) whereas the seeds of *Arabidopsis* mutant *lpa* and maize mutant *lpa1-241* exhibit reduced germination under special conditions (Kim et al., 2011; Doria et al., 2009). In addition, rice mutant *lpa* has no negative effects on seed weight, germination or plant growth (Kuwano et al., 2009). These results indicate that roles of endogenous PA in seed vigor are different in crops, but no report on wheat is found, although it is one of the most important crops in the world.

On the other hand, PA, as a chelator and antioxidative agent, can inhibit Fe-driven formation of ROS by chelating Fe^{2+} (Graf et al., 1987). PA does not scavenge H_2O_2 but can protect DNA from oxidative damage caused by H_2O_2 or H_2O_2 and Cu(II) (Midorikawa et al., 2001). In addition, PA decreases the germination capacity of maize mutant *lpa1-241* by preventing oxidative stress in seeds (Doria et al., 2009). Together, ROS stimulate the germination of plant seeds while PA reduces oxidative stress in seeds. However, whether this characteristic is related to its antioxidative capacity is not clear. In the present study, we aim to investigate the effect of exogenous PA on germination, early growth of wheat seedlings and the roles of ROS in this process.

Results

Exogenous PA inhibits the early growth of wheat seedlings

In order to detect the influences of exogenous PA on seed germination and early seedling growth of wheat, various concentration of PA were used to treat wheat seeds. A dose-dependent inhibition of seedlings growth by PA is observed (Fig 1). Shoot and root length of wheat seedlings treated by exogenous 1 mM PA are 32.7 % and 25.2 % of the control, respectively (Table 1). On the contrary, coleoptile length is not affected by exogenous PA (Table 1). Besides that PA has no significant effect on fresh weight, germination rate, germination energy and germination index (Table 1). Therefore, exogenous PA has little effect on seed germination but restrains markedly early seedlings growth in wheat.

Exogenous PA suppresses the production of $O_2^{\cdot-}$ but not H_2O_2

We next explored the mechanism of the suppressing early growth of wheat seedlings by exogenous PA. From figure 2A, $O_2^{\cdot-}$ content in leaves is decreased noticeably to 47.1% of control by 1 mM exogenous PA in wheat seedlings. Moreover 1 mM exogenous PA also decreases significantly the $O_2^{\cdot-}$ content in wheat roots (Fig 2C). On the contrary, the exogenous PA has no influence on H_2O_2 production in wheat leaves and roots (Fig 2B, D). Accordingly, exogenous PA might inhibit early growth of wheat seedlings by decreasing the apoplast $O_2^{\cdot-}$.

$O_2^{\cdot-}$ recovers the inhibition of wheat early seedling growth by exogenous PA

To further explore whether the decrease of $O_2^{\cdot-}$ production is the key cues in suppressing the early development of wheat seedlings by exogenous PA, exogenous $O_2^{\cdot-}$ also was added to treat wheat seedlings. As shown in the table 2 and Fig 3, exogenous $O_2^{\cdot-}$ alone inhibits significantly early growth of shoots and roots whereas improves notably the suppression effects on early growth of wheat seedlings by exogenous PA. For instance, shoot length of the wheat seedlings treated by 1 mM PA alone or combined with exogenous $O_2^{\cdot-}$ are 41.6 % and 73.2 % of control, respectively. Similarly, root length treated by 1 mM PA alone or combined with exogenous $O_2^{\cdot-}$ are 20.1% and 33.2 %. Therefore, inhibition of shoot and root growth in wheat seedlings occurred at about 1 mM PA and the inhibition can be relieved by $O_2^{\cdot-}$.

Next, effects of exogenous $O_2^{\cdot-}$ on the production of $O_2^{\cdot-}$ and H_2O_2 in roots and leaves were detected. Decrease of $O_2^{\cdot-}$ production in roots and leaves caused by exogenous PA are both raised markedly by exogenous $O_2^{\cdot-}$ (Fig 4A, C). On the contrary, exogenous $O_2^{\cdot-}$ has little effect on H_2O_2 in roots and leaves of wheat seedlings (Fig 4B, D). From these results, we speculate that $O_2^{\cdot-}$ but not H_2O_2 plays a key role in the early development of shoots and roots elongation of wheat seedlings and PA inhibits the growth of wheat seedlings by decreasing $O_2^{\cdot-}$ content.

