The Cross Talk Between ROS And NO In The Viability Decline 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), two common active molecules, are both involved in changes in viability after liquid nitrogen (LN) storage, but the relationship between these two molecules has not been examined in plant cryopreservation until now. In this study, the pollen of *Paeonia lactiflora* 'Fen Yu Nu' with significantly decreased viability after cryopreservation was used as the material. We studied the effects of the two regulators on each other and their biosynthesis and scavenging indices 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 changes in the ROS and NO content had a significant effect on post-LN pollen viability. The 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. Down-regulated NO had a positive effect on the ROS content, and it significantly affected the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and its regulatory gene *RBOHJ*. It also significantly affected catalase (CAT) and substrates related to the ascorbic acid (AsA)-glutathione (GSH) antioxidant cycle system. These results indicate that there was a positive interaction between ROS and NO in pollen cryopreservation. The NOS biosynthesis pathway is one of the ROS-regulated NO pathways, and the NADPH oxidase, CAT and AsA-GSH antioxidant cycle systems are the key sites of regulation of the ROS content by NO.

Key Message

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

Introduction

Pollen carries all of 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 have shown that cryopreservation is one of the key ways to preserve germplasm for a long time (Zhang et al., 1993; Souza et al., 2015; Engelmann, 2004) and it has been successfully applied to the long-term preservation of pollen of many species (Dmura and Akihama, 1980; Mercier, 1995; Li, 2005; Zhang, 2007; Li, 2010; Borghezan et al., 2011; Xu, 2014; Dinato et al., 2018). However, some problems have been found in practical applications. Pollen viability mainly shows three different trends after cryopreservation, and in most cases, it was significantly decreased (Zhang, 2007; Xu et al., 2014; Ren et al., 2019a; Li, 2019). According to previous studies, the decrease in pollen viability after cryopreservation is mainly due to osmotic stress and low-temperature stress during preservation in liquid nitrogen (LN). Other studies have found that reactive oxygen species (ROS) and nitric oxide (NO) may play a key role in this process (Ren et al., 2020b; Ren et al., 2021).

ROS and NO are signal transduction molecules in plant and animal cells that respond to stress and regulate a variety of physiological phenomena (Wilkins et al., 2011). Studies on plant stress have not only clarified the changes in ROS and NO but also discussed the relationship and the mechanism of interaction between these two factors. In the occurrence of programmed cell death (PCD) of *Nicotiana tabacum* var. samsun. suspension cells induced by chitosan, a large amount of ROS synthesis was induced along with the production of NO (Zhang et al., 2012). NO production was enhanced by ROS production during the occurrence of PCD in suspension cells of *Nicotiana tabacum* callus induced by sphingine and dihydrosphingine (Da Silva et al., 2011). The relationship between these two molecules and their possible interaction pathways were further proven by exogenous addition experiments. The study of Esim and Atici (2014) showed that exogenous spraying of NO could improve the activity of antioxidant enzymes and reduce the ROS level of *Zea mays* under low temperature stress, which plays an important role in improving cold resistance. Xiao's (2014) study found that exogenous NO donor sodium nitroferricyanide (III) dihydrate (SNP) increased the activity of NADPH and NADP⁺, while NO scavenger carboxy-PTIO potassium salt (c-PTIO), NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) and hydrogen peroxide (H₂O₂) scavenger dimethylthiourea (DMTU) inhibited the effect induced by the SNP, indicating that interactions between ROS and NO in *Cucumis sativus* seedlings under cold stress, NADPH and NADP⁺ activity may be the critical factors.

