Cryopreservation of seeds of blue waterlily (*Nymphaea caerulea*) using glutathione adding plant vitrification solution, PVS+

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Abstract

Nymphaea caerulea is a valuable freshwater aquatic plant, not only because of its ornamental value but also its extractions for chemical and medical uses. It is necessary to store its seeds as backup resources. Cryopreservation is the reliable, cost-effective method for long-term preservation of botanical genetic banks, especially for recalcitrant plants. In this study, we demonstrated that due to unable to tolerate desiccation and low-temperature, *N. caerulea* is recalcitrant. Since viability was lost before 6 months storage, *N. caerulea* seeds were not appropriate to long-term store by traditional storage method. The only way to long-term store *N. caerulea* seeds is cryopreservation. However, Plant Vitrification Solution 3 (PVS3; 50% w/v sucrose, 50% w/v glycerol), a commonly used plant vitrification solution, was ineffective on *N. caerulea* seeds cryopreservation. The maximum survival rate of cryopreserved seeds treated by PVS3 was only 23%. Oxidative stress induced by reactive oxygen species (ROS) accumulation within seeds was the reason to cause inefficiency of PVS3 treated seeds cryopreservation. We developed a new plant vitrification solution (PVS+), by adding glutathione (GSH)

into PVS3. PVS+ rescued the inefficiency by decreasing ROS accumulation and elevated

survival rate of cryopreserved seeds to 97%. Our results showed some inefficiencies of plant tissue cryopreservation may be caused by ROS-induced oxidative stress. Antioxidants can reduce ROS-induced oxidative stress and improve seeds survival after cryopreservation. In conclusion, *N. caerulea* seeds were identified as recalcitrant and successfully cryopreserved. Suppressing ROS accumulation during cryopreservation may be a potential strategy to improve survival rate of recalcitrant seeds after cryopreservation.

1. Introduction

Nymphaea caerulea (also known as blue waterlily), a freshwater aquatic plant, is close related to human society, from ancient Egypt culture (Emboden, 1978) to modern human life. With its growing capacious ornamental market (Kulus and Zalewska, 2014), and extractions for medical and chemical uses (Agnihotri *et al.*, 2008; Fossen *et al.*, 1999), *N. caerulea* is valuable and needs to be conserved. Seed storage is an inexpensive and useful technique for ex-situ conservation of plants

(Bonner, 1990). Seeds can broadly divide into two different storage characteristics base on their desiccation and low-temperature tolerances. Seeds can tolerate

desiccation and low-temperature belong to orthodox, otherwise recalcitrant (Berjak *et al.*, 1989; Pammenter and Berjak, 1999). Seed banks use traditional method to store seeds (most of them are orthodox). In seed banks, seeds are dried to a low water content (< 10%, fresh water basis) and placed in sealed container at low temperature (normally -20℃). It is predicted that orthodox seeds stored in seed banks will remain viable for many years. Recalcitrant seeds, on the other hand, due to desiccation and low-temperature intolerance are not suitable for long-term storing by using traditional method.

Cryopreservation, a method to store bio-material at ultralow temperature (often in liquid nitrogen, -196℃), is regarded as the pragmatic approach to provide the secure and cost-efficient long-term preservation of recalcitrant seeds (Walters *et al.*, 2013; Walters *et al.*, 2008). It is believed that tissues and cells pause all metabolic activities at ultra-low temperature. Therefore, seeds presumably can be permanently stored in liquid nitrogen. However, directly immerse seeds in liquid nitrogen would form bulky ice crystals within cells and cause seeds to die (Wesley-Smith *et al.*, 2014; Wesley-Smith *et al.*, 2015). To overcome this difficulty, seeds need to become vitrification before immerse in liquid nitrogen. Vitrification is the physical process that supercools a highly concentrated

cryoprotective solution to ultralow temperatures (for example, -196℃) and finally solidifies into a metastable glass, without forming crystallized ices at a practical cooling rate. Thus, vitrification is an effective freeze-avoidance mechanism. Vitrification in plant tissues including seeds is usually achieved by preculturing plant tissues in plant vitrification solution (PVS).

