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Abscisic acid and reactive oxygen species were involved in slightly acidic electrolyzed water-promoted seed germination in watermelon

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ABSTRACT

Seed germination is a vital process in seedling stage. The delayed seed germination and irregular emergence frequently occur in watermelon. It is critical to exploit technologies for promoting seed germination. This study aims to explore the effects of slightly acidic electrolyzed water (SAEW) on the seed germination of watermelon. The results showed that SAEW significantly improved the germination indexes and caused a mass of changes to nutrient substances and enzymatic activities in watermelon seeds. In addition, SAEW treatment decreased abscisic acid (ABA) contents, while increased gibberellin (GA) contents of watermelon seeds by regulating the gene expressions of metabolic related enzymes. Thereinto, SAEW remarkably upregulated the transcriptional level of *CYP707A1* and *CYP707A2* involved in ABA catabolism, which inhibited the accumulation of ABA. Moreover, SAEW could inhibit reactive oxygen species (ROS) accumulation in seeds and improve the seed germination which was identical to the effect of DMTU (dimethyl thiourea, ROS inhibitor) on the seed germination. In addition, the expressions of *RbohC* and *RbohD* involved in ROS accumulation were notably depressed by SAEW. This study demonstrated that exogenous SAEW could inhibit the accumulation of ABA content and decrease the accumulation of ROS, which finally improves the germination ability of watermelon seeds.

1. Introduction

Seed germination is one of the most critical phases during seedling establishment, which converts dormant seeds into active seedlings (Baskin et al., 2019; Hubbard et al., 2012). There are a number of factors controlling seed germination including seed vigor, temperature and humidity of germinating environment (Malcolm et al., 2003; Huehne and Bhinija, 2012; Dahlquist-Willard et al., 2016; Jiao et al., 2016; Yu et al., 2016; Guan et al., 2020). Phytohormones include auxin, ABA (abscisic acid), ethylene, GA (gibberellin), CTK (cytokinin), BR (brassinosteroid), and so forth, which could regulate seed dormancy and germination in plants (Miransari and Smith, 2014). It has long been known that GA could break seed dormancy and promote germination (Kucera et al., 2005; Wang et al., 2011). However, ABA is the only hormone known to maintain seed dormancy and delay seed germination (Cutler et al., 2010; Hubbard et al., 2010). Furthermore, more recent researches have shown that reactive oxygen species (ROS) played roles in seed germination (Lin et al., 2013; Ortiz-Espin et al., 2017; Luo et al., 2021). It was revealed that ROS played important roles in the radicle elongation and endosperm weakening as a non-enzymatic player during seed germination (Yang et al., 2020).

Watermelon (*Citrullus lanatus*) is a highly popular fruit crop with a great economic value and widely cultivated in China, Turkey and Iran with its annual production of over 119 million tons in 2017 (FAOSTAT, 2017). A mass of studies about watermelon have focused on the improvement of nutritional quality, saccharinity and survival rate of grafting as well as the tolerance to biotic and abiotic stresses (Dabirian et al., 2017; Li et al., 2017; Filippos et al., 2019; Jawad et al., 2020; Wei et al., 2020; Zhang et al., 2020). However, there is a scarce study on the seed germination of watermelon. Due to the specific structure and reduced viability of watermelon seeds stored in the natural condition, the delayed seed germination and irregular emergence frequently occur,

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especially for triploid varieties of watermelon. Triploid watermelon seeds are smaller than diploid varieties in size, which causes a limited number of reserves to support germination and seedling growth of triploid watermelon (Pratibha et al., 2020). Prompt and uniform seed germination is crucial for taking full advantage of production resources in commercial agriculture (Chen and Arora, 2013). Therefore, improving the germination rate and emergence quality of watermelon is important, which is beneficial to the growth of later watermelon seedlings and bringing watermelon for the market ahead of time. Up to now, there are few reports on the positive impacts of exogenous substances to the germination of watermelon in natural conditions.

Slightly acidic electrolyzed water (SAEW) is a novel disinfectant generated by the electrolysis of dilute sodium chloride or hydrochloric acid solutions or both in an electrolytic cell without a separating membrane. SAEW, colourless, odourless, and harmless to humans, has been directly used on food surfaces to extend shelf-life in Japan and America (Jung et al., 2017; Wang et al., 2017; Ye et al., 2017; Huang et al., 2021). Meanwhile, SAEW is regarded as an environmental friendly measure for broad-spectrum microbial decontamination in recent years (Ye et al., 2017; Wang et al., 2018). In this research, the effects of SAEW on the germination in watermelon seeds were investigated. We evaluated the effects of SAEW on germination rate, germination energy, germination index and vitality index of watermelon seed. Furthermore, analyses of plant hormone content were used to identify the significant phytohormone responding to SAEW treatment in watermelon seeds. The pharmacology experiment was conducted to preliminarily establish the connection between SAEW-induced germination and ROS. These results may help us to understand the germination mechanisms of watermelon seed, as well as to further reveal the physiological regulatory functions not merely the sterilization of SAEW in plants.

2. Materials and methods

2.1. Preparation of slightly acidic electrolyzed water (SAEW)

SAEW was prepared by electrolysis of a 6% HCl solution using a generator (HD-240L, Fuqiang-Want Sanitary Accessories Ltd, Shanghai, China) with a non-membrane electrolytic chamber at a voltage of 220 V. SAEW was prepared on the day of the experiments. After that, the pH and the available chlorine concentration of were respectively determined by a pH meter (FE-28, METTLER TOLEDO, Shanghai, China) and chlorine concentration test paper (Newstar paper, Q/HSSC 202-2016, Hangzhou, China) .

2.2. Plant material and treatments

Seeds of watermelon (*Citrulus lanatus* L. cv. 8424), a kind of triploid hybrid variety, were chosen as the research objective. Watermelon seeds were purchased from 'Hefeng Seed Industry Co., Ltd of China'. The hundred-grain weight and seed germination on the label of seeds were 5.20 g and 85% respectively. Firstly, seeds were respectively soaked in deionized water (Control, Con) or different concentrations of SAEW (20, 40, 60, 80 ppm) at 25℃ for 5 h. After that, the effect of SAEW on seed germination was performed by cultivating seeds at 25℃ on cotton gauze soaked with deionized water or different concentrations of SAEW in 90 mm plastic petri dish (30 seeds per dish) at 25℃ all night in an incubator. The germination indices were measured at indicated time (24, 48, 72, 96 and 120 h). The germination energy was calculated by a formula: GE (%) $=M/N\times100$. GE is the germination energy (%), parameter M is the number of germinating seeds in five days (120 h) with different treated concentrations of SAEW, and N is the total number of seeds from the germination test. Germination rate (%), GR $= A/B\times100$. Parameter A is the number of germinating seeds, and B is the total number of seeds from the germination test. Germination index (GI) $= \sum(x/y)$, parameter x is the number of germinating seeds in x day, and y is the corresponding days. Finally, Vitality index (VI) $=$ GI$\times$L, parameter GI is the germination index, and L is the total length of radicle in x day.

