Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v34i3.12660

α-Tocopherol levels in natural and artificial aging of soybean seeds

Maria Izabel Krüger Giurizatto[1*], Osvaldo Ferrarese-Filho[2], Maria de Lourdes Lucio Ferrarese[2], Antonio Dias Robaina[3], Manoel Carlos Gonçalves[3] and Cláudia Andréa Lima Cardoso[4]

*[1]Agência Estadual de Defesa Sanitária Animal e Vegetal, Laboratório de Análise de Sementes Oficial de Dourados, Rodovia Dourados/ Itahum, km 12, Cx. Postal 533, Dourados, Mato Grosso do Sul, Brazil. [2]Universidade Estadual de Maringá, Maringá, Paraná, Brazil. [3]Universidade Federal da Grande Dourados, Dourados, Mato Grosso do Sul, Brazil. [4]Universidade Estadual do Mato Grosso do Sul, Dourados, Mato Grosso do Sul, Brazil. *Author for correspondence: E-mail: m.kruger@terra.com.br*

ABSTRACT. Tocopherols are well known constituents of vitamin E, and the main antioxidants in soybean. Are natural antioxidants and stabilizers that can inhibit lipid degradation, reducing non-enzymatic oxidation of these compounds during storage of seeds, germination and initial development of seedlings. The objective of this work was to determine the level of α-tocopherol in four soybean seeds cultivars naturally and artificially aged. Seeds of four soybean cultivars stored from 0 to 180 days in a dry chamber (natural aging) and subjected to high temperature and humidity (artificial aging) were analyzed for α-tocopherol content. The quantification of α-tocopherol in the soybean seeds was performed by high performance liquid chromatography (HPLC) combined with the Soxhlet extraction method. Significant differences in α-tocopherol levels in seeds were observed for all cultivars and storage times. The α-tocopherol contents of the soybean seeds showed linear correlations with an increasing period of storage for all the cultivars studied. However, the artificially aged seeds had a higher content of α-tocopherol than those naturally aged.

Keywords: (*Glycine max* (L.) Merrill), α-tocopherol, storage.

Teores de α-tocoferol no envelhecimento natural e artificial de sementes de soja

RESUMO. Tocoferóis são conhecidos constituintes da vitamina E, sendo os principais antioxidantes lipofílicos na soja. Eles são substâncias antioxidantes naturais e estabilizadoras, capazes de inibir a degradação de lipídios limitando a oxidação não enzimática destes compostos durante o armazenamento das sementes, a germinação e o desenvolvimento inicial das plântulas. O objetivo deste trabalho foi determinar o conteúdo de α-tocoferol nas sementes de quarto cultivares de soja envelhecidas natural e artificialmente. As sementes de quatro cultivares de soja envelhecidas em armazenamento de zero a 180 dias em câmara seca (envelhecimento natural) e submetidas a altas temperaturas e umidade relativa (envelhecimento artificial) foram analisadas quanto ao teor de α-tocoferol. A quantificação de α-tocoferol nas sementes de soja foi efetuada por cromatografia líquida de alta eficiência (CLAE) associada ao método de extração Soxhlet. Diferenças significativas nos teores de α-tocoferol nas sementes foram observadas para todas as cultivares e tempos de armazenamento. O teor de α-tocoferol das sementes de soja mostrou resposta linear crescente com o aumento dos períodos de armazenamento. Entretanto, as sementes envelhecidas artificialmente apresentaram maior teor de α-tocoferol do que as envelhecidas naturalmente.

Palavras-chave: (*Glycine max* (L.) Merrill), α-tocoferol, armazenamento.

Introduction

Vitamin E includes eight compounds found in nature: four tocopherols and four tocotrienols named and identified by the prefixes α-, β-, γ- and δ-tocopherol. Tocopherols are natural antioxidants and stabilizers that can inhibit lipid degradation. Together with other nutritional substances present in soybean seeds (*Glycine max* (L.) Merrill), tocopherols have important effects on human health, such as preventing cardiovascular disease and cancer and improving the immune system (CARRÃO-PANIZZI; ERHAN, 2007). Tocopherols are synthesized in plants, located in the plastids, and accumulate to varying degrees in all tissues. Seeds contain the highest levels of tocopherols (SATTLER et al., 2004).