PVS3 is commonly used solution, and has been applied to successfully cryopreserve different plant tissues (shoot tip, callus, embryonic axis, and seeds) from various plant species (Sakai and Engelmann, 2007; Sakai *et al.*, 2008).

When seeds are treated with PVS3 for vitrification and immerse in liquid nitrogen for cryopreservation, they are under large osmotic and cold stresses, which induce oxidative stress. Oxidative stress is prompted by accumulation of copious reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), which are highly reactive and toxic chemical species. Seeds activate self-defensive mechanism programmed cell death (PCD) reaction in order to protect themselves from deterioration of ROS. However, too many ROS cause manifold irreversible PCD and eventually lead seeds to death (Petrov *et al.*, 2015). In nature, plants produce variety of antioxidants, for example carotenoids, vitamin C, and glutathione (GSH) to balance oxidative stress caused by ROS. GSH is deemed a vital

antagonist against ROS-induced oxidative stress in most organisms, including plants (Foyer *et al.*, 1997; Kranner *et al.*, 2006) and is applied to improve shoot tip as well as callus survival after cryopreservation (Wang and Deng, 2004; Zhang *et al.*, 2015).

In this paper, we first investigated the storage characteristic of the *N. caerulea*. Next we tested if seeds of *N. caerulea* could be successfully cryopreserved by preculturing in PVS3. Finally, we explored the way to improve the inefficiency of *N. caerulea* seeds cryopreservation using PVS3. The purpose of this paper was to successfully cryopreserved *N. caerulea* seeds.

2. Materials and methods

2 - 1. plants materials

Seeds were manually extracted from freshly harvested mature fruits of *N. caerulea*, which provided by Dr. Cecilia Koo Botanic Conservation Center (KBCC, Taiwan). After extraction, seeds were rinsed and cleaned three times (5 min each), twice in tap water, and lastly in distilled water and instantly surface dried with hand towel papers.

2 - 2. Desiccation and low-temperature tolerance test

Water content (WC) of seeds was determined gravimetrically for seed groups (100 seeds as a group, repeated 3 times), which were weighed before and after drying in an oven at 100℃ for 3 days. Water content was expressed mean percentage of seed weight (g H_2O per g fresh seed weight). % water content = (original WC-WC after drying)/(original WC) X 100.

In desiccation tolerance test, 20 seeds were randomly selected and desiccated to 10% or 5% WC. After reaching to selected WC, 20 seeds were put into Eppendorf with 1 mL dd water and cultured in 25℃, 12/12 (light/dark) cycle incubator for 30 days. The germination rate of each Eppendorf was recorded after 30 days. This experiment was repeated 3 times.

In low-temperature tolerance test, 20 seeds were randomly selected and sealed in a cryovial storing at -20℃ or LN for 1 day. 20 seeds were put into Eppendorf with 1 mL dd water and cultured in 25℃, 12/12 (light/dark) cycle incubator for 30 days. Recorded the germination rate of each Eppendorf after 30 days. This experiment was repeated 3 times.

2 - 3. Storage using traditional method in long term

60 seeds were randomly selected as a group. Each group was sealed in a Ziploc

sealed plastic bag with a saturated tissue paper to maintain moisture in bag. There were 3 groups to store in each combination of 3 different temperatures (25℃, 4℃, and -20℃) and 8 various storing times (1 w, 2 w, 1 m, 2 m, 3 m, 4 m, 5 m, and 6 m). After storage, 60 seeds were separated into 3 Eppendorfs (each contained 20 seeds) with 1 mL dd water and cultured in 25℃, 12/12 (light/dark) cycle incubator for 30 days. The germination rate of each Eppendorf was recorded after 30 days.