In the time-course experiment of germination, watermelon seeds were respectively soaked in deionized water (Con) or 60 ppm SAEW for 1, 2, 5 and 10 h. After that, the time effect of SAEW on seed germination was performed by cultivating seeds at 25℃ on cotton gauze soaked with deionized water (Con) or 60 ppm SAEW in 90 mm plastic petri dish (30 seeds per dish) at 25℃ all night in an incubator. The germination indices were measured at indicated time (24, 48, 72, 96 and 120 h).

2.3. Assay of antioxidant enzyme activity

Fresh seeds of watermelon (0.5 g) with different treatments were homogenized with 10 mL precooled sodium phosphate buffer (50 mM, pH 7.8). After that, the mixed liquor was centrifuged with 12,000 rpm at 4℃ for 20 min. The collected supernatant solution was used for enzyme activity assays.

Superoxide dismutase (SOD) and peroxidase (POD) activities were analyzed with the methods described by Han et al. (2007) with some changes. One unit of SOD (U) represented the amount of enzyme required to inhibit 50% nitro blue tetrazolium (NBT) reduction which was measured at 560 nm. U/g FW was represented the activity of SOD. The oxidation rate of guaiacol in presence of H_2O_2 at 470 nm for 3 min was used to perform the POD activity of watermelon seeds. The absorbance was kept track for every minute and expressed as U/min•g•FW. The activity of catalase (CAT) was determined by Huang et al. (2006) with measuring the reduction of H_2O_2 at 240 nm for 30 s.

2.4. Quantification of soluble sugar, starch, protein and fat

Fresh samples of the shelled watermelon seeds (1 g) were ground in a mortar with 2.5 mL of distilled water. The homogenates were centrifuged at 13,000 rpm for 30 min. Then, the supernatant was used to analyze the contents of soluble sugar. The soluble sugar content was determined using the sulfuric acid anthrone method with a spectrophotometer (UV-5200 spectrophotometer, Shanghai Metash Instruments Co., Ltd, Shanghai, China) at a wavelength of 630 nm (Morris, 1948).

For the analysis of starch content, frozen shelled watermelon seeds were thoroughly ground with liquid nitrogen. The obtained residues were washed with 80% ethanol and filtered again and then dissolved in 20 mL hot distilled water. The mixture was incubated in boiling water bath for 15 min. After incubation, the ice-cooled perchloric acid was added to the mixture and continued ice-cooled for 15 min. After that, the mixture was filtered and the residues were dissolved in 10 mL of distilled water and then incubated in a hot water bath at 100℃ for 15 min. The resulting solutions were extracted with 2 mL of perchloric acid (4.6 mol•L^{-1}) for 15 min and then filtered. The filtrates were collected and washed with distilled water three times. The resulting solution was adjusted to 100 mL with distilled water to analyze starch content (Lv et al., 2021).

The shelled watermelon seeds samples (0.05 g FW) were ground in a mortar with liquid nitrogen, and the powder transferred with 3 mL of a phosphate buffered solution (pH 7.0) into centrifuge tubes. After 15 min centrifugation at 13,000 g (4℃), 0.1 mL of the supernatant was combined with 5 mL of Coomassie brilliant blue G-250 solution. Two minutes later, the soluble protein content (mg•g^{-1} FW) was determined at a wavelength of 595 nm (Su et al., 2017).

The fat content was evaluated according to the modified method of Mohammed et al., 2020. The shelled watermelon seeds samples were homogenized in a solvent mixture of chloroform/ methanol (2:1, v/v) and the mixture was centrifuged at 5,000 g for 20 min. The residues were dissolved in 2 mL of toluene/ethanol mixture (4/1, v/v). The extracted lipids were concentrated by a rotary evaporator and extracted fat was weighed in vials to calculate the fat content.

2.5. Determination of phytohormone contents

Seeds were respectively soaked in deionized water (Con) and 60 ppm SAEW at 25℃ for 5 h. After that, the effect of SAEW on seed germination was performed by cultivating seeds at 25℃ on cotton gauze soaked with deionized water or different concentrations of SAEW in 90 mm plastic petri dish (30 seeds per dish) at 25℃ all night in an incubator. The seeds were sampled at indicated time (0, 24, 48 and 72 h). Endogenous bioactive IAA, ABA, GA_1, GA_3, GA_4, GA_7 and Zeatin concentrations in watermelon seeds of the investigated cultivars were measured using HPLC-MS/MS following the protocols described in Pérez-Jiménez et al. (2014) and Camilo et al. (2019), respectively.

2.6. Localization of hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}) by 3,3'-diaminobezidine (DAB) and nitroblue tetrazolium (NBT)

On account of the high oil and fatty acid concentrations of the seeds, ROS staining in the seeds is difficult. Thus, radicles (germinated for 72h) were used for further ROS staining processes.

DAB was dissolved in sterilized water (pH=3.8) and produced a solution with a final concentration of 1 mg•ml^{-1} according to Luo et al., 2021. Seeds were soaked in deionized water (Con) and 60 ppm SAEW (SAEW) for 5 h. Then, radicles were incubated in DAB-staining solution for 12 h. After that, the stained radicles were washed with 75% ethanol and photographed.

Histochemical localization of superoxide radicals by NBT staining in watermelon was performed according to Zhang et al., (2014). Seeds were soaked in deionized water (Con) and 60 ppm SAEW (SAEW) for 5 h. Then, radicles of seed were incubated 1 mM NBT in 50 mM phosphatic buffer solution (PBS, pH 7.5) at 37℃ for 30-60 min. After that, the stained radicles were washed with 75% ethanol and photographed.

2.7. Determination of hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}) concentrations in plants

H_2O_2 content was detected as described in Yu et al., (2019). Seeds were soaked in deionized water (Con) and 60 ppm SAEW (SAEW) for 5 h. Then, 0.1 g radicles were ground in liquid nitrogen and extracted with 1 ml cooled acetone. After having an ice bath for 5 minutes, the samples were centrifuged for 10 min at 10,000 rpm at 4°C. Then, 1.5 ml liquid supernatant was mixed with 0.1 ml 5% (w/v) titanium sulfate and 0.2 ml ammonia. The miscible liquid was centrifuged for 10 min at 10,000 rpm at 4°C. After that, the precipitate was washed with the cooled acetone until it became colorless. 3ml of 2M H_2SO_4 was used to dissolve the precipitate, and then immediately measured the value of A_{415}. The H_2O_2 content was calculated through a standard curve produced from a series of H_2O_2 standards at known concentrations.

O^{2-} content in this study was quantified according to Luo et al., 2021. Seeds were soaked in deionized water (Con) and 60 ppm SAEW (SAEW) for 5 h. Then, 0.1 g radicles were ground in liquid nitrogen, and the superoxide anion content was measured by manufacturer's instructions of superoxide anion test kit (BC1290; Solarbio Corp.).