Exogenous PA decreases content of $O_2^{\cdot-}$ by reducing activity of NADPH oxidase

To assess the causes of decrease of $O_2^{\cdot-}$ production by exogenous PA, we detected the activities of NADPH oxidase (generating ROS) and total SOD and POD (scavenging ROS). Our results show that exogenous PA not only decreases the total activities POD (Fig 5B) but also decreases the activity of NADPH oxidase (Fig 5C) but has no influence on activity of SOD (Fig 5A) and exogenous $O_2^{\cdot-}$ can recover in part the effects of PA (Fig 5B, C). Results above have shown that $O_2^{\cdot-}$ is decreased significantly by PA (Fig 2). Our data suggest that the decrease of NADPH oxidase activity, rather than POD activity, leads to the depression of $O_2^{\cdot-}$. In addition, the exogenous $O_2^{\cdot-}$ alone had little effect on NADPH oxidase but improved remarkably activities of SOD and POD in order to scavenge the redundant $O_2^{\cdot-}$ (Fig 5A-C). Otherwise, exogenous PA decreases markedly the content of MDA due to the decreasing of $O_2^{\cdot-}$ in wheat seedlings. While exogenous $O_2^{\cdot-}$ alone had little effect on the content of MDA in wheat seedlings in the present or absent of PA (Fig 5D).

Discussion

As a chelator, PA is a well known important anti-nutrition factor attributed to block the absorption of metal ion such as P, Ca, Mg, Zn, and Fe in haplo-stomach animal (Raboy, 2002). In order to eliminate the anti-nutrient function of PA in edible seeds, some investigations on the maize, *Arabidopsis*, bean and rice mutant *lpa* have been reported in recent years (Aluru et al., 2011; Campion et al., 2009; Kim et al., 2011; Kuwano et al., 2009). Compared to wild type, the whole kernel concentrations of P, K, Mg, Fe, Zn, and Mn (besides Ca) are comparable or higher (Lin et al., 2005) but inorganic ions are improved significantly in *lpa* seeds. For example *lpa1-241* mutant is a 10-fold increase in inorganic phosphorus level without affecting the total amount of phosphorus stored in the kernel than in wt seed (Pilu et al., 2003, 2005). Crops *lpa* mutants have different effects on germination capacity (Aluru et al., 2011; Campion et al., 2009; Kim et al., 2011; Kuwano et al., 2009) namely PA has played a key role in germination capacity of seeds. In our knowledge, effects of exogenous PA on germination capacity of wheat seeds have not been reported. Therefore, we applied the exogenous PA to treat wheat seeds to

Table 1. Exogenous PA inhibits early growth of wheat seedlings but not seeds germination capacity.

Treatment (mM)	Root length (cm)	Shoot height (cm)	Coleoptile length (cm)	Fresh weigh (g)	Germination rate (%)	Germination energy (%)	Germination index (%)
0	7.76±0.79	7.09±0.25	2.43±0.06	0.16±0.02	95.24±3.30	91.43±5.72	76.39±5.67
1 PA	1.96±0.29**	2.32±0.40**	1.96±0.29ns	0.09±0.01**	80.00±3.00ns	80.00±3.00ns	66.93±0.73ns

$P < 0.05$ (*) and $P < 0.01$ (**) are considered as significant and very significant.

Table 2. The inhibitory effects of exogenous PA are released by $O_2^{\cdot-}$.

Treatment (mM)	Root length (cm)	Shoot length (cm)	Coleoptile length (cm)	Fresh weigh (g)
0 PA	9.24±0.64	9.04±0.37	2.88±0.08	0.18±0.01
1 PA	1.86±0.74**	3.76±0.10**	1.51±0.16ns	0.09±0.01**
0.1 $O_2^{\cdot-}$	0.36±0.15*	2.10±0.63*	2.69±0.18ns	0.06±0.01
0.1 $O_2^{\cdot-}$+1 PA	3.07±0.14*	6.54±0.14*	2.26±0.23ns	0.12±0.04**

$P < 0.05$ (*) and $P < 0.01$ (**) are considered as significant and very significant.

detect the influences of exogenous PA on germination ability and seedlings early growth in the paper.