2 - 4. Cryopreservation procedure (PVS3 and PVS+)

(1). Preculture : Seeds were cultured in different solutions at 25℃ for various times. Solutions and times were different as various experiments demanded. In PVS3 treated experiment, solution was PVS3 (50% sucrose + 50% glycerol, w/v; (Nishizawa *et al.*, 1993)), times were 1, 3, 6, 9, 12 and 24 hr. In PVS+ experiment, solutions were PVS3 + 0.8mM GSH (Sigma – Aldrich, USA), PVS3 + 0.08mM GSH (PVS+) and PVS3 + 0.008mM GSH, time was 9 hr.

(2). Cryopreservation (LN) : Seeds after preculture were immediately put into cryovial and directly plunged into liquid nitrogen, last for 1 day. (3). Rewarming : Seeds removed from liquid nitrogen were directly rewarmed at 38 ℃ water bath for 5 min.

(4). Recovery : Seeds were put into Eppendorf with 1mL dd water and cultured in

25℃, 12/12 (light/dark) cycle incubator for 30 days. Recorded the germination rate of each Eppendorf after 30 days.

Every experiments were repeated 3 times.

2 – 5. Extraction and determination of H_2O_2

The extraction and determination of H_2O_2 content was modified according to (Zhang et al., 2015) method. The 1g seeds (about 200 seeds) were ground in 1.8 mL of 4℃ dd water centrifuged at 5000g at 4℃ for 10 min. Add 1.0mL of 16% H_2SO_4, 0.2 mL of 20% KI, and 60μL of 50mL $Na_2MoO_4 \cdot 2H_2O$ to 0.5 mL supernatant for a

reaction time of 5 min. The absorbance of the solution was measured at 405nm by iMark™ Microplate Absorbance Reader.

2 – 6. Assessment of survival and statistical analysis

The effect of different experiments was assessed by calculating germination rate percentages of control and cryopreserved seeds. Seeds were recorded as germination only on both stem and root appearing. The results, presented as percentage of germination rate, were analyzed using t-test function, Excel software, Microsoft co. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, all were considered

statistically significant.

3. Results

3 - 1. The seed of *N. caerulea* is recalcitrant and not suitable to store in long-term by traditional storage method

In order to successfully preserve seeds, it is necessary to figure out the proper storage characteristic of seeds of *N. caerulea*, which is still unknown so far. Storage characteristic can be assessed by two methods. (1) If seeds can still survive under desiccation situation (WC <10%). (2) If seeds can still survive under sub-zero degree Celsius. Seeds can survive on both situations are considered as orthodox,

otherwise, recalcitrant. The survival rate of *N. caerulea* seeds shapely decreased from fresh seeds (88%) to desiccated seeds. When water content dropped to 10% and 5% the survival rate of seeds significantly decreased to 12% and 13%, respectively (Fig. 1a). After subzero degree Celcius treatment (-20℃ and liquid

nitrogen), the survival rate of seeds were both 0% (Fig. 1b). The results indicated that *N. caerulea* seeds were sensitive to desiccation and low-temperature. To further explore whether *N. caerulea* seeds can be stored in long-term using

traditional storage method, seeds were stored in concealed plastic bag with moisture at RT, 4 ℃ and -20℃ from 1 week to half year. Seeds stored in RT still have high survival rate (>90%) before the 1-month storage, and decreased greatly to 20 - 30% after storing for 1 months. Like RT, seeds that were stored at 4℃ have high survival rate (>90%) before the 1-month storage, and started progressively dropping after storing for 1 month. Seeds stored in -20℃ did not survive after storage (fig. 1c). If we considered survival rate of fresh seeds (88%) as normal viability of seeds, *N. caerulea* seeds could only be stored in short-term (less than 1 month at both RT and 4 ℃) under traditional storage method. Because they are unable to tolerate desiccation and subzero temperature, and could only be stored in short-term under a traditional storage method, seeds of *N. caerulea* is recalcitrant.

