

Optimal seed water content and freezing method for cryopreservation of *Areca catechu* L. seeds

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Accepted 7th June, 2020.

Abstract. *Areca catechu* is an important cash crop in tropical regions of Asia and Central Africa. The seed is susceptible to low humidity and temperatures as recalcitrant seeds, so it cannot be stored by conventional methods, only can be sowed soon after collecting. The aim of this study was to explore a long-term effective preservation method with cryopreservation for *A. catechu* seeds. Mature seeds responses to drying were tested. Further, we analyzed the effects of liquid nitrogen freezing on the microstructure and biochemical indicators of the seeds. Desiccation of seeds with moisture content (MC) from 55 to 20% reduced their germination rate from 90% to 40% in significance. Stored at 25°C and 4°C for 2 months, the germination rates declined with increasing of seed moisture content. The optimum safe MC in *A. catechu* seeds is 35%. When stored in liquid nitrogen, the vigor of seeds with MC of 35% treated by direct freezing was highest compared to vitrification and step freezing. Direct freezing had no effect on the seed microstructure, but enhanced free radical scavenging activity, and weakened the seed's antioxidant activity. Notably, freezing had no effect on dehydrogenase explaining the high vigor observed. The vitality of seeds with 35% MC after cryopreservation was 95.67%. Liquid nitrogen preserves biochemical indicators and maintains high vitality; therefore, cryopreservation is an effective method for *A. catechu* seeds.

Keywords: *Areca catechu*, recalcitrant seed, liquid nitrogen, moisture content.

INTRODUCTION

Areca catechu L., a member of the Palmae family, is a perennial tropical woody plant common in tropical Pacific, Southeast Asia, South Asia and parts of East Africa. It is the main economic crops in these regions mainly as a food source and for medicinal purposes (Zhang *et al.*, 2008; Yang *et al.*, 2018). Chewing *A. catechu* is a traditional custom going back to over 2,000 years, and the species is used by about 600 to 700 million people worldwide (Huang, 2017). *A. catechu* seed is the main part used for medicinal purposes. The seeds are used in

promoting digestion, lowering blood pressure, as anti-depressants, anti-parasitic and bacteriostatic agents (Sun *et al.*, 2017). In China, *A. catechu* has been used for medicinal purposes for about 1,700 years (Zhou *et al.*, 2017). Further, *A. catechu* is the world's fourth-most consumed item after nicotine, ethanol, and caffeine (Zhou *et al.*, 2017).

Storage of dried seeds is the most economical and practical method for the preservation of crop germplasm. However, current storage methods are not effective for *A.*

catechu seeds (Lu *et al.*, 2016), therefore, preservation is challenging. Traditional *in situ* conservation and *in vitro* tissue culture preservation methods are expensive and are susceptible to the external environment factors, thus they are not effective for long-term stable conservation of germplasm resources (Chen *et al.*, 2013). Cryopreservation is characterized by minimal space requirements and low maintenance costs, therefore, it is the preferred method for long-term preservation of germplasm resources. In addition, it is considered the only viable method for the long-term preservation of recalcitrant seeds (Berjak *et al.*, 2014; Florent, 2010).

Cryopreservation refers to the storage of biological samples in liquid nitrogen (LN, -196° C) or LN vapor (LNV, about -165 to -190°C) (Wang *et al.*, 2020). Cryopreservation reduces cell metabolic activity while preserving the cell viability and morphogenic ability. This ensures long-term preservation of samples (Zhang *et al.*, 2015). The National Center for Genetic Resources Conservation, Fort Collins, Colorado, reported that materials cryopreserved in LN could be stored for about 3400 years (Marcin *et al.*, 2015). The use of low temperature in cryopreservation of seed materials leads to longer storage periods of the seeds (Marcin *et al.*, 2015). Cryopreservation has been effective in long-term storage of many plant species from tropical climate regions like *Dipterocarpus turbinatus* (Zeng *et al.*, 2018), *Archontophoenix alexandrae* (Shao, 2006), and *Manilkara zapota* L. (Wen *et al.*, 2013), *Myristica fragrans* (Wu *et al.*, 2019), *Ananas comosus* L. (Edilene *et al.*, 2016), and *Livistona chinensis* (Wen, 2011).