Exogenous PA inhibits early growth of wheat seedlings owe to decreasing of $O_2^{\cdot-}$.

Shoot and root growth is the important reflection of seedlings growth. Results have shown that, in contrast with the control, exogenous PA represses significantly shoot and root length whereas has no effect on the germination rate, germination energy and germination index (Table 1). In addition, exogenous PA decreases markedly the production of $O_2^{\cdot-}$ both in shoots and leaves of wheat seedlings. On the contrary, there is little difference on the production of H_2O_2 in wheat seedlings between present and absent PA (Fig 2). It is the most important that exogenous $O_2^{\cdot-}$ alleviates the inhibitory effect of phytic acid on the root and shoot growth (Table 2). Therefore, exogenous PA inhibits the growth of shoots and roots by lowering the apoplast $O_2^{\cdot-}$.

To date, more and more researches have shown that ROS is an important modulator of plant development, such as tip growth of root hair and pollen tubes (Muller et al., 2009; Joo et al., 2005). Liszkay et al. (2004) have reported that $O_2^{\cdot-}$ generating is demonstrated in the growing zone of the root hair and auxin-induced inhibition of growth is accompanied by a reduction of $O_2^{\cdot-}$ generating. In addition, it is well known that root hair initiation and tip growth is two different processes during development of root growth. An immediate transient burst of $O_2^{\cdot-}$ and H_2O_2 are occurred during seed imbibitions (*Pisum sativum*) whereas a second increase in $O_2^{\cdot-}$ but not H_2O_2 production occurred at the final stages of germination, coinciding with radical elongation (Kranner et al., 2010). Therefore, $O_2^{\cdot-}$ plays a vital role in growth of root hair. Moreover, exogenous PA inhibits growth of roots by decrease of $O_2^{\cdot-}$ but not H_2O_2 (Fig 2 and Fig 4). Therefore, $O_2^{\cdot-}$ maybe plays more important role in root tip growth than in root initiation.

Exogenous PA decreased $O_2^{\cdot-}$ by inhibition of NADPH oxidase

More and more papers have summarized that ROS derived from NADPH oxidase play vital roles in root tip growth (Gapper and Dolan, 2006; Swanson and Gilroy, 2010). A major step forward in defining a role for ROS in this process came with the achievement that root of Arabidopsis mutant *rhd2* (ROOT HAIR DEFECTIVE2) have decreased levels of ROS and are 20% shorter of wide type (Foreman et al., 2003, Renew et al., 2005). After cloning of the gene, they are found to reside in a gene encoding the respiratory burst oxidase homolog C of Arabidopsis (AtrbohC), a homolog of the gp^{91phox} catalytic subunit of the mammalian NADPH oxidase responsible for ROS production. To date, AtrbohD and AtrbohF have been found participated an ABA related growth inhibition process (Kwak et al., 2003). Besides in Arabidopsis, NOX derived ROS also control cell expansion in maize (*Zea mays*) roots (Liszkay et al., 2004). Therefore, ROS derived from NADPH oxidase contribute to root growth of not only Arabidopsis but also maize. Our results have shown that PA depresses $O_2^{\cdot-}$ (Fig 2 and Fig 4) owe to inhibiting of NADPH oxidase (Fig 5.). Besides eliminating the formation of ROS driven by Fe, PA also inhibits the formation of ROS generated from the plasma membrane NADPH oxidase. On the other side, ROS originated from NADPH oxidase may determine the root growth of wheat. In a word, exogenous PA has no influence on germination capacity but restrains markedly the growth of shoots and roots. In order to explore the mechanism, our results show that PA inhibits growth of shoots and roots owe to the decrease of $O_2^{\cdot-}$ but not H_2O_2. In addition, PA depresses $O_2^{\cdot-}$ by inhibition of its formation generated by NADPH oxidase. However, cloning of NADPH oxidase homolog in wheat and which of them gives rise to growth of shoots and roots should be researched in the future.