In 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 is involved in the changes in viability after LN storage. Zhang (2015), in the cryopreservation of calli from *Agapanthus praecox*, found that the ROS content significantly increased after preservation with LN, and it had a significant effect on cell activity after cryopreservation by inducing oxidative stress and programmed cell death. In cryopreservation of protocorms of *Dendrobium nobile*, Jiang (2019a) found that NO was produced during the cryopreservation process and had dual regulatory effects on cell activity after preservation in LN. Other studies on cryopreservation of *Dendrobium nobile* protocorms showed that there was a certain relationship between ROS and NO (Jiang, 2019b). However, the existing studies on how these two molecules work have not been 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 viability after preservation in LN. Ren (2020b), in the cryopreservation of *Paeonia lactiflora* pollen, found that the content of ROS was significantly increased after preservation in LN, and ROS had a significant impact on post-LN viability by mediating the occurrence of PCD. In the cryopreservation of *Paeonia lactiflora*, NO was also found to be involved in the changes in 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 the materials. We compared and analyzed the changes of ROS and NO contents before and after cryopreservation, and studied their effects on each other and the related synthetases by using their modulators 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 air humidity was appropriate for drying, a bud that was about to open was selected and the anther was gently removed and placed at 4 °C. Then, the anthers were placed at room temperature (23±2 °C) for 24 h to completely disperse the pollen. The pollen was collected with an 80 mm aperture sieve (Ren *et al.*, 2019b).

The collected pollen (0.1 g/package) was divided into tin foil packets, placed in a 2 mL cryotube, and then directly put into LN for storage. After 3 months of storage, the cryotube (containing the tinfoil-wrapped pollen) was rewarmed under running water or thawed at room temperature.

Pollen moisture content and viability determination

The moisture content of fresh pollen was determined by the 105 °C constant temperature drying method (Ren *et al.*, 2019b).

Pollen variability was evaluated by the pollen germination rate. The pollen germination rate before and after cryopreservation was detected by the hanging drop culture method. The medium was 10% sucrose and 0.1% boric acid. The germination conditions were 25 °C for 4 h without light. A pollen tube length greater than twice the pollen grain diameter was the criterion for pollen germination. Four replicates were performed for every treatment, and three fields were randomly selected for each replicate (at least 300 grains). 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 diaminofluorescein-FM diacetate (DAF-FM DA) fluorescence staining. Pollen (0.01 g) was added to 200 µL DAF-FM DA (100 µmol/L) fluorescent probe (Biyuntian Biotechnology Co., Ltd., S0019) in the dark and thoroughly mixed. Then, the cells were incubated at 37 °C for 30 min without light and centrifuged at 2000 rpm for 20 s, and the fluorescent dye was discarded. The fluorescently loaded pollen was washed twice with 500 µL PBS (pH= 7.4), and 1 mL PBS (pH= 7.4) was used to resuspend the precipitated pollen. A flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to measure the fluorescence values in the FITC channels. The actual fluorescence measurement value was the relative content of NO, and each treatment was repeated 3 times.

Pollen ROS content determination

ROS content was determined by fluorescence staining with 2',7'-2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Xu, 2014). A 0.01 g sample of pollen was added to 200 µL DCFH-DA (100 µmol/L)

fluorescent probe (Sigma Chemical Co., St Louis, MO, USA, D6883-50MG) without light and thoroughly mixed. The pollen was incubated at 37 °C for 30 min in the dark, centrifuged at 2000 rpm for 20 s, and the dye solution was discarded. The pollen was washed twice with 500 µL PBS (pH= 7.4), and then it was resuspended in 1 mL PBS (pH= 7.4). The fluorescence was measured in the FITC channel by flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). The actual fluorescence measurement value was taken as the NO content, and every treatment was repeated 3 times.

Pollen NOS-like activity determination

Nitric oxide-like synthase (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, 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), and the mixture was incubated at 37 °C for 30 min without light. Flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to detect the fluorescence value in the FITC channel, taking the actual fluorescence measurement value as the NOS-like activity, and each treatment was repeated 3 times.