Breeding soybeans to increase tocopherol levels (mainly α and γ-tocopherol) is considered important for nutritional and functional concerns regarding food stability and human health. Genetic variability in tocopherol levels was observed in more than 1,000 soybean genotypes, and only three seed varieties had high α-tocopherol levels (UJIIE et al., 2005). According to these authors, weather

conditions and changes can affect the tocopherol concentrations in soybean seeds.

In general, seed performance declines as storage time increases, particularly for soybean seeds. This aging or loss of vigor is demonstrated by delayed germination and emergence, slower growth, increased susceptibility to environmental stress, and finally, by a decline in germination. Seed aging is therefore a problem for agriculture, and it is worth investigating the mechanisms or events that lead to loss of viability and vigor. Various events or processes have been suggested to be causative mechanisms, including chromosomal damage, loss of several enzymes, degradation of the respiratory system, losses in ATP production/storage capacity, and membrane deterioration (PARRISH; LEOPOLD, 1978).

Seed longevity is an important issue for the agricultural and ecological prospects of soybean farming, and lipid oxidation may be a significant factor in this process. There is a known inverse correlation between lipid oxidation and seed longevity during natural and artificial aging. Seed deterioration during storage and natural aging occurs mainly due to factors that increase respiratory intensity. Therefore, antioxidants such a α-tocopherol could reduce respiratory intensity by blocking the entry of oxygen into the internal tissues of seeds and therefore reduce seed deterioration.

Artificial seed aging in the laboratory is based on the considerable increase in seed deterioration at high temperature and relative humidity. Under these conditions, lower-quality seeds will deteriorate faster than more vigorous ones, with effects on germination after a period of accelerated aging (TORRES; MARCOS FILHO, 2001).

Studies to assess the levels of α-tocopherol in soybean cultivars are relevant because of this compound's importance in reducing seed deterioration during storage. The objective of this work was to determine the level of α-tocopherol in four soybean seeds cultivars naturally and artificially aged.

Material and methods

The study was performed with four soybean cultivars (factor 1): BRS 285, BRS 246 RR, MSOY 7908 RR, and BMX Titan RR, provided by both private and public entities from the State of Mato Grosso do Sul (Brazil). Analyses of the physiological quality of the seeds were made in the Official Iagro Laboratory for Seed Analysis at the School of Agricultural Sciences/UFGD in the town of Dourados, Mato Grosso do Sul State. Measurement of α-tocopherol levels was performed using a high performance liquid chromatography in the Chemistry Laboratory of the Biodiversity Research Center (CPBIO) at the UEMS.

Soybean seeds aged during storage (natural aging) and by exposure to high temperatures and humidity (accelerated aging) were analyzed for α-tocopherol levels. To measure the α-tocopherol levels, the seeds were aged by two methods (factor 2): natural and artificial aging. Natural aging was performed in a dry chamber for 0, 60, 120 and 180 days (factor 3) with controlled temperature and relative humidity (± 15°C and 60% RH). Artificial aging of seeds after the four storage times was performed by the accelerated aging method. The seeds were uniformly distributed on an aluminum mesh and placed inside plastic gearbox-type boxes (mini-chambers). The boxes were capped and taken to an oven with a temperature of 41°C for 48h (MARCOS FILHO, 2005).

The quantification of α-tocopherol in the soybean seeds was performed by high performance liquid chromatography (HPLC) combined with the Soxhlet extraction method (LIM et al., 2007). Three samples (100 mg each) of soybean seeds from each treatment were placed into a Soxhlet apparatus and extracted using 100 mL of hexane solvent with 0.01% BHT. After a six-hour extraction, the samples were placed in ultrasound for 3 hours. The extracts were then filtered through qualitative filter paper, the filtrate obtained was re-filtered through a 0.22 μm membrane, and the content was immediately analyzed using a Varian 210 chromatograph with diode array detection, absorbance at 200-800 nm, a C-18 reverse-phase column (25 cm x 4.6 mm x 5 μm), and a guard column (2.5 cm x 3 mm) packed with the same stationary phase as the analytical column. Isocratic elution was performed with 50% methanol and 50% acetonitrile. The analysis time was 10 minutes, the pump flow rate was 1.5 mL $min.^{-1}$, and the injected volume was 10 μL. Chromatographic grade methanol (Dynamics and Vetec) and hexane (Vetec) were used as solvents, in addition to ultra-purified water (Pure Water System, Scholar-UV, Human UP 900).

