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Full Length Research Paper

Physiological and biochemical responses of ultra-dry storage of *Elymus dahuricus* seeds

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The silica gel drying method was utilized to reduce the moisture content of *Elymus dahuricus* seeds from an original content of 9.03 to 7.69, 6.10, 4.97, 4.47, 3.83, 2.58 and 1.26%. After sealing the seeds in aluminum foil bags, they were placed at -4°C, 4°C, room temperature and 45°C to store for 12 months and to identify their physiological and biochemical indicators. Results indicate that ultra-dry storage can: 1) Improve the storability and membrane permeability of *E. dahuricus* seeds, 2) increase the activity of *E. dahuricus* SOD, POD and CAT, and 3) lower MDA content. Moderate ultra-dry storage at room temperature can reach the conserved effect of low-temperature without intense ultra-drying. Therefore, the moderate ultra-dry can be use as effective measure for the conservation of *E. dahuricus* germplasm resources.

Key words: *Elymus dahuricus*, ultra-dry storage, moisture content.

INTRODUCTION

Effective protection of plant germplasm resources needs the combination of *in situ* and *ex situ* conservation. The former preserves the increase in diversity of germplasm resources with continued evolution; while the purpose of *ex situ* conservation is to maintain the genetic stability of germplasm resources, and the applied conservation techniques will minimally reduce the possibility of mutations, selection, random drift and genetic contamination (Cheng, 2004). Seeds of most plants can be stored for long-term under low temperature and low moisture content; therefore, seed repository has become a primary method of *ex situ* conservation (Chen and Zheng, 2004; Chen et al., 2009). Seed storage temperature and seed moisture content are the key factors to maintain the seed vigor and viability during storage (Huang et al., 2009). It is easier and more reliable to control temperature than moisture content; therefore, seed storage conditions are most often governed by the use of low temperature and ultra-low temperature. International Plant Genetic Resources Committee proposed that storage conditions maintain a moisture content of 5 ± 2% and a low temperature of -18°C. These conditions have proven to be ideal for the long-term conservation of germplasm resources throughout the world. Among the collected 6.1 million accessions of plant germplasm resources, about 90% of them are stored in thousands of low temperature germplasm repositories (Chen, 1995).

However, the high cost of low-temperature germplasm repository, high technical demand, guarantee for power and high annual operating costs have limited the construction and operation of low temperature germplasm repositories. Subsequently, more efficient seed storage methods are needed, which can save energy, reduce expenditures and provide adequate protection of germplasm in storage. To address these needs, FAO/International Plant Genetic Resources Institute (FAO/IPGRI) initiated and funded global research efforts on ultra-dry storage of seeds in the late 1980s. Research locations included the United Kingdom Reading Laboratory and Botany Institute at the Chinese Academy of Science. They used appropriate drying method to reduce the moisture content of seed below 5% in order to substitute low-temperature repository in part or fully. Ultra-drying can improve the storability and stability of seeds, indicating that the moisture content should not be as low as possible (Wang et al., 2001). Studies show that when the moisture content of seeds decreases to a certain value,

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the seed vigor will decrease (Ladbrooke et al., 1969; Ma et al., 2009). Excessive ultra-dry is useless when used to prolong the life of seeds, and may even promote their deterioration. A lot of studies show that the optimal moisture content varies widely for seeds of different species, from 2 to 6% (Eillis et al., 1988). This may be related to the chemical substances existing in seeds. When seeds contain a high content of hydrophobic compounds, they will then have a lower optimal moisture content for storage and a stronger desiccation tolerance. Additionally, the optimal moisture content will change with differing storage temperatures. Ellis et al. (1990) reported that when the moisture content of *Brassica campestris* seeds decreases to 1.5% or less, the seeds will remain viable and their storage life will be extended. However, the authors suggest that the optimal moisture content of *B. campestris* is 2.8%, *Brassica juncea* is 3.8%, and that *Arachis hypogaea* is 2.0% (Ellis, 1990). Starch levels are also known to affect the desiccation tolerance of seeds. For example, the desiccation tolerance of *Sorghum bicolor*, *Hordeum vulgare* and *Triticum aestivum* seeds is high, while the desiccation tolerance of paddy rice (*Oryza sativa* L.) seeds is low (Jiang, 2010)

This study examined *Elymus dahuricus* seeds to evaluate the impact of ultra-dry storage on their physiological and biochemical characteristics under different temperature conditions. The objective was to provide a theoretical basis for the conservation of *Elymus* germplasm resources.

