The Cross Talk Between ROS and NO in The Viability Declination of Cryopreserved-Pollen From *Paeonia Lactiflora*

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Research Article

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Abstract

Reactive oxygen species (ROS) and nitric oxide (NO), as two common active molecules, are both involved in the changes of viability after liquid nitrogen (LN) stored, but the relationship between these two molecules has not been involved in plant cryopreservation up to now. In this study, the pollen of *Paeonia lactiflora* 'Fen Yu Nu' with significantly decreased viability after cryopreservation as materials, by studied the effects of the two regulators on each other and their biosynthesis and scavenging indexes, to explore the interaction between ROS and NO in pollen cryopreservation and its mechanism.

The results showed that: the contents of ROS and NO increased significantly with the decrease of pollen viability after cryopreservation, and regulated ROS and NO content had a significant effect on post-LN pollen viability; the changes of ROS content positively regulated the endogenous NO content, and had significant effects on the expression level of NOS-like enzyme regulation gene *CSU2* and its activity; while regulated NO had a positive effect on ROS content, and significantly affected the expression of NADPH oxidase and its regulatory gene *RBOHJ*, and also significantly affected catalase (CAT) and the substrate related to ascorbic acid (AsA)- glutathione (GSH) antioxidant cycle system. These results indicate that there was a positive interaction between ROS and NO in the pollen cryopreservation, NOS biosynthesis pathway was one of the ROS regulated NO pathways, NADPH oxidase, CAT and AsA-GSH antioxidant cycle system were the key sites for NO regulated ROS content.

Key Message

There was a positive interaction between ROS and NO in the declination of viability of *Paeonia lactiflora* 'Fen Yu Nu' pollen after cryopreservation.

Introduction

Pollen carries all the male genetic information of plants, and its safe and effective preservation has great significance for the conservation of plant germplasm resources. A large number of studies show that the cryopreservation was one of the key ways to preserve germplasm for a long time (Zhang *et al*., 1993; Souza *et al*., 2015; Engelmann, 2004), which has been successfully applied to the long-term preservation of pollen of many species (Li, 2005; Zhang, 2007; Shang, 2005; Li, 2010; Xu, 2014). However, some problems have been found in the practical application, pollen viability mainly showed three different trends after cryopreservation, and in most cases, it was significant decreased (Ren *et al*., 2019a). According to existing studies, the decrease of pollen viability after cryopreservation is mainly due to osmotic stress and low temperature stress during preservation of liquid nitrogen (LN). Further studies have found that reactive oxygen species (ROS) and nitric oxide (NO) may played a key role in this process (Ren *et al*., 2020b; Ren *et al*., 2021).

ROS and NO as the signal transductions molecules in plant and animal cells, which response to stress and regulated a variety of physiological phenomenas (Wilkins *et al*., 2011). Studies in plant stress have

not only clarified the changes of ROS and NO, but also discussed the relationship and the mechanism of action between these two factors. In the occurrence of PCD of *Nicotiana tobacillus* suspension cells induced by chitosan, a large amount of ROS synthesis was induced with the production of NO (Zhang *et al*., 2012). NO production was enhanced by ROS production during the occurance of PCD in suspension cells of *Nicotiana tobacillus* callus induced by sphingine and dihydrosphingine (Da Silva *et al*., 2011). The relationship between these two molecules and their possible action pathways were further proved by exogenous addition experiments. The study of Esim and Atici showed that exogenous spraying of NO could improved the activity of antioxidant enzymes and reduced the ROS level of *Zea mays* under low temperature stress, which played an important role in improving cold resistance (Esim and Atici, 2014). Xiao's study found that exogenous NO donor SNP increased the activity of NADPH and $NADP^+$, while NO scavenger c-PTIO, NADPH oxidase inhibitor DPI and H_2O_2 scavenger DTU inhibited the effect induced by the SNP, indicated that interaction between ROS and NO in *Cucumis sativus* seedlings under cold stress, NADPH and $NADP^+$ activity may be the critical sites (Xiao, 2014).

In the plant cryopreservation, it has been found that the stress involved in the process of LN preservation has a certain influence on the production of intracellular ROS and NO, and the production of ROS and NO was involved in the changes of viability after LN storage. Zhang in the cryopreservation of callus from *Agapanthus praecox*, found that ROS content significantly increased after preservation with LN, and it had a significant effect on cell activity after cryopreservation by induced oxidative stress and programmed cell death (Zhang *et al*., 2015). In cryopreservation of protocorms of *Dendrobium nobile*, Jiang found that NO produced during the cryopreservation process, and had dual regulatory effects on cell activity after preservation of LN (Jiang *et al*., 2019). Further studies on cryopreservation of *Dendrobium nobile* protocorms showed that there was a certain relationship between ROS and NO (Jiang *et al*., 2019). But the existing studies on how these two molecules work has not be addressed in plant cryopreservation.

In addition, studies on ROS and NO in pollen cryopreservation are relatively rare, and most of the existing studies only focus on the production of ROS and its effects on the viability after preserved in LN. Ren in the cryopreservation of *Paeonia lactiflora* pollen found that the content of ROS was significantly increased after preservation with LN, and ROS had a significant impact on the post-LN viability by mediating the occurrence of PCD (Ren *et al*., 2020b). And in the cryopreservation of *Paeonia lactiflora*, NO was also found to be involved in the changes of pollen viability after preservation with LN by regulating PCD (Ren *et al*., 2021). Thus, in this study, the pollen of *Paeonia lactiflora* 'Fen Yu Nu' with significantly decreased viability after cryopreservation was used as materials, compared and analyzed the changes of ROS and NO contents before and after cryopreservation, and studied the effects on each other and related synthetase by using their modulators respectively, to clarify the relationship between ROS and NO in pollen cryopreservation and their possible pathways of action.

Materials And Methods

Pollen collection and cryopreservation

Pollen of *P. lactiflora* 'Fen Yu Nu' was collected at the International Peony Garden in Luoyang, Henan province, on April 29, 2019. When the dry and humidity of air was appropriate, selected the bud that is about to open, gently picked the anther and brought it back under the 4℃. Then placed the anthers at room temperature (23±2℃) for 24 h to completely dispersed the pollen. The pollen was collected with an 80 mm aperture sieve (Ren *et al*., 2019b).

Divide the collected pollen (0.1 g/ package) into tin foil, placed it in a 2 mL cryopreserved tube, and then directly put it into LN for storage. When needed, the pollen was thawed by running water (Thaw) or thawing at room temperature (LN).

Pollen moisture content and viability determination

Pollen moisture content was determined by 105℃ constant temperature drying method (Ren *et al*., 2019b).

Pollen germination rate was detected by hanging drop culture method. The medium was 10% sucrose and 0.1% boric acid. The culture condition was under 25℃ constant temperature for 4 h. Pollen tube length greater than twice of the pollen grains diameter was the criterion for pollen germination. Four replicates every treatment, and three fields were randomly selected for each replicate. The germination number was counted with a 20X eyepiece (Leica DM-500), and the mean value of the results was taken (Ren *et al*., 2019b).

Pollen NO content determination

NO content was determined by DAF-FM DA fluorescence staining. The pollen (0.01 g) was added with 200 μL 100 μmol/L DAF-FM DA fluorescent probe (Biyuntian Biotechnology Co., Ltd., S0019) in the dark state and thoroughly mixed. Then incubated at 37℃ for 30 min without light, centrifuged at 2000 rmp for 20 s, then discarded the dye. Washed the fluorescent loading pollen twice with 500 μL PBS (pH= 7.4), 1 mL PBS (pH= 7.4) resuspended the precipitation pollen. Used flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) to measure the fluorescence values at FITC channels, the actual fluorescence measurement value as the relative content of NO, and each treatment repeated 3 times.