2.8. RNA extraction and real time quantitative PCR (qRT-PCR)

Watermelon seeds were respectively soaked in deionized water (Con) or 60 ppm SEAW for 5 h. After that, the seed were cultivated at 25℃ on cotton gauze soaked with deionized water (Con) or 60 ppm SAEW in 90 mm plastic petri dish (30 seeds per dish) at 25℃ all night in an incubator. Then, the seed were sampled at indicated germination time (0, 24, 48 and 72 h). The RNA extraction and qRT-PCR were performed followed the report of Wu et al., (2019). RNA purity was verified based on the ratio (>1.9) of 260/280 nm absorbance using a spectrophotometer. The primer sequences of different genes were designed by Primer Premier 5 software and listed in Table S1. Three independent biological experiments were performed with three replications.

2.9. Statistical analysis

Statistical analyses were performed through SPSS statistical software package (version 11.0). And the differences among treatments were analyzed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test, with $P < 0.05$ as the threshold. Before the ANOVA, all the data passed the normality test ($P > 0.05$).

3. Results

3.1. Effect of slightly acidic electrolyzed water (SAEW) on the germination of watermelon seeds

To compare the effects of SAEW on the seed germination, the watermelon seeds were respectively soaked at 20, 40, 60 and 80 ppm SAEW for 5 h (Fig. 1). Besides, the seeds immersed in deionized water were used as control (Con). There were no differences in the germination rate among various treatments until the germination time reached 48 h (Fig. S1A). As shown in the embedded histogram of Fig. S1A, the germination rate was gradually increased by elevated concentrations of SAEW at 48 h. Thereinto, the germination rate was reached 93.33% when the seeds were treated with 60 ppm SAEW for 48 h. However, the germination rate was only reached 16.67% in Con. In addition, the germination energy, germination index and vitality index of watermelon seeds performed the similar tendency with germination rate, which were gradually increased by SAEW (Fig.S1B-D). Thereinto, the germination energy, germination index and vitality index were respectively increased by 48.28%, 47.49% and 69.35% with 60 ppm SAEW-treatment compared with the control. All the results revealed that 60 ppm SAEW could significantly promote the germination of watermelon seeds.

After the verification of SAEW-treated concentration, the time effect of 60 ppm SAEW on the seed germination was measured by the germination rate, the germination energy, the germination index and the vitality index. As shown in Fig.S2A, the germination rate was increased with the extension of germination time. Thereinto, the significant differences were mainly performed at 48 and 72 h. It was shown that the germination rate, germination energy, germination index and vitality index peaked with 60 ppm SAEW treatment for 5 h. When the treated time was prolonged to 10 h, the germination rate and germination energy were reduced by 60 ppm SAEW (Fig.S2 A and B). Above all, 5 h was the optimum time for SAEW to promote the germination of watermelon seeds.

3.2. Effect of slightly acidic electrolyzed water (SAEW) on the concentrations of nutrient substances and activities of enzymes

The process of germination could cause a mass of changes to nutrient substances in seeds. As shown in Fig.S2A, the content of soluble sugar was increased as the time prolonged and peaked at 48 h. Moreover, the soluble sugar content in the seeds treated with SAEW was significantly higher than it in control at 48, 72 and 96 h. Thereinto, the content of soluble sugar at 48 h was increased by 48.87% with SAEW treatment. On the contrary, the changing trends of starch and protein were decreased as the time prolonged. In addition, SAEW further accelerated the reduction of starch and protein contents after germination for 48 h (Fig. S2B and C). The contents of starch and protein were respectively reduced by 47.75% and 26.40%. Beyond that, the fat content was gradually reduced as time went by and not influenced by SAEW treatment (Fig.S2D). All the results showed that 60 ppm SAEW had the capacity for expediting the germination of watermelon seeds.

Seed germination could also cause the change of enzymatic activities. In order to further explored the effect of SAEW on enzymes, the activities of SOD, POD and CAT were determined in the seed of watermelon. As the time of germination increased, the activity of SOD increased and reached the peak on 48 h in both the Con and the SAEW