Pollen NDAPH oxidase activity determination

Nicotinamide adenine dinucleotide phosphate (NDAPH) enzyme activity was determined according to the NADP⁺/NADPH detection kit (S0179) provided by Biyuntian Biotechnology Co., Ltd. The pollen (0.01 g) was homogenized in an ice bath with 400 µL NADP⁺/NADPH extraction buffer and centrifuged at 12,000 rpm at 4 °C for 10 min. Then, 200 µL glucose-6-phosphate dehydrogenase (G6PDH) working solution was added to 100 µL extraction solution and incubated at 37 °C for 10 min without light. Then, 10 µL chromogen solution was added, mixed well and incubated for 60 min at 37 °C without light. The absorbance was measured at 450 nm with an ultraviolet spectrophotometer. Each treatment was repeated three times, and the average of the results was taken.

Pollen antioxidants determination

Superoxide dismutase (SOD) activity was determined by the nitrogen blue tetrazole (NBT) reduction method (Li et al., 2000). Pollen (0.05 g) was homogenized with 1 mL of 0.05 mol/L phosphoric acid buffer in an ice bath and centrifuged at 10,000 rpm at 4 °C for 15 min. Then, 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-Na₂ (100 mol/L) and 300 µL riboflavin solution (20 µmol/L) were added to 300 µL extraction solution without light. The mixture was subjected to 4000 lx ray reaction for 15 min, and the reaction was terminated in darkness. The absorbance value was determined by spectrophotometry at a wavelength of 560 nm. Every treatment was repeated three times, and the average of the results was taken.

Catalase (CAT) activity was measured according to Prochakova's (2001) method with slight modifications. Pollen (0.05 g) was homogenized with 1 mL 0.05 mol/L phosphate buffer in an ice bath and centrifuged at 10,000 rpm at 4 °C for 15 min. Then, 1.5 mL phosphoric acid buffer (pH= 7.0) and 1.0 mL distilled water were added to 200 µL of the extraction solution and 300 µL hydrogen peroxide solution

(0.1 mol/L) was added to initiate the reaction. The absorbance value was measured at a wavelength of 240 nm by a spectrophotometer. Each treatment was repeated three times, and the mean value was taken.

Glutathione reductase (GR) activity was determined according to the glutathione reductase assay kit (S0055) provided by Biyuntian Biotechnology Co., Ltd. with slight modifications. Phosphate buffer solution (1 ml, 0.01 mol/L) was added to 0.03 g pollen and it was ground to a homogenate in an ice bath and then centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was collected for the determination of GR activity, and every treatment was repeated three times.

Ascorbic acid reductase (APX) activity was determined using the method of Nakano and Asada (1981) with slight modifications. APX enzyme extraction reagent (1 ml, 50 mmol/L PBS+2 mmol/L AsA+5 mmol/L EDTA) was added to 0.03 g pollen, and it was homogenized in an ice bath and centrifuged at 10,000 rpm at 4°C for 20 min. To 50 µL of the extract, we 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%) to initiate the reaction. The absorbance was measured at 290 nm with a spectrophotometer. Each treatment was repeated three times, and the results were averaged.

Ascorbic acid (AsA) content was determined by Kampfenkel's (1995) method with some modifications. The pollen (0.03 g) with 1.5 mL trichloroacetic acid (10%) was homogenized in an ice bath and centrifuged at 10,000 rpm at 4°C for 10 min. Then, 200 µL sodium dihydrogen phosphate solution (150 mmol/L) and 200 µL ddH₂O were added to 200 µL of the supernatant extract, and it was mixed for at least 30 s. Then, we added 10% trichloroacetic acid solution, 44% phosphoric acid solution and 4% 2,2-dipyridine solution in turn, 400 µL each. The reaction was mixed and initiated by adding 200 µL FeCl_3 (3%) and then incubated at 37 °C for 1 h. The absorbance was measured at 525 nm by a spectrophotometer, each treatment was repeated 3 times, and the mean value of the results was taken.