Effective cryopreservation of seeds requires identification of the hydration window, which refers to the safe range of moisture content at which seeds can be exposed to cryogenic temperatures without affecting their viability (Elena *et al.*, 2016). High moisture content damages or kills seeds during liquid nitrogen cryogenic freezing process. On the other hand, low moisture content causes dehydration damage (Zeng *et al.*, 2014). Currently, no studies on the safe moisture range for *Palmae* species seed cryopreservation, while the semi-lethal moisture content of *Archontophoenix alexandrae* seeds is 30% (Shao *et al.*, 2006), the safe moisture range of *Cocos nucifera* L. embryos is 25 to 65% (Nan *et al.*, 2012). However, the effect of *A. catechu* seeds storage and their tolerance to low temperature and low humidity has not been investigated.

In this context, the aim of this study was investigated different water contents and preservation methods to recommend effective methods for *A. catechu* seed preservation.

MATERIALS AND METHODS

Plant material

A. catechu seeds were collected from Hainan Province,

China. Selected ripe fruits with orange peel, full size, and no cracks, then removed the fiber pulp and took out seeds. The seeds were refrigerated at 10°C until further use, with initial moisture content (MC) of 55%, a vitality of 95%, and a 90% germination rate. Notably, the seeds were not air-dried after collection, and refrigerator storage time was less than one week.

The MC was determined using a gravimetric method. The weight of three replicate samples of 10 seeds each was measured before and after heating in a drying oven (130 ± 3°C) for one hour (Zhang *et al.*, 2005). The percentage MC was expressed as the ratio of the fresh weight to the dry weight after oven drying.

The viability of the seeds was evaluated using the 2,3,5-triphenyl tetrazolium chloride (TTC) method (Zhang *et al.*, 2005).

Storage experiment

Seeds with MC of 55% were placed in sealed bags and stored at 25 and 4°C, seeds with water content of 35 and 26% were sealed and stored at 4°C, and the germination rate of the seeds was determined after 60 days. Three replications of 10 seeds each were used each time.

Reduction of moisture content

The seed MC was reduced by rapid drying method. The seeds were placed in a desiccator containing silica gel with a volume ratio of silica gel to seeds of 200: 1 for different periods based on the initial MC of the seeds, and the predicted drying speed to obtain seeds with different gradients of water. Three replications of 10 seeds each were used each time.

Further, the seeds were dried at room temperature for 0, 1, 3, 5, 27, and 122 h, and seeds with water content of 55 ± 1%, 45 ± 1%, 40 ± 1%, 35 ± 1%, 30 ± 1%, 25 ± 1% were obtained.

Cryopreservation experiments

Dehydrated seeds were divided into four groups; one group was a control used for the determination of viability, physiological, and biochemical parameters without any freezing treatment. The other three groups were frozen in liquid nitrogen (LN). *A. catechu* seeds were cryopreserved by vitrification (Sakai *et al.*, 1990), step freezing, and direct freezing. All treatments were done in triplicates of 30 to 50 seeds each. Further, we conducted seed-vigor testing to determine the optimal freezing method for LN cryopreservation of *A. catechu* seeds.

Seed vitrification: A cryoprotective loading solution consisting of 2.0 M glycerol and 0.4 M sucrose was

added to a cryotube containing the seeds. Cryovials were incubated for 20 min at room temperature ($25 \pm 2^\circ\text{C}$), then the loading solution was replaced with a plant vitrification solution 2(PVS2). After incubation of the cryotube in an ice bath for 30 min, the loading solution was replaced with pre-cooled fresh PVS2. Seeds were immediately transferred into LN for storage. The PVS2 (Sakai *et al.*, 1990) comprised 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide and 0.4 M sucrose in MS medium (pH 5.7).

Seed step freezing: The test seeds were immersed in a cryotube containing PVS2 ($25 \pm 2^\circ\text{C}$) and incubated at 4°C for 30 min. The seeds were then immediately transferred to a -20°C freezer and incubated for 1 hour. Further, seeds were transferred into LN for storage (Chen *et al.*, 2013).

Direct freezing of seeds: The seeds were placed in a cryotube and transferred into LN for storage (Ma *et al.*, 2007).

Thaw of seeds: After 24 hours in LN, cryovials were removed and rapidly thawed in a 40°C water bath for 3 min. Seeds in the step-frozen vitrification groups were washed with liquid MS medium+1.2 M sucrose (pH 5.7) for 15 min and then rinsed with sterile water for 5 min (Chen *et al.*, 2013).