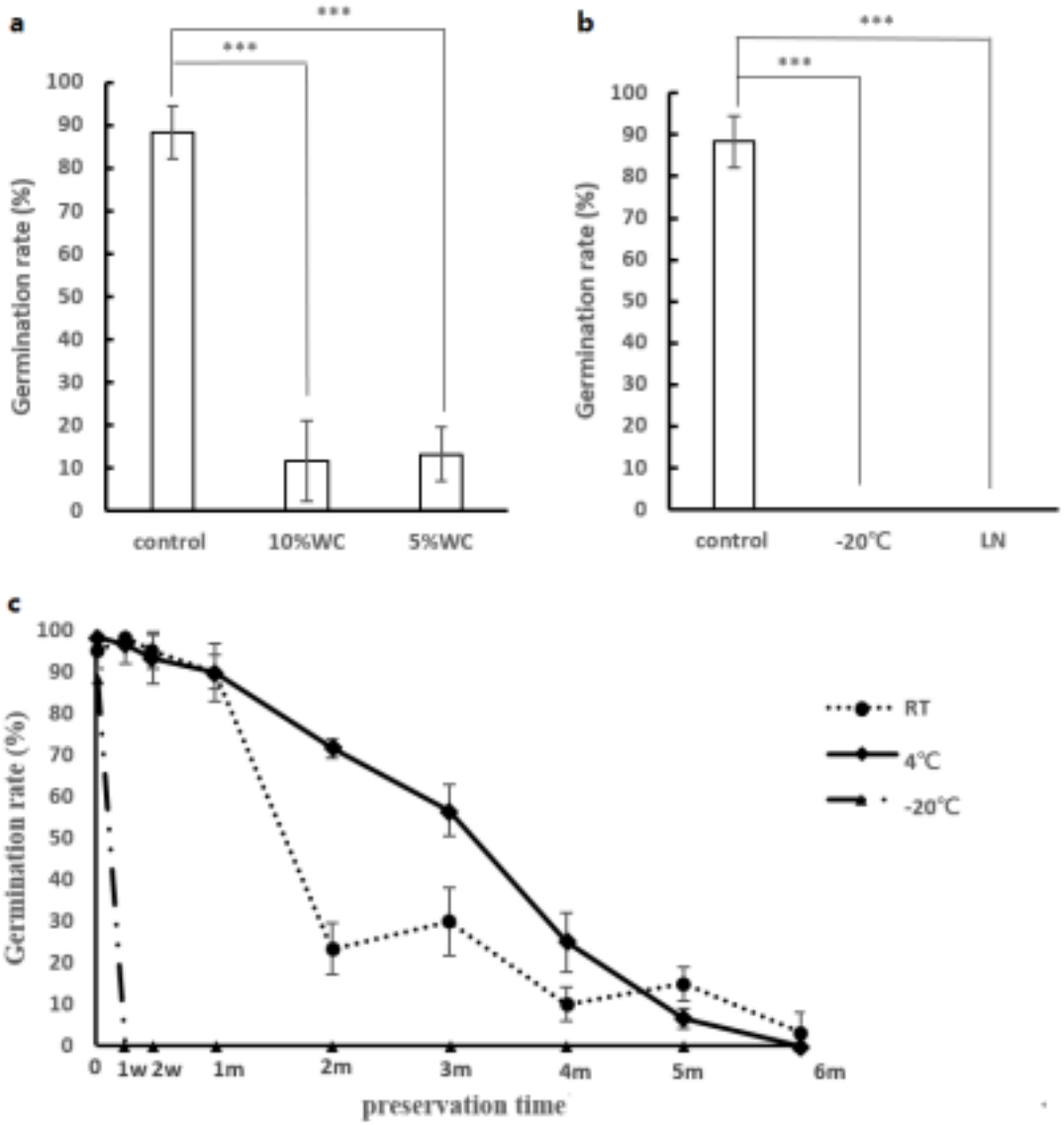

Fig. 1. Stress tolerance tests of *N. caerulea* seeds and traditional storage at different temperatures. (a) Desiccation tolerance test of seeds. Control represent fresh seeds. 10%WC and 5%WC represent water content of seeds air-dried to 10% and 5%, respectively. (b) Low-temperature tolerance test of seeds. Control represent seeds were treated at room temperature. -20℃ and LN represent seeds were treated at -20℃ and -196℃ (liquid nitrogen), respectively. (c) Seeds were sealed in Ziploc sealed plastic bags with moisture and placed at different temperatures (RT, 4℃, -20℃) last from 1

week to 6 months. WC, water content. LN, liquid nitrogen, RT, room temperature. Significant differences are indicated as ***P < 0.005.