MATERIALS AND METHODS

E. dahuricus seeds were collected from Qinghai in 2007 with an initial moisture content of 9.03%, germination percentage of 56% and fresh seeds percentage of 38%.

Ultra-dry processing and storage conditions

The silica gel drying method was utilized at room temperature. Specifically, *E. dahuricus* seeds were placed in nylon mesh bags which were then buried in 120℃ dry silica gel at a 1:10 ratio (seeds to gel mass). Dehydration was done at a room temperature and the silica gel was changed every 12 h. Seeds were weighed frequently to the desired moisture contents of 1.26, 2.58, 3.83, 4.74, 4.97, 6.10, 7.69 and 9.03%. In November 2008, the seeds with the aforementioned moisture contents were sealed with aluminum foil bags and store at -4℃, 4℃, room temperature (15 to 30℃) and 45℃ for about 12 months, and subsequently, the physiological and biochemical indicators affecting storage were determined.

Standard germination percentage and the determination of germination potential

Following the Testing Procedure of Forage Seed (GB/T 2930.1~2930.11-2001), three layers of filter paper were used as the germination bed, on which fifty seeds were placed per plate with four replications. For germination, the following conditions were maintained: 25℃ temperature, 8 h of light and germinations counts at the 5^{th} and 12^{th} days. The percentage of normal seedlings was calculated. The percentage of normal seedlings germinating within a specified time is referred to as the germination potential.

Determination of electrical conductivity of the leaching solution

For the determination of electrical conductivity of the leaching solution, three replications of 50 seed count weights were utilized. The seeds were washed three times with de-ionized water to absorb the moisture from the seed surface. The seeds were then placed in a covered 250 ml triangular flask to which 200 ml de-ionized water was added. Controls flasks of 200 ml de-ionized water were included in each replication. Flasks were placed in 20℃ plant growth chambers for 24 h. The electrical conductivity of each flask was determined with a EC215 conductivity meter. The conductivity of the leaching solutions was determined by subtracting the conductivity of deionized water in the control flask from the conductivity of each leaching solution, and then dividing by the sample's seed weight.

Preparation of enzyme extracting solution

Three replications of 0.5 g of seeds were washed with distilled water. Then, 8 ml of 50 mmol/L, pH 7.0 of ice-cold phosphate buffer was added which was then ground to a homogenized state. The solution was then centrifuged at 4000 r/min for 20 min at 4℃. The resulting supernatant was then frozen.

Determination of enzyme activity of antioxidant system

The determination of superoxide dismutase (SOD) activity was done according to plant T-SOD Elisa Kit (Nanjing Jiancheng Biology Engineering Corporation, China). When the SOD suppression ratio of per 1 mg tissue protein in 1 ml reaction solution reaches 50%, its corresponding SOD content is regarded as a SOD activity unit (U). SOD activity (U/mgprot) = $(OD_c - OD_t) \times V_F \times (50\% \times OD_c \times V_s \times C)^{-1}$, where, OD_t is the absorbance of test tube; OD_c is the absorbance of control tube; V_F is the total volume of reaction solution (ml); V_s is the volume of enzyme solution used for determination (ml) and C is the protein content (mg/ml).

The determination of superoxide dismutase (POD) activity was done according to plant POD Elisa Kit (Nanjing Jiancheng Biology Engineering Corporation, China). The principle of POD was used to catalyze hydrogen peroxide reaction, by measuring changes of absorbance at 420 nm to reach its enzyme activity. POD activity (U/gprot) = $(OD_t - OD_c) \times V_F \times (12 \times W \times V_s \times t \times C)^{-1}$, where, OD_t is the absorbance of test tube; OD_c is the absorbance of control tube; V_F is the total volume of reaction solution (ml); W is the cuvette diameter (1 cm); V_s is the volume of enzyme solution used for determination (ml); t is the reaction time (30 min) and C is protein content (mg/ml).

