

Effects of Different Storage Temperatures and Times on Germination and Antioxidant Responses of *Jatropha curcas* L. Seeds

S. Gao^{1*}, R. Yan², and F. Chen²

ABSTRACT

The effects of different storage temperatures and times on germination rate, MalonDiAldehyde (MDA), SuperOxide Dismutase (SOD), PerOxiDase (POD) and Catalase (CAT) activities in *Jatropha curcas* L. seed were investigated. The results showed that germination rates of seeds stored at room temperature and 4°C for 1 month was significantly higher than those stored for 3 months at -20 and -80°C. MDA contents increased significantly for 1 and 3 months storage with decreasing temperatures. SOD activity decreased gradually down to -80°C for 1 and 3 months storage. POD activity fluctuated slightly for 1 month, but increased apparently for 3 months storage with decreasing temperatures. CAT activity declined rapidly as storage temperatures decreased, particularly for 3 months storage. A significant interaction of storage temperatures and times was found for the activity of SOD and POD, and there was no significant interaction on germination rate, MDA content, and CAT activity. Electrophoretic analysis showed that the observed changes of SOD, POD and CAT isoenzyme bands under different storage temperatures were consistent with the changes of enzyme activities assayed in extract solutions. These results suggested that SOD, POD, and CAT may be involved in regulating the level of reactive oxygen species under different storage temperatures and times.

Keywords: Antioxidant enzyme, Germination rate, Isoenzyme pattern, Malondialdehyde.

INTRODUCTION

Cold storage is a widely adopted technology to preserve post-harvest life of plant seeds by slowing respiration and other metabolic processes. But, low temperature can induce the production and accumulation of Reactive Oxygen Species (ROS), which are often indicated in plant as a prime cause of seed deterioration and loss of ability to germinate (Tommasi *et al.*, 2006; Tomonari *et al.*, 2009; Xin *et al.*, 2010). In order to balance the level of ROS, plant cells operate a sophisticated enzymatic and non-enzymatic antioxidant system to counteract the ROS. The major

ROS-scavenging system includes low molecular scavengers, such as ascorbate and glutathione, and enzymatic scavengers, such as SuperOxide Dismutase (SOD), PerOxydase (POD), and Catalase (CAT). Their cooperative activities play an important role in resistance to oxidative injuries and minimizing cell damages (Bailly, 2004). Plant cell membranes are rich in polyunsaturated fatty acids and can easily suffer from lipid peroxidation in the presence of ROS. Therefore, MalonDiAldehyde (MDA) may serve as an indicator of lipid peroxidation under low temperature storage (Sung, 1996; Chiu *et al.*, 2002). Thus, these findings are important to

¹ Institute of Ecological Forestry, Faculty of Forestry, Sichuan Agricultural University, Wenjiang 611130, Chengdu, Sichuan, People's Republic of China.

* Corresponding author, e-mail: shun1220@yahoo.com

² College of Life Sciences, Sichuan University, 610064, Chengdu, People's Republic of China.



understand the behavior of those enzymes under different environmental conditions.

Jatropha curcas L., belonging to the family *Euphorbiaceae*, is cultivated as a medicinal plant in many tropical and subtropical countries (Debnath and Bisen, 2008). In many plant species, the relationship between physiological and biochemical changes and variations of antioxidant enzymes activities during different temperatures storage has been investigated (Pukacka and Ratajczak, 2005; Tommasi et al., 2006; Tomonari et al., 2009; Xin et al., 2010). However, the effects of storage conditions ranging from room temperature to extreme low temperature are still unclear. The aim of this study was to investigate seed germination, changes of MDA contents and antioxidant enzyme activities, as well as patterns of antioxidant isoenzymes of *Jatropha curcas* under different storage temperatures.

MATERIALS AND METHODS

Plant Materials and Chemicals

Mature *Jatropha curcas* seeds were collected in August, 2007 from more than 10 individual wild trees in Panzhihua, Sichuan Province, China. These seeds were dried naturally until reaching moisture content of approximately 5.46 %. Seeds were selected and stored in plastic mesh baskets with labeled (No. 20070822) at four temperatures levels: Room Temperature (RT, $15\pm 2^{\circ}\text{C}$), 4, -20 and -80°C , with three replicates of 500 seeds each. The analyses described below were carried out at 1 and 3 months of storage. Methionine, and Nitro Blue Tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of reagent grade or higher.