3 – 2. Cryopreservation of *N. caerulea* seeds using PVS3 was not effective enough After realizing *N. caerulea* seeds is recalcitrant and could not be long-term stored by traditional storage method, it was rational to try to store seeds using cryopreservation. Seeds could not be cryopreserved without becoming vitrification. PVS3 is commonly used plant vitrification solution. The result showed that *N. caerulea* seeds started to decrease viability after preculturing in PVS3 for 6 hours. However, if seeds were not precultured long enough, there was not enough time for PVS3 to penetrate into seeds to achieve cryoprotecting effect.

In cryopreservation, seeds survived after preculturing 3 hours and achieved maximum survival rate (23%) at 9 hours (fig. 2). Preculturing 9 hours was a suitable time, which on one side long enough to have cryoprotecting effect to achieve maximum survival rate, while on the other side not so long to prevent osmotic damages from PVS3.

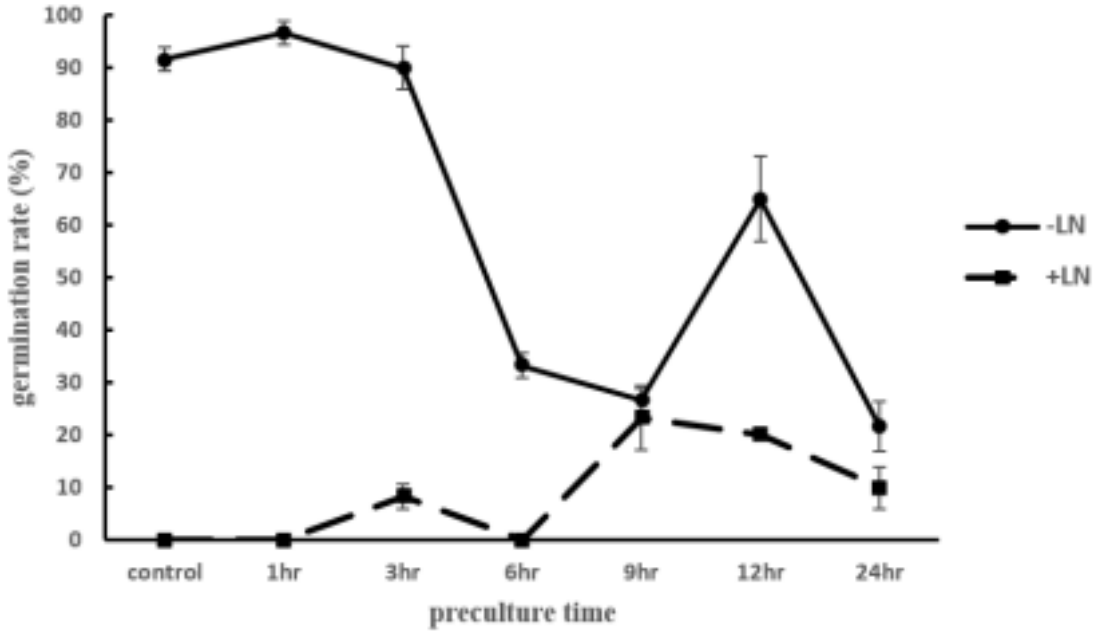

Fig. 2. Germination rate of N. caerulea seeds cryopreservation using PVS3 treatment. Seeds were precultured for various times ranging from 1 hour to 24 hours. Control represent survival rate of fresh seeds without PVS3 treatment. LN, liquid nitrogen.