The determination of CAT activity was altered according to Camak and Horst (1991) method. Fifty microlitres supernatant of enzyme was placed in a test tube, adding 3.4 ml phosphate buffer (pH 7.0) with a concentration of 25 mmol/L and 200 µl H_2O_2 at a concentration of 100 mmol/L. The solution was then reacted at 25℃ to determine the dynamic changes of A_{240}. A_{240} was calculated within one minute at 25℃ and subsequently, three times at one minute interval to determine the changes in levels of A_{240}. The results were calculated according to the method of Li (2000): CAT activity (U/g·min) = $\Delta A_{240} \times V_T \times (0.1 \times V_S \times t \times W_F)^{-1}$, where, V_T is the total volume of crude enzyme extracting solution (ml); V_S is the volume of enzyme solution used for determination (ml); W_F is the fresh weight of sample (g) and t is the period between addition of H_2O_2 and the last reading.

Table 1. The impact of ultra-dry storage on the germination percentage of *E. dahuricus* seeds for 12 months.

Moisture content (%)	Germination percentage (%)			
	-4 (℃)	4(℃)	Room temperature	45(℃)
1.26	94^{a}	88^{ab}	86^{ab}	84^{a}
2.58	90^{abc}	90^{ab}	80^{bc}	86^{a}
3.83	88^{abc}	90^{ab}	88^{ab}	86^{a}
4.47	88^{abc}	86^{ab}	92^{a}	88^{a}
4.97	84^{c}	86^{ab}	90^{a}	84^{a}
6.10	92^{ab}	90^{ab}	92^{a}	80^{a}
7.69	86^{bc}	90^{ab}	90^{a}	80^{a}
9.03 (CK)	86^{bc}	82^{b}	78^{c}	0b

Different small letters in the same column mean significance at $P < 0.05$.

Determination of malondialdehyde (MDA) content

Five milliliters of trichloroacetic acid (TCA) (concentration of 10%) and 1 ml thiobarbituric acid solution (concentration of 0.5%) was added to 1 ml of the prepared supernatant (prepared with 10% TCA solution). For the control, 1 ml of phosphate buffer was used in place of the supernatant. After holding in water at 95℃ for 30 min, the mixture was immediately placed in an ice bath to stop the reaction. It was then centrifuged at 4000 r/min for 10 min. OD values of the supernatant were then determined at the wavelengths of 532, 600 and 450 nm. MDA concentration (μmol/L) = 6.45 (OD_{532} - OD_{600}) - 0.56 OD_{450}, MDA content (nmol·g^{-1}) = (C×A×V) / (a × W), where, C is the MDA concentration (μmol/ml); V is the leaching solution volume (ml); W is the seed weight (g); a is the volume of determined enzyme solution (ml) and A is the reaction volume (ml).

RESULTS

The impact of ultra-dry storage on germination of *E. dahuricus* seeds

The germination of *E. dahuricus* seeds following 12 months of storage under variable moisture contents at -4℃, 4℃, room temperature and 45℃ without using an ultra-dry process have significantly lower germination percentage than those with ultra-dry storage (Table 1). At 45℃ storage for 12 months, seeds without ultra-dry process completely lost their ability to germinate.

The impact of ultra-dry storage on the conductivity of *E. dahuricus* seeds

The conductivity of the leaching solution *of E. dahuricus* seed after 12 months of being hermetically stored using variable moisture contents at -4, 4, room temperature and 45℃ increased with increasing temperature. Specifically, when stored at room temperature and 45℃, in the range of 2.921 to 4.645%, the conductivity of the leaching solution is very low but the conductivity of the ultra-dry seeds' leaching solution significantly increases. When the storage temperature is lower, the difference between ultra-dry and non-ultra-dry is not significant (Figure 1).