Materials and methods

Plant material

"Zhengmai 9023", 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.

PA 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

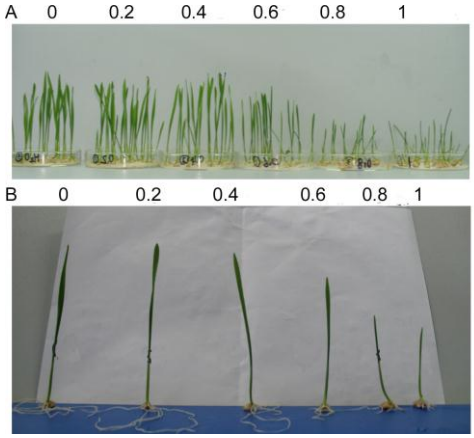


Fig 1. Morphous changes of wheat seedlings are treated by different cocentration of exogenous PA. Concentration of exogenous PA is gradually increased from 0 mM to 1 mM. The morphurs of roots (B) and shoots (A) are shown in the figure 1. PA inhibits shoot and root growth in dose dependent.

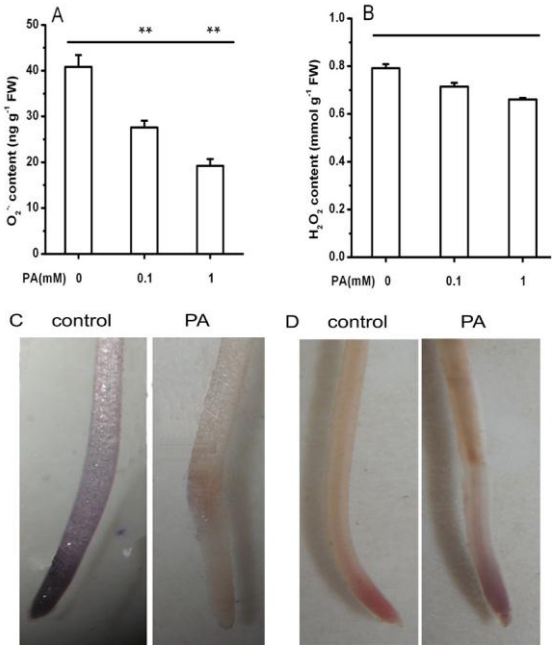


Fig 2. Exogenous PA decreases remarkedly contents of $O_2^{\cdot-}$ and H_2O_2. Contents of $O_2^{\cdot-}$ and H_2O_2 in shoots treated by 0.1 and 1 mM PA are shown in A and B, respectively. Uniformly, the the $O_2^{\cdot-}$ and H_2O_2 contents in roots treated by 1 mM are shown in C and D.

temperature-controlled incubator with 1h light and 1h darkness at 25℃ for germination and early growth. During the whole process, the seeds or seedling were treated by different concentration of PA or in combination with 0.1 mM $O_2^{\cdot-}$ every day. $O_2^{\cdot-}$ was generated by 26 mM L-methionine, 4μM riboflavin and 20μM EDTA-Na_2 when methionine and riboflavin of solutions are irradiated (Yeh et al. 2007). Number of germinated wheat seeds was recorded every day. Germination percentage, length of root, shoot, coleoptile and physiological indexes of six-day-seedlings were determined. Germination energy was represented the germination rate at the third day. Germination index (GI) was calculated by the equation: $GI = \sum (Gt / Dt)$. Gt represents the germination number at t days and Dt was the corresponded germination day.