3 – 3. Glutathione rescued the inefficiency outcome of *N. caerulea* seeds cryopreservation using PVS3 treatment

N. caerulea seeds were not successfully cryopreserved using PVS3 treatment. ROS induced oxidative stress might be the reason to cause failure of cryopreservation of PVS3 treated seeds. Low temperature stress induced plant tissues to accumulate large amount of ROS, and eventually might occur programmed cell death. To investigated if ROS caused the inefficiency, We added GSH, an antagonist against ROS-induced oxidative stress, into PVS3 to develop new plant vitrification

solutions. The purpose was to use GSH to decrease ROS accumulation caused by low-temperature stress, and to test if survival rate of cryopreserved seeds can be elevated after treating with the new plant vitrification solution. Data showed that seeds treated with various concentration GSH added PVS exhibited different enhancement in viability of both treated control and cryopreservation. In treated control, different concentration GSH added PVS increased survival rate of seeds 57 – 60%. In cryopreservation, different concentration GSH added PVS increased survival rate of seeds 48 – 63%. 0.08mM GSH added PVS, which was named plant vitrification solution + (PVS+), had the best improvement to increase survival rate of cryopreserved seeds (97%) (fig. 3). It seemed like GSH could actually promote survival rate of cryopreserved *N. caerulea* seeds.

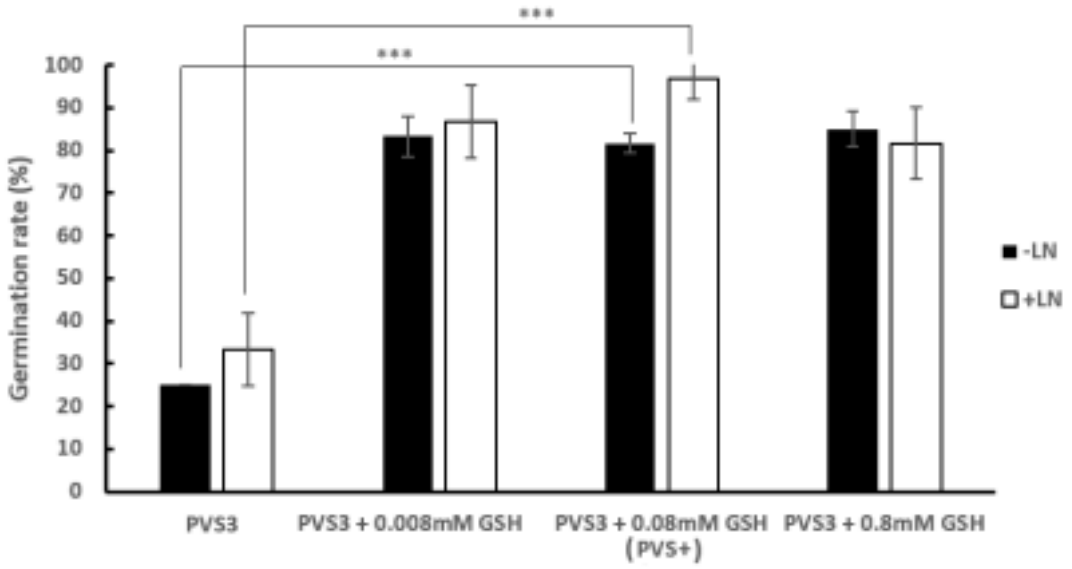

Fig. 3. Various concentration GSH added PVS treatment promoted survival rate of *N.*

caerulea seeds after cryopreservation. 0.08mM GSH added PVS had the best improvement to increase survival rate of cryopreserved seeds, and was named PVS+. GSH, glutathione. LN, liquid nitrogen. Significant differences are indicated as ***P < 0.005.

3 – 4. Amount of H_2O_2 within seeds increased after cryopreservation and decreased after adding GSH.