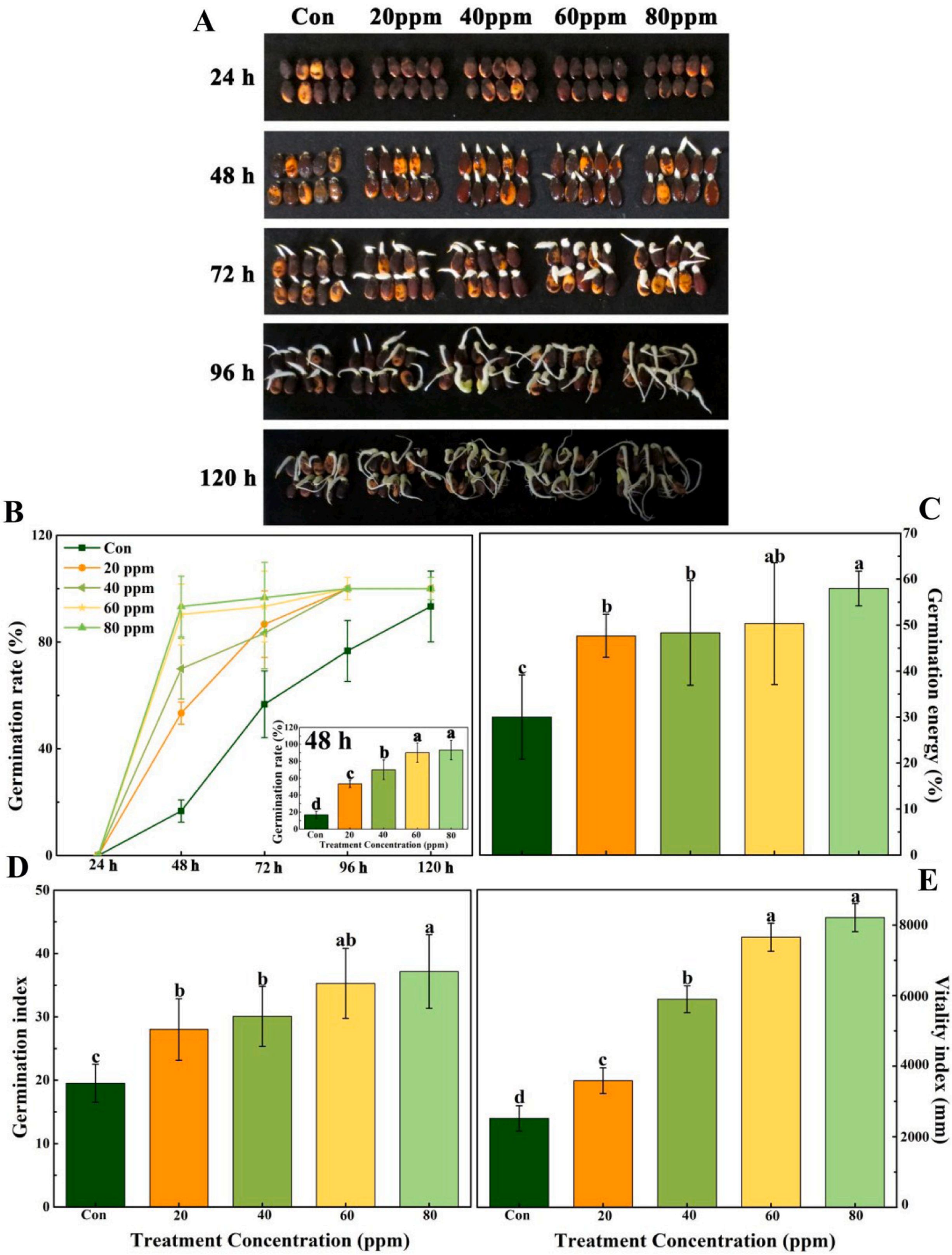

Fig. 1. The effects of different concentrations of slightly acidic electrolyzed water (SAEW) on watermelon seed germination. (A) performed the phenotype of seed germination with different treatments. The germination rate (B), germination energy (C), germination index (D) and vitality index (E) influenced by different concentrations of SAEW were shown. Watermelon seeds were respectively soaked in deionized water (Control which was presented as Con) or different concentrations of SAEW (20, 40, 60, 80 ppm) at 25℃ for 5 h. The germination indices were measured at indicated time (24, 48, 72, 96 and 120 h). Data are shown as the means ± SE (n=30). Different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range test.

treatment. Meanwhile, the activity of SOD was significantly improved by SAEW except 24 h. The activity of SOD was respectively increased over 50% at different germination times (Fig.S3A). In addition, the activities of POD and CAT had the similar change trend. With extended time of germination, the activities of POD and CAT were gradually increased, which remained constantly after 72 and 48 h respectively. Thereinto, the activity of POD was nearly tripled by SAEW after germinated 48 h and remained constantly after 96 h (Fig.S3B and C). In conclusion, the up-regulation of SOD, POD and CAT activities was induced by SAEW, which was benefit for seed germination.

3.3. Effect of slightly acidic electrolyzed water (SAEW) on the concentrations of endogenous phytohormone in watermelon seed

ABA could induce and maintain seed dormancy and GA is responsible for the induction of seed germination (Shu et al. 2016; Tuan et al. 2018). In order to figure out whether these plant hormones contributed to the SAEW-induced germination, the concentrations of ABA, GA, IAA and Zeatin were detected severally. As shown in Fig.2A, ABA concentration was markedly decreased by SAEW. Particularly at 24 h, the ABA concentration was reduced by 45.18% with SAEW treatment. Meanwhile, the total GA concentration was gradually improved with the germinating time (Fig.2B). The total GA was the sum of GA1, GA3 and GA4, which was separately shown in Fig.S4A-C. At 48h, the total GA concentration was improved by 64.85% with SAEW treatment. Then, the ratio of GA to ABA was remarkably risen by SAEW. Besides, the concentration of Zeatin was consistent with the changing trend of GA, which was gradually increased as germination time went by and further improved by SAEW (Fig.S4D). In addition, the trend of IAA concentration first increasing and then decreasing was showed with the germination time, and SAEW-induced the increase of IAA concentration was shown at 48 and 72 h (Fig.S4E). In conclusion, the SAEW-induced germination maybe caused by the changing of endogenous ABA and GA.

3.4. Effect of slightly acidic electrolyzed water (SAEW) on the expressions of key genes involved in abscisic acid (ABA)/gibberellins (GA) biosynthesis and catabolism

The endogenous ABA and GA level in seed is determined by a balance between its biosynthesis and catabolism. In order to further probe into the interrelation between ABA/GA and SAEW-induced germination, the transcriptional level of relative genes involved in ABA/GA biosynthesis and catabolism were analyzed respectively (Fig.3A). The expression of *ZEP*, encoded a single copy zeaxanthin epoxidase gene that functions in first step of the biosynthesis of ABA, was reduced by SAEW. As the extension of germination time, the expression of *ZEP* showed a descending trend gradually in both the Con and the SAEW-treatment group. *NCEDs* encodes 9-cis-epoxycarotenoid dioxygenase which is a key enzyme in the biosynthesis of ABA (Tan et al., 2003; Lefebvre et al., 2006). Then, the expressions of *NCED2, NCED3* and *NCED6* showed the same changing tendency as the *ZEP*, which were declined by degrees no matter in the Con or in SAEW treatment. Thereinto, the expressions of *NCED3* were further lowered by SAEW, but the expressions of *NCED2* and *NCED6* were lower in the control than it in SAEW-treatment. In addition, the expression of *AAO3*, coded the aldehyde oxidase delta isoform catalyzing the final step in ABA biosynthesis, were gradually reduced as the germination of seed and slightly lowered at 48 and 72 h in the control. What's more, the expressions of *CYP707A1* and *CYP707A2*, encoded a protein with ABA 8'-hydroxylase activity involved in ABA catabolism, showed an upward tendency as the extension of germination time and were further significantly elevated by the SEAW treatment, which may contribute to the reduction of ABA content in seeds.

When it comes to the GA biosynthesis and catabolism, the expressions of *GA3ox1, GA3ox2, GA13ox* and *GA20ox2*, encoded gibberellin oxidases involved in GA biosynthesis, were gradually increased as the process of germination and further induced by SAEW (Fig.3B). Meanwhile, the expressions of *GA2ox3, GA2ox6* and *GA3ox1* involved in GA catabolism showed a decline tendency induced by SAEW. However, the effect of SAEW on the expressions of GA-related genes was not significant. In conclusion, the SAEW-induced germination of watermelon seeds may arise from the accelerating catabolism of ABA by increasing the transcriptional levels of *CYP707A1* and *CYP707A2*, which ultimately led to a lower ABA accumulation to break the seed dormancy.