Seed Germination

J. curcas seeds were surface sterilized with 0.5% potassium permanganate solution

for 10 min, and then rinsed several time with sterile water. Seeds were sown in vinyl discs filled with soils for germination and watered using 1/10 strength Hoagland nutrient solution. The soil used in this study consisted of river sand, perlite, and vermiculite. The trays were labelled with sampling dates (No. 20070922 and 20071122) and were arranged in a complete randomized design on a table kept with an even light supply. Germination experiment was performed in three replicates with 50 seeds. Germination experiment was carried out in the greenhouse, at temperature of $30/25^{\circ}\text{C}$ day/night, relative humidity 70%, and light intensity $250\ \mu\text{M m}^{-2}\text{ s}^{-1}$ for one week. Germinated seeds and rotted seeds were counted every other day and the rotten seeds were removed. Seeds were regarded as germinated when the healthy, white radicles had emerged through the seed coat and reached more than 2 mm in length. The Germination Rate (GR) is the proportion, expressed as percentage of geminated seeds to the total number of viable seeds that were tested (Yang et al., 2007). GR was calculated for each treatment using the following equation: $GR = n/N \times 100\%$, where n is the number of germination, and N represents the total number of tested seeds.

Lipid Peroxidation Assay

MDA was determined by the Sung Method (1996). One gram seed was homogenized with 4 mL of 0.1% (w/v) TriChloroacetic Acid (TCA) and centrifuged at 12,000 rpm for 10 minutes. To 1 mL aliquot of the supernatant, 4 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid was added. The mixture was heated at 95°C for 30 minutes and then cooled in running water. The supernatant was centrifuged at 12,000 rpm for 10 minutes, and the absorbance was recorded at 532 and 600 nm. MDA content was calculated using an extinction coefficient ($\epsilon = 155\ \text{mM}^{-1}\text{ cm}^{-1}$) and expressed as mmol per gram Fresh Weight ($\text{mmol g}^{-1}\text{ FW}$).

Protein Extraction and Estimation

Seeds (0.3 g) were ground with liquid nitrogen and homogenized in 2 mL of 50 mM sodium phosphate buffer (pH 7.0) including 0.5 mM EDTA (Gao *et al.*, 2010). The homogenate was centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant was used for protein determination and enzyme assays. Protein concentration was assayed by the Lowry Method, using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Assay of Antioxidant Enzymes

SOD activity was assayed in a 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM methionine, 1.17 mM riboflavin, 56 mM NBT and 50 μ L enzyme extract. The absorbance of solution was tested by measurement of its capacity of inhibiting the photochemical reduction of NBT at 560 nm. One unit of SOD was defined as the enzyme activity that reduced the photoreduction of nitroblue tetrazolium to blue formazan by 50% (Gao *et al.*, 2010). SOD activity was expressed as enzyme Units per gram Fresh Weight (U g^{-1} FW). POD activity was determined by measuring the increase in the absorbance at 470 nm (Sakharov and Bautista, 1999). The mixture consisted of 2.8 mL of guaiacol (3%), 100 μ L H_2O_2 (2%) and 100 μ L enzyme extract. One unit of POD activity was defined as an increase in absorbance of 1.0 per minute. POD activity was expressed as enzyme Units per gram Fresh Weight (U g^{-1} FW). CAT activity was determined by the decrease in absorbance at 240 nm. The activity was assayed for 1 minute in a 3 mL reaction solution composed of 2.8 mL phosphate buffer (50 mM, pH 7.0), 100 μ L H_2O_2 (1%) and 100 μ L of crude extract. One unit of CAT activity was defined as the amount of enzyme which caused 1 μ L H_2O_2 decomposition in one minute (Montavon *et al.*, 2007). CAT activity was expressed as

enzyme Units per gram Fresh Weight (U g^{-1} FW).

