Oxidative stress with the damage of scavenging system: a mechanism for the nutrients loss in rice seeds during post-harvest storage

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ABSTRACT

Rice seeds with different moisture contents were stored in simulated storage facilities at different temperatures for 300 days. Measurement of biochemical properties and the observation of embryo and endosperm structures showed that high temperature and moisture content accelerated the deterioration in rice seeds and damaged the cell structures. Two hypotheses were proposed to explain the mechanisms of deterioration in rice seeds. The study of scavenging systems supported that scavenging system performed well and external storage conditions partly contributed to the deterioration in the early stage while the self-protection metabolism was damaged to some extent with the malfunction of scavenging systems in the later stage of storage. In the practice, it is an energy-saving way to prevent deterioration of rice seeds by moisture removal and staged low-temperature storage.

1. Introduction

Rice (Oryza sativa L.) seeds, which have high productivity and value-added nutrients, are one of the staple foods in human diet (Bhullar & Gruissem, 2013; Xu et al., 2011). To avoid the social problem and humanitarian crisis caused by food shortage, a large number of storage facilities have been established to reserve rice seeds worldwide (Wang, Zhang, & Lu, 2018). In addition, the grain storage is a good way for the government to mitigate the fluctuation of food price in market. In China, the storage period of rice seeds in national grain reserve depots could reach 1–2 years (Zhang et al., 2007). As a result, the long-term storage not only causes the quantity loss, but also negatively impacts the quality of grain. Yang, Zhang, Lü, Cao, and Chen (2014) reported that various factors impacted the quantity loss and quality loss of rice seeds during post-harvest storage included cell respiration, activities of insects, and growth of microorganism. The intensive cell respiration is considered as the major factor leading to internal deterioration and causing quality loss (Yang et al., 2014; Ye et al., 2011).

Respiration in storage could reduce the nutritional value of rice seeds by consuming nutrients, including protein, lipid, and carbohydrate. In addition, intensive respiration would accelerate the oxidation and internal deterioration in rice seeds, causing peroxidation of lipid and protein (Özdemir, Bor, Demiral, & Türkan, 2004). Accordingly, the unsaturation degree of lipid and amino acid profile would be negatively impacted. Last but not the least, some degradation products of lipid and protein in rice seeds may trigger inflammation and even cause cancer in human bodies (He & Lu, 2015; Rubió, Motilva, & Romero, 2013). For example, malondialdehyde (MDA), which is an inducer of cancer, increased more than two-fold in wheat seeds after 300-day storage (Wang et al., 2018). Hence, to reduce the quality loss in long-term storage, it is essential to understand the cell metabolism of rice seeds and alleviate the internal deterioration.

In rice seeds, enzymatic and nonenzymatic scavenging systems are responsible to remove free radicals and alleviate internal deterioration. The enzymatic scavenging system, consisting of antioxidants, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), could catalyze the conversion of oxidants to non-oxidative compositions (Wang et al., 2014). In rice seeds, enzymatic and nonenzymatic scavenging systems are responsible to remove free radicals and alleviate internal deterioration. The enzymatic scavenging system, consisting of antioxidants, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), could catalyze the conversion of oxidants to non-oxidative compositions (Wang et al., 2014). Besides, in the nonenzymatic...
scavenging system, glutathione-ascorbate cycle driven by NADPH, could directly react with oxidants (Xi, Liu, Yang, Wu, & Zheng, 2010). Hence, internal deterioration in stored grain could be explained in two possible ways. Firstly, accumulated free radicals exceed the scavenging ranges of two systems although these two scavenging systems operate properly. Secondly, rice seeds are exposed to oxidative stress during storage due to the malfunction of scavenging systems. Our previous study has reported the functions of scavenging systems in wheat seeds during storage (Wang et al., 2018). The studies of Xu et al. (2011) and Ye et al. (2011) explored the changes of scavenging systems in the germination process of rice seeds. However, studies on the performance of scavenging system and its relation with the deterioration in rice seeds during long-term storage were rare.

Some strategies, such as pre-treatment by moisture removal (Ondier, Siebenmorgen, & Mauromoustakos, 2010), atmosphere adjustment by inert gas (Wang et al., 2018), and low temperature storage (Park, Kim, Park, & Kim, 2012), have been proven to be possible way to reduce the quantity and quality loss caused by internal deterioration during storage. For example, the low temperature could limit the respiration of grain seeds and extend the storage period (Lin et al., 2010). The study of Wang et al. (2018) reported that although atmospheric adjustment by nitrogen gas effectively delay the deterioration in grain seeds, such a strategy is too expensive to be widely applied. To our knowledge, the energy inputs of storage strategies have not been widely considered in the storage of rice seeds.

