Changes in soybean seeds as affected by accelerated and natural aging

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Abstract
Changes occurring in seed during aging are very significant for seed quality, i.e. seed longevity. The rate at which the seed aging process takes place depends on the ability of seed to resist degradation changes and protection mechanisms, specific for each plant species. Six soybean varieties developed at Institute of Field and Vegetable Crops Novi Sad, Serbia, were submitted to accelerated aging for three and five days, and natural aging for six and twelve months, under controlled and conventional storage conditions. The content of malondialdehyde, superoxide dismutase and peroxidase activities were studied in relation to seed germination. Lipid peroxidation, as well as the decrease in superoxide dismutase and peroxidase activities (especially pronounced by applied accelerated aging) occurred with both types of aging. Duration of aging period, type of storage and characteristics of soybean varieties affected the degree of seed damage and the ability of seed to resist the negative consequences of aging.

Keywords: Glycine max (L.) Merr., seed aging, lipid peroxidation, superoxide dismutase and peroxidase activities, seed germination

Introduction
Unfavorable environmental conditions and seed storage can cause oxidative stress in plant tissue, and development of superoxide radical, hydrogen peroxide, and hydroxyl radical, which are the most active, toxic and destructive products of oxidative stress. The composition of fatty acids is the most important factor which determines oils susceptibility to oxidation. The types of fatty acids present in oil, and in particular number of their double bonds, determine the type and extent of chemical reactions which occur during the storage time [1]. Lipid peroxidation (LP) is oxidative damage of cell membranes, lipoproteins and other molecules containing lipids, caused by oxidative stress. Once initiated, reaction of LP continues auto-catalytically and progressively leads structural and functional substrate changes [2]. According to the degree of reactivity, the most reactive amino acids sensitive to oxidative damages are: cysteine, histidine, tryptophan, methionine and fenilalanin [3].

Some biochemical changes strongly influencing the quality and viability of seed take place inside the soybean seed during aging [4]. The chemical structure of soybean seed (20-22% of oil) enables some specific processes, very often degrading in nature. Lipid autooxidation and increase of free fatty acid content during storage are the most often mentioned reasons for accelerated damage of seed of oily plant species [5]. Accumulation of active oxygen species and free radicals has often been considered as one of the most important factors of seed aging [6].

Processes pertaining to peroxidation cause changes that accelerate seed aging, so their removal or decrease would positively influence the seed viability [7]. Low concentrations of free radicals are the “stress signals” for adaptable reaction of antioxidative plant system [8]. It
was determined that under oxidative stress the genes responsible for antioxidative enzymes synthesis are activated via \( \text{H}_2\text{O}_2 \) as a signal molecule [9, 10]. Therefore, besides being cytotoxic, individual free radicals (depending on their concentration) also play role as signal molecules in biochemical plant response under oxidative stress, caused by different environmental factors [11]. In studies of several authors, special attention was paid to enzymatic activities due to their possible usage as significant indicators of seed viability and longevity. Activity of the system of free radicals detoxification of sunflower seed kept at 10 °C and 25 °C in correlation with seed germination and enzymatic activity of aged seed was very low [12].

The aim of this research was to determine the effect of aging on seed viability and biochemical changes in seed of tested soybean varieties, and possibility to predict the speed of seed deterioration during storage by applying accelerated aging test.

**Materials and methods**

Six soybean (\( \text{Glycine max (L.) Merr.} \)) varieties of different maturity groups: Afrodita and Lasta (0 maturity group), Balkan and Novosadjanka (I maturity group), Vojvodjanka and Morava (II maturity group), developed at Institute of Field and Vegetable Crops, Novi Sad, were used in this research.

Accelerated aging: The seeds were placed in metal dishes, on metal sieve into water bath at 42 °C, and relative humidity of 100%. Testing was performed after three and five days, in four replications.

Natural aging: Seed was stored in two ways. 1) seed was kept in cool chamber (controlled conditions) at 4 °C and relative humidity of 80 to 85%, 2) seed was kept under conventional storage conditions (uncontrolled conditions). Testing was performed after six and 12 months of storage.

Germination of fresh seed (harvested seed), and that of artificially and naturally aged seed were estimated by standard laboratory germination test, using four replications [13]. Extraction of malondialdehyde (MDA) from soybean seed was done by using solution of thiobarbituric acid (TBA), trichloroacetic acid (CCl_3COOH) and perchloric acid (HClO_4), and concentration was determined spectrophotometrically at 532 nm [14]. Soybean seed (0.5 g) were homogenized in mortar with 4.5 ml extraction solution and incubated in water bath at 90 °C for 20 minutes. After incubation, solutions were cooled to stop the reaction and centrifuged for 10 min at 5500 r/min. MDA concentration i.e. intensity of lipid peroxidation was expressed as nmol of MDA g^-1 of fresh mass.