Pollen ROS content determination

ROS content determined by fluorescence staining with DCFH-DA (Xu, 2014). A weight of 0.01 g pollen added with 200 μL 100 μmol/L DCFH-DA fluorescent probe (Sigma Chemical Co., St Louis, MO, USA, D6883-50MG) without light and thoroughly mixed. The pollen was incubated at 37℃ for 30 min in the dark, centrifuged at 2000 rmp for 20 s, and discarded the dye solution. Washed the pollen twice with 500 μL PBS (pH=7.4), then 1mL PBS (pH=7.4) resuspended the fluorescent loading pollen. Fluorescence was measured at FITC channel by flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA), taken the actual fluorescence measurement value as the NO content, and every treatment repeated 3 times.

Pollen NOS-like activity determination

NOS-like activity was determined by a kit (S0025) provided by Biyuntian Biotechnology Co., Ltd. Pollen (0.01 g) was added to 100 μL NOS detection buffer, and then added 79.6 μL Milli-Q water, 10 μL arginine solution, 10 μL NADPH solution (0.1 mM) and 0.4 μL DAF-FM DA solution (5 mM), then incubated at 37℃ for 30 min without light. Used the flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) detected the fluorescence value at the FITC channel, taken the actual fluorescence measurement value as the NOS-like activity, and each treatment repeated 3 times.

Pollen NDAPH oxidase activity determination

NDAPH enzyme activity was determined according to the NADP+/NADPH detection kit (S0179) provided by Biyuntian Biotechnology Co., Ltd. Ground the pollen (0.01 g) to homogenate in ice bath with 400 μL $NADP^+$/NADPH extract, and centrifuged at 12,000 rpm at 4°C for 10 min. Added with 200 μL G6PDH working solution into 100 μL extraction solution, incubate at 37°C for 10 min without light. Then add 10 μL chromogen solution, mixed well and incubated for 60 min under the constant temperature of 37°C without light. Measured the absorbance at 450 nm with an ultraviolet spectrophotometer, each treatment repeated three times, and the average of the results was taken.

Pollen antioxidants determination

SOD determined by nitrogen blue tetrazole (NBT) reduction method (Li et al., 2000). Homogenated 0.05 g pollen with 1 mL of 0.05 mol/L phosphoric acid buffer in ice bath, and 4℃ centrifuged at 10,000 rpm for 15 min. Added 1.5 mL phosphoric acid buffer (0.05 mol/L), 1.5 mL methionine solution (130 mmol/L), 300 μL NBT solution (750 mol/L), 300 μL EDTA-Na_2 (100 mol/L) and 300 μL riboflavin solution (20 μmol/L) into 300 μL extract solution without light. Mixed and subjected to 4000 Lx ray reaction for 15 min, and the reaction was terminated in darkness. Determined the absorbance value by spectrophotometer at the wavelength of 560 nm, every treatment repeated three times, and the average of the results was taken.

CAT was measured according to the Prochakova's method and with slight modifications (Prochazkova *et al.*, 2001). Pollen (0.05 g) was homogenated with 1 mL 0.05mol/L phosphate buffer in ice bath, and centrifuged at 10,000 rpm at 4℃ for 15 min. Added 1.5 mL phosphoric acid buffer (pH= 7.0) and 1.0 mL distilled water into 200 μL extract solution. Mixed and added 300 μL hydrogen peroxide solution (0.1 mol/L) to initiate the reaction. The absorbance value was measured at the wavelength of 240nm by the spectrophotometer. Each treatment repeated three times, and the mean value was taken.

GR activity was determined according to the glutathione reductase assay kit (S0055) provided by Biyuntian Biotechnology Co., Ltd. and with slight modifications. A liquid of 1 mL phosphate buffer solution (0.01 mol/L) was taken to ground 0.03 g pollen into homogenate in ice bath, and then centrifuged at 4℃ at 10,000 rpm for 15min. The supernatant was collected for the determination of GR activity, every treatment repeated three times.

APX activity was determined using the method of Nakano and Asada with slight modification (Nakano and Asada, 1981). A liquid of 1 mL APX enzyme extraction reagent (50 mmol/L PBS+2 mmol/L AsA+5 mmol/L EDTA) was taken to ground 0.03 g pollen into homogenate in ice bath, and centrifuged at 10,000 rpm at 4℃ for 20 min. In 50 μL supernatant extract, added 2 mL PBS (50 mmol/L, pH=7.0), 500 μL AsA solution (5 mmol/L) and 500 μL EDTA-Na_2 (1 mmol/L), then added 50 μL H_2O_2 (30%) initiated the reaction. Measured the absorbance at 290 nm with a spectrophotometer, each treatment repeated three times, and the results were averaged.

AsA content was determined by Kampfenkel's method and with some modifications (Kampfenkel *et al*., 1995). Ground 0.03 g pollen with 1.5 mL trichloroacetic acid (10%) into homogenate in ice bath, and centrifuged at 10,000 rpm at 4℃ for 10 min. Added 200 μL sodium dihydrogen phosphate solution (150 mmol/L) and 200 μL ddH_2O into 200 μL supernatant extract, mixed at least 30 s. Then added 10% trichloroacetic acid solution, 44% phosphoric acid solution and 4% 2, 2-dipyridine solution in turn, 400 μL each. Mixed and initiated reaction by added 200 μL $FeCl_3$ (3%), then incubated at 37℃ for 1 h. Measured the absorbance value at 525 nm by spectrophotometer, each treatment repeated 3 times, and the mean value of the results was taken.

GSH content was determined by 2-nitrobenzoic acid (DTNB) reduction method (Griffith, 1980). A liquid of 1.5 mL trichloroacetic acid (10%) was taken to ground 0.03 g pollen into homogenate in ice bath, and centrifuged at 10,000 rpm at 4℃ for 10 min. Added 0.5 mL ddH_2O, 1 mL phosphate buffer (1 mmol/L, pH= 7.7) and 0.5 mL DTNB (4 mmol/L) into 0.5 mL supernatant extract, mixed well and reacted for 10 min at 25℃. Measured the absorbance at 412 nm by spectrophotometer, each treatment repeated three times, and the average of the results was taken

qRT- PCR analysis of pollen ROS and NO related genes

Pollen total RNA extracted by the Plant RNA Rapid Extraction Kit (RN38-EasySpin Plus) provided by Beijing Aidlab Biotechnologies Co., Ltd.; cDNA was synthesized by the Rever Tra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Osaka, Japan); primers were designed by the IDT online software (https://sg.idtdna.com/Primerquest/Home/Index) and synthesized by Beijing Ruiboxingke Biological Technology Co., Ltd (Tab. S1 and Tab. S2).

According to the instructions of SYBR Premix EX Taq (Takara, Otsu, Japan) on the MiniOpticon™ real-time PCR detection system (Bio-Rad®, Hercules, CA) performed the real-time quantitative PCR. The amplification protocols for qRT-PCR included an initial denaturing step (95°C for 30 s), followed by 40 cycles of 95°C for 10 s, Tm(°C) for 15 s, and 72°C for 15 s; 65°C for 5 s and 95 °C for 5 s (Wan *et al*., 2019). The reaction system was 20 μL, including 1 μL cDNA, 2 μL forward primer (10 μmol/L), 2 μL reverse primer (10 μmol/L), 5 μL ddH_2O, and 10 μL SYBR® Premix Ex Taq II (Tli RNase Plus) (2X). The expression level of the target gene was calculated by the $2^{-\Delta\Delta Cq}$ method, and each treatment was repeated 3 times.