Determination of $O_2^{\cdot-}$ content

$O_2^{\cdot-}$ was determined according to the method developed by Elstner and Heupel (1976). Wheat seedlings (0.5 g FW) were ground in 4 ml of sodium phosphate buffer (pH 7.8, 50 mM) using a mortar and pestle at 4°C. The extracts were centrifuged at 11 000 g for 15 min, and the resulting supernatants were used for $O_2^{\cdot-}$ determination. The supernatant (1 ml) was first incubated at 25°C for 30 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). Then, 2 ml of this reaction mixture was incubated with 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM 2-naphtylamine at 25°C for 30 min. The absorbance was measured at 530 nm after centrifugation at 13, 000 g for 10 min. A calibration curve was established using sodium nitrite. The results are expressed as l μg·g^{-1} FW.

Determination of H_2O_2 content

Wheat seedlings (0.2g FW) were ground in 1.4 ml of cold 5% trichloroacetic acid (TCA) (Patterson et al., 1984), The extracts were centrifuged at 12, 000 g for 15 min, the supernatants were used to detect H_2O_2 content using H_2O_2 colorimetric detection kits (Nanjing Jiancheng Company of biological technology, China) according to the instructions of manufacturer (Zhang, 2009).

In-situ detection and quantification of $O_2^{\cdot-}$ and H_2O_2

$O_2^{\cdot-}$ was detected by NBT staining of roots according to the method described by Dunand et al. (2007). 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, pictures were taken from 20 mm top of the roots. Settings were identical for all the pictures in the same experiment. H_2O_2 was detected by DAB staining of roots according to the method described by Tewari et al. (2008).

Determination of SOD activity

The total activity of SOD was assayed according to Meloni et al. (2003), 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 polyvinylpyrolidone (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 nitroblue tetrazolium (NBT). One unit of SOD activity was defined as the amount of sample to reach an inhibition of 50% of the NBT reduction rate.

Determination of POD activity

POD activity was measured by use of guaiacol (Zhang, 2009). 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 mins after admixing of 0.8 mL extracts and the reaction solution. Reaction solution was contained 50 mL 0.2 M phosphate buffer (pH6.0), 28 μL

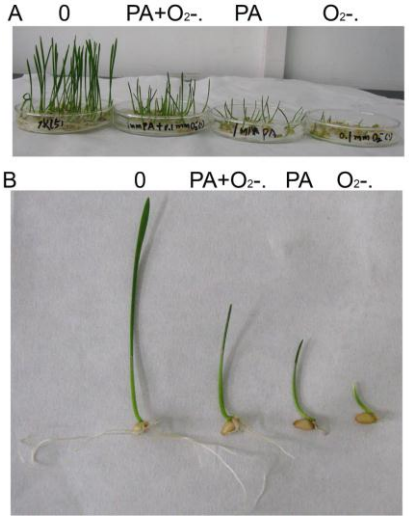


Fig 3. The growth inhibitory effects of exogenous PA are released by $O_2^{\cdot-}$. Seeds were grown in water (control) or solutions contained 1 mM PA, 0.1 mM $O_2^{\cdot-}$ ($O_2^{\cdot-}$) or 1 mM PA and 0.1 mM $O_2^{\cdot-}$(PA + $O_2^{\cdot-}$).

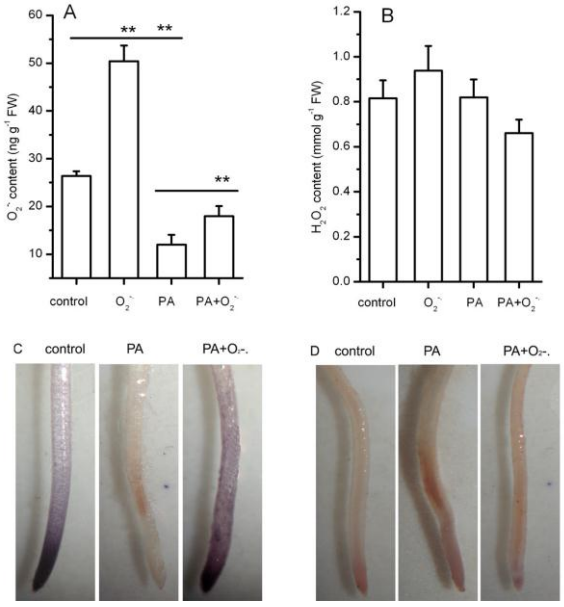
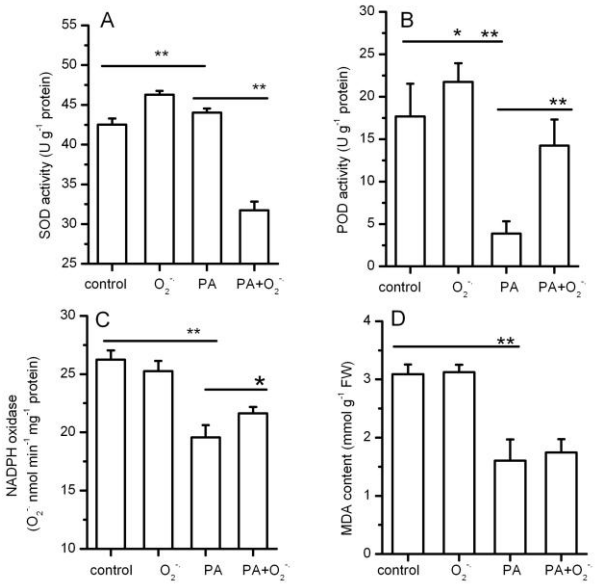