Superoxide dismutase (SOD) and peroxidase (Px) were determined using extract of 1g of soybean seed and 5 ml 0.1M phosphate buffer pH 7. The same extract was used for determination of protein content in seed in order to express activities of the tested enzymes. Obtained homogenate was centrifuged at 4500 r/min at 5 °C for 10 minutes.

SOD activity [U mg^-1 protein] was determined using spectrophotometer method [15], on the basis of reaction of auto-oxidation of adrenaline to adrenochrome. Change of adrenaline solution absorbance was measured at 480 nm in carbonate buffer pH 10.2.

The activity of Px [U mg^-1 protein] was determined on the basis of transformation of guaiacol into tetraguaiacol and by measuring change of absorbance at 436 nm in phosphate buffer [14].

Enzyme assay and MDA content values were average over at least three replications for each treatments and variety. Linear regressions between germination and enzyme activity/MDA content were tested for slope significances, using software Statistica 8.0.
Results and discussion

Intensity of LP increased during natural aging of soybean seed. It was determined that MDA content in seed under controlled and conventional storage conditions was significantly increased after six and twelve months of seed storage, in the majority of the studied varieties (Fig. 1a). Obtained results confirmed the possibility of determining the levels of lipid peroxidation in seed via determination of MDA derivatives content. These results were in agreement with the results which mention that MDA content was significantly increased in embryos and cotyledons of peanut seed after three days of artificial aging [16]. MDA content in axes markedly increased with accelerated aging, and increased by 80 and 34%, respectively, by the 15th and 20th days of accelerated aging, as compared with non-aged axes [17]. Due to differences existing among varieties, it can be noticed that performances of genotypes also influenced the peroxidative changes during seed storage. The above mentioned results are in accordance with the results obtained by other researchers dealing with damages caused during seed storage. Seed susceptibility to peroxidative changes differed, depending on seed fatty acid composition, and lipid peroxidation can be considered as one of the indicators of individual soybean genotype susceptibility to oxidative stress [18]. During storage, a number of physiological and physicochemical changes occur, termed aging [19].

Figure 1: Lipid peroxidation (a) and activity of superoxide dismutase (b) and peroxidase (c) during natural aging of soybean seed (FS-fresh seed; CC-controlled condition and CS-conventional storage conditions, after 6 and 12 months)
SOD activity (Fig. 1b) decreased during natural aging. Significant decrease in SOD activity in seed of all tested varieties occurred after six months of storage, both under controlled and conventional storage conditions in relation to SOD activity in the fresh seed. Decreasing trend of SOD activity after 12 months of natural aging in seed of all tested varieties continued in both variants of seed storing.

After six months of natural soybean seed aging, a very significant decrease in Pxx activity in seed of all tested varieties was also determined, in relation to the activity of this anti-oxidative enzyme in fresh seeds, under both controlled and conventional storage conditions (Fig. 1c). Decreasing trend of Pxx activity after 12 months of natural aging in seed of all tested varieties was continued under both conditions of seed storage.

Increased intensity of LP resulted in decreased seed germination of the tested soybean varieties (Fig. 2a). The lowest decrease rate was observed six months after seed storage (-0.7435 and -0.6798), and somewhat higher rate was observed 12 months after storage under controlled conditions (-0.8724). The greatest germination decrease, in relation to increased LP intensity, was observed in seed stored for 12 months under conventional storage condition (-1.2094). Shorter storage period, as well as the controlled storage conditions slowed down the process of fatty acid peroxidation in soybean seed. However, in the case of prolonged storage, especially under conditions of variable temperature and air humidity, the decline rate of seed viability increased with the changes in lipid peroxidation intensity. Based on the obtained relations between increased lipid peroxidation and decreased seed germination, it can be pointed out that storage conditions, in longer time period, were more pronounced in expressing negative influence on soybean seed viability.
Different storage conditions, first of all temperature and relative air humidity significantly affected soybean seed germinability [20]. The same authors concluded that optimal conditions for soybean seed storage were temperature not higher than 25 °C and relative air humidity ranging from 55% to 65%. In testing the influence of aging on sunflower seed viability, seed germination decreased with prolonged sunflower seed storage period under conventional storage conditions [21]. Both soybean seed germination and SOD activity decreased linearly in relation to storage condition and duration (Fig. 2b). The lowest decrease rate was noticed in seed stored for six months under controlled conditions (0.161), and the highest in seed stored for 12 months under conventional storage conditions (0.4706). Under oxidative stress conditions, SOD appears to regulate the effects of environmental conditions on the intensity of stress in plant cells. Increase in SOD activity in fresh soybean seed embryos was significantly inhibited in aged seed [22], in regard to decrease SOD activity in aged sunflower seed [23]. SOD plays a key role in inhibition of oxidative stress caused by different effects [24, 25].