Analysis of Activity Gel Electrophoresis

PAGE for isoenzymes assay was performed with 8% acrylamide gel. SOD isoenzymes were detected by the procedure described by Beauchamp and Fridovich (1971). The gel equilibrated with 50 mM phosphate buffer (pH 7.5) containing 2.8×10^{-5} M riboflavin, 0.028 M *N,N,N,N*-TetraMethyl EthyleneDiamine (TEMED) for 30 minutes. The gel was washed in distilled water for 1 minute and submerged in a same solution (mentioned above) containing 2.45 mM NBT for 10-20 minutes with gentle agitation in the presence of light, the enzymes appeared as colorless bands in a purple background. POD isoenzymes were shown by the Ros Barcelo Method (1987). The gels were rinsed in water and then stained in a solution containing 0.06% (v/v) H_2O_2 , 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till the brown color displays. CAT isoenzymes were shown by the Woodbury Method (Woodbury *et al.*, 1971). Gels were firstly incubated in 0.01% H_2O_2 for 10 minutes, then transferred into 4% soluble starch for 1 hour and finally stained in a 0.5% (m/v) FeCl_3 and 0.5% $\text{K}_3\text{Fe}(\text{CN})_6$ (m/v) solution (1:1) for 10 minutes. Under these treatments, positions with CAT activity became transparent while the rest parts of the gel turned to be deep blue. When the maximum contrast was achieved, the reaction was stopped by rinsing the gel with deionized water.

Statistical Analysis

All values shown in this paper were the mean of three assays carried out for each value. Data were tested at significant levels of $P < 0.05$ using two-factor analysis of variance using SPSS 16.0 software.



RESULTS AND DISCUSSION

Effects of Storage Temperatures and Times on Germination Rate

Figure 1 shows percentage of seed germination for 1 and 3 months storage under different temperatures. The germination rate of fresh seeds is about 85%. The percentages of seed were 58 and 60% at room temperature and 4°C, respectively, for 1 month storage. The germination rate dropped to 48% at -20°C, and to 24% at -80°C. Compared to data for 1 month, a decreasing trend in germination rate was observed for 3 months storage, except for -80°C. Analysis of variance showed that there was no significant interaction between storage temperature and time for germination rate (Table 1). Earlier reports indicated that low temperature can preserve tissue viability for 1 year, but did not preserve their capability to seed

germination (Chiu *et al.*, 2002). Moreover, the loss of germination capability of *Shorea robusta* seeds was related to a drop in the antioxidant system efficiency (Chaitanya *et al.*, 2000). Therefore, lower seed germination rate and the changes of the antioxidant enzymes in *Jatropha curcas* seeds indicate that oxidant damage under low temperature storage may be attributed to the formation of excessive ROS as well as the changes in SOD, POD and CAT activities. These findings might be consistent with the results that excessive production of ROS appears and, consequently, leads to the decrease in their viability in *Fagus sylvatica* seeds under relatively low temperature storage (Pukacka and Ratajczak, 2005).

Effects of Storage Temperatures and Times on MDA Content

Plant cells are potentially susceptible to

Table 1. Correlation analysis between storage temperatures and times on the effects of seed germination, MDA, SOD, POD and CAT.

Parameters	Germination rate	MDA ^a	SOD ^b	POD ^c	CAT ^d
Time	0.003*	0.679	0.000*	0.000*	0.210
Temperature	0.000*	0.006*	0.000*	0.000*	0.020*
Time×Temperature	0.054	0.438	0.007*	0.000*	0.562

^a MalonDiAldehyde, ^b SuperOxide Dismutase, ^c PerOxiDase, ^d Catalase, * indicates statistical significance according to two-factor analysis of variance (P< 0.05).

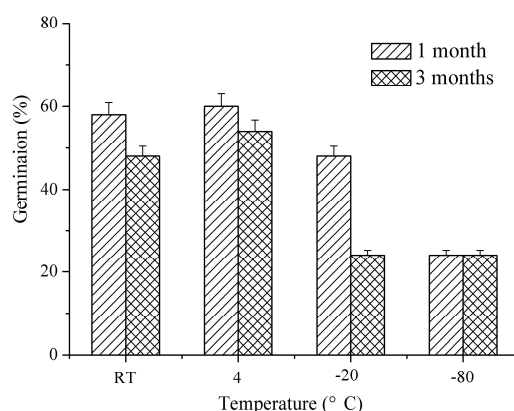


Figure 1. The changes of germination rate of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. RT: Room Temperature.