The glutathione (GSH) content was determined by the 2-nitrobenzoic acid (DTNB) reduction method (Griffith, 1980). First, 1.5 mL trichloroacetic acid (10%) was added to 0.03 g pollen, it was homogenized in an ice bath and centrifuged at 10,000 rpm at 4 °C for 10 min. Then, 0.5 mL ddH₂O, 1 mL phosphate buffer (1 mmol/L, pH= 7.7) and 0.5 mL DTNB (4 mmol/L) were added to 0.5 mL of the supernatant extract, mixed well and reacted for 10 min at 25 °C. The absorbance was measured at 412 nm by a spectrophotometer. Each treatment was repeated three times, and the average of the results was taken.

qRT-PCR analysis of pollen ROS and NO related genes

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

According to the instructions of the SYBR Premix EX Taq (Takara, Otsu, Japan) on the MiniOpticon™ real-time PCR detection system (Bio-Rad®, Hercules, CA), real-time quantitative PCR was performed. 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 ddH₂O, and 10 µL SYBR® Premix Ex Taq II (Tli RNase Plus) (2X). The expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method, and each treatment was repeated 3 times.

Addition of ROS and NO exogenous regulator

In pollen without running water treatment after preservation in LN, 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 was added at a ratio of 1:100 (*w/v*) and thoroughly mixed. After incubation for 10 min in the dark at 37 °C, the pollen incubated with the regulator was washed three times with phosphate buffer (PBS, pH= 7.4) at 0.01 mol/L. After centrifugation at 2000 rpm for 30 s, the precipitated pollen was used for the determination of various indices, and three repeats were set for each treatment.

Statistical analysis

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

Results

Changes in pollen viability before and after cryopreservation

The moisture content of the pollen of *Paeonia lactiflora* 'Fen Yu Nu' was 8.10%, and the fresh pollen viability was 22.73%. However, cryopreservation significantly reduced the pollen viability (Table 1); in particular, the viability of pollen thawed to room temperature after preservation with LN was significantly lower than that of fresh pollen, decreased by 6.31%, while the viability of pollen thawed by running water after LN storage was only reduced by 1.93% compared with that of fresh pollen.

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

Cultivar	Moisture content / %	Germination percentage / %		
		Fresh	LN	Thaw
<i>P. lactiflora</i> '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). Fresh: the pollen without cryopreservation; LN: the pollen thawed at room temperature after cryopreservation; Thaw: the pollen thawed with running water after cryopreservation.				

Changes in ROS and NO contents in pollen before and after cryopreservation

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

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

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

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

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.

Changes in viability, ROS and NO contents in pollen with and without exogenous ROS regulator treatments after cryopreservation

The ROS scavengers AsA and GSH at appropriate concentrations not only significantly increased pollen viability after LN storage (Fig. 2- A) but also significantly inhibited the endogenous ROS content (Fig. 3- A,

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

Changes in viability, ROS and NO contents in pollen with and without exogenous NO regulator treatments after cryopreservation

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

Changes in NO related genes and substances in pollen with and without exogenous ROS regulator treatments after cryopreservation

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

The 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 LN preserved pollen without treatment, respectively (Fig. 6).

Changes in ROS related genes and substances in pollen with and without exogenous NO regulator treatments after cryopreservation

After cryopreservation, the addition of exogenous NO carrier SNP significantly upregulated the expression levels of the *RBOHJ*, *CAT1*, *DHAR2* and *GSTU8* genes but significantly downregulated the expression levels of *APX3*, *GPX6* and *GSH2*. However, the addition of the NO scavenger c-PTIO after cryopreservation significantly downregulated 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 storage significantly promoted the activation of NADPH enzymes ($P < 0.05$), and the addition of the NO scavenger c-PTIO significantly inhibited the activity of NADPH enzymes (Fig. 8). After cryopreservation, the appropriate concentration of NO carrier SNP significantly inhibited the activities of the enzyme ROS scavenging substances CAT, APX, and GR and the content of the nonenzyme ROS scavenging substance AsA ($P < 0.05$) but significantly increased the SOD activity of pollen. However, the addition of the NO scavenger c-PTIO not only significantly