The cell membrane allows the cell to communicate with the extracellular environment, and its functionality is essential for the regulation of many cellular activities, such as, regulation of the cell's material communication and transport, and enzyme activity. The integrity of the cell membrane is the basis of seed vigor, if the membrane is damaged, the organelles may become distorted and disintegrated, and the destruction of various enzyme molecules will result in the decrease of seed vigor. The stability of membrane structures is reflected in the selective permeability of the cell membrane. The loss of seed vigor is due to cell membrane damage, which results in the leakage of cytoplasm which increases the conductivity of the leaching solution (Cheng, 2005) When the moisture content of *E. dahuricus* seed decreases to 1.26%, the conductivity increases as compared to moisture contents of 2.58 and 3.83%. Additionally, the fluidity of the membrane and its ability to maintain its integrity decreases. After storage, the leakage of intracellular electrolytes of ultra-dry seeds is significantly lower than that of the non-ultra-dry seeds. This indicates that the selective permeability of the cell membrane in ultra-dry seeds is better conserved than that of non-ultra-dry seeds.

The impact of ultra-dry storage on peroxidase (POD) activity of *E. dahuricus* seeds

The POD activity of seeds increases and then decreases as moisture content increases when *E. dahuricus* seeds are stored using variable moisture contents at -4℃, 4℃, room temperature and 45℃ for 12 months. When the moisture content of seeds is 3.83%, POD activity reaches its peak of 0.989 U/mgprot. The POD activity of seeds without an ultra-dry process is lower than seeds that have gone through a moderate ultra-dry process. Under the high-temperature conditions, POD activity is extremely low, only 0.0185 U/mgprot (Figure 2A).

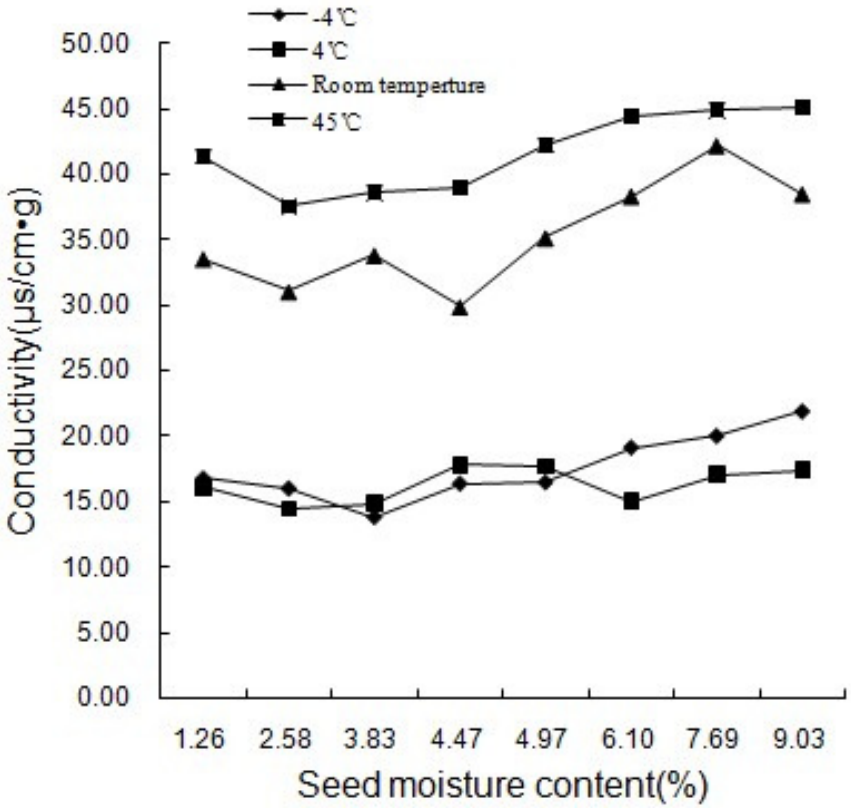

Figure 1. Changes of electronic conductivity of *E. dahuricus* seeds at different temperatures and different seed moisture content over 12 months.

The impact of ultra-dry storage on superoxide dismutase (SOD) activity of *E. dahuricus* seeds

The SOD activity of *E. dahuricus* seeds stored under variable moisture contents at -4℃, 4℃, room temperature and 45℃ appears to decrease and then increase. When the seed moisture content is 1.26%, the maximum SOD activity is 1.07 U/mgprot. At room temperature and high temperature storage at 45℃, the SOD activity first increases and then decreases. These SOD changes occur in a moisture content range of 3.83 to 4.97% (Figure 2B).