Physiological and biochemical indicators

The seeds in the control and optimal frozen groups were placed in a freeze grinder for enzyme extraction for physiological and biochemical indicator studies. Dehydrogenase and superoxide dismutase (SOD) activities were determined by the nitrogen blue tetrazolium method (Liu, 2010). In addition, catalase (CAT) activity was measured using the phosphate buffer method (Yin *et al.*, 2008). The activity of α -amylase was determined by the amylase kit iodine-starch colorimetry (the kit is Nanjing Jiancheng Bioengineering Institute Amylase Kit C016), while the polyphenol oxidase (POD) activity was evaluated using the guaiacol method (Liu, 2010). Thiobarbituric acid (TBA) method was used to determine free *malondialdehyde* (MDA) content (Wang *et al.*, 2015). The Coomassie brilliant blue colorimetric assay was used to determine the soluble protein content (Zhang *et al.*, 2012), and seed conductivity was measured using conductivity meter (Zhang, 1999).

Observation of seed microstructure

The seeds in the control and optimal frozen groups were cut exposing the embryo-containing part, and then carry out paraffin section treatment. The stained sections were

observed with a biological microscope and the changes in the seed embryo cells recorded.

Statistical analysis

SAS software was used for all statistical analysis of the data. Analysis of variance (ANOVA) was used to assess the significance of different treatments, as was the *F*'s test for pair-wise comparisons. *F*'s test was performed at a significance level of $P > F < 0.05$. Separate ANOVAs and *F*'s tests were performed for germination and physiological and biochemical indexes. Error lines indicate standard errors (SEs) of the mean within an isolated treatment.

RESULTS

Effect of moisture content and storage time on seed viability

We observed that the viability of *A. catechu* seeds with MC more than 30% was over 90%. The MC of 26% reduced the viability to 63.33%, while seed viability below 40% was observed for a 20% MC (Figure 1). Therefore, the appropriate MC of *A. catechu* seeds should not be below 26%.

The germination rate of the seeds decreased with increase in storage time. After 60 days of storage, seeds with a water content of 55% had a germination rate of less than 20% at 25°C , and a germination rate of 50% at 4°C . After storage at 4°C for 60 days, the germination rate of seeds with MC of 35% decreased from 96 to 63.67% while the germination rate of the seeds with a MC of 26% decreased from 63.33 to 35% (Figure 2). These findings show that the optimum safe MC in *A. catechu* seeds is 35%.

Effect of freezing method on viability of seeds with different moisture contents

Seed storage moisture content and freezing methods are key factors in achieving effective cryopreservation. Before cryopreservation, when the MC of the seeds was higher than 30%, it had no significant effect on seed viability even when water content was decreased. While water content below 30% significantly decreased seed vitality. Compared with before freezing, the reduction of water content after cryopreservation significantly decreased the viability of seeds. However, the seeds with 35% MC after direct freezing showed 95.77% viability, which was comparable to 96.97% viability before freezing. These findings showed that *A. catechu* seeds with MC of 35% can be stored by direct freezing on cryopreservation (Figure 1).