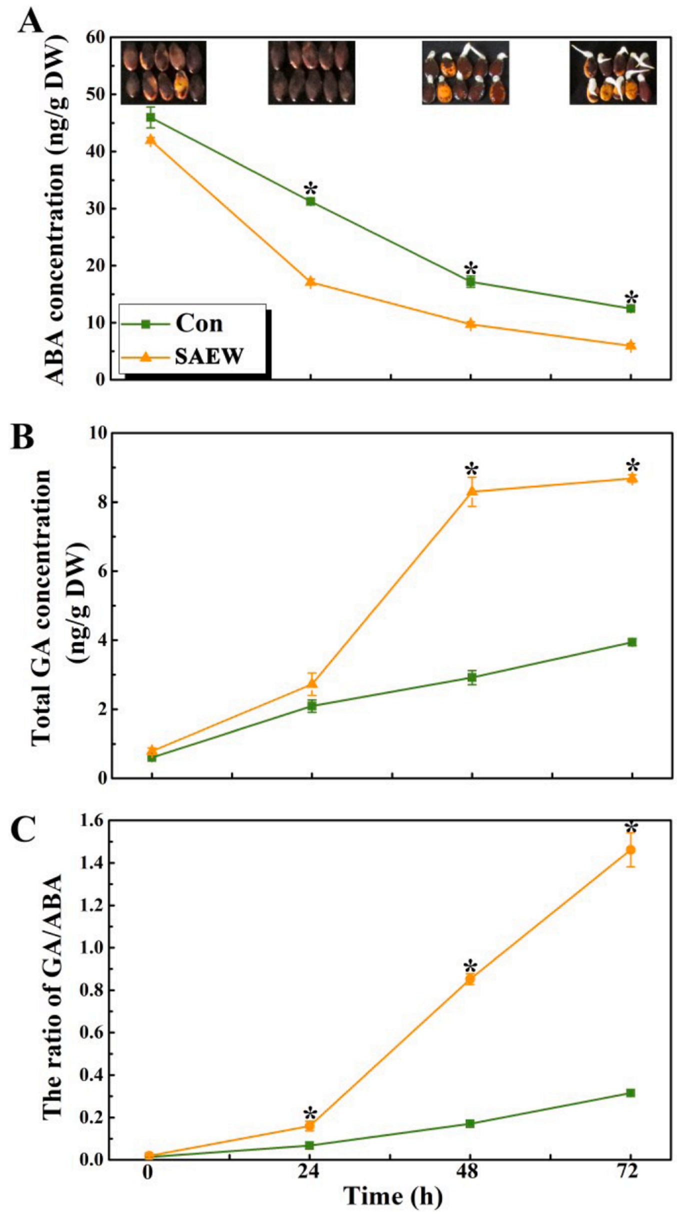

Fig. 2. The analyses of endogenous abscisic acid (ABA) and gibberellin (GA) concentrations in watermelon seed after soaked in slightly acidic electrolyzed water (SAEW). The concentrations of ABA (A), total GA (B) and the ratio of GA/ABA (C) influenced by 60 ppm SAEW were shown. Watermelon seeds were respectively soaked in deionized water (Control) or 60 ppm SAEW at 25℃ for 5 h. The concentrations of plant hormones were measured at indicated time (0, 24, 48 and 72h). Data are shown as the means ± SE (n=3). The asterisk indicated significant differences at $P < 0.05$ according to Duncan's multiple range test and the significant differences were compared between Control and 60 ppm SAEW treatment at indicated time.

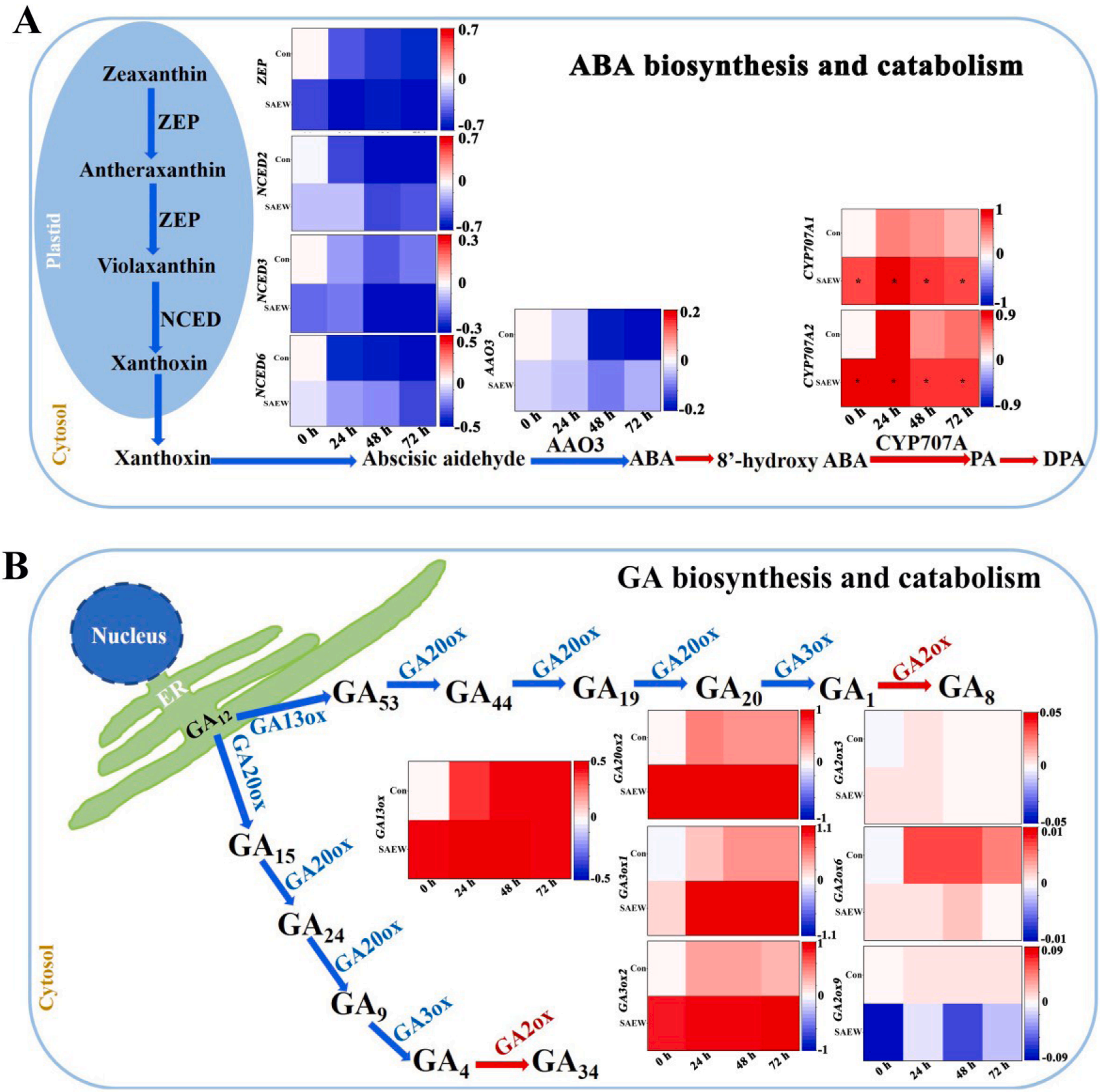

Fig. 3. The effects of slightly acidic electrolyzed water (SAEW) treatment on the expressions of genes involved in biosynthesis and catabolism of abscisic acid (ABA) and gibberellin (GA) . The key genes of ABA (A) and GA (B) influenced by 60 ppm SAEW were shown. Watermelon seeds were respectively soaked in deionized water (Con) or 60 ppm SEAW at 25℃ for 5 h. The expressions of genes were measured at indicated time (0, 24, 48 and 72 h). Data are shown as the means ± SE (n=9). The asterisk indicated significant differences at $P < 0.05$ according to Duncan's multiple range test and the significant differences were compared between Con and 60 ppm SAEW treatment at indicated time.