This work, aiming at identifying the mechanisms for oxidative stress in rice seeds during storage and developing economically feasible storage strategies, measured the basic characteristics of rice seeds during 300 days storage and proposed two possible explanations for the oxidative stress. Both enzymatic and nonenzymatic scavenging systems were studied to confirm the hypothesis for oxidative stress in rice seeds. Electronic microscope was employed to detect the physical changes of embryo and endosperm in rice seeds during storage. The energy inputs of different storage strategies were calculated. The knowledge and techniques of this work would be useful to prevent internal deterioration and reduce quality loss of rice seeds during storage.

2. Material and methods

2.1. Materials

Rice seeds, which were harvested in 2016, with different moisture contents (11%, 13%, and 15%) were obtained from China Grain Reserves Corporation (Henan, China). Simulated pilot-scale warehouses (Height: 1.0 m; Length: 2.5 m; and Width: 2.0 m) equipped with air conditioner (Figure 1(a)) were used in this study to store rice seeds.

2.2. Experimental design and parameters analysis

2.2.1. Experimental design

This study was carried out in five steps. The first step was to explore the effects of temperatures (10°C, 20°C, and 30°C) and moisture contents (11%, 13%, and 15%) on basic biochemical properties, including germination efficiency (%), acid value, content of soluble saccharides, and protein content, of rice seeds. The second step was to identify the oxidative stress in rice seeds during storage by measuring four biochemical parameters, including superoxide anion radicals (O$_2^-$), MDA, conductivity, and activity of cytochrome oxidase (CCO). In the third step, electron microscopes were used to observe the microstructures of embryo and endosperm in rice seeds. In the fourth step, changes of enzymatic and nonenzymatic scavenging systems in rice seeds during storage were measured. Mechanisms were proposed to explain the internal deterioration and oxidative stress in rice seeds during storage. The last step was to compare different storage modes for rice seeds and evaluate the energy input of these strategies in practice. Moisture contents of rice seeds before and after moisture removal were set as 15% and 11%, respectively, in four storage modes.

All experiments and tests, in this study, were carried out in triplicate. The results were expressed as mean ± standard deviation.

2.2.2. Sampling

To accurately reflect the actual changes of rice seeds during long-term storage, this study was carried out in pilot-scale warehouses with 5.0 m$^3$ storage capacity. The sampling process, hence, should be performed in a good way to increase the reliability and accuracy of experimental results. In this work, 10 samples were collected from different locations (four corners and central point at bottom layer and top layer) in the pilot-scale warehouse every 30-day. Collected samples (about 150 g) were sealed in plastic bags and sent for analysis immediately (Wang et al., 2018).

2.2.3. Measurement of basic biochemical properties

Since rice seeds after storage are mainly used for food processing or seeding, changes of germination efficiency, and nutrient compositions during storage should be considered (Lu, He, & Liu, 2015; Lu, Liu, Wang, & Liu, 2017). In this work, acid value, content of soluble saccharides, and protein content, which could reflect the changes of main nutrient compositions in rice seeds, were measured.

Process of measuring germination efficiency (%) was listed as follows: 100 rice seeds evenly placed on a Petri dish with sterilized wet filter paper were kept in an incubator at 30 ± 1°C. After 10 days, the number of germinated rice seeds was counted and the germination efficiency was calculated according to Equation 1.

\[
G = \frac{N}{100} \times 100\% \quad (1)
\]

where G refers to the germination efficiency (%) and N is the number of germinated rice seeds after 10-day incubation.

Acid value was measured by using 0.1 mol/L potassium hydroxide (KOH) solution to neutralize the lipid. In this work, lipid was extracted by ethanol at 70°C and phe-nolphthalein indicator was used to identify the degree of naturalization. The acid value was expressed as mg KOH. Protein content (%) in rice seeds was measured by using 660 nm Protein Assay Kit (Catalog Number: 22,662) purchased from Thermo Fisher Scientific (USA). Soluble saccharides were extracted by mixed solution of ethanol: distilled water (v:v = 8:2) and the absorbance of supernatant after centrifugation was measured at 480 nm (Aghaleh, Niknam, Ebrahimzadeh, & Razavi, 2009; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of soluble saccharides in rice seeds was expressed as percentage.
2.2.4. Measurement of oxidative stress

Four parameters, including content of MDA, activity of CCO, content of superoxide anion radicals (O$_2^-$), and conductivity of rice seeds, were measured to reflect the oxidative stress in storage. Contents of MDA and O$_2^-$, expressed as μmol/g and μg/g, respectively, were measured by using Lipid Peroxidation (MDA) Assay Kit (Catalog Number: ab118970) purchased from Abcam and Superoxide Anion Assay Kit (Catalog Number: CS1000-1KT) purchased from Sigma-Aldrich. Activity of CCO, which is a parameter reflecting the metabolic activities of rice seeds, was measured by using Cytochrome Oxidase Activity Colorimetric Assay Kit (Catalog Number: K287) purchased from Biovision (Wang et al., 2018). To measure the conductivity, 40 rice seeds were weighted and put in an Erlenmeyer flask filled with 200 mL distilled water for 24 h at room temperature (25 ± 1°C). The blank sample was 200 mL distilled water without rice seeds. The conductivity (mS/m) was measured by using a conductivity meter and further calculated by Equation 2.

\[ C = \frac{C_1 - C_2}{W} \]  

where C refers to conductivity of rice seeds; $C_1$ and $C_2$ represent the conductivities of distilled water with rice seeds and without rice seeds, respectively. W is the weight of rice seeds.