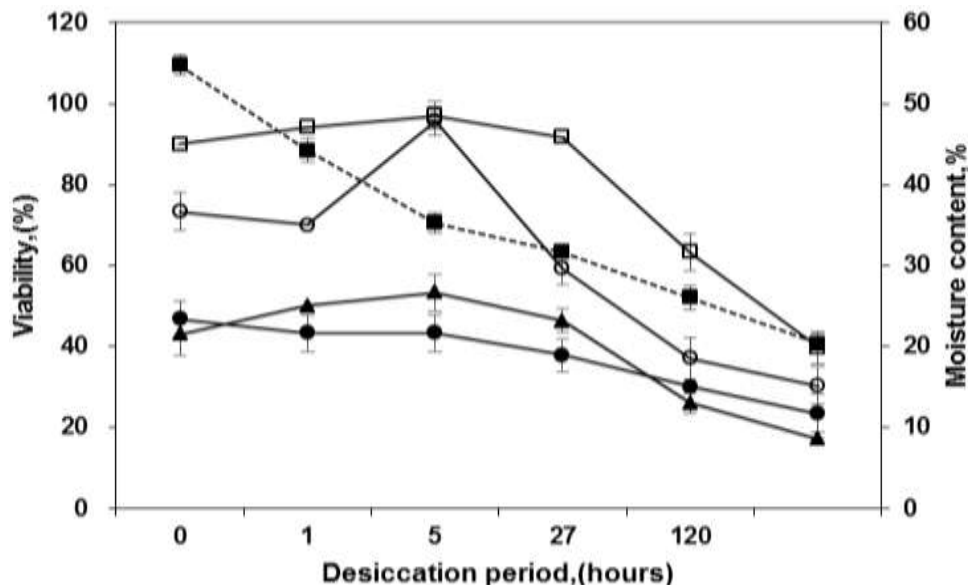


Figure 1. Effect of moisture content and freezing method on seed viability before and after liquid nitrogen exposure for *A. catechu*. Viability was determined before (\square) and after cryopreservation by vitrification (\bullet), step freezing (\blacktriangle) and direct freezing (\circ) at various moisture contents (\blacksquare). Values plotted are the mean of three replicates \pm SE values. Error line indicates 1.96 times of the standard deviation. The moisture content and viability of seeds decreased with the extension of desiccation period. When stored in liquid nitrogen, the viability of seeds decreased with the decrease of moisture content. The seeds with 35% water content under the direct freezing had the highest vitality, which was not significantly different from that before cryopreservation treatment.

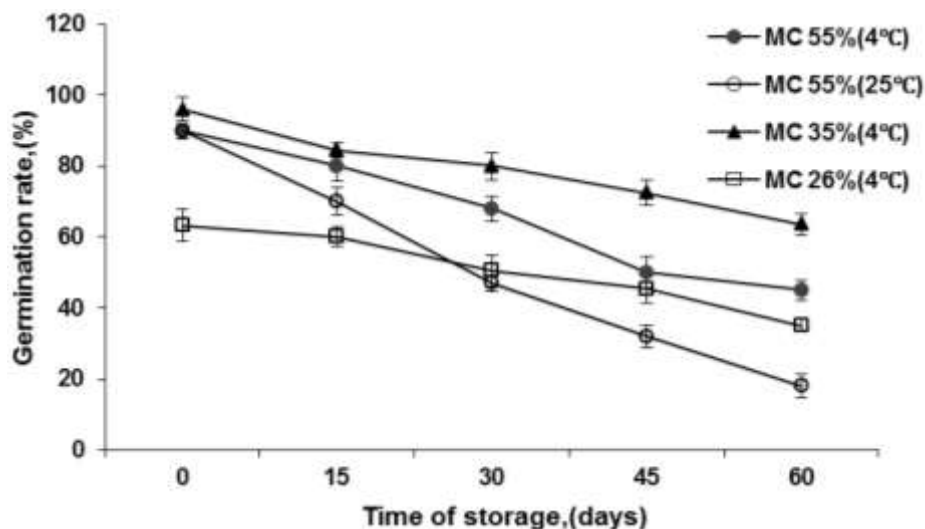


Figure 2. Effect of moisture content and storage time on seed germination rate. Error line indicates 1.96 times of the standard deviation. The germination rate of seeds decreased with the extension of storage time. Seeds with moisture content (MC) of 35% were stored at 4°C, and the germination rate was always higher than 60%. Seeds with MC of 55% were stored at 25°C, the germination rate dropped sharply, even below 20%.

Effect of cryopreservation on physiological and biochemical indexes of seeds

The physiological and biochemical indexes of seeds were

changed after direct freezing treatment. The CAT activity and SOD activity of seed were significantly decreased, while the relative conductivity and MDA content were significantly increased. The freezing of liquid nitrogen