To further investigate the oxidation effect, We measured the amount of H_2O_2, one of ROS indicator, within *N. caerulea* seeds at different treatments. The data showed that in normal state, fresh seeds contained 150.07 μmol·g^{-1} H_2O_2. After preculturing PVS3 and PVS+ for 9 hours, seeds still contained similar amount of H_2O_2 (at 171.48 and 172.55 μmol·g^{-1}, respectively). However, the amount of H_2O_2 within seeds significantly increased to 526 μmol·g^{-1} after cryopreservation. Even precultured using PVS3, amount of H_2O_2 of seeds dropped a little bit to 484.05μmol·g^{-1} but still remained at high level after cryopreservation. After precultred by PVS+, amount of H_2O_2 of seeds decreased to 216.44 μmol·g^{-1} near the normal state (fig. 4). This finding indicated that amount of H_2O_2 of seeds would significantly increase even treated with PVS3 after cryopreservation, and GSH could decrease H_2O_2 formation. Antioxidants significantly decreased ROS

induced by cryopreservation

and therefore increased seeds viability after cryopreservation, and suggested that inhibiting ROS-induced oxidative stress might be a useful method to improve seeds viability after cryopreservation.

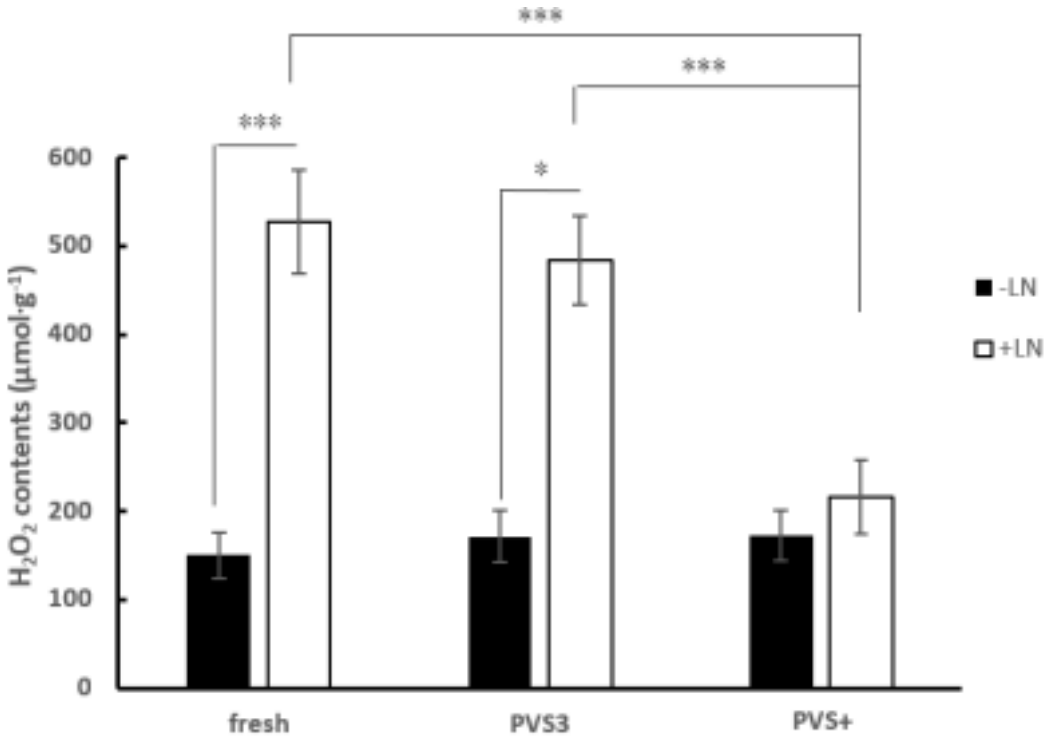

Fig. 4. H_2O_2 content in seeds of different treatments with or without cryopreservation. LN, liquid nitrogen. Data used Bonferroni's correlation. Values labeled with the star represent significantly different at *, P value <0.05 ; ***, P value <0.005.