2.3. Observation of microstructures

Microstructures of embryo and endosperm in rice seeds were observed by using a JEM-1400 Transmission Electron Microscope (JEOL, Japan) and a S-4800 Scanning Electron Microscope (Hitachi, Japan), respectively. The observation of endosperm was performed at 2,000× magnification while the observation of embryo was performed at 10,000×, 30,000×, and 50,000× magnification according to the instruction manual (Wang et al., 2018). In the observation of endosperm, changes of protein matrix (PM), polyhedron (Po), mycelium (M), broken starch (BS), and semi-gelatinized matrix (HG) were focused on. Changes of cell wall (CW), intercellular space (IC), mitochondria (M), nucleus (N), nucleolus (Nu), plasma membrane (PM), vesicle (VE), protein bodies (PB), lipid bodies (L), and some other subcellular structures were observed to evaluate the damage of long-term storage to embryo in rice seeds (Wang et al., 2018).

Figure 1. Storage facilities and storage modes for rice seeds: (a) Facilities for rice seeds storage; (b) Description of storage modes.

Figura 1. Instalaciones de almacenamiento y modos de almacenamiento de semillas de arroz: (a) Instalaciones para el almacenamiento de semillas de arroz; (b) Descripción de los modos de almacenamiento.
2.4. Analysis of scavenging systems in rice seeds

Essential enzymes and antioxidants in both enzymatic scavenging system and nonenzymatic scavenging system were measured in this work. Activities of three major enzymes, SOD, CAT, and POD in enzymatic scavenging system were measured by using Superoxide Dismutase (SOD) Colorimetric Activity Kit (Catalog Number: EIASODC) and Catalase Colorimetric Activity Kit (Catalog Number: EIACATC) purchased from Thermo Fisher Scientific and Peroxidase Activity Assay Kit (Catalog Number: ab115895) purchased from Abcam. Two main antioxidants, ascorbic acid (ASC) and glutathione (GSH), of the nonenzymatic scavenging system were measured by using Ascorbic Acid Assay Kit (Catalog Number: ab65346) and Glutathione Assay Kit (Catalog Number: ab65322) purchased from Abcam.

Deactivation rates of enzymes or reduction rates of antioxidants were calculated according to Equation 3. The correlation of storage conditions and performance of scavenging system was explored to assess the self-protection systems in rice seeds during long-term storage.

\[ D = \frac{X_0 - X_t}{t} \times 100\% \]  

where \( D \) is the deactivation rate of enzyme or reduction rate of antioxidant; \( X_0 \) and \( X_t \) are enzyme activities or antioxidant contents on Day 0 and Day \( t \), respectively. The last parameter, \( t \), refers to the storage period (days) of rice seeds.

2.5. Comparison of strategies to alleviate internal deterioration

According to the results of this work and previous studies, low moisture content, low temperature, and atmosphere adjustment are three effective methods to alleviate internal deterioration in rice seeds during long-term storage (Tananu Wong & Malia, 2011; Wang et al., 2018). However, due to the high investment and operation cost of atmosphere adjustment strategy, this strategy might not be economically feasible in some developing countries (Kobayashi et al., 2010). Hence, the alleviation of internal deterioration by atmosphere adjustment was not considered in this study. Four storage modes assessed by this work were shown in Figure 1(b). In Mode 1, moisture content of rice seeds was reduced to 11% and the dried rice seeds were stored at normal temperature (30 ± 1°C). In Mode 2, rice seeds with 15% moisture content were stored at low temperature (10 ± 1°C). In Mode 3, rice seeds were subjected to moisture removal before storage and then preserved at low temperature for 300 days. In Mode 4, after moisture removal, rice seeds were stored in two consecutive stages, normal temperature storage (180 days), and low temperature storage (120 days). Four storage modes were assessed according to the deterioration of rice seeds during storage and the electricity consumption of storage facilities.