3.5. The endogenous reactive oxygen species (ROS) of watermelon seeds was changed by slightly acidic electrolyzed water (SAEW)

Several researches revealed that ROS identified as non-enzymatic factors play a key but largely elusive role in the repression of seed germination in some plant species like *Arabidopsis thaliana* and lettuce (Müller et al., 2009; Lin et al., 2013; Ortiz-Espin et al., 2017; Choudhury et al., 2017; Luo et al., 2021; Zhang et al., 2014; Yang et al., 2020). In order to ensure the roles of ROS in the SAEW-induced germination, the ROS accumulation in different treatments was examined by the content analyses and staining of H_2O_2 and O^{2-} respectively. As it shown in Fig.4A-C, the H_2O_2 and O^{2-} contents were significantly reduced by SAEW. The lighter brown and bluish violet were found in SAEW treatment (Fig.4A). Meawhile, the results of content determination were in accord with the staining evidence. The contents of H_2O_2 and O^{2-} were markedly decreased by SAEW at 48 h and 96 h. Thereinto, compared with the control, the content of H_2O_2 and O^{2-} was reduced by 54.29% and 55.56% at 48 h respectively with SAEW treatment.

Most of *Rbohs* are shown involved in the process of the response to abiotic stress and stomatal closure (Kámán-Tóth et al., 2019; Ma et al.,

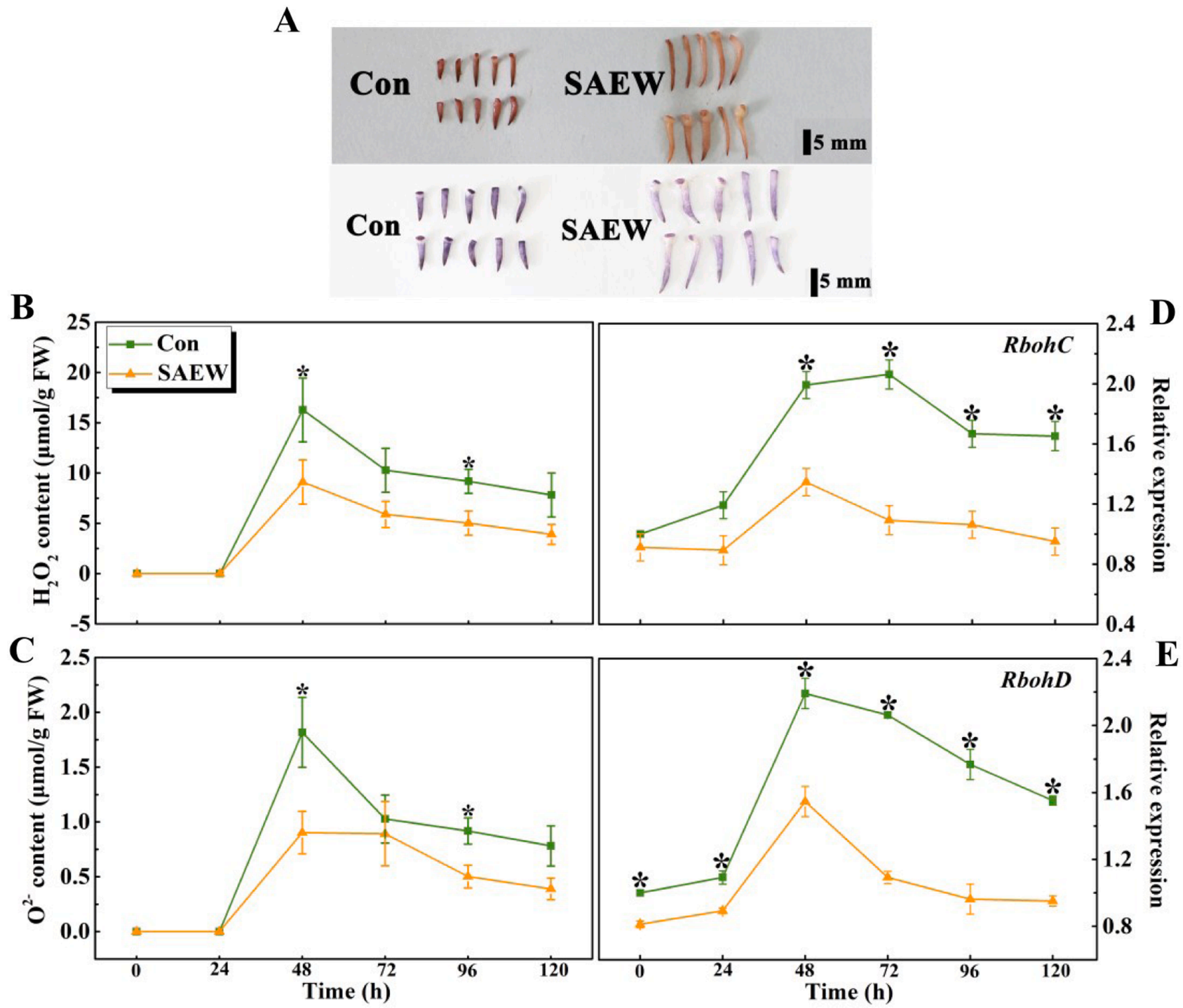

Fig. 4. The effects of slightly acidic electrolyzed water (SAEW) on the endogenous reactive oxygen species (ROS). Histochemical staining of O^{2-} (superoxide radicals) by NBT (nitro blue tetrazolium) and H_2O_2 (hydrogen peroxide) by DAB (3,3'-diaminobezidine) were shown in (A). The contents of H_2O_2 (B) and O^{2-} (C) were influenced by SAEW. (D) and (E) showed the relative expressions of *RbohC* and *RbohD* with different treatment. Watermelon seeds were respectively soaked in deionized water (Control) or 60 ppm SAEW at 25°C for 5 h. The concentrations of ROS and the expressions of genes were measured at indicated time (0, 24, 48, 72, 96 and 120 h). Data are shown as the means ± SE (n=9). The asterisk indicated significant differences at $P < 0.05$ according to Duncan's multiple range test and the significant differences were compared between Control and 60 ppm SAEW treatment at indicated time. And the histochemical staining was performed at 72 h in the radicles of watermelon seeds.

2012; Scuffi et al., 2018; Wang et al., 2016). However, only *RbohB* and *RbohC* have been identified so far in the process of seed development and germination (Müller et al., 2009; Yang et al., 2020). In the study, *RbohC, RbohD, RbohF* and *RbohH* were expressed in watermelon seeds (Fig.4D, E and Fig.S5). Meanwhile, the relative expressions of *Rbohs* were detected at indicated time. Thereinto, the expressions of *RbohC* and *RbohD* were up-regulated first but then them went down with the extension of germination time (Fig.4D and E). The expressions of *RbohC* were significantly reduced by SAEW after 48 h and the expressions of *RbohD* were markedly inhibited from 0 h to 120 h. That's to say, *RbohD* had lower transcriptional level after seeds imbibed with SAEW than it with deionized water (Con). Beyond that, the expressions of *RbohF* and *RbohH* were measured and shown in Fig.S5. However, it revealed that the expressions of *RbohF* and *RbohH* were not significantly affected by SAEW. To sum up, the reduced ROS accumulation may give rise to the promoted germination caused by SAEW in watermelon.