Addition of ROS and NO exogenous regulator

The pollen without running water treatment after preserved in LN was respectively added with 0.8 mmol/L NO carrier SNP (228710-5G, Sigma Chemical Co., St Louis, MO, USA), 0.8 mmol/L NO scavenger c-PTIO (C221-10MG, Sigma Chemical Co., St Louis, MO, USA), 200 mmol/L ROS scavenger AsA (A7506-25G, Sigma Chemical Co., St Louis, MO, USA) or 0.16 mmol/L ROS scavenger GSH (G6013-5G, Sigma Chemical Co., St Louis, MO, USA) solution at a ratio of 1:100 (*w/v*), and was thoroughly mixed. Incubated for 10 min in the dark at 37°C, then washed the pollen incubated with the regulator three times with phosphate buffer of 0.01 mol/L (PBS, pH= 7.4). After centrifugated 2000 rpm for 30 s, the precipitated pollen was used for the determination of various indexes, three repeats were set for each treatment.

Statistical analysis

Flow data was processed and analyzed with FlowJO software; SPSS 17.0 (Version 17.0 SPSS Inc., Chicago, IL, USA) was used for one-way ANOVA analysis and correlation analysis; Microsoft Excel 2013 software (Microsoft Corp., Richmond, CA, USA) was used to draw charts and tables.

Results

Changes of pollen viability during cryopreservation and the relationship with ROS and NO

The moisture content of the pollen of *Paeonia lactiflora* 'Fen Yu Nu' was 8.10%, and its fresh pollen viability was 22.73%. However, cryopreservation significantly reduced pollen viability (Tab. 1), especially the viability of the pollen that has not unfrozen by running water after preservation with LN (LN) was significantly lower than the fresh pollen, which decreased by 6.31%; while, the viability of pollen thawing by running water after LN storage (Thaw), was only reduced by 1.93% compared with the fresh pollen.

Cryopreservation resulted significant changes in the ROS and NO content of *Paeonia lactiflora* 'Fen Yu Nu' pollen ($P < 0.05$). The ROS content of pollen was significantly higher than the fresh pollen after LN stored with and without running water thawing treatment, which respectively increased by 42.85% and 47.38% (Fig. 1- A, B, C, D).

The NO content of *Paeonia lactiflora* 'Fen Yu Nu' pollen with and without running water treatment after LN preserved were significantly higher than that of the fresh pollen ($P < 0.05$), and 92.71% and 94.14% higher than the control, respectively (Fig. 1- E, F, G, H).

Before and after cryopreservation, pollen viability was negatively correlated with the content of ROS and NO ($P < 0.05$).In addition, ROS content was positively correlated with NO content ($P < 0.01$) (Tab. 2).

Interaction between ROS and NO during pollen cryopreservation

ROS scavengers AsA and GSH at appropriate concentrations not only significantly increased the pollen viability level after LN stored (Fig. 2- A), but also significantly inhibited the endogenous ROS content (Fig.

3- A, B, C, D). At the same time, the addition of ROS scavengers AsA and GSH significantly reduced the endogenous NO content of pollen ($P < 0.05$), which were 31.23% and 25.96% lower than that of without ROS scavengers treatment (Fig. 3- E, F, G, H).

The appropriate concentration of NO carrier SNP had a significant inhibitory effect on pollen viability after LN storage (Fig. 2- B), but increased the content of endogenous NO; while the addition of NO scavenger c-PTIO increased pollen viability after preservation with LN, and significantly inhibited the content of endogenous NO (Fig. 4- A, B, C, D). In addition, added appropriate concentration of NO carrier SNP after LN stored significantly increased the ROS content of pollen ($P < 0.05$); while the addition of NO scavenger c-PTIO significantly inhibited the accumulation of endogenous ROS content in pollen after LN stored (Fig. 4- E, F, G, H).

The interaction pathways of ROS and NO during pollen cryopreservation

Compared with the LN preserved pollen, the addition of ROS scavenger AsA after preservation with LN significantly down-regulated the expression of *CSU2* (Fig. 5-B), which was the regulatory gene of NOS biosynthesis pathway, but had no significant effect on the expression of *NIA* and *CYP94A1* (Fig. 5-A, C), the regulatory gene of nitrate reductase biosynthesis pathway; while the addition of GSH had no significant effect on the expression of these three genes (Fig. 5).

ROS scavengers AsA and GSH had significant inhibitory effects on the activities of NOS-like enzymes after cryopreservation ($P < 0.05$), which were decreased by 51.16% and 54.59% compared with the control, respectively (Fig. 6).

After cryopreservation, the addition of exogenous NO carrier SNP significantly up-regulated the expression levels of *RBOHJ*, *CAT1*, *DHAR2* and *GSTU8* genes, but significantly down-regulated the expression levels of *APX3*, *GPX6* and *GSH2*. However, the addition of NO scavenger c-PTIO after cryopreservation significantly down-regulated the expression levels of *RBOHJ*, *SODA*, *APX3*, *DHAR2*, *GSTU8*, *GPX6*, and *GSH2*, but had no significant effect on the expression levels of *CAT1* (Fig. 7).

The addition of exogenous NO carrier SNP after LN stored significantly promoted the activation of NADPH enzyme ($P< 0.05$); and the addition of NO scavenger c-PTIO significantly inhibited the activity of NADPH enzyme (Fig. 8). After cryopreservation, the appropriate concentration of NO carrier SNP significantly inhibited the activities of enzyme ROS scavenging substances CAT, APX, GR and the content of non-enzyme ROS scavenging substances AsA ($P < 0.05$), but significantly increased the SOD activity of pollen. However, the addition of NO scavenger c-PTIO not only significantly improved the activities of enzyme scavengers CAT, APX and GR (Fig. 9-B, C, D), but also significantly promoted the accumulation of non-enzymatic AsA and GSH (Fig. 9-E, F).

Discussion

Effects of ROS on NO and its pathways in pollen cryopreservation

A large number of studies have shown that ROS involved in the regulation of NO production. In the study of *Medicago truncatula* ender low temperature stress, it was found that H_2O_2 treatment accelerated the accumulation of NO content, which played a key role in improved the efficiency of replacing oxidase (AOX) and alleviated the damage of photosystem under low temperature stress (Arfan *et al*., 2019). In the root development of *Arabidopsis thaliana*, exogenous H_2O_2 increased NO levels by 8 times (Wang *et al*., 2010). Exogenous H_2O_2 also triggered NO production in guard cells and other leaf cells of *Phaseolus aureus* (Lum *et al*., 2002). In this study, exogenous ROS scavengers AsA and GSH at appropriate concentrations significantly reduced the endogenous NO content of pollen after cryopreservation (Fig. 3-E, F, G, H). It can be seen that ROS was involved in the regulation of intracellular NO content during cryopreservation of *P. lactiflora* pollen.

The biosynthesis of NO in plant cells during stress response was mainly achieved through L-arginine dependent pathway and nitrate reductase pathway (Ma and Berkowitz, 2016). Among them, the NOS-like enzyme synthesis pathway dependent on L-arginine was the most widely studied one (Leitner *et al*., 2009). Although nitric oxide synthase (NOS) has not been found in plants, but it has been reported that NOS inhibitors have a significant inhibitory effect on NO signaling in plants, indicating the existence of NOS enzymes in plants similar to those found in mammals (Tewari *et al*., 2013). Previous studies have shown that the accumulation of H_2O_2 inducesd NO production by increased the expression of nitrate reductase involved in NO biosynthesis (Lin *et al*., 2012; Wang *et al*., 2013). However, in this study, the addition of ROS scavengers AsA and GSH at appropriate concentrations did not significantly affected the regulatory genes involved in the nitrate reduction pathway involved in NO biosynthesis, and only AsA significantly down-regulated the expression level of the regulatory gene *CSU2* involved in the NOS-like enzyme biosynthesis pathway dependent on L-arginine (Fig.5); while ROS scavenging agent with appropriate exogenous concentration significantly reduced the activities of NOS-like enzymes in the cryopreserved-pollen at the biochemical level (Fig. 6). These results indicate that in pollen cryopreservation, ROS participates in the regulation of intracellular NO content changes, which may mainly by acting on NOS-like enzymes involved in NO biosynthesis.