The results were analyzed using an analytical curve prepared with α-tocopherol (Sigma Chemical Co, St Louis, USA, with 96% purity). Standards were prepared at different concentrations of α-tocopherol (1 to 100 mg L^{-1}) for the calibration curve. A graph was made using concentration x area of substance analyzed to obtain the concentration of α-tocopherol in the samples. The results were expressed as μg of α-tocopherol g^{-1} of soybean seed sample.

The experimental design was completely randomized in a 4 x 4 x 2 factorial scheme (cultivar x storage time x age) with four replicates. The data on

α-tocopherol levels were subjected to analysis of variance by the F-test at 5% probability, and the means were compared by the Newman Keuls test at 5% probability. Correlation and regression analyses were performed using SAEG software (RIBEIRO JUNIOR; MELO, 2008). Regression equations were fit to the significant features, and the best-fitting model was selected by the coefficient of determination.

Results and discussion

Table 1 shows the ANOVA summary for α-tocopherol levels in seeds subjected to natural and artificial aging and storage. The results indicate that all the parameters were sensitive to the causes of variation and that the α-tocopherol levels were influenced by cultivar, aging method, seed storage time, and interaction among the variables. There was no significant difference for the variable AT in the interaction between the factors EN*CV.

Regression analysis for α-tocopherol levels of the four cultivars in relation to seed storage times was not significant for 0, 60, and 120 days of storage. Only the results obtained after 180 days of storage were significantly different among the four cultivars. The cultivars MSOY 7908 RR and BMX Titan RR showed higher α-tocopherol levels at the 180-day storage time, and BRS 285 had the lowest level (578 μg g^{-1}) (Table 2).

Table 1. ANOVA summary for α-tocopherol levels in seeds of four soybean cultivars subjected to natural and artificial aging and storage. Dourados, Mato Grosso do Sul State, 2009.

Sources of variation	Degrees of freedom	Mean squares
		α-tocopherol
Aging (EN)	1	115.320.00*
Cultivar (CV)	3	540.68*
EN*CV	3	9.84^{NS}
Storage time (TA)	3	179.785.60*
EN*TA	3	42.534.64*
CV*TA	9	271.78*
EN*CV*TA	9	163.36*
Residue	96	63.78
CV(%)		1.56

*Significant and; ^{NS}not significant at 5% probability by F test.

Table 2. Levels of α-tocopherol in seeds of four soybean cultivars aged naturally for four storage times. Dourados, Mato Grosso do Sul State, 2009.

Storage	Levels α-tocopherol (mg g^{-1})			
time (days)	BRS 285	BRS 246 RR	MSOY 7908 RR	BMX Titan RR
0	430 $^{a\,D}$	435 $^{a\,D}$	437 $^{a\,D}$	435 $^{a\,D}$
60	462 $^{a\,C}$	462 $^{a\,C}$	463 $^{a\,C}$	467 $^{a\,C}$
120	555 $^{a\,B}$	557 $^{a\,B}$	557 $^{a\,B}$	554 $^{a\,B}$
180	578 $^{c\,A}$	587 $^{b\,A}$	603 $^{a\,A}$	603 $^{a\,A}$

Means followed by the same lowercase letter in each row and the same uppercase letter in each column do not differ significantly according to the Newman Keuls test at 5% probability.

To identify the genotypes with high levels of α-tocopherol, Ujiie et al. (2005) analyzed 909 soybean cultivars and 200 wild-type specimens from the germplasm bank. The authors found three genotypes with this characteristic; however, they believe that further studies are necessary to determine how α-tocopherol is influenced by environmental conditions. According to these authors, the α-tocopherol levels are significantly influenced by temperature during seed formation.

Carrão-Panizzi and Erhan (2007) found a wide variation of α-tocopherol levels, ranging from 11 ppm (by the cultivar Davis) up to 191 ppm (by the cultivar IPB-T), when they analyzed the composition of 89 Brazilian soybean cultivars produced in the State of Paraná. Comparing 16 production sites in Minas Gerais and Goiás State using the cultivar MG BR 46 Conquista, the authors found the highest α-tocopherol levels in soybean seeds at Conquista, Minas Gerais State and Chapadão do Céu, Goiás State. Lower α-tocopherol levels were observed at Uberlândia, Minas Gerais State and Cristalina, Goiás State. The authors concluded that the α-tocopherol level in seeds from soybean cultivars is influenced by genetic traits and environmental factors at different production sites.

Significant differences in α-tocopherol levels in seeds were observed for all cultivars and storage times (Table 2). At 180 days of storage, the α-tocopherol levels were higher in all the cultivars studied, varying from 578 up to 603 μg g^{-1}. Moreover, the four cultivars showed significant variation in α-tocopherol levels among the four storage times, and the highest value was recorded at 180 days of storage.