3. Results and discussion

3.1. Effects of storage on biochemical properties

3.1.1. Germination efficiency

As shown in Figure 2(a), long-term storage had unfavorable effects on the germination capacity of rice seeds since their germination efficiencies decreased with the extension of storage periods. Figure 2(a) indicated that after 300 days storage at 30°C, germination efficiencies of rice seeds with 11% and 15% moisture contents decreased by 19.4% and 66.9%, respectively. In addition, germination efficiencies of rice seeds with 13% moisture content decreased by 12.1% and 44.1%, respectively, when the storage temperatures were 10°C and 30°C. Therefore, both high storage temperature and high moisture content could accelerate the decrease of germination efficiency. With the serious damage of germination capacity, rice seeds after long-term storage would not be used for seedling on farm (Chauhan & Johnson, 2011; Farooq et al., 2011). In the practice, to maximize the economic benefits of storage industry, rice seeds with high germination efficiencies are expected. According to previous studies, both embryo and endosperm could impact the germination efficiency of grain seeds (Bandeira, Evangelista, & Gloria, 2012; Sheoran, Olson, Ross, & Sawhney, 2005). The damage of either embryo or endosperm during storage, hence, would negatively impact the germination efficiency of rice seeds. It was reported that the nutrients degradation in endosperm and the deactivation of essential enzymes in embryo are two common reasons for the low germination efficiency of plant seeds (Berger, Grini, & Schnittger, 2006). The decrease of germination efficiency (Figure 2(a)) suggested that long-term storage may cause the damage of embryo and endosperm in rice seeds.

3.1.2. Acid value

Figure 2(b) showed that the acid value in rice seeds (13% moisture content) stored at 10°C, 20°C, and 30°C increased by 0.30, 0.051, and 0.072 (KOH)/(g/100 g) daily, respectively, suggesting that temperature is a critical parameter impacting the deterioration in rice seeds. In addition, comparison of acid values of rice seeds with different moisture contents revealed that rice seeds with high moisture content had more serious deterioration. For example, when the storage temperature was 20°C, acid values of rice seeds with 11%, 13%, and 15% moisture contents increased by 125.77%, 223.22%, and 262.20%, respectively. Since the acid value is a parameter reflecting the deterioration of lipid deterioration in grain and grain products, the increase of acid value might make the rice seeds not suitable for eating (Mezouari & Eichner, 2007). Considering the great effects of moisture content and storage temperature on the change of acid value, it is supposed to be a possible way to prevent the deterioration of rice seeds during storage by controlling the temperature and moisture content (Genkawa, Uchino, Inoue, Tanaka, & Hamanaka, 2008).

3.1.3. Contents of soluble saccharides and protein

Saccharides, which are the main ingredients in endosperm, have been regarded as main energy source and carbon source in the germination process (Schramm, Abadie, Hua, Xu, & Lima, 2007). The main soluble saccharides in rice seeds include maltose, glucose, and sucrose. In the food industry, content of soluble saccharides in grain seeds would impact the nutrient value of grain products (Rivas, Conde, Moure, Dominguez, & Parajó, 2013; Saldivar, Wang, Chen, & Hou, 2011). In this study, it was discovered that the content of soluble saccharides decreased during the storage. As shown in Figure 2(c), when the moisture content was 15% and temperature was 30°C, the content of soluble saccharides decreased by 41.99% in 300-days storage. The degradation of soluble saccharides was
mainly caused by the respiration, which converted saccharides to carbon dioxide and produced energy, of rice seeds during the storage (Asatsuma et al., 2005). Besides soluble saccharides, protein in rice seeds also degraded with the extension of storage period. Figure 2(d) showed that at the end of storage period, protein content in rice seeds with 15% moisture was reduced to 4.15% at 30°C. Accelerated respiration in rice seeds caused by high moisture content and temperature may be one of the reasons for the degradation of soluble saccharides and protein (Stone, 1996; Wang et al., 2018). As a result, with the degradation of soluble saccharides and protein, nutrient value of rice seeds was negatively impacted.

3.2. Oxidative stress and internal deterioration

3.2.1. Contents of $O_2^-$ and MDA

As reported by previous study, content of $O_2^-$ in cells would determine the oxidative stress (Wang et al., 2018). In this study, during the 300 days storage, content of $O_2^-$ increased gradually, suggesting that the oxidative stress became more serious with the extension of storage period. The increase rates were impacted by the moisture content and storage temperature. As shown in Figure 3(a), when the storage temperature changed from 10°C to 30°C, increased rate of $O_2^-$ content in rice seeds with 11% moisture content changed from 0.014 to 0.027 µg/(g.d). Similar trend was observed when the moisture content of rice seeds increased from 11% to 13% (Figure 3(a)). Therefore, high moisture content and high storage temperature would increase the oxidative stress in rice seeds.