damage caused by excess ROS due to their high amount of polyunsaturated fatty acids in membrane phospholipids. MDA, as the final product of lipid peroxidation, has often been used as an indicator of the level of lipid peroxidation (Sung, 1996; Chiu *et al.*, 2002). In the present study, MDA content was measured as marker commonly used for assessing lipid peroxidation for 1 and 3 months under different storage temperature (Figure 2). MDA content increased 2.6, 21.7 and 61.4% for 1 month at, respectively, 4, -20, and -80°C, compared to that at room temperature. The values increased 38.1, 130.4, and 204.5% for 3 months at 4, -20, and -80°C, respectively. The levels of MDA content stored for 3 months were less than that stored for 1 month at room temperature and 4°C. However, the opposite results were observed at -20 and -80°C. The levels of MDA in our experiment were lower for 1 and 3 months under room temperature and 4°C storage (Figure 2) accompanied with the increased CAT activity and the higher seeds germination rate at room temperature and 4°C (Figures 2 and 7), which suggested a certain relationship among the MDA levels, CAT activity and seeds germination rate. The increased MDA content (Figure 2),

increased SOD (Figure 3), and POD activities (Figure 5) were also observed at RT and 4°C storage for 3 months. There was no significant interaction between storage temperature×time on MDA content (Table 1). Our results indicate that plant cells can develop a broad range of defense responses, such as increase in antioxidant enzyme activities, to cope with the cellular level of ROS. However, the increased MDA levels seem to be correlated to the lower seeds germination rate (Figures 1 and 2). Our findings suggest that ROS-scavenging enzymes may not be enough to prevent oxidative damage or excessive production of ROS and, furthermore, ROS can cause a decline in seed viability not only by lipid peroxidation but also by the changes in antioxidant enzymatic systems. Effects of Storage Temperatures and Times on SuperOxide Dismutase (SOD) Activity

SOD, a key enzyme catalyzing superoxide free radical dismutation into H₂O₂ and O₂, has been implicated as a component against the toxicity of oxygen and this defensive action was affected by temperature (Bailly, 2004). Changes of SOD activities for 1 and 3 months under different storage temperatures are shown in Figure 3. SOD

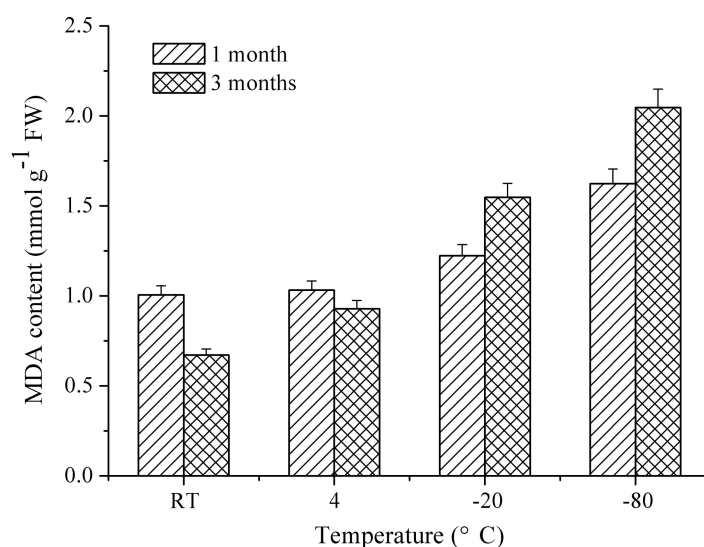


Figure 2. The changes of MalonDiAldehyde (MDA) content of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. RT: Room Temperature.

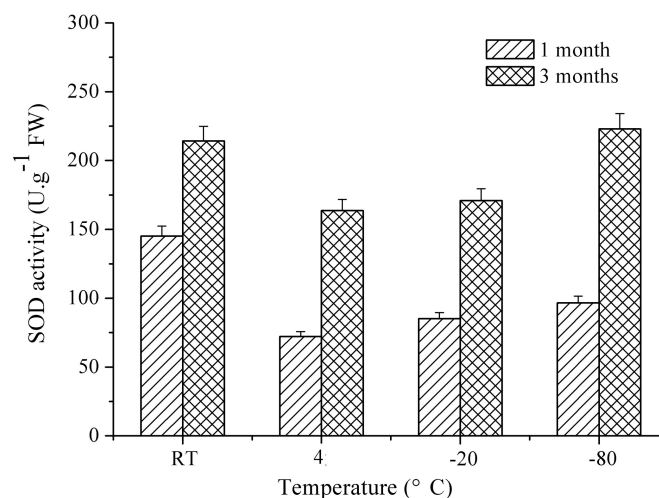


Figure 3. The changes of SuperOxide Dismutase (SOD) activity of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. RT: Room Temperature.