4. Discussion

This study aimed at discovering a method to long-term store *N. caerulea* seeds. First

step was to realize the storage characteristic of *N. caerulea* seeds. We found that survival rate of *N. caerulea* seeds dropped significantly after desiccation and low temperature treatment. Furthermore, *N. caerulea* seeds could only sustain normal viability for 1 month storage using traditional storage method. It is worthy to note that the reason of survival rate of seeds using traditional storage method stored at RT dropped sharply after 1 month was because seeds started to germinate and rot after 1 month storage. Seeds stored in 4℃ did not germinate and rot during storing period. Germination time of seeds stored under both RT and 4℃ became increasingly long (data not show), which may imply that the qualities of seeds were getting worse while storing time getting longer. These findings suggested that it was not appropriate to long term store *N. caerulea* seeds using traditional storage method. Inability to tolerate desiccation and low-temperature, and short-term storage under traditional storage condition, all these characteristics indicated that *N. caerulea* seeds is recalcitrant. The result is consistent with previous studies which indicated *N. caerulea* close relative - *Nymphaea alba* is recalcitrant as well (Hay *et al.*, 2000).

Unlike previous studies showed that various plant tissues were successfully cryopreserved using PVS3 (Gonzalez-Arnao *et al.*, 2009; Sajini *et al.*, 2011). *N. caerulea* seeds cryopreservation using PVS3 did not be considered as a success.

Maximum survival rate of cryopreserved *N. caerulea* seeds reached only 23% when seeds were precltured in PVS3 for 9 hours. The cryoprotective performance was not good if we considered germination rate (88%) of fresh seeds as normal viability of seeds. The reason caused PVS3 failed to successfully cryopreserve *N. caerulea* seeds is ROS induced oxidative stress. Seeds are under extremely low temperature stress while cryopreservation. This stress causes seeds to induce ROS-induced oxidative stress, which is harmful to seeds. Data showed that within seeds the amount of H_2O_2, one of ROS indicator, hugely surged after cryopreservation. Even precultured by PVS3, the amount of H_2O_2 still significantly increased after cryopreservation. This might explain why PVS3 treated seeds lost a lot of viability after cryopreservation. However, if seeds precultured by PVS+ (PVS3 adding 0.08mM GSH) for 9 hours, the amount of H_2O_2 in seeds remained at normal level and seeds still have high survival rate after cryopreservation. The result indicated that *N. caerulea* seeds viability might be determined by ROS-induced oxidative stress, and using antioxidant to eliminate ROS accumulation could erase damages caused by oxidative stress. Our findings are agree with results of (Zhang et al., 2015) for cryopreservation of embryonic callus in *Agapanthus praecox*. They found that ROS (including H_2O_2) accumulated within embryonic callus after cryopreservation. This ROS-induced oxidative stress eventually cause apoptosis-like programmed cell death to damage cells. Antioxidants (such as

GSH) act as scavenger to eliminate intracellular H_2O_2 and decrease damages caused by oxidative stress in cryopreservation.

Recalcitrant seeds cryopreservation still remains some challenges. In addition to some issues scientists have already focused on, for example the role of water in cryopreservation of seeds (Zaritzky, 2015), the large size problem of recalcitrant seeds (Tweddle *et al.*, 2003), finding drying rate (Berjak *et al.*, 1993; Pammenter *et al.*, 1998)

and finding cooling rate (Dussert *et al.*, 1998). Oxidative stress problem in seeds cryopreservation also drew scientists' attention recently (Berjak *et al.*, 2011; Skyba *et al.*, 2012; Varghese *et al.*, 2012). Our findings provided an another evidence to showed that oxidative stress caused by cryopreservation is harmful to cryopreserved plant tissues. Antioxidants, ROS antagonists, rescue the damage and achieve plant tissues cryopreservation.

5. Conclusion

This study indicated that *N. caerulea* belongs to recalcitrant, which is sensitive to desiccation and low-temperature and not suitable to long-term store by traditional storage method. PVS3 treatment was ineffective on *N. caerulea* seeds

cryopreservation. Oxidative stress induced by ROS accumulation was the reason to cause inefficiency of seeds cryopreservation. GSH, an antioxidant, rescued the inefficiency by decreasing ROS accumulation. After decreasing damaging caused by low-temperature induced oxidative stress, *N. caerulea* seeds were successfully cryopreserved. Our results may

suggest a new direction to improve recalcitrant plant cryopreservation.

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