Effects of NO on ROS and its pathways in pollen cryopreservation

Up to now, a large number studies have shown than the changes of NO play an important role in ROS changes of cells. Xiao found that NO played a positive regulatory role on ROS in *Cucumis sativus* seedlings under cold stress (Xiao, 2014). Jiang found NO produced positive effect to ROS content during the preculture stage of vitrification-cryopreservation in *Dendrobium* protocorm-like bodies (Jiang, 2019). However, some studies have found that NO plays a negative regulatory role in the accumulation of ROS. He found that the addition of NO carrier SNP reduced the excess H_2O_2 in the root tips of *Arachis hypogaea* during the process of aluminum stress induced PCD; while the addition of NO scavenger c-PTIO produced the opposite effect to that of SNP (He *et al*., 2019). This difference in NO regulation of endogenous ROS in botanical cells may be due to the dual role of NO in the occurrence of physiological and biochemical phenomena, which depends on a variety of factors, such as cell type, cell redox status,

and local NO flux and dose (Wang *et al*., 2010), but the specific mechanism of regulatory differences remains to be further explored. In this study, the addition of appropriate concentration of NO carrier SNP after LN stored significantly promoted the accumulation of endogenous ROS; while added appropriate concentration of NO scavenger c-PTIO significantly reduced the content of endogenous ROS (Fig. 4- E, F, G, H). It can be seen that NO plays a positive role in the regulation of intracellular ROS accumulation during pollen cryopreservation. This is consistent with the findings of Jiang in the cryopreservation of *Dendrobium nobile* procorms, when the intracellular concentration of NO decreased, the accumulation of H_2O_2 also significantly decreased, it indicated that NO plays a positive regulatory role on ROS (Jiang *et al*., 2019).

In plant response to stress, further studies showed that NADPH oxidase involved in ROS production was one of the key pathways in NO regulation of intracellular ROS concentration. Yun in plant immune reactions found that NO affected ROS synthesis through S- nitrosolation NADPH oxidase (Yun *et al*., 2011). Wang's study also showed that NO may control the production of ROS through protein S-nitrosylation (Wang *et al*., 2013). In this study, it was found that the addition of NO carrier SNP significantly promoted the activation of NADPH oxidase and up-regulated the regulatory gene *RBOHJ* in pollen after LN storage; and the addition of NO scavenging agent c-PTIO after preservation with LN inhibited the activity of NADPH oxidase and the relative expression of its regulatory gene *RBOHJ* (Fig.7-8). These results indicates that NO participated the regulation of endogenous ROS accumulation by acting on NADPH oxidase during pollen cryopreservation.

In addition, antioxidant system may be another key pathway for NO to regulated the intracellular ROS concentration. Murgia in callus suspension cells of *Arabidopsis thaliana* and *Nicotiana tobacum* found that NO carrier SNP inhibited the ROS scavenging system, thus accelerated the intracellular ROS accumulation (Murgia *et al*., 2004). Similarly, NO carrier SNP inhibited antioxidant enzymes of *Zinnia elegans* xylem (Ferrer and Ros Barceló, 1999), and a similar inhibitory effect of SNP on antioxidant enzymes in the leaves of oxidizing damaged *Solanum tuberosum* (Beligni and Lamattina, 2002), then reduced the endogenous ROS content. In this study, it was found that the addition of appropriate concentration of NO carrier SNP after LN stored had significant inhibitory effects on ROS scavenging substances CAT, APX, GR and AsA, and significantly down-regulated the expression levels of related regulatory genes *APX3*, *GPX6* and *GSH2*; and the addition of NO scavenging agent c-PTIO significantly promoted the accumulation of CAT, APX, GR, AsA and GSH contents, and had a certain down-regulation effect on the expression levels of anti-oxidase related regulatory genes *SODA*, *APX3*, *DHAR2*, *GSTU8*, *GPX6* and *GSH2* (Fig.7, 9). It suggested that during the cryopreservation of pollen, NO regulated endogenous ROS content mainly by affected CAT activity and the related substrates of AsA-GSH circulatory system. This is consistent with the findings of Jiang in the cryopreservation of protocorms from *Dendrobium nobile*, NO participated in the regulation of endogenous ROS changes by effected the activity of endogenous ROS scavenging substances, thus participated in the changes of its viability (Jiang *et al*., 2019).

In a word, there was an interactive relationship between ROS and NO in pollen cryopreservation, which was consistent with a large number of existing research results (de Pinto *et al.*, 2002; Neill *et al.*, 2002; Zaninotto *et al.*, 2006). The regulation of NO content by ROS was mainly by acted on NOS-like enzymes involved in NO biosynthesis; the effect of NO on ROS was mainly through the action of NADPH oxidase involved in ROS generation and the AsA-GSH antioxidant cycle involved in ROS scavenging. This is consistent with the findings of *Murgia* in suspension cells of *Arabidopsis thaliana* and *Nicotiana tobacum* and Arfan in *Medicago truncatula* under low temperature stress (Murgia *et al.*, 2004; Arfan *et al.*, 2019).

Conclusion

This study first investigated the interaction and its pathways between ROS and NO during pollen cryopreservation. It is found that there was a two-way interaction between ROS and NO during pollen cryopreservation. Among them, the NADPH oxides involved in ROS production and the antioxidant cycling systems of AsA-GSH and CAT responsible for intracellular ROS scavenging were the key sites of NO regulated the changes of ROS; the biosynthesis pathway of NOS-like enzymes involved in NO production was one of the important pathways for ROS regulated the change of endogenous NO content (Fig.10).

Declarations

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Author contribution statement: Ruifen Ren designed the research, completed the experiments analyzed the data and drafted the manuscript. Hao Zhou offered some help on the material collection. Lingling Zhang offered some help on the technical aspects of the experiment. Xueru Jiang offered some help on the research design. Yan Liu conceived the project, supervised the analysis and critically revised the manuscript. All authors read and approved the manuscript.

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Tables

Table. 1 Moisture content and germination percentage of *P. lactiflora* pollen

Cultivar	Moisture content / %	Germination percentage / %		
		Fresh(CK)	LN	Thaw
P. lactiflora 'Fen Yu Nu'	8.10±0.59	22.73±1.64a	16.42±0.82b	20.80±2.27a

Notes: Values of moisture content represent the mean (± SE) over 3 detections; values of germination percentage represent the mean (± SE) over 12 detections. Different letters indicate significant differences among cryopreserved time at the 0.05 level (P< 0.05, Duncan's multiple range test).

Table.2 Correlation analysis among pollen viability, ROS content and NO content before and after cryopreservation

	Pollen viability	ROS content	NO content
Pollen viability	1.000		
ROS content	-0.742*	1.000	
NO content	-0.740*	0.854**	1.000

Notes: * was significantly correlated at 0.05 level; ** was significantly correlated at 0.01 level.