The impact of ultra-dry storage on catalase (CAT) activity of *E. dahuricus* seeds

Under different storage temperatures, the CAT activity of the ultra-dry seeds was higher than seeds without an ultra-dry process, and CAT activity of ultra-dry seeds does not change significantly with increasing moisture contents. The impact of temperature on the CAT activity of seeds with different moisture content was irregular. At low temperatures, the CAT activity of seeds at all moisture contents was lower than the activity observed at room temperature and under high temperature. At 4℃, CAT activity was the lowest (Figure 2C).

The **impact of ultra-dry storage on malondialdehyde (MDA) content of *E. dahuricus* seeds**

Malondialdehyde (MDA) is the end product of lipid peroxidation and its gradual accumulation in seed occurs as seed deteriorates during storage (McDonald, 1999). As temperatures increased, the MDA content of *E. dahuricus* seeds increased after a 12 month storage period at variable water contents. Particularly, under high temperature conditions of 45℃, the MDA content was significantly higher than contents observed under other temperatures. Water content had no effect on MDA content (Figure 2D).

DISCUSSION

Moisture content and storage temperature are the main factors that affect the life of seeds and the seed germination percentage is a key indicator to measure quality (Shi et al., 2009). When a species is stored at different temperature, it's safe moisture content ranges will differ. Others have shown that the life of seeds is closely