SAEW could significantly influence the ROS accumulation (Fig.4). Therefore, the role of ROS in SAEW-induced germination in watermelon seeds were further explored. DMTU (dimethyl thiourea), an inhibitor of ROS, was used to soak the watermelon seeds. As shown in Fig.5, DMTU and SAEW could significantly promote the germination of seeds. Furthermore, the co-treatment of SAEW+DMTU accelerated the promotion of germination. The germination energy, germination index and vitality index were markedly increased by SAEW+DMTU. It suggested that the reduced accumulation of ROS may contribute to the SAEW-induced germination.

4. Discussion

To improve the seed germination of watermelon, some treatments

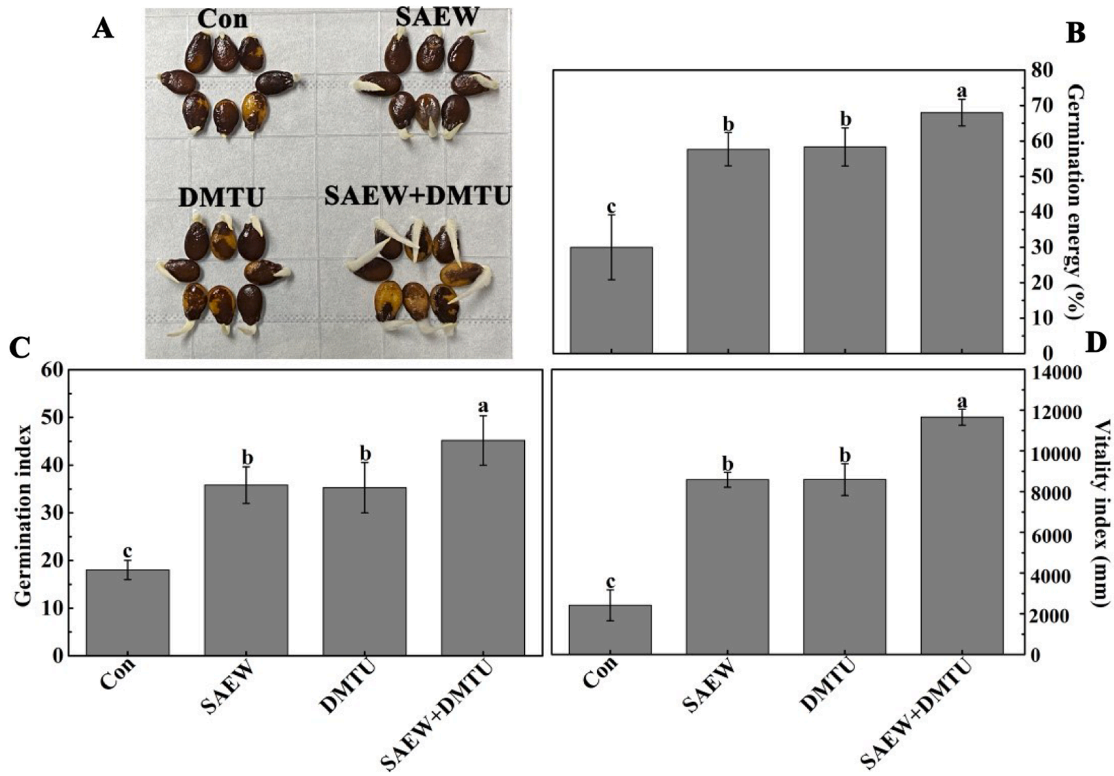

Fig. 5. The effects of reactive oxygen species inhibitor (N,N'-Dimethylthiourea, DMTU) and slightly acidic electrolyzed water (SAEW) on the germination of watermelon seeds. The phenotype (A), germination energy (B), germination index (C) and vitality index (D) were shown. Watermelon seeds were respectively soaked in deionized water (Control), 60 ppm SAEW, 60 mM DMTU and SAEW+DMTU mixture at 25℃ for 5 h. The germination-related indicators were measured. Data are shown as the means ± SE (n = 30). Different letters indicated significant differences at $P < 0.05$ according to Duncan's multiple range test.

including seed coat removal, scarifcation, and seed nicking have been proved to have the capacity of enhancing seed germination and seedling vigor. However, some varieties of watermelon like triploid seeds still had lower seedling vigor than that of diploids (Grange et al., 2000; Acharya et al., 2020). Therefore, the novel methods to improve and uniform seed germination and seedling vigor are needed. Up to now, some exogenous substances such as salicylic acid, oxygenated brackish water and plasma activated water were shown to markedly improve the seed germination in sunflower (*Helianthus annuus* L.), wheat (*Triticum aestivum* L.), alfalfa (*Medicago sativa*) and mung bean (*Vigna radiata*) (Huang et al., 2020; Zhu et al., 2020; Machado-Moreira et al., 2020). However, the research of using exogenous substances to improve the seed germination of watermelon is scarce until now. This study assessed the roles of SAEW in the seed germination of watermelon by the analyses of germination index, nutrient contents, endogenous plant hormone contents and the expression level of related genes. These data manifested that SAEW had the capacity for the promotion of seed germination may be caused by accelerating the catabolism of ABA by the induced-expressions of *CYP707A1* and *CYP707A2*, which further led to a lower ABA accumulation than it in the control. Meanwhile, the decreased-expressions of *RbohC* and *RbohD* caused a less accumulation of ROS and further broke the seed dormancy (Fig.6).

4.1. Abscisic acid catabolism was involved in the slightly acidic electrolyzed water-induced the promotion of seed germination in watermelon

The germination capacity of watermelon seeds can be reflected by germination parameters, including germination rate, germination energy, germination index and vitality index (Wen et al., 2018). In this study, the germination parameters of SAEW-treatment were significantly higher than those of control, indicating that SAEW could promote seed germination of watermelon (Fig.1). As the SAEW concentration increased, the acceleration of seed germination was increased by degrees. Compared with the control, 60 ppm SAEW could induce seed germinated one day in advance (Fig.1). In addition, when the time of seed soaked with 60 ppm SAEW from 1 to 10 h, the germination parameters were improved gradually and then decreased (Fig.S1). Meanwhile, SAEW caused a mass of changes to nutrient substances and enzymatic activities in watermelon seeds (Fig.S2-S3), which performed the ability of SAEW to accelerate the seed germination. To sum up, soaked in 60 ppm SAEW with 5 h could significantly promote water seed germination.