activity decreased 50.3, 41.4 and 33.4% for 1 month at, respectively, 4, -20, and -80°C, compared to that at room temperature. The activity decreased 23.6 and 20.1% for 3 months at 4 and -20°C, respectively, but increased 4.2% at -80°C. Compared to the result for 1 month, SOD activity increased 47.6, 127.1, 101.2 and 130.8% for 3 months at room temperature, 4, -20, and -80°C. SOD activities decreased significantly for 1 month under 4°C storage condition (Figure 3). A significant interaction of storage temperature \times time was found for the activity of SOD (Table 1). This is in accordance with the observed results in the pulp samples under 6°C storage condition (Huang *et al.*, 2005). These results may be attributed to the lower production of O_2^- in cells or slow metabolism for a short period under low temperature. However, SOD activities increased remarkably for 3 months under 4°C storage condition compared to those for 1 month. Increased SOD activity as observed in our studies may be either due to increased production of reactive oxygen species or a protective measure adopted by *Jatropha curcas* seeds against extreme low temperature. Our findings are in line with the fact that an up-regulating SOD activity in *Blanquilla* pears was observed during cold storage (Larrigaudière *et al.*, 2004).

SOD comprises a family of metalloenzymes in the forms of many different isoforms. SOD isoenzymes are complicated since they are regulated at different times and places by various kinds of biotic and abiotic stress (Bailey, 2004). Electrophoresis analysis revealed the presence of at least three SOD isoforms with distinct activities for 1 and 3 months under different temperatures, but no new band was observed (Figure 4). The intensity changes of SOD isoenzymes bands were in accordance with the changes of their activities assayed in extract solutions (Figures 3 and 4). Effects of Storage Temperatures and Times on PerOxiDase (POD) Activity

Plants peroxidases, which are encoded by multigenic families, have been implicated in a broad range of physiological processes (Passardi *et al.*, 2005). Figure 5 shows the changes in POD activity for 1 and 3 months under different temperatures. POD activity increased 2.0 and 16.5% for 1 month at, respectively, -20 and -80°C compared to the control, but it decreased 47.6% at 4°C. When stored for 3 months, POD activities increased significantly by 106.0, 185.6, and 218.1% at 4, -20, and -80°C, respectively. In addition, a notable increase in POD activity was observed for 3 months at all storage temperatures in comparison with that for 1

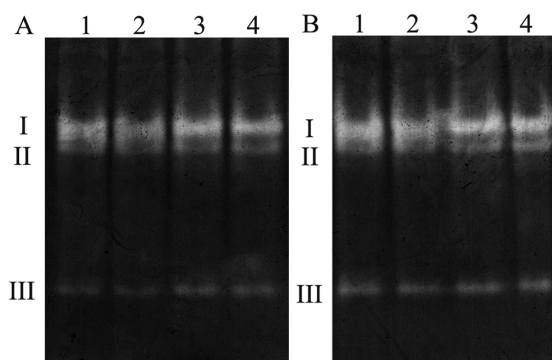


Figure 4. Patterns of SuperOxide Dismutase (SOD) isoenzymes of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. (A) 1 month, (B) 3 months. Lanes from 1 to 4 were room temperature, 4, -20 and -80°C, respectively.