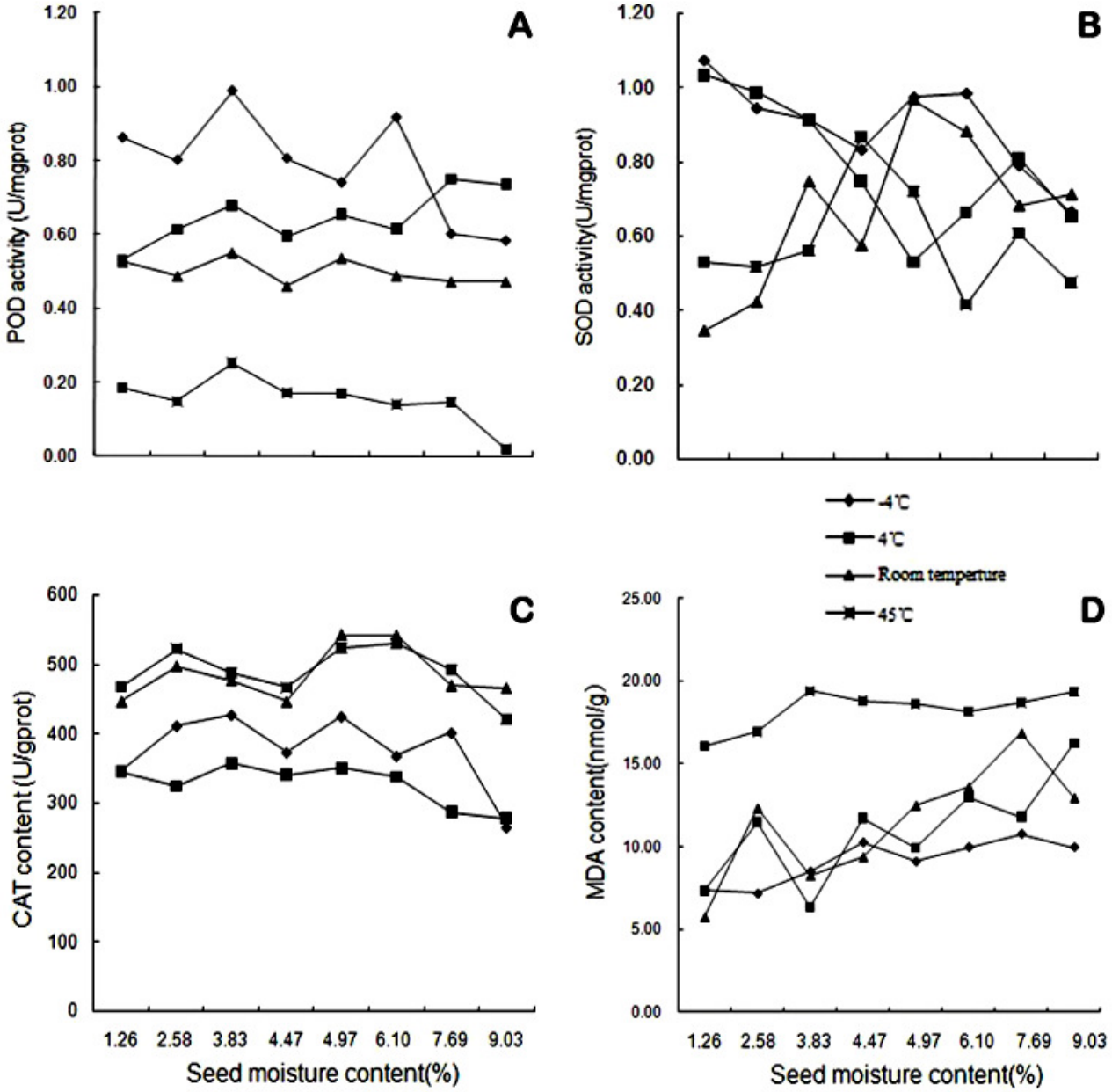

Figure 2. Changes of POD (A), SOD (B), CAT (C) and MDA (D) activity/content of *E. dahuricus* seeds at different temperatures and different seed moisture content over 12 months.

related to moisture content and storage temperature; and there exists a critical value of moisture (Chen et al., 2006). The study of Tong (2009) on the ultra-dry storage of Huabei *Ceratoides latensand* and *Haloxylon persicum* seeds showed that when *C. arborescens* and *H. persicum* seeds are stored at 4℃, room temperature and 35℃, the optimum moisture content of the former is 5.89 to 3.78, 4.91 to 3.78% and 2.69%; while the moisture content of the latter is 4.24 to 5.23, 3.25 and 3.25%. The optimum seed moisture content g enerally decreases as temperature increases.

The current study shows that when stored at room temperature and moisture content in the range of 1.26 to 7.69%, *E. dahuricus* seeds can maintain a higher germination percentage after being stored for 12 months. These results are similar to the germination percentages that result from ultra-drying *E. dahuricus* seeds followed by storage at low temperatures. The method of moderate ultra-dry at room temperature is an effective way for the storage of *Elymus* germplasm resources, thereby

reducing the cost of conservation needed for storage at low temperature.

The membrane system is vital for the regulation of material exchange, cell transport, and it also affects enzyme activity. Once the membrane system has been damaged, it will cause abnormal metabolic changes, thus accelerating the loss of seed vigor and germination percentage (Chen and Zheng, 1991). The research on seed of *C. arborescens* and *H. persicum* shows that after being stored for a certain time, intracellular electrolyte leakage of ultra-dry seeds is significantly lower than that of non-ultra-dry seeds (Tong, 2009). This indicates that the membrane of ultra-dry seeds is more stable than that of non-ultra-dry seeds. Moderate moisture content of ultra-dry seeds can protect the integrity of membrane structures, but when the moisture content is too low, protection is reduced.

Results of this study showed that storage of *E. dahuricus* seeds at room and high temperatures accelerated aging, and increases conductivity of non-ultra-dry and ultra-dry seeds as compared to storage at lower temperatures. This effect may be due to cell membrane permeability reductions that resulted from temperature changes rather than variations in moisture content. For confirmation, this would require further study.

Antioxidant enzymes such as POD and CAT in plant cells can remove active oxygen caused by plant stress (Su, 2008), thus, effectively preventing its accumulation in plants. The destruction of antioxidant enzymes or the reduction of their activity will accelerate seed aging.

The results show that when the seed moisture content drops to a certain level, the intracellular moisture will enter into a state of flux, the respiratory metabolism will decrease and lipid peroxidation will be partially inhibited. However, the scavenging system of free radicals remains intact. Therefore, when seed initiates germination, the scavenging system of free radical recovers rapidly which is able to remove toxins that accumulated during storage. The removal of toxins and free radicals prevents lipid peroxidation to maintain seed activity and improve seed storage life (Koster et al., 2000). The enzyme activity of *E. dahuricus* seeds, after 12 months of storage under different temperatures, appears to remain functional under moderate ultra-dry moisture levels; and these conditions may directly or indirectly inhibit lipid peroxidation. Excessive drying may promote lipid peroxidation as evidenced by decreased germination.

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