Figures

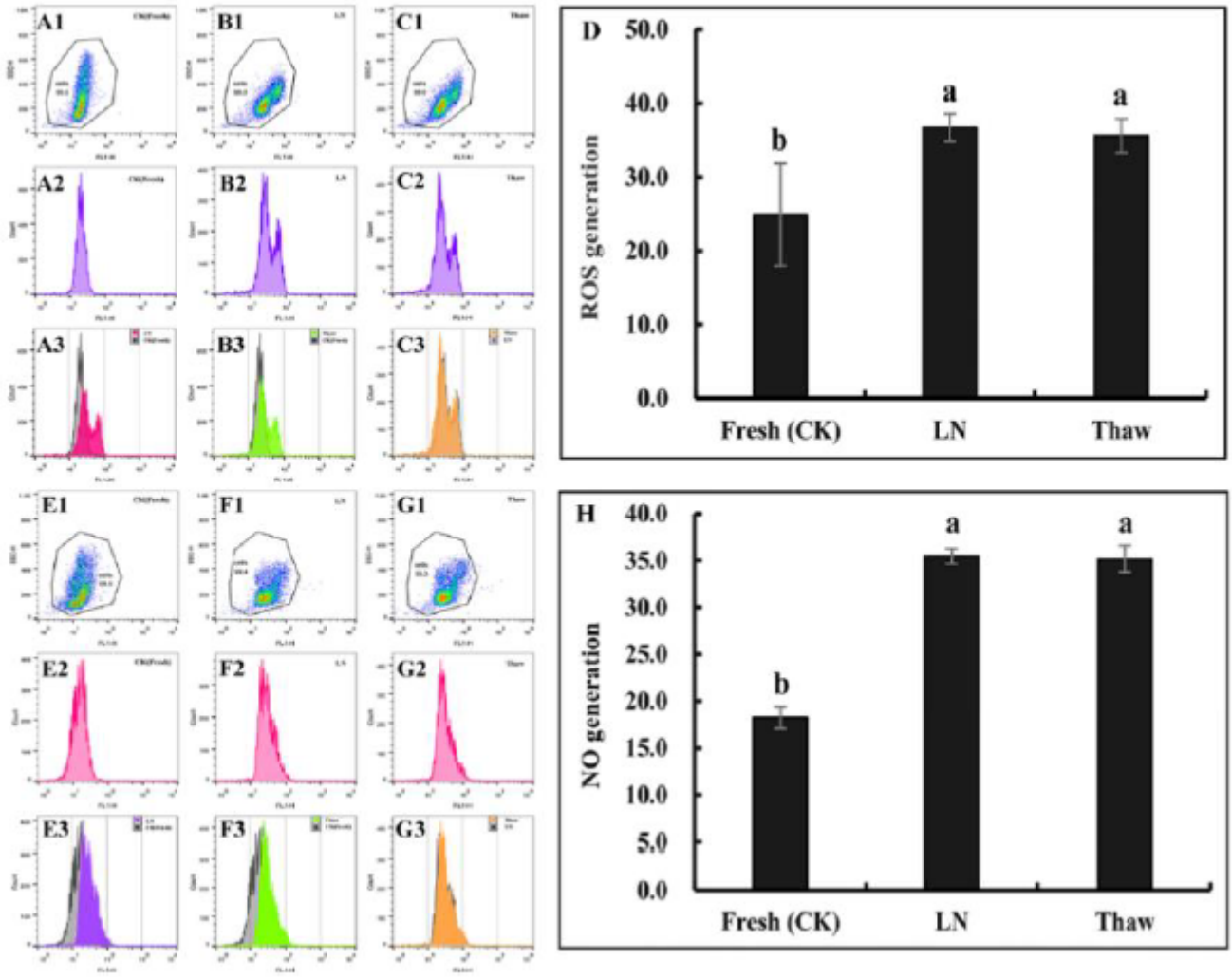

Figure 1

Changes of ROS and NO content in P. lactiflora pollen before and after cryopreservation. Notes: The statistical difference was based on the 0.05 level. A and E- fresh pollen (CK); B and F - LN preserved pollen (LN); C and G - the LN preserved pollen with thaw treatment (Thaw); D- the ROS content; H- the NO content of pollen. 1- the flow cytometry maps; 2- the flow spectrogram of DCFH-DA fluorescence (ROS fluorescent probe); 3- the comparison spectrograms.

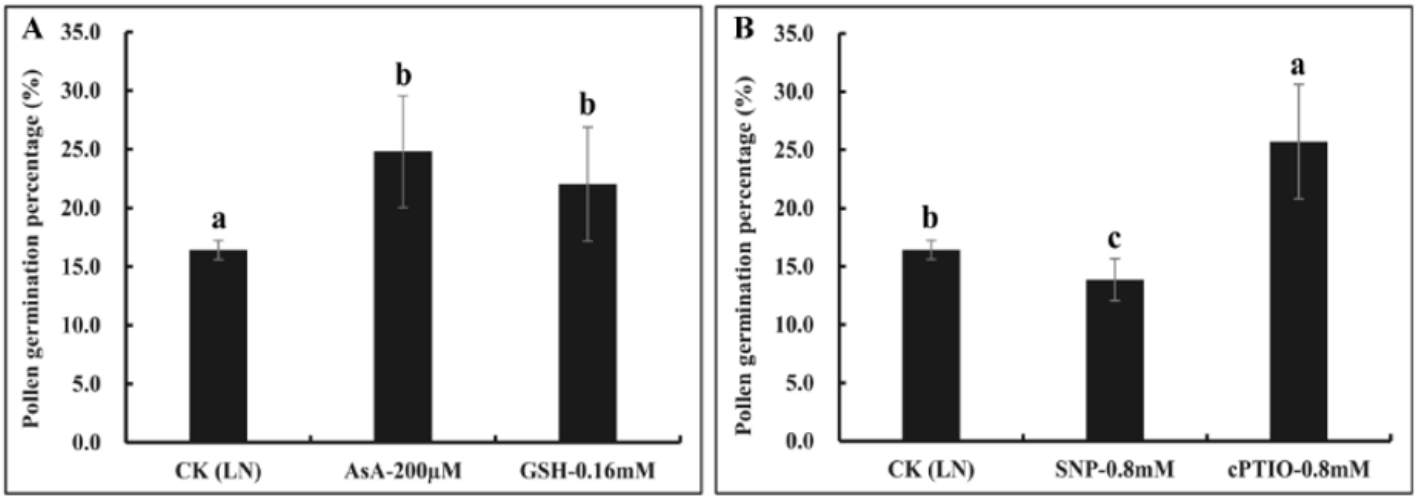

Figure 2

Changes of pollen vaibilty in P. lactiflora with and without exogenous regulator treatments after cryopreservation. Notes: The statistical difference was based on the 0.05 level. A- pollen viability changes with and without ROS regulator treatments; B- ROS content changes with and without ROS regulator treatments; C- pollen vaibility changes with and without NO regulator treatments; D- NO content changes with and without NO regulator treatments.