improved the activities of the enzyme scavengers CAT, APX and GR (Fig. 9-B, C, D) but also significantly promoted the accumulation of nonenzymatic 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 are involved in the regulation of NO production. In a study of *Medicago truncatula* under low-temperature stress, H₂O₂ treatment accelerated the accumulation of NO, which played a key role in improving the efficiency of replacing oxidase (AOX) and alleviating the damage to the photosystem under low-temperature stress (Arfan et al., 2019). In the root development of *Arabidopsis thaliana*, exogenous H₂O₂ increased NO levels by 8 times (Wang et al., 2010). Exogenous H₂O₂ 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). ROS were involved in the regulation of intracellular NO content during cryopreservation of *P. lactiflora* pollen.

The biosynthesis of NO in plant cells during the stress response was mainly achieved through the L-arginine-dependent and nitrate reductase pathways (Ma and Berkowitz, 2016). Among them, the NOS-like enzyme synthesis pathway dependent on L-arginine is the most widely studied (Leitner et al., 2009). Although nitric oxide synthase (NOS) has not been found in plants, 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 H₂O₂ induces NO production by increasing 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 affect the regulatory genes involved in the nitrate reduction pathway for NO biosynthesis. Only AsA significantly downregulated the expression level of the regulatory gene *CSU2* involved in the NOS-like enzyme biosynthesis pathway that is dependent on L-arginine (Fig. 5), while ROS scavenging agents at appropriate exogenous concentrations significantly reduced the activities of NOS-like enzymes in cryopreserved pollen at the biochemical level (Fig. 6). These results indicate that in pollen cryopreservation, ROS participate in the regulation of intracellular NO content, which mainly acts on NOS-like enzymes involved in NO biosynthesis.

Effects of NO on ROS and its pathways in pollen cryopreservation

To date, a large number of studies have shown that changes in NO play an important role in ROS changes in cells. Xiao (2014) found that NO played a positive regulatory role on ROS in *Cucumis sativus* seedlings under cold stress. Jiang (2019b) found that NO produced a positive effect on ROS content during the preculture stage of vitrification-cryopreservation in *Dendrobium* protocorm-like bodies.

However, some studies have found that NO plays a negative regulatory role in the accumulation of ROS. He (2019) found that the addition of NO carrier SNP reduced the excess H₂O₂ 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. 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 mechanisms causing the regulatory differences remains to be further explored. In this study, the addition of an appropriate concentration of NO carrier SNP after LN storage significantly promoted the accumulation of endogenous ROS, while the addition of an appropriate concentration of NO scavenger c-PTIO significantly reduced the content of endogenous ROS (Fig. 4- E, F, G, H). NO plays a positive role in the regulation of intracellular ROS accumulation during pollen cryopreservation. This is consistent with the findings of Jiang (2019b) in the cryopreservation of *Dendrobium nobile* procorms. When the intracellular concentration of NO decreased, the accumulation of H₂O₂ also significantly decreased, indicating that NO plays a positive regulatory role in ROS.

In the plant response to stress, previous studies showed that NADPH oxidase involved in ROS production was one of the key pathways involved in NO regulation of intracellular ROS concentration. Yun (2011), in plant immune reactions, found that NO affected ROS synthesis through S-nitrosylation of NADPH oxidase. Wang's (2013) study also showed that NO might control the production of ROS through protein S-nitrosylation. In this study, it was found that the addition of NO carrier SNP significantly promoted the activation of NADPH oxidase and upregulated the regulatory gene *RBOHJ* in pollen after LN storage, and the addition of the 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 indicate that NO participates in the regulation of endogenous ROS accumulation by acting on NADPH oxidase during pollen cryopreservation.