MDA, as a product of lipid deterioration, is another symbol of the oxidative stress in rice seeds (Djanaguiraman, Prasad, & Seppanen, 2010). Previous studies have demonstrated that accumulation of MDA in food products would reduce the nutrients value since MDA is an inducing factor of cancer, inflammation, cardiovascular diseases in human body (Chole, Patil, Basak, Palandurkar, & Bhowate, 2010; Jiang & Xiong, 2016). In this study, with the increase of oxidative stress in rice seeds, accumulation of MDA was observed. Figure 3(b) showed that accumulation of MDA was accelerated by the high storage temperature and moisture content. The highest content of MDA in rice seeds after 300 days storage was 0.487 µmol/g. Similar phenomenon was observed in previous study which explored the accumulation of MDA in wheat seeds during storage at different conditions (Wang et al., 2018). Since rice is a staple food in many countries, intake of stored rice seeds with high content of MDA will cause serious health-related problems and threaten the public health (Chole et al., 2018). Therefore, the alleviation of oxidative stress during storage is not only

Figure 2. Changes of biochemical characteristics and nutrients loss in rice seeds during storage: (a) Germination efficiency of rice seeds; (b) Acid value of rice seeds; (c) Content of soluble saccharides in rice seeds; (d) Content of protein in rice seeds.

Figura 2. Cambios en las características bioquímicas y pérdida de nutrientes en las semillas de arroz durante el almacenamiento: (a) Eficiencia de germinación de las semillas de arroz; (b) Valor ácido de las semillas de arroz; (c) Contenido de sacáridos solubles en las semillas de arroz; (d) Contenido de proteína en las semillas de arroz.
Figure 3(a and MDA. In addition, high storage temperature and high moisture content were partly responsible for the nutrients loss in rice seeds (Tefera, 2012). As shown in Figure 3(c), the reduction of activity of CCO was observed after 300 days storage. It was reported that the damage of cell structure and the oxidative stress could reduce the activity of CCO and further prohibit the cell metabolisms (Hüttemann et al., 2012). As shown in Figure 3(d), the reduction of activity of CCO mainly occurred in the late stage of storage. For example, in the early stage (Day 0–Day 150), the activity of CCO only decreased by less than 23.75% while the reduction of activity could reach 90.98% in the late stage (Day 150–Day 300). Since the activity of CCO is a parameter reflecting the biological activity of cells, these results suggested that the biological activities of rice seeds were negatively impacted in the late stage of storage. In addition, deactivation of CCO caused by the damage of cell structure and the oxidative stress is partly responsible for the decrease of germination efficiency of rice seeds presented in Figure 2(a) (Turrens, 2003).

The discussion above demonstrated that high storage temperature and high moisture content partly contributed to the deterioration and nutrients loss in rice seeds. Measurement of oxidative stress parameters showed that unfavorable conditions could accelerate the accumulation of \( \text{O}_2^- \) and MDA. In addition, high storage temperature and high moisture content were partly responsible for the damage of cell structure and deactivation of CCO in the late stage of storage. Therefore, the decrease in CCO activity would be partly responsible for the decrease in the germination efficiency of rice seeds presented in Figure 2(a) (Turrens, 2003).
rice seeds. Hence, to prevent the serious deterioration in rice seeds during storage, temperature and moisture content are two critical control points.

3.3. Microstructures of embryo and endosperm during storage

3.3.1. Microstructure of embryo

The embryo of rice seeds before storage was shown in Figure 4(a). In Figure 4(a-A) and (a-C), a couple of mitochondria, which are responsible for the cell metabolisms, were observed. In addition, the embryo contained complete nucleus membrane, nucleus, cell wall, protein bodies, lipid bodies, and vesicle, suggesting that the rice seeds before storage were well protected (Figure 4(a-A), (a-B) and (a-D)).

After 300 days storage at 10°C, embryo of rice seeds (11% moisture content) did not have obvious change (Figure 4(b)). This result confirms that the low moisture content and the low temperature could prevent the damage of embryo in rice seeds during storage. Figure 4(c-A) indicates that the protein bodies, mitochondria, and plasma membrane degraded seriously when the storage temperature reached 30°C. In addition, the damage of nucleus and the cohesion of chromatin were observed in embryo after storage (Figure 4(c-B)). As shown in Figure 4(d), when the rice seeds with high moisture content were stored at 30°C, more serious deterioration was observed. Figure 4(d-A) showed that the cell wall was damaged seriously and Figure 4(d-B) demonstrated that the nucleus was totally disappeared and dissolved in the cytoplasm. Since embryo plays an essential role in the germination of rice seeds, the damage of embryo’s structure not only causes nutrients loss, but also limits the usage of rice seeds after storage (Kaneko, Itoh, Ueguchi-Tanaka, Ashikari, & Matsuoka, 2002).