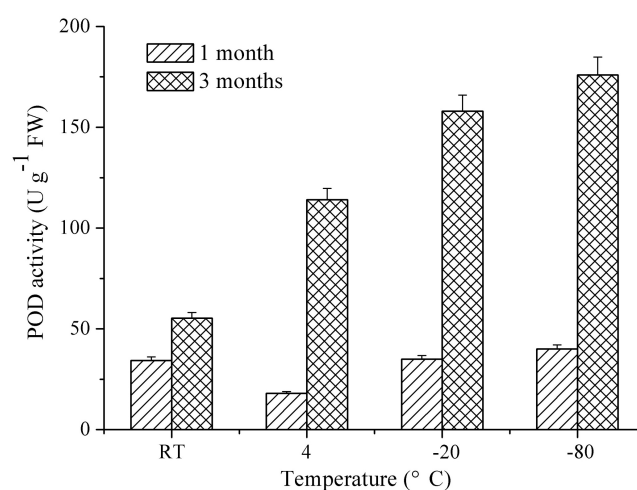


Figure 5. The changes of PerOxydase (POD) activity of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. RT: Room Temperature.

month. In our study, POD activities are induced significantly for 3 months under lower temperature conditions, but show no remarkable changes for 1 month under these conditions, except for 4°C (Figure 5). A significant interaction of storage temperature×time was found for the activity of POD (Table 1). Our findings suggest that the higher POD activities under -20 and -80°C conditions is related to the increase of MDA content and the loss of seed germination rate. Changed POD activities have been observed in other plant species under lower temperature storage (Chiu *et al.*, 2002; Larrigaudière *et al.*, 2004). Electrophoresis analysis shows that at least two isoforms are detected and the intensity of POD activity observed appeared to be closely correlated with the changes of POD

isoform activity (Figure 6). The intensities of POD isoenzymes show different changes during low temperature stress (Figure 6). It can be seen from the bands of POD isoenzymes in native PolyAcrylamide Gel Electrophoresis (PAGE) that the intensities of these isoforms depend on the storage temperature and times. These results indicate that the increased POD activities are involved in protecting cells against low temperature stress.

Effects of Storage Temperatures and Times on Catalase (CAT) Activity

CAT, which catalyzes the decomposition of hydrogen peroxide into dioxygen and water, is the major ROS-scavenging enzyme in all

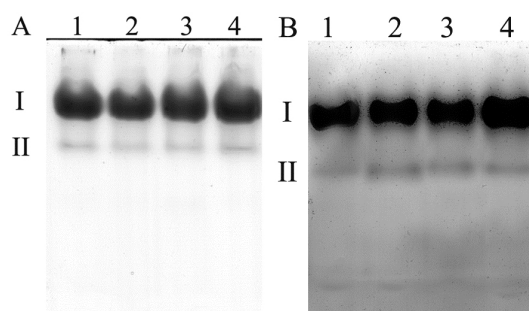


Figure 6. Patterns of PerOxydase (POD) isoenzymes of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. (A) 1 month, (B) 3 months. Lanes from 1 to 4 were room temperature, 4°C, -20 and -80°C, respectively.

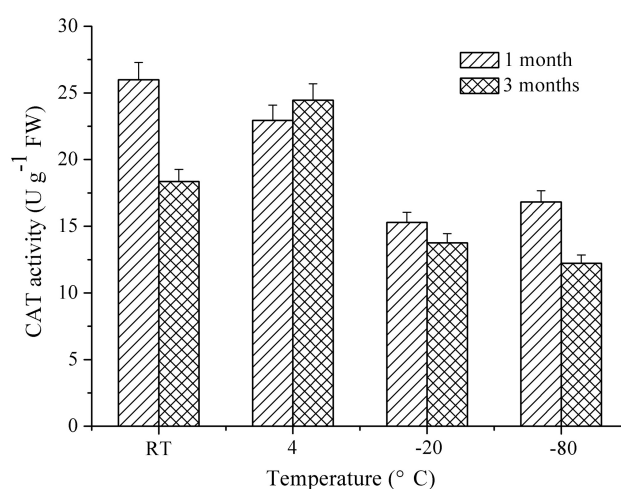


Figure 7. The changes of Catalase (CAT) activity of *Jatropha curcas* seeds for 1 and 3 months under different storage temperatures. RT: Room Temperature.