By observing Px activity during soybean seed storage (Fig. 2c), as well as the seed germination, the most pronounced dependence of the mentioned parameters was noticed after 12 months under conventional storage conditions (R² = 0.6149), with the greatest rate of germination decline (3.7548). Decrease of germination was not correlated with decrease in Px activity in seed stored for six months under controlled conditions. Peroxidase activity was also gradually reduced during natural and induced seed aging [22].

Extreme conditions such as 40 °C and 100% of relative air humidity caused some biochemical changes in seed as well as reduction in seed germination. After three days of accelerated aging, obtained seed germination was on the level of six-month naturally aged seed germination, both under controlled and conventional storage condition (Table 1). Seed germination after five days of accelerated aging was the same as the germination of seed stored for 12 months under conventional storage conditions.
Table 1. Seed germination and biochemical parameters in soybean seed depending on different aging treatments (AA-accelerated aging after 3 and 5 days; CC-controlled condition and CS-conventional storage after 6 and 12 months)

<table>
<thead>
<tr>
<th>Aging treatment</th>
<th>Seed germination (%)</th>
<th>Lipid peroxidation (nmol MDA g⁻¹ fresh mass)</th>
<th>Peroxidase activity (U mg⁻¹ protein)</th>
<th>Superoxide dismutase activity (U mg⁻¹ protein)</th>
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<tbody>
<tr>
<td>CS6</td>
<td>76.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>50.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS12</td>
<td>56.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC6</td>
<td>81.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CC12</td>
<td>72.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>AA5</td>
<td>55.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>87.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>35.3&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>AA3</td>
<td>79.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;de&lt;/sup&gt;</td>
<td>46.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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Values not connected by same letter in same column are significant different at level p<0.05

Intensity of lipid peroxidation in seed after three days of accelerated aging was on the level of intensity of lipid peroxidation in seed after 12 months for both storage conditions. After applying accelerated aging test of five days, intensity of lipid peroxidation in seed was significantly different regarding all aging treatments. Extreme conditions of three-day accelerated aging test resulted in such activity of SOD in seed as that obtained in seed after six months for both storage conditions. Therefore, using comparisons between artificial and natural aging, no significant relations regarding peroxidase activity in seed were determined. In their research, many authors have observed changes in germinability, changes in biochemical parameters of artificially aged seed, as well as certain relations to natural aging [21, 26], not only in soybean, but in other oil crops as well.

Possibility to predict the longevity of stored soybean seed has practical significance. Multiple regressions were implemented to build a model for estimation of seed germination during conventional storage, based on five-day accelerated aged test. Model was built based on germination and antioxidative enzyme activity data of soybean seed exposed to five-day accelerated age test. In order to estimate seed germination after six months under conventional storage condition, obtained regression was based on seed germination (b = 0.551) and SOD activity in soybean seed (b = 0.405). Seed germination (b = 0.775) and Px activity in seed (b = 0.630) gave the best model to estimated seed germination after 12 months under conventional storage condition. Both models have similar values for adjusted R² (for first above mentioned model R² = 0.628 and for second one R² = 0.684). Model for estimation

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of seed germination during storage can be based on accelerated aging test, and can be helpful in making a decision on duration of soybean seed storage (Figure 3). If observation included more parameters of oxidative stress and seed viability, the model of prediction of seed deterioration would be more precise. The symptoms observed during accelerated aging can be used to characterize the degree of aging, which changes in the opposite direction as compared to storability and there is a possibility of predicting the length of seed storage using accelerated aging test [27, 23]. Accelerated aging of seed, high temperature and high relative humidity lead to the loss of seed vigor and eventually seed viability, which is an excellent method for determination of vigor changes during seed storage [17].

Conclusions

1. Content of MDA in soybean seed was increased by prolonged storage, on the basis of which it can be concluded that lipid peroxidation was more intensive in aged seed. Such seed had decreased germination revealing negative influence of oxidative stress on seed viability.
2. The most intensive lipid peroxidation was observed five days after application of accelerated aging test.
3. MDA content in seed exposed to three-day accelerated aging was almost the same as the MDA content in seed stored for 12 months under conventional storage conditions.
4. The activities of superoxide dismutase and peroxidase decreased during soybean seed aging, which was especially pronounced when accelerated aging was applied.
5. Based on the results given in this paper, it was possible to estimate the degree of changes in stored seed by applying accelerated aging test.

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