![Figure 4](image_url)

Figure 4. Microstructure of embryo in rice seed during storage: (a) Embryo of rice seeds before storage; (b) Embryo of rice seeds (11% moisture content) storage at 10°C; (c) Embryo of rice seeds (11% moisture content) storage at 30°C; (d) Embryo of rice seeds (15% moisture content) storage at 30°C (Magnification times of a-A, a-B, a-C, a-D, b-A, b-B, c-A, c-B, d-A, and d-B are 10,000×, 30,000×, 50,000×, 10,000×, 10,000×, 50,000×, 30,000×, 30,000×, 30,000×, and 30,000×, respectively). (CW: Cell wall; IC: Intercellular space; M: Mitochondria; PB: Protein bodies; L: Lipid bodies; PM: Plasma membrane; Cm: Cytoplasmic matrix; VE: Vesicle; N: Nucleus; Nu: Nucleolus; RER: Endoplasmic reticulum).
3.3.2. Microstructure of endosperm

The changes of endosperm, which mainly provides nutrition in the form of starch, would impacts the nutrition profile of rice seeds (James, Denyer, & Myers, 2003). As shown in Figure 5, micro-structures of endosperm and endosperm surface layer could be changed obviously under certain conditions. Figure 5(a-A) indicated that endosperm of rice seeds before storage was rich with protein matrix and polyhedral starch granules. In addition, the protein matrix and the starch granules were integrated closely. Figure 5(a-B) shows that the endosperm surface layer had complete structure and clear lines. As shown in Figure 5(b), when the rice seeds with low moisture content (11%) were stored at low temperature (10°C), the micro-structures of endosperm and endosperm surface layer did not have obvious change.

However, as shown in Figure 5(c), after storage, the nutrition in endosperm degraded and the micro-structure was damaged to some extent. Figure 5(c-A) demonstrated that the storage at high temperature (30°C) damaged the microstructure of starch granules and enlarged the interstitial space in endosperm. The integration structure of starch granules and protein matrix was damaged and some starch granules were exposed. In addition, some microorganism colonies were observed on the endosperm surface layer (Figure 5(c-B)). The main reason is that with the damage of physical structure of rice seeds, high temperature supported the growth of bacteria and fungi on endosperm surface layer and accelerated the formation of microorganism colonies (Siripatrawan & Makino, 2015).

As shown in Figure 5(d), the micro-structure of endosperm was seriously damaged after storage of rice seeds at 30°C. Figure 5(d-A) indicates that the borderline between starch granules was unclear and the integration of starch granules and protein matrix became much loose. Besides the
microorganism colonies, some starch granules were observed on the endosperm surface layer (Figure 5(d-B)). The main reason for the exposure of starch granules is that with the damage of endosperm surface layer and the integration structure of starch and protein, the force of combing starch granules decreased seriously (Fujita et al., 2011). Similar phenomenon was reported by the study of Wang et al. (2018) that explored the changes of micro-structure in wheat seeds during storage. Comparison of Figure 5(a), (c), and (d) indicated that both high temperature and high moisture content accelerated the damage of endosperm in rice seeds during long-term storage.

### 3.4. Changes of intracellular scavenging systems

Although biochemical assay and micro-structure measurement were conducted to evaluate the deterioration of rice seeds during storage, the changes of intracellular scavenging systems, which protect rice seeds from deterioration, during storage have not been fully studied yet. There are two hypotheses for the relation between scavenging system and deterioration in rice seeds. First, the scavenging systems performed well in rice seeds during the storage while excessive oxidative stress caused the deterioration. Second, scavenging systems were deactivated during storage and the damage of oxidative stress to rice seeds became serious.

![Figure 6](image-url)

**Figure 6.** Damage of scavenging system in rice seed during storage: (a) Activity of SOD in rice seed; (b) Activity of CAT in rice seed; (c) Activity of POD in rice seed; (d) Content of GSH in rice seed; (e) Content of ASC in rice seed.

**Figura 6.** Daño del sistema de eliminación en las semillas de arroz durante el almacenamiento: (a) Actividad de SOD en semillas de arroz; (b) Actividad de CAT en semillas de arroz; (c) Actividad de POD en semillas de arroz; (d) Contenido de GSH en semillas de arroz; (e) Contenido de ASC en semillas de arroz.
without the protection of scavenging systems. Essential enzymes and antioxidants in the scavenging systems were measured to test these two hypotheses.

3.4.1. Enzymatic scavenging system

Figure 6 indicated that with the extension of storage period, activities of three main enzymes in scavenging system decreased gradually. As shown in Figure 6(a), the activities of SOD under different storage conditions decreased by 25.70% and 88.63%. Comparison of Figures 6(a) and 3(a) indicated that the increase of $O_2^-$ content was in accordance with the decrease of SOD activity. That main reason for such a phenomenon is that SOD could alleviate intracellular oxidative stress by converting $O_2^-$ to $O_2$ or $H_2O_2$ (Thounaojam et al., 2012). With the deactivation of SOD during storage, the conversion efficiency of $O_2^-$ in rice seeds was reduced to a lower level. Similar phenomena were also observed in Figures 6(b, c). Under the most unfavorable condition (30°C and 15% moisture content) in this work, the activities of SOD, CAT, and POD decreased by 88.63%, 89.00%, and 73.39%, respectively. With the serious deactivation of scavenging enzymes, the oxidative stress in rice seeds increased accordingly (Sharma, Jha, Dubey, & Pessarakli, 2012).