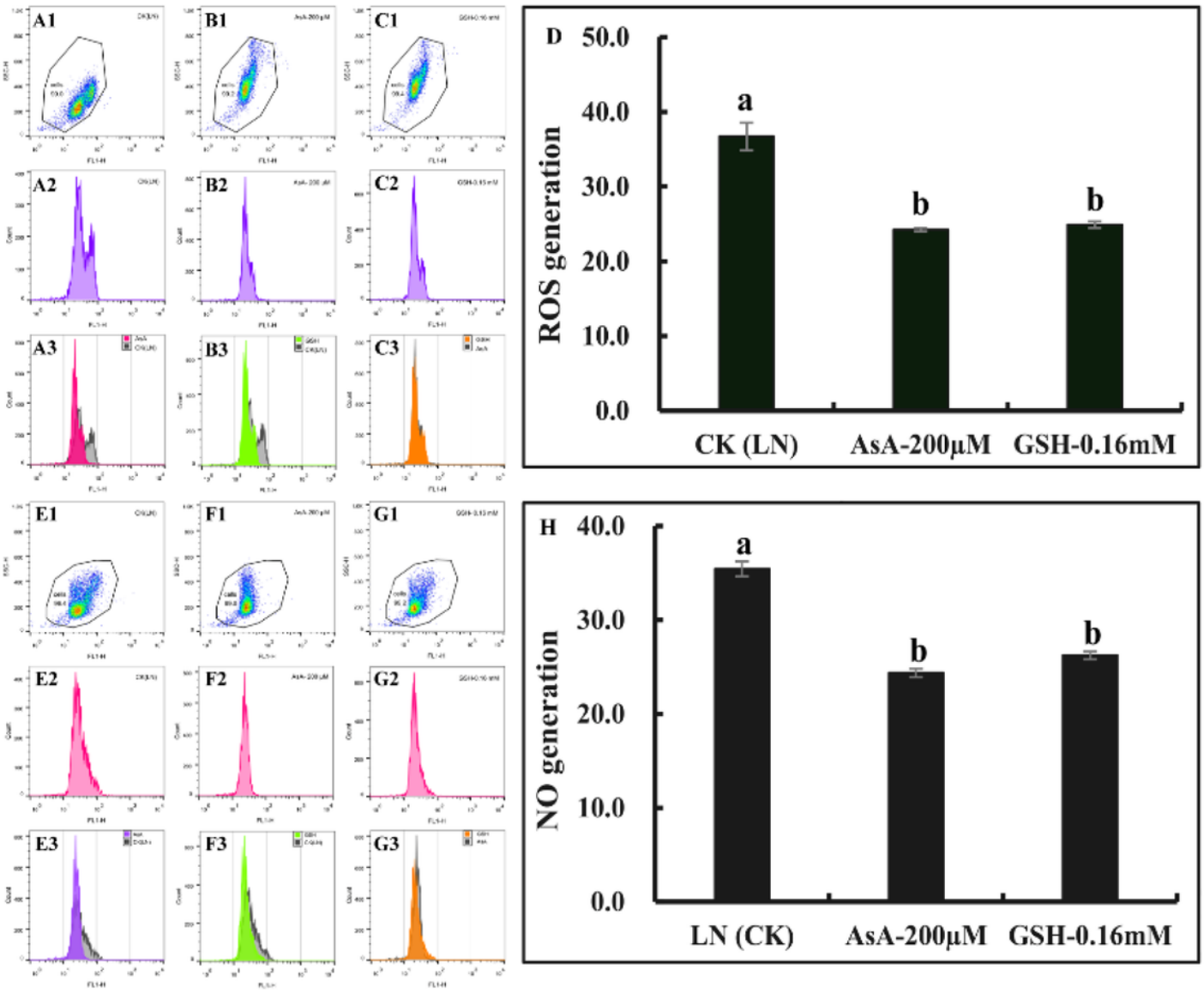

Figure 3

Changes of endogenesis ROS and NO content before and after exogenous ROS regulator treatments. Notes: The statistical difference was based on the 0.05 level. A-CK(LN) of ROS; B-LN+SNP treatment of ROS; C-LN+ c-PTIO treatment of ROS; D-ROS content changes with and without ROS regulator treatments; E- CK(LN) of NO; F- LN+SNP treatment of NO□G-LN+ c-PTIO treatment of NO; H-NO content changes with and without ROS regulator treatments; 1- the flow cytometry maps; 2- the flow spectrogram of DCFH-DA fluorescence (ROS fluorescent probe); 3- the comparison spectrograms.

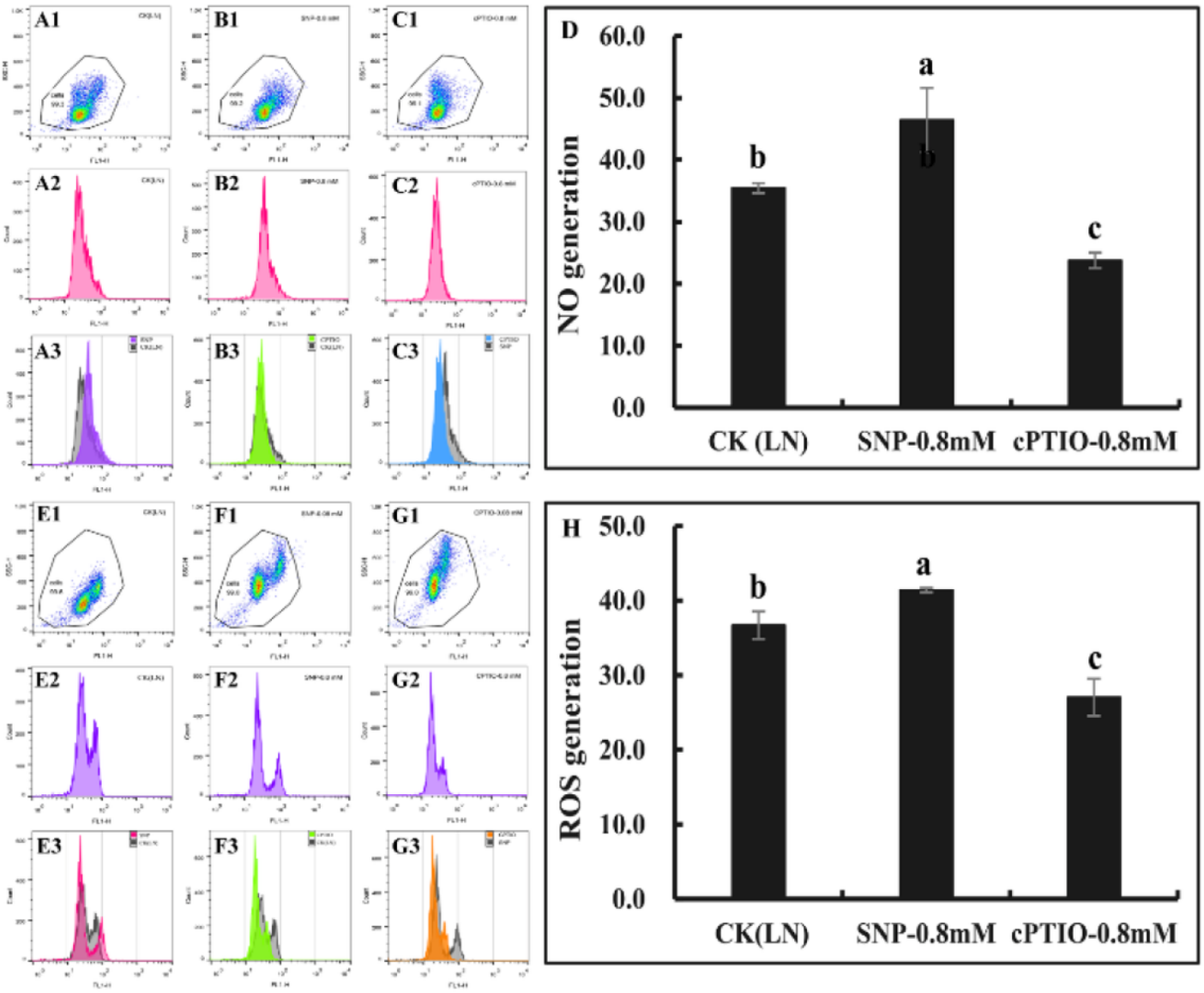

Figure 4

Changes of endogenesis NO and ROS content before and after exogenous NO regulator treatments. Notes: The statistical difference was based on the 0.05 level. A-CK(LN) of NO; B-LN+SNP treatment of NO; C-LN+ c-PTIO treatment of NO; D-NO content changes with and without NO regulator treatments; E-CK(LN) of ROS; F- LN+SNP treatment of ROS；G-LN+ c-PTIO treatment of ROS; H-ROS content changes with and without NO regulator treatments; 1- the flow cytometry maps; 2- the flow spectrogram of DCFH-DA fluorescence (ROS fluorescent probe); 3- the comparison spectrograms.