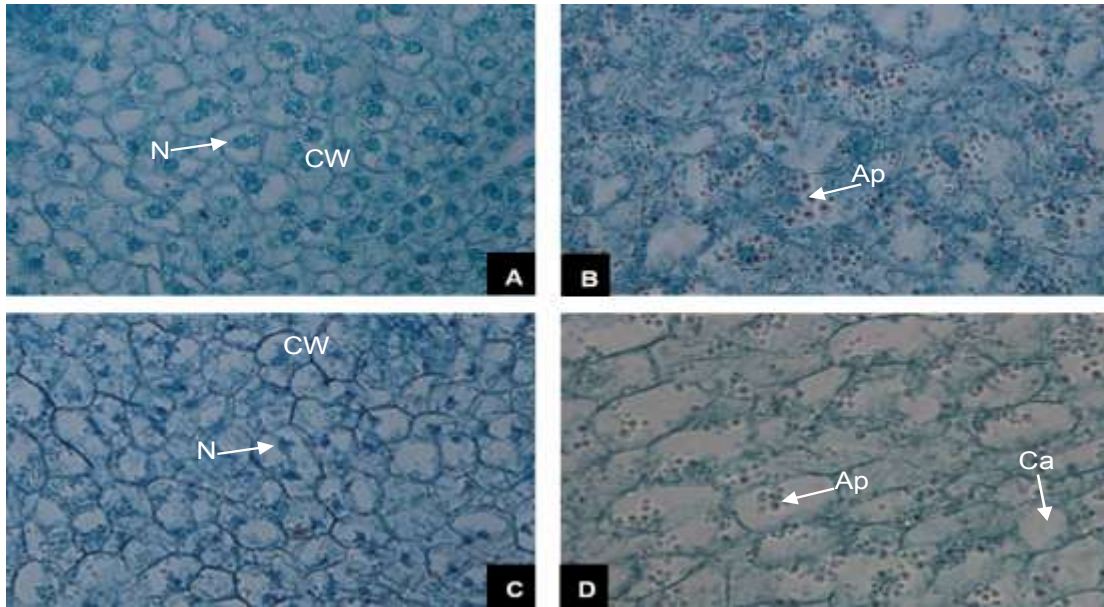


Figure 3. Effect of cryopreservation on seed structure before and after liquid nitrogen exposure for *Areca catechu*. Nu: Nucleus; CW: Cell Wall; Ap: Starch Grains; Ca: Cavity. A-B: Control cells, neatly arranged, with obvious nucleus, containing a lot of organelles and starch granules. C-D: Cryopreservation cells, the nucleus was not obvious, the starch granules reduced, and the cavity appears.

Table 1. Effect of cryopreservation by direct freezing on physiological and biochemical indexes of seeds before and after liquid nitrogen exposure.

	Control	Cryopreservation	<i>Pr>F</i>	<i>F</i>
Conductivity (%)	18.53 ± 0.01	56.36 ± 0.02	<.0001	649.25
MDA (μmol/g)	6.81 ± 0.43	10.83 ± 0.52	0.0011	70.4
POD (U/g)	1633.33 ± 7.02	5843.97 ± 17.74	<.0001	97391.1
CAT (U/g)	127.35 ± 0.47	77.97 ± 0.01	<.0001	21720.9
SOD (U/g)	701.32 ± 13.52	587.59 ± 1.03	0.0003	140.76
α-amylase (U/g)	3.25 ± 1.91	2.20 ± 1.92	0.6111	0.3
Protein (%)	3.63 ± 0.01	3.23 ± 0	0.5284	0.48
Dehydrogenase (μg/ml)	35.14 ± 0.49	34.21 ± 1.47	0.4451	0.72

F's test was performed at a significance level of $Pr>F < 0.05$. The physiological and biochemical indexes of seeds were changed after direct freezing treatment. The CAT activity and SOD activity of seed were significantly decreased, while the relative conductivity and MDA content were significantly increased. The α-amylase activity, protein content and dehydrogenase activity did not change significantly.

damages the seeds, resulting in an increase in cell membrane permeability and an increase in active oxygen levels, thereby weakening the antioxidant capacity of the enzyme and a sharp drop in enzyme activity. The α-amylase activity, protein content and dehydrogenase activity did not change significantly, indicating that liquid nitrogen freezing had no significant effect on them, which was consistent with the seeds viability test results (Table 1).

Effect of cryopreservation on seed microstructure

After direct freezing, the seed microstructure had some

changes. Before cryopreservation, the embryo cells were intact and neatly arranged. There was a circular cell nucleus in the center, with regularly nuclear membrane, and it is rich in vacuoles and a large number of organelles (Figure 3A). Further, the endosperm cell space was not clear and the starch granules were uniformly distributed (Figure 3B). After freezing treatment, the embryonic cells were loosely arranged, the color of the nucleus was lightened, some of the nucleus material was scattered, and the organelles were blurred (Figure 3C). However, the endosperm cell space was clear with a low number and content of starch granules, and cavities appear in some cells (Figure 3D). After direct freezing,

the embryos remained intact with no separation from the wall. These findings indicate that cryopreservation did not significant damage *A. catechu* seed structure.