It was also observed that the deactivation of enzymes mainly occurred in the later stage of the 300 days storage. Under the most unfavorable condition (30°C and 15% moisture content) in this work, the activities of SOD, CAT, and POD decreased by 62.20%, 65.07%, and 52.81%, respectively, in later stage (Day 150–Day 300) while only decreased by 26.43%, 23.92%, and 20.59% in the early stage (Day 0–Day 150). The serious deactivation of enzymes in the later stage was partly attributed to the damage of cell structures caused by the deterioration in rice seeds. For example, with the dissolution of cell wall, enzyme inhibitors might come into the cells and reduce the enzymes activities, reducing the activity of enzymes (Le Gall et al., 2015).

3.4.2. Nonenzymatic scavenging system

The glutathione-ascorbate cycle, which is able to detoxify intracellular oxidants, plays an important role in the nonezymatic scavenging system of rice seeds (Das & Roychoudhury, 2014). The conversion, driven by NADPH, between GSH and ASC could reduce $H_2O_2$ (Thounaojam et al., 2012). As shown in Figure 6, nonenzymatic scavenging system of rice seeds was damaged as well during the storage. Same as the enzymatic scavenging system, the damage of nonenzymatic scavenging system mainly happened in the later stage of storage. Figure 6(d) showed that under different storage conditions, the decrease of GSH content in the early stage ranged between 5.55% and 14.79% while could reach 55.17% in the later stage of storage. Similar phenomenon was observed in the changes of ASC content in rice seeds during storage (Figure 6(e)). Under the most unfavorable condition (30°C and 15% moisture content) in this work, contents of GSH and ASC decreased by 69.95% and 73.61%, respectively, after 300 days storage.

According to the results above, unfavorable conditions could seriously damage the scavenging systems in rice seeds during the late stage of storage. Hence, both two hypotheses are reasonable in some extent. In the early stage of long-term storage, the scavenging systems in rice seeds performed well while the unfavorable conditions, such as high temperature, humidity, and moisture content, mainly contributed to the intracellular deterioration, supporting the first hypothesis. During this stage, with the protection of scavenging systems, the deterioration in rice seeds was not serious. The second hypothesis is more reasonable to the changes of rice seeds in the later stage.

3.5. Comparison of strategies for rice seeds storage

As shown in Table 1, although moisture removal or low-temperature storage could limit the deterioration process to some extent individually, the integration of moisture removal and low-temperature storage is more effective in the protection of rice seeds. The statistical analysis showed that after 300 days storage, rice seeds of Mode 1 were significantly different ($p < 0.05$) from those of Mode 3 and Mode 4 in terms of germination efficiency and deterioration parameters (Tables 1 and 2). Hence, in the industrial implementation, storage conditions with low temperature are more likely to be applied (Tefera et al., 2011). Interestingly, according to the statistical analysis, Mode 3 and Mode 4 are not significantly different ($p > 0.05$) in the changes of deterioration parameters (Table 2), suggesting that the storage at normal temperature in early stage did not cause serious deterioration in rice seeds. Similar phenomenon was also observed in the measurement of biochemical properties of rice seeds stored by Mode 3 and Mode 4 (Table 1).

As shown in Tables 1–3, the energy input of Mode 3 was much higher than that of Mode 4 while Mode 3 and Mode 4 had no significant difference ($p > 0.05$) in terms of deterioration alleviation. Since the serious deterioration was mainly observed in the late stage of storage, the staged low-temperature storage in Mode 4 successfully reduced the energy input without causing serious deterioration in rice seeds. Hence, the later stage is a critical control point of rice seeds storage. According to this
Tabla 2. Deterioro de semillas de arroz almacenadas empleando diferentes estrategias (Modo 1: almacenamiento a temperatura normal después de eliminar la humedad; Modo 2: almacenamiento a baja temperatura; Modo 3: almacenamiento a baja temperatura después de eliminar la humedad; Modo 4: almacenamiento intermitente a baja temperatura después de eliminar la humedad).