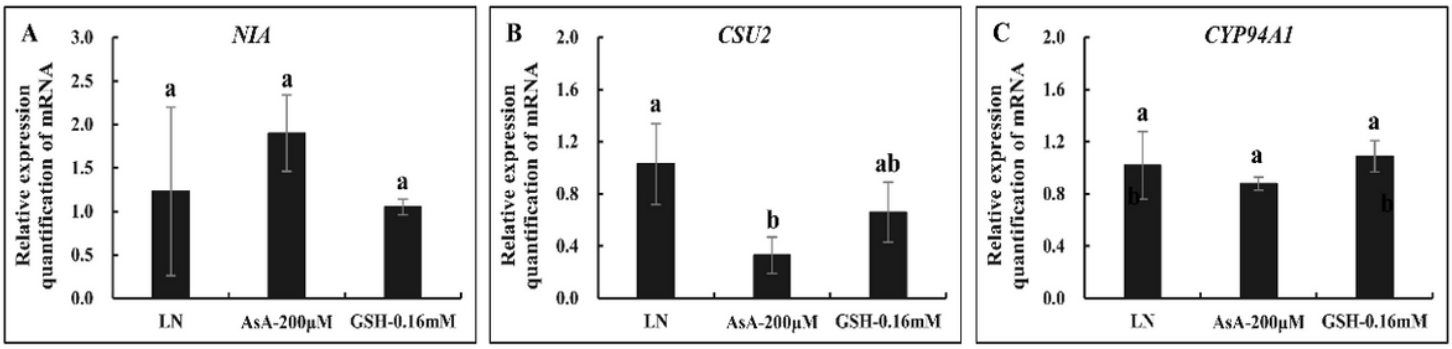

Figure 5

Changes of NO related genes in pollen with and without ROS regulter after cryopreservation. Notes: The statistical difference was based on the 0.05 level. A- NIA; B-CSU2; C- CYP94A1.

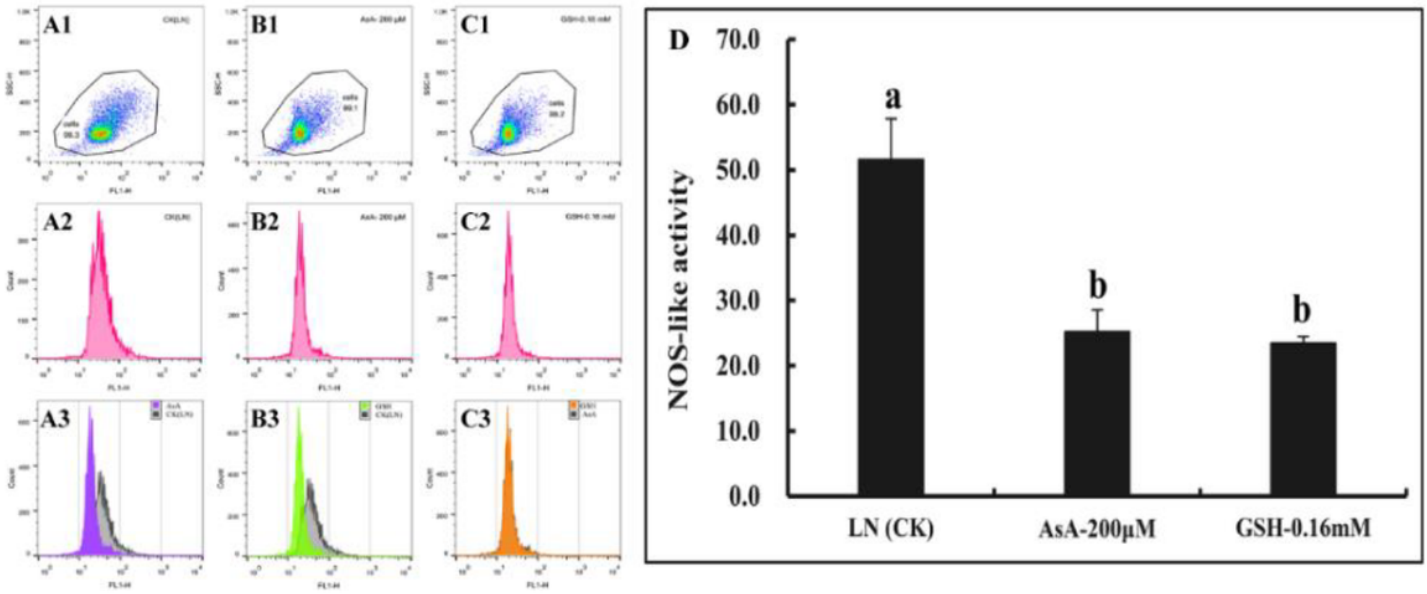

Figure 6

Changes of activity of NOS-like enzyme before and after exogenous ROS regulator treatments. Notes: The statistical difference was based on the 0.05 level. A- LN(CK); B-LN+ AsA; C-LN+GSH; D- the changes of activity of NOS-like enzyme before and after exogenous ROS regulator treatments; 1- the flow cytometry maps; 2- the flow spectrogram of DAF-FM DA fluorescence (NOS-like fluorescent probe); 3- the comparison spectrograms.

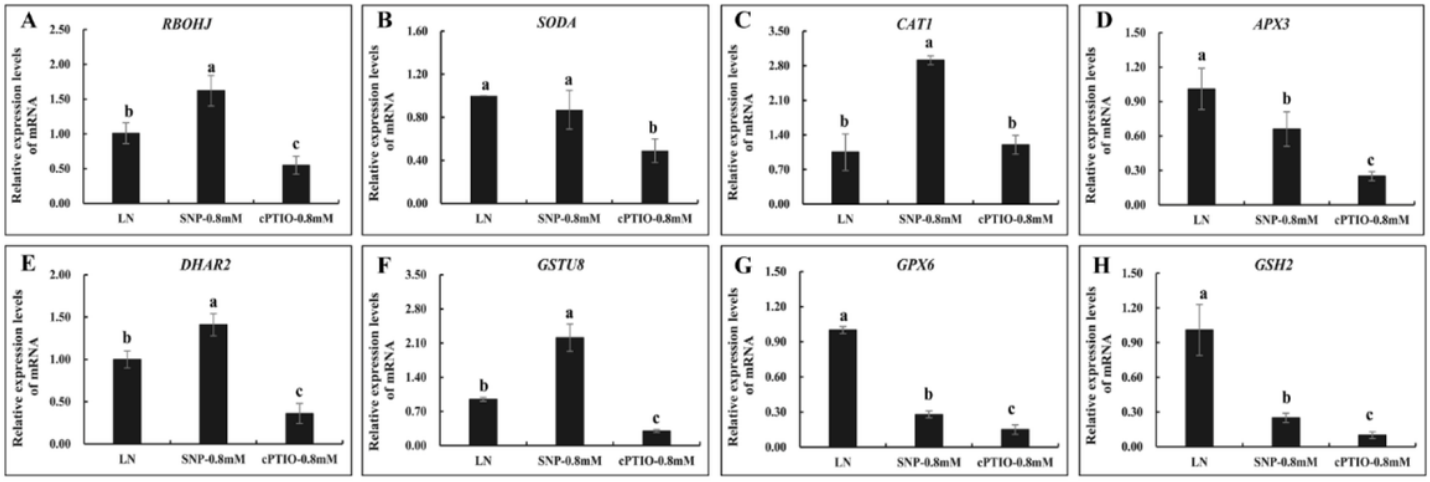

Figure 7

Changes of ROS related genes in pollen with and without NO regulter after cryopreservation. Notes: The statistical difference was based on the 0.05 level. A- RBOHJ; B- SODA; C- CAT1; D-APX3; E-DHAR2; F-GSTU8; G- GPX6; H-GSH2.