In addition, the antioxidant system may be another key pathway by which NO regulates the intracellular ROS concentration. Murgia (2004), in callus suspension cells of *Arabidopsis thaliana* and *Nicotiana tabacum*, found that NO carrier SNP inhibited the ROS scavenging system, thus accelerating intracellular ROS accumulation. Similarly, NO carrier SNP inhibited antioxidant enzymes in *Zinnia elegans* xylem (Ferrer and Ros Barceló, 1999), and a similar inhibitory effect of SNP on antioxidant enzymes in the leaves damaged *Solanum tuberosum* (Beligni and Lamattina, 2002) and reduced the endogenous ROS content. In this study, it was found that the addition of an appropriate concentration of NO carrier SNP after LN storage had significant inhibitory effects on the ROS scavenging substances CAT, APX, GR and AsA and significantly downregulated the expression levels of the related regulatory genes *APX3*, *GPX6* and *GSH2*; additionally, the addition of the NO scavenging agent c-PTIO significantly promoted the accumulation of CAT, APX, GR, AsA and GSH and had a certain downregulatory effect on the expression levels of the antioxidant-related regulatory genes *SODA*, *APX3*, *DHAR2*, *GSTU8*, *GPX6* and *GSH2* (Figs. 7, 9). This result suggested that during the cryopreservation of pollen, NO regulated endogenous ROS content mainly by affecting CAT activity and the related substrates of the AsA-GSH circulatory system.

This is consistent with the findings of Jiang (2019b) in the cryopreservation of protocorms from *Dendrobium nobile*. NO participated in the regulation of endogenous ROS changes by affecting the activity of endogenous ROS scavenging substances, thus participating in the changes in viability.

In summary, there was an interactive relationship between ROS and NO in pollen cryopreservation, which is 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 mediated by NOS-like enzymes involved in NO biosynthesis; the effect of NO on ROS was mainly mediated 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 (2004) in suspension cells of *Arabidopsis thaliana* and *Nicotiana tobacum* and Arfan (2019) in *Medicago truncatula* under low temperature stress.

Conclusion

This study first investigated the interaction and its pathways between ROS and NO during pollen cryopreservation. 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 changes in ROS; the biosynthesis pathway of NOS-like enzymes involved in NO production was one of the important pathways by which ROS regulated changes in endogenous NO content.

Declarations

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Conflict of interest: The authors declare that they have no conflict of interest.

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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Figures

Figure 1

Changes in ROS and NO contents in *P. lactiflora* pollen before and after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: Fresh pollen; B: Pollen thawed at room temperature after cryopreservation; C: Pollen thawed with running water after cryopreservation; D: ROS content; E: Fresh pollen; F: Pollen thawed at room temperature after cryopreservation; G: Pollen thawed with running water after cryopreservation; H: NO content. 1: the flow cytometry maps; 2: the flow spectrogram of fluorescence; 3: the comparison spectrograms. Fresh: fresh pollen without cryopreservation; LN: pollen thawed at room temperature after cryopreservation; Thaw: pollen thawed with running water after cryopreservation.

Figure 2

Changes in pollen viability in *P. lactiflora* with and without exogenous ROS and NO regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: Pollen viability changes with and without ROS regulator treatments after cryopreservation; B: Pollen viability changes with and without NO regulator treatments after cryopreservation. LN: pollen thawed at room temperature after cryopreservation; AsA-200 µM: pollen supplemented with 200 µM AsA after cryopreservation; GSH-0.16 mM: pollen supplemented with 0.16 mM GSH after cryopreservation; SNP- 0.8 mM: pollen supplemented with 0.8 mM SNP after cryopreservation; cPTIO- 0.8 mM: pollen supplemented with 0.8 mM cPTIO after cryopreservation.

Figure 3

Changes in pollen endogenesis ROS and NO content in *P. lactiflora* with and without exogenous ROS regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: Pollen thawed at room temperature after cryopreservation; B: Pollen supplemented with 200 µM AsA after cryopreservation; C: Pollen supplemented with 0.16 mM GSH after cryopreservation; D: ROS content; E: Pollen thawed at room temperature after cryopreservation; F: Pollen supplemented with 200 µM AsA after cryopreservation; G: Pollen supplemented with 0.16 mM GSH after cryopreservation; H: NO content. 1: the flow cytometry maps; 2: the flow spectrogram of fluorescence; 3: the comparison spectrograms. LN: pollen thawed at room temperature after cryopreservation; AsA-200 µM: pollen supplemented with 200 µM AsA after cryopreservation; GSH- 0.16 mM: pollen supplemented with 0.16 mM GSH after cryopreservation.