Fig 4. $O_2^{\cdot-}$ recovers decrease of $O_2^{\cdot-}$ content caused by PA. Whatever in shoots (A) or roots (B), decrease effects of $O_2^{\cdot-}$ content by exogenous PA (1 mM) can be released partially by exogenous $O_2^{\cdot-}$ (0.1 mM). On the contrary, exogenous PA and $O_2^{\cdot-}$ have little influence on the content of H_2O_2 both in shoots (C) or roots (D).

H2O2 and 19 μL guaiacol. One unit of POD activity was defined as the amount of sample to change 0.01 per min.

Determination of MDA content

The MDA content was determined according to the method of Dhindsa et al. (1981), Wheat seedlings (0.5 g FW) were ground in 2 ml of sodium phosphate buffer (pH 7.8, 50 mM). The homogenate was centrifuged at 12,000g for 20 min and 0.5 ml



of the supernatant fraction was mixed with 2 ml 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was heated at 90℃ for 20 min, cooled and then centrifuged at

Fig 5. Effects of exogenous PA and $O_2^{\cdot-}$ on the activities of SOD, POD, NADPH and MDA content. Exogenous PA decreases activities of POD (B) and NADPH oxidase (C) and MDA content (D) and $O_2^{\cdot-}$ can release partially the inhibitory effects on POD and NADPH oxidase by PA. $O_2^{\cdot-}$ plus PA decrease activity of SOD (A). On the contrary, MDA content in wheat with exogenous PA has little change by $O_2^{\cdot-}$. Seeds were treated by the same way as Fig 3. $P < 0.05$ (*) and $P < 0.01$ (**) are regarded as significant and very significant.

12,000g for 15 min. The absorbance was recorded at 450 nm, 532 nm and the value for non-specific absorption at 600 nm was subtracted. The extinction coefficient of 155 $mmol \cdot l^{-1} \cdot cm^{-1}$ was used to calculate the content of MDA.

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℃) Na-phosphate buffer (pH 6.0, 10 mM), the homogenization 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 (Baumgarten, 1985). Crude germinated seed homogenates (0.2 ml) were precipitated with acetone (9:1 acetone: homogenate) at -20 ℃ for 15 min. Precipitated proteins were recovered by centrifugation at 14,000 g for 10 min at 4℃. Protein pellets were resuspended in buffer (50 mM Tris-HCl, 0.1 mM $MgCl_2$, 0.25 M Suc, 0.1% Triton-X-100, pH 8.0) and were used for photometry assay of NADPH oxidase activity. The NADPH oxidase activity was determined by nitroblue tetrazolium (NBT) reduction. The reaction contained 0.5 ml protein solution and 0.5 ml 730 μM NBT, addition of 1ml 100 μM NADPH to initiate reaction, NBT reduction was determined at 530 nm, an extinction coefficient of 12.8 mM^{-1} cm^{-1} was used for calculation of the oxidase activities.

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.

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