ABA and GA are the two major phytohormones regulating seed dormancy and germination of plants. ABA could induce and maintain seed dormancy and GA is responsible for the induction of seed germination (Shu et al. 2016; Tuan et al. 2018). Moreover, the degree of seed dormancy correlates with endogenous ABA levels in imbibed seeds rather than in dry seeds in various species, such as in *Arabidopsis thaliana*, lettuce, barley and tobacco (Ali-Rachedi et al., 2004; Gonai et al., 2004; Jacobsen et al., 2002; Grappin et al., 2000). In addition, the ABA accumulation in dry seed decreased rapidly after seed imbibition (Okamoto et al., 2006). In this study, it revealed that SAEW could dramatically decrease the ABA accumulation while increase the GA content (Fig.2), which gave rise to the accelerated germination. The endogenous ABA and GA level in seed is determined by a balance between its biosynthesis and catabolism. The ABA is mainly biosynthesized by *NCEDs, ZEP, AAO* and catabolized by *CYP707As* (Nambara and Marion-Poll, 2005). Meanwhile, the GA is biosynthesized by *GA3ox, GA13ox, GA20ox* and catabolized by GA2ox respectively (Tuan et al., 2020). In addition, some previous studies have revealed the roles of genes encoding these enzymes in regulating seed GA levels and germination in several plant species including *Arabidopsis thaliana* and wheat (Ogawa et al., 2003; Mitchum et al., 2006; Kashiwakura et al., 2016;

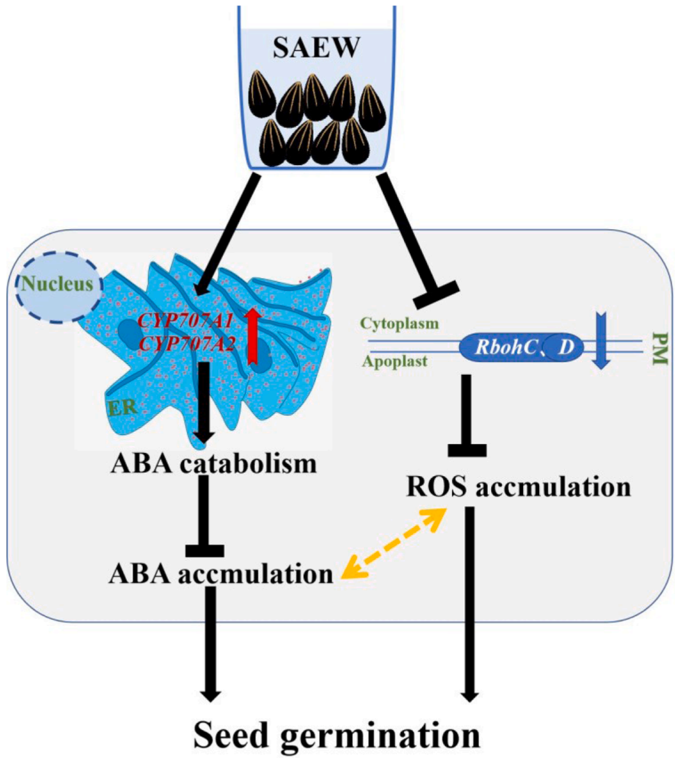

Fig. 6. The mechanistic model of slightly acidic electrolyzed water (SAEW)-induced germination in watermelon seeds. The germination of seeds was significantly promoted by 60 ppm SAEW. SAEW could increase the expressions of *CYP707A1* and *CYP707A2*, which accelerated the ABA catabolism and then reduced the accumulation of ABA. In addition, the expressions of *RbohC* and *RbohD* were further decreased, which gave rise to the reduced accumulation of ROS. Ultimately, the germination of watermelon seeds was improved by the imbibition with SAEW. However, the interrelation between ABA and ROS in seed germination, pointed by the yellow double-headed arrow, need to be further explored. ER stood for the endoplasmic reticulum and PM represented the plasma membrane.

Izydorczyk et al., 2018). According to the expression analyses of primary genes, it suggested that SAEW mostly decreased the transcriptional levels of genes involved in ABA biosynthesis such as *ZEP, NCED3* and *AAO3* (Fig.3A). What's more, the transcriptions of *CYP707A1* and *CYP707A2* were signally increased by SAEW, which may contribute to the reduction of ABA content in seeds. In addition, as the time went by, the expressions of *CYP707A1* and *CYP707A2* were gradually increased in both Con and SAEW treatment (Fig.3A). It was consistent with the result of previous study which revealed that the transcription of *CYP707A2* accumulated predominantly in the dry seed and immediately up-regulated after seed imbibition (Kushiro et al., 2004). Beyond that, transcriptions of *GA3ox, GA13ox, GA20ox*, which were responsible for GA biosynthesis, were mostly induced by SAEW and gradually elevated with the extension of time (Fig.3B). All the results suggested that SAEW-induced germination may arise from the depressed ABA accumulation while raised GA accumulation. Moreover, SAEW reduced ABA content in seeds mainly by the promotion of ABA catabolism.

4.2. Reactive oxygen species may play a role in the accelerated seed germination caused by slightly acidic electrolyzed water

ROS are increasingly identified as non-enzymatic factors in seed germination in some plant species like *Arabidopsis thaliana* and lettuce (Müller et al., 2009; Zhang et al., 2014; Choudhury et al., 2017; Yang et al., 2020). In this study, ROS accumulation was reduced by SAEW treatment, which was performed by the analyses of H_2O_2 and O^{2-} concentrations (Fig.4). Moreover, we found that DMTU could significantly improve the seed germination, which was in accordance with the promoted germination caused by SAEW treatment (Fig.5). That's to say, the reduced ROS accumulation may contribute to the improvement of germination induced by SAEW. The plasma membrane NADPH oxidases, encoded by the *Rbohs* family, are key enzymes for the production of ROS. Most of *Rbohs* are shown involved in the process of the response to abiotic stress and stomatal closure (Ma et al., 2012; Wang et al., 2016; Scuffi et al., 2018; Kámán-Tóth et al., 2019). However, only *RbohB* and *RbohC* have been identified so far in the process of seed development and germination (Müller et al., 2009; Yang et al., 2020). In the study, *RbohC, RbohD, RbohF* and *RbohH* were expressed in watermelon seeds (Fig.4D, E and Fig.S6). Thereinto, the expressions of *RbohC* and *RbohD* were up-regulated first but then them went down with the extension of germination time (Fig.4D and E). Furthermore, SAEW had the capacity for inhibition of *RbohC* and *RbohD* transcript, which may lead to the reduced accumulation of H_2O_2 and O^{2-} (Fig.4B and C). However, *RbohF* and *RbohH* were not significantly influenced by SAEW (Fig.S6). To sum up, the reduced ROS accumulation may give rise to the promoted germination caused by SAEW in watermelon. Even more to the point, the previous research demonstrated that the increased expression of *RbohD* is essential for ABA-mediated induction of ROS accumulation (Kwak et al., 2003). However, the relationship among endogenous ABA, ROS and seed germination in watermelon need to be further explored.

5. Authors' contributions

XW and KC contributed to the conception of the study. XW designed and performed the experiments. XW analyzed the data and wrote the manuscript. CW and ZB helped with graphical edit. ZY and LM modified the grammatical and format errors. LX and EB critically reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this research.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110581.

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