The regression equations that best represent seed behavior for each cultivar with relation to α-tocopherol levels during storage are presented in Figure 1. Regression analysis performed between α-tocopherol levels and the four storage periods resulted in a direct relationship between the two variables, with R^2 values of 0.9414 for the BRS 285 cultivar and 0.9719 for the BMX Titan RR cultivar. The α-tocopherol level of the seeds increased linearly with longer storage time for all the cultivars, with differences between the cultivars found at 180 days of storage (Table 2).

The results are consistent with those described by Simontacchi et al. (1993) who used two-dimensional chromatography to determine that α-tocopherol was predominant in young corn, wheat, barley, and pea plants, whereas other tocopherols appeared as the plants aged. In soybeans and beans, they observed an increased concentration of α-tocopherol in leaves over aging.

The α-tocopherol levels in seeds increased linearly with longer storage periods in both natural and artificial aging. However, artificially aged seeds

showed higher levels beginning at 120 days of storage (Table 3). This can also be observed by the regression equation where artificially aged seeds showed higher levels of α-tocopherol than those naturally aged (Figure 2). There was no significant difference in the levels of α-tocopherol between natural and artificial aging at 0 and 60 days of storage. However, after 120 days of storage, the α-tocopherol contents increased (25%) for the artificially aged soybean seeds, and at 180 days of storage, the difference in α-tocopherol levels between natural and artificially aged seeds was 23%.

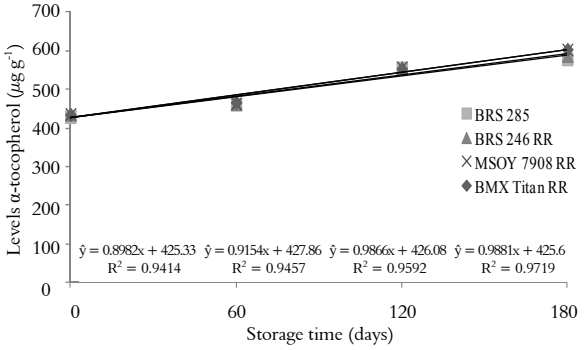

Figure 1. Levels of α-tocopherol (μg g^{-1}) in seeds from four soybean cultivars aged naturally for four storage times. Dourados, Mato Grosso do Sul State, 2009.

According to Munneé-Bosch (2005), α-tocopherol levels undergo significant changes during plant growth and in response to environmental stress as a result of changes in the expression of genes related to the degradation and recycling pathway.

Changes in α-tocopherol levels during plants' responses to environmental stress are characterized by two phases. First (phase I), there is an increase in the synthesis of α-tocopherol, which is followed by a second phase (phase II) in which the levels fall (MUNNEÉ -BOSCH, 2005). According to this author, the initial levels of α-tocopherol contribute to seed protection by reducing the levels of reactive oxygen species (ROS), especially H_2O_2, O_2^-, and OH, and by inhibiting lipid peroxidation, thereby preventing oxidative damage. When the stress is severe, α-tocopherol degradation exceeds its synthesis, and the levels decrease (phase II). Consequently, lipid peroxidation increases, followed by cell death because the deficiency of α-tocopherol is not compensated by other protective mechanisms. In stress-tolerant plants, only the first phase is observed (unless the stress is very severe), while in stress-sensitive plants, only the second phase is observed.

The α-tocopherol, in cooperation with other antioxidants, plays an important role in reducing the levels of ROS (mainly O_2^- and OH) in the photosynthetic membranes. Additionally, it limits the extent of lipid peroxidation by reducing lipid peroxyl radicals to the corresponding hydroperoxides. Therefore, α-tocopherol contributes to the preservation of an appropriate redox state in chloroplasts to maintain the structure and function of the thylakoid membrane during plant development and plant responses to stress (SATTLER et al., 2004; MUNNEÉ-BOSCH, 2005).

Table 3. Levels of α-tocopherol in seeds from four soybean cultivars aged naturally and artificially for four storage times. Dourados, Mato Grosso do Sul State, 2009.

Storage time (days)	Levels of α-tocopherol (mg g^{-1})	
	Natural aging	Artificial aging
0	435 ^{Ad}	434 ^{Ad}
60	466 ^{Ac}	461 ^{Ac}
120	495 ^{Bb}	617 ^{Ab}
180	531 ^