aerobic organisms (Bailly, 2004). Figure 7 shows the changes of CAT activity for 1 and 3 months under different storage temperatures. As shown in Figure 7, CAT activity declined 11.5, 41.2, and 35.3% compared to the control for 1 month at 4, -20 and -80°C, respectively. The activity increased 33.5% for 3 months at 4°C, but decreased 25.0 and 33.4% at -20 and -80°C, respectively. CAT activity decreased for 3 months at room temperature, -20, and -80°C, except at 4°C. CAT activity decreased significantly for 1 and 3 months at low temperature stress, but the activities increased 33.5% for 3 months at 4°C. Our findings suggest that the increased CAT activities, the lower levels of MDA, and the higher seed germination rate are related (Figures 1, 2, and 7). There was no significant interaction of storage temperature × time on CAT activity

(Table 1). However, our results are consistent with the deficiency in CAT activities linked to lower germination rate of barley and corn seeds (Bettaieb *et al.*, 2007). Electrophoresis analysis suggested that there was at least one CAT isoenzyme in seeds under different temperatures (data not shown). The intensity changes of CAT isoform agreed with the enzyme activities assayed in extract solutions. CAT, which is part of the defense mechanism against maintaining ROS levels, plays important roles in protecting plant cell from damage due to low temperature stress (Bailly, 2004). However, CAT activities were inhibited under -20 and -80 °C storage conditions (Figure 7). The mechanism of extreme temperature inhibition of CAT activities is complex and needs further study.

CONCLUSIONS

In conclusion, the effects of different storage temperatures and times on seed germination rate, antioxidant enzymes, and PAL activities in *Jatropha curcas* seeds were tested. Differences were observed among these parameters at different storage temperatures and times. POD activities in this study are found to be more sensitive to low temperatures, and SOD activities are increased with the extension of storage period. However, CAT activities are inhibited by decreasing storage temperatures. These results suggest that POD, SOD, and CAT activities are subjected to regulation depending on the storage temperatures or storage period or both. Moreover, a significant interaction of storage temperature \times time was found for the activities of SOD and POD. However, there was no significant interaction of storage temperature \times time on both MDA content and CAT activity. Such responses may be of considerable value in understanding the response mechanisms of seed storage and gaining insights into storage temperature-time interactions during the storage.

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اثر های درجه حرارت و زمان انبارداری روی جوانه زنی و واکنش آنتی اکسیدانی *Jatropha curcas* L. بذر گیاه

س. گاوو، ر. یان، ف. چن

چکیده

در این پژوهش، اثر های درجه حرارت و زمان های مختلف انبارداری روی نرخ جوانه زنی، مقدار مالون دی آلدیهاید (MDA)، superoxide dismutase (SOD)، پراکسیداز (POD)، و فعالیت های کاتالاز (CAT) در بذر *Jatropha curcas* L. بررسی شد. نتایج نشان داد که نرخ جوانه زنی بذرهایی که برای یک ماه در درجه حرارت اتاق و ۴ درجه سانتی گراد انبار شده بودند به طور معنی داری بیشتر از آنهایی بود که به مدت ۳ ماه در درجه ۲۰- و ۸۰- درجه سانتی گراد نگهداری شده بودند. نیز، با کاهش درجه حرارت در زمان های ۱ و ۳ ماه انبارداری، مقدار MDA به طور معنی داری افزایش یافت ولی فعالیت SOD به تدریج تا ۸۰- درجه سانتی گراد برای همین مدت انبار داری کاهش یافت. در مورد انبارداری ۱ ماهه، فعالیت POD تا حدودی نوسان داشت ولی در مورد ۳ ماه انبارداری، ظاهراً با کاهش درجه حرارت فعالیت آن افزایش داشت. فعالیت CAT با کاهش درجه حرارت انبار به سرعت کم شد، به ویژه در انبارداری ۳ ماهه. افزون بر این، بین درجه حرارت انبار و زمان انبار داری برهمکنش و تعامل معنی داری برای فعالیت SOD و POD مشاهده شد ولی برهمکنش مشابهی در مورد جوانه زنی، محتوای MDA و فعالیت CAT دیده نشد. تجزیه الکتروفورزی نشان داد که تغییرات مشاهده شده در SOD، POD، و باند های ایزوآنزیم CAT تحت درجه حرارت های مختلف انبارداری با تغییرات فعالیت آنزیم های محلول عصاره گیری همخوان بود. بر پایه این نتایج می توان گفت که SOD، POD، و CAT ممکن است در تنظیم سطح اکسیژن فعال در درجه حرارت ها و مدت های مختلف انبارداری دخالت داشته باشند.