DISCUSSION

Mature dehydration is the terminal event of seed development, which plays an adaptive role for seeds to survive in storage and ensure species reproduction. The difference between recalcitrant seeds and orthodox seeds is that orthodox seeds exhibit low water content when they fall off and can be further dried to a moisture content of 1 to 5% without damage. On the contrary, recalcitrant seeds do not undergo mature dehydration; therefore, the moisture content is relatively high when the seeds are shed. In addition, recalcitrant seeds are not resistant to dehydration throughout the development process and are typically sensitive to low temperatures. Under the conditions suitable for orthodox seed storage, the lifespan of recalcitrant seeds is usually a few days to a couple of weeks (Fu *et al.*, 2004). The *A. catechu* seeds with high initial moisture content have poor dry tolerance, according to Roberts (Roberts, 1973) who classified them as recalcitrant seeds. This could be because these seeds do not undergo natural dehydration when they mature, protective molecules are not synthesized, and all cells are highly metabolically active. Once dehydrated, the cells cannot dedifferentiate and metabolize, as normal cells are damaged (Roberts, 1973).

Most mature seeds of Palmae plants have high-water content. For instance, the semi-lethal water content of *Archontophoenix alexandrae* seeds is 30% while *Livistona chinensis* seeds are not resistant to dehydration (Wen, 2011). In this study, we evaluated the dehydration tolerance and low temperature sensitivity of *A. catechu* seeds through dehydration and storage experiments. The results show that *A. catechu* seeds are sensitive to low temperature and dryness when the MC is below 26%, so the appropriate MC of *A. catechu* seeds should not be below 26%.

Generally, seeds with high water content are damaged or even die during cryogenic freezing with liquid nitrogen (Zeng *et al.*, 2014). However, in this study, we found that *A. catechu* seeds with MC of 35 to 55% after direct freezing, showed more than 70% survival rate while its seed vigor reached 95.67% for 35% MC. This is explained by the high lipid content in *A. catechu* seeds (Zhou *et al.*, 2010). A previous study reported that seeds with high oil content (mazzard cherry (*Prunus avium* L.)) or high lipid content (*Fagus sylvatica* L.) are more tolerant to liquid nitrogen freezing at higher water contents (Marcin *et al.*, 2015; Stanislaw *et al.*, 2014). Therefore, it is possible that the observed differences are as a result of differences in the oil or lipid content in the seeds (Bernardo *et al.*, 2001; Hor *et al.*, 2005).

Vitrification method is the most widely used cryopreservation freezing method, as it is suitable for

freezing materials with high water content (Berjak *et al.*, 2014; Elif *et al.*, 2012). However, the viability of *A. catechu* seeds with 30 to 55% MC after cryopreservation through vitrification and step-freezing method is lower compared with that of the direct freezing method. This difference may have resulted from high PVS2 concentration, or long soaking time of the refrigerant. However, the cause of this difference in vitality should be explored.

An increase in conductivity, MDA content and POD activity in *Areca catechu* seeds after freezing in LN, were observed because MDA is associated with POD activity. A previous study reported that MDA is a metabolite implicated in plant stress response and its accumulation is caused by the peroxidation of cell membrane lipids. Reactive oxygen species activity produces hydrogen peroxide, which promotes increase in POD activity (Byron *et al.*, 2013). Further, plant tissues produce a high levels of free radicals when subjected to external stress, causing the membrane lipid peroxidation and ultimately MDA formation. An increase in MDA content causes a large amount of extravasation of ions in the cells, resulting in an increase in the relative conductivity of plant tissues (Pan 2004). Changes in seed biochemical indicators as a result of dehydration (Pammenter *et al.*, 2014), freezing of LN (Byron *et al.*, 2013), or aging during storage (Heloisa *et al.*, 2016) is a complex process. Both the vigor and biochemical indicators indicate that it is scientifically feasible to preserve the *A. catechu* seeds for cryopreservation.

ACKNOWLEDGEMENTS

This study was funded by the National Natural Science Foundation of China (No. 81603399) and CAMS Initiative for Innovative Medicine (CAMS-I2M) (No. 2016-I2M-2-003).

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