Figure 4

Changes in pollen endogenesis ROS and NO content in *P. lactiflora* with and without exogenous NO regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: Pollen thawed at room temperature after cryopreservation; B: Pollen supplemented with 0.8 mM SNP after cryopreservation; C: Pollen supplemented with 0.8 mM cPTIO after cryopreservation; D: NO content; E: Pollen thawed at room temperature after cryopreservation; F: Pollen supplemented with 0.8 mM SNP after cryopreservation; G: Pollen supplemented with 0.8 mM cPTIO after cryopreservation; H: ROS content. 1: the flow cytometry maps; 2: the flow spectrogram of fluorescence; 3: the comparison spectrograms. LN: pollen thawed at room temperature after cryopreservation; SNP- 0.8 mM: pollen supplemented with 0.8 mM SNP after cryopreservation; cPTIO- 0.8 mM: pollen supplemented with 0.8 mM cPTIO after cryopreservation.

Figure 5

Changes in NO related genes in *P. lactiflora* pollen with and without exogenous ROS regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: NIA; B: CSU2; C: CYP94A1. LN: pollen thawed at room temperature after cryopreservation; AsA-200 μ M: pollen supplemented with 200 μ M AsA after cryopreservation; GSH- 0.16 mM: pollen supplemented with 0.16 mM GSH after cryopreservation.

Figure 6

Changes in NOS-like enzyme in *P. lactiflora* pollen with and without exogenous ROS regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: Pollen thawed at room temperature after cryopreservation; B: Pollen supplemented with 200 μ M AsA after cryopreservation; C: Pollen supplemented with 0.16 mM GSH after cryopreservation; D: activity of NOS-like enzyme. 1: the flow cytometry maps; 2: the flow spectrogram of fluorescence; 3: the comparison spectrograms. LN: pollen thawed at room temperature after cryopreservation; AsA-200 μ M: pollen supplemented with 200 μ M AsA after cryopreservation; GSH- 0.16 mM: pollen supplemented with 0.16 mM GSH after cryopreservation.

Figure 7

Changes in ROS related genes in *P. lactiflora* pollen with and without exogenous NO regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: *RBOHJ*; B: *SODA*; C: *CAT1*; D: *APX3*; E: *DHAR2*; F: *GSTU8*; G: *GPX6*; H: *GSH2*. LN: pollen thawed at room temperature after cryopreservation; SNP- 0.8 mM: pollen supplemented with 0.8 mM SNP after cryopreservation; cPTIO- 0.8 mM: pollen supplemented with 0.8 mM cPTIO after cryopreservation.

Figure 8

Changes in ROS production (NADPH oxidase) in *P. lactiflora* pollen with and without exogenous NO regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. LN: pollen thawed at room temperature after cryopreservation; SNP- 0.8 mM: pollen supplemented with 0.8 mM SNP after cryopreservation; cPTIO- 0.8 mM: pollen supplemented with 0.8 mM cPTIO after cryopreservation.

Figure 9

Changes in ROS scavenger in *P. lactiflora* pollen with and without exogenous NO regulator treatments after cryopreservation. Notes: The significant difference was based on the 0.05 level. A: SOD; B: CAT; C: APX; D: GR; E: AsA; F: GSH. LN: pollen thawed at room temperature after cryopreservation; SNP- 0.8 mM: pollen supplemented with 0.8 mM SNP after cryopreservation; cPTIO- 0.8 mM: pollen supplemented with 0.8 mM cPTIO after cryopreservation.

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