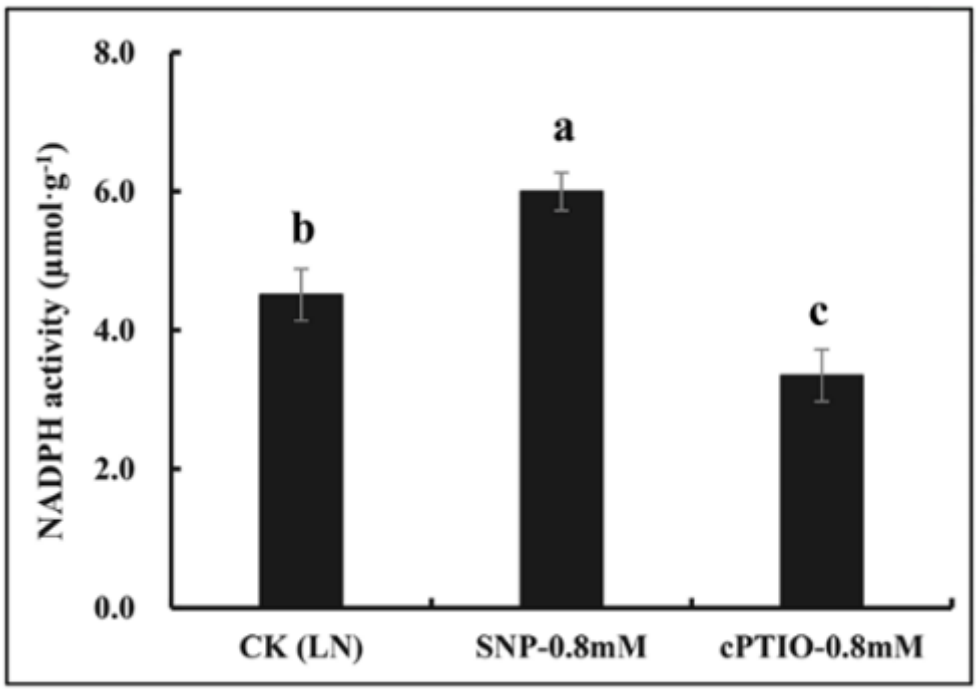

Figure 8

Changes of enzyme of ROS production (NADPH oxidase) before and after exogenous NO regulator treatments. Notes: The statistical difference was based on the 0.05 level.

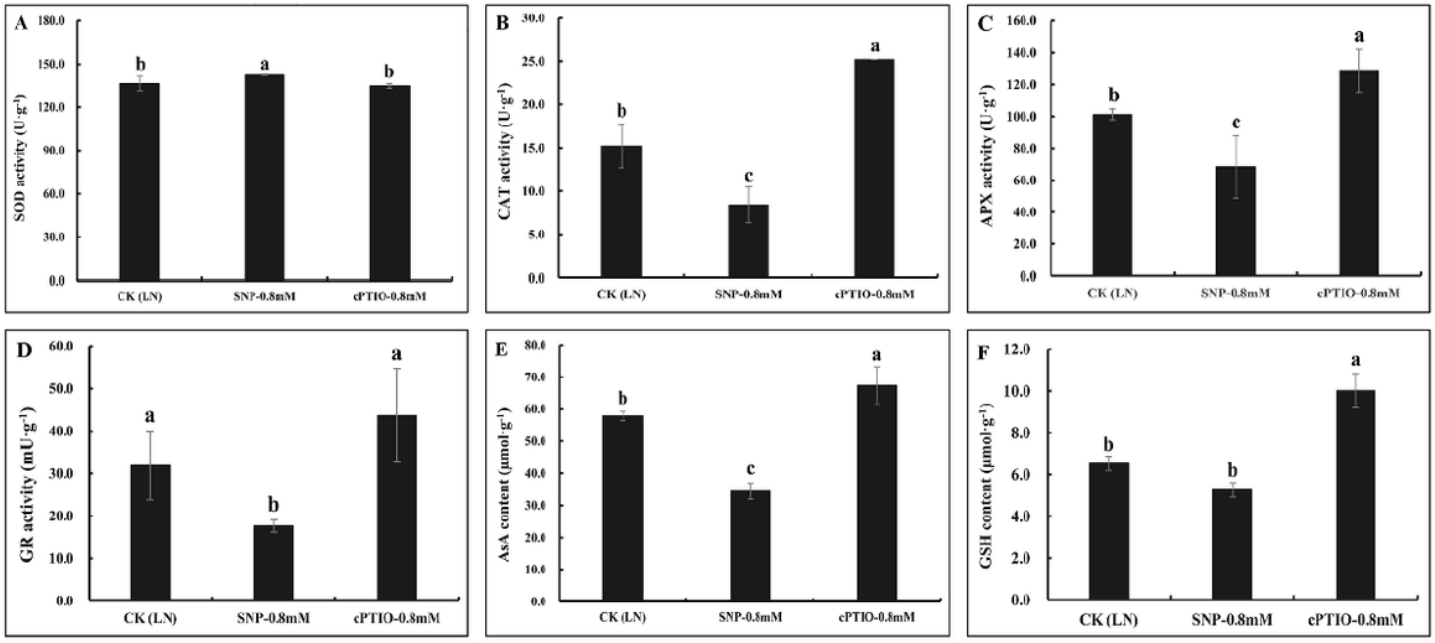

Figure 9

Changes of index of ROS clearance before and after exogenous NO regulator treatments. Notes: The statistical difference was based on the 0.05 level. A-SOD; B-CAT; C-APX; D-GR; E-AsA; F-GSH.

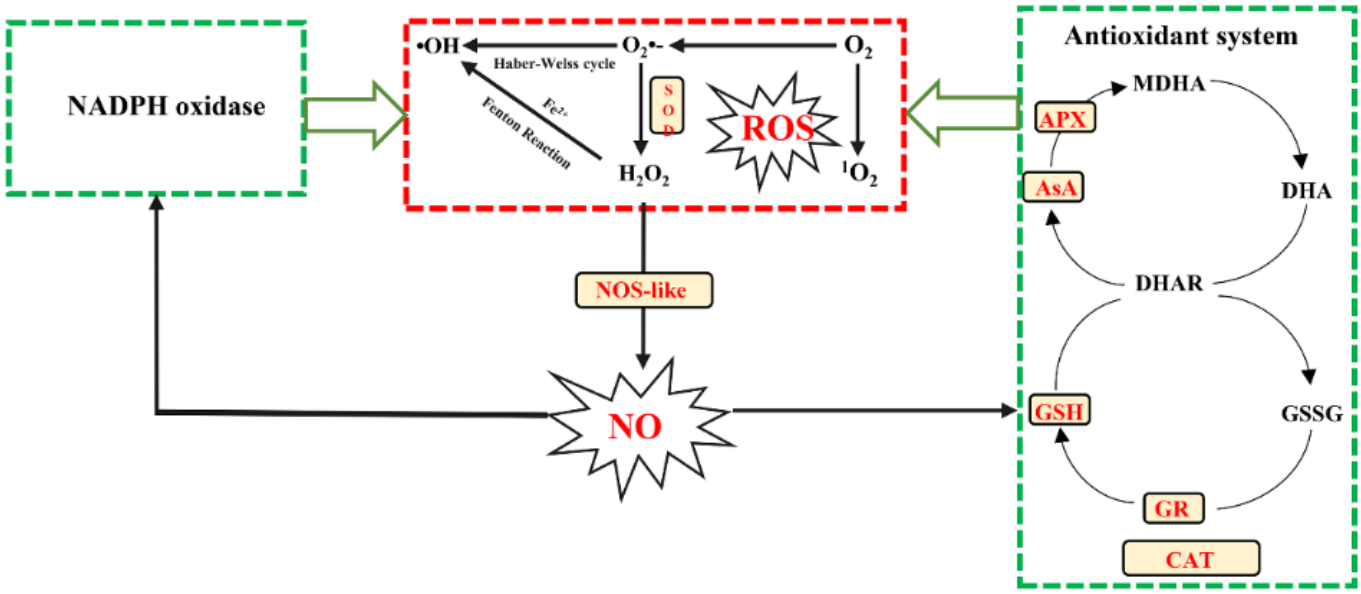

Figure 10

The schematic of interaction between ROS and NO in cryopreservation of pollen.

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