| Modo     | Día 0 | Día 1 (300 días) | Día 2 (300 días) | Día 3 (300 días) | Día 4 (300 días) |
|----------|-------|------------------|------------------|------------------|------------------|
|          | O$_2^-$ (µg/g) | MDA (µmol/g) | Conductividad (mS/m) | CCO (mmol/min) |
| Modo 1   | 20.67 ± 1.02   | 0.251 ± 0.004$^d$ | 61.7 ± 3.1$^a$ | 0.158 ± 0.008$^b$ |
| Modo 2   | 28.47 ± 0.58$^e$ | 0.420 ± 0.008$^a$ | 94.9 ± 1.9$^a$ | 0.099 ± 0.009$^d$ |
| Modo 3   | 26.96 ± 0.82$^{c, b}$ | 0.340 ± 0.018$^b$ | 86.1 ± 3.0$^c$ | 0.115 ± 0.009$^c$ |
| Modo 4   | 24.79 ± 0.91$^{b, c}$ | 0.301 ± 0.021$^b$ | 80.8 ± 1.8$^c$ | 0.132 ± 0.012$^{c, d}$ |
|          | 25.27 ± 0.69$^{b, c}$ | 0.327 ± 0.019$^{c, d}$ | 82.3 ± 2.5$^{c, d}$ | 0.125 ± 0.008$^{c, d}$ |

*MDA* y “CCO” se refieren a malondialdehído y citocromo oxidasa, respectivamente.

Tabla 3. Evaluación de diferentes estrategias de almacenamiento (Modo 1: almacenamiento a temperatura normal después de eliminar la humedad; Modo 2: almacenamiento a baja temperatura; Modo 3: almacenamiento a baja temperatura después de eliminar la humedad; Modo 4: almacenamiento intermitente a baja temperatura después de eliminar la humedad).

| Modo     | Día 0 | Día 1 (300 días) | Día 2 (300 días) | Día 3 (300 días) | Día 4 (300 días) |
|----------|-------|------------------|------------------|------------------|------------------|
| Energy input of moisture removal (kW) | 40   | 0    | 40   | 40   |
| Energy input of storage (kW) | 30   | 330  | 330  | 150  |
| Biochemical properties | Germination efficiency | 115.64% | 118.40% | 16.65% | 18.28% |
| Acid value | 157.91% | 162.26% | 155.44% | 155.83% |
| Content of soluble saccharides | 122.04% | 116.35% | 19.78% | 113.57% |
| Content of protein | 129.41% | 143.25% | 124.22% | 125.49% |
| Deterioration parameters | Content of O$_2^-$ | 127.40% | 123.33% | 116.62% | 118.20% |
| Content of MDA | 140.24% | 126.18% | 116.61% | 123.24% |
| Conductivity | 134.98% | 128.34% | 123.64% | 125.03% |
| Activity of CCO | 137.34% | 127.22% | 116.46% | 120.89% |

*MDA* y “CCO” se refieren a malondialdehído y citocromo oxidasa, respectivamente.

4. Conclusions

In conclusion, (1) high temperature and moisture content could accelerate the deterioration and reduce the nutrients value of rice seeds; (2) microstructure of embryo and endosperm was damaged during the long-term storage as a consequence of deterioration; (3) study of the scavenging systems confirmed the hypotheses that scavenging system performed well and external storage conditions partly contributed to the deterioration in the early stage while the self-protection metabolism was damaged to some extent with the malfunction of scavenging systems in the later stage of storage; (4) Moisture removal followed by staged low-temperature storage is an energy-saving way to protect the properties of rice seeds during long-term storage.

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References

Aghaleh, M., Niknam, V., Ebrahimzadeh, H., & Razavi, K. (2009). Salt stress effects on growth, pigments, proteins and lipid peroxidation in Salicornia persica and S. europaea. Biologia Plantarum, 53(2), 242–248.

Asatsuma, S., Sawada, C., Itoh, K., Okito, M., Kitajima, A., & Mitsui, T. (2005). Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. Plant and Cell Physiology, 46(6), 858–869.

Bandeira, C. M., Evangelista, W. P., & Gloria, M. B. A. (2012). Bioactive amines in fresh, canned and dried sweet corn, embryo and endosperm and germinated corn. Food Chemistry, 131(4), 1355–1359.

Berger, F., Grini, P. E., & Schnittger, A. (2006). Endosperm: An integrator of seed growth and development. Current Opinion in Plant Biology, 9(6), 664–670.

Bhullar, N. K., & Gruissem, W. (2013). Nutritional enhancement of rice for human health: The contribution of biotechnology. Biotechnology Advances, 31(1), 50–57.

Chauhan, B. S., & Johnson, D. E. (2011). Row spacing and weed control timing affect yield of aerobic rice. Field Crops Research, 121(2), 226–231.

Chole, R. H., Patil, R. N., Basak, A., Palandurkar, K., & Bhowate, R. (2010). Estimation of serum malondialdehyde in oral cancer and precancer patients. Journal of Cancer Research and Therapeutics, 6(4), 487.

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Environmental Science, 2, 53.

Djanaguiraman, M., Prasad, P. V., & Seppanen, M. (2010). Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiology and Biochemistry, 48(12), 999–1007.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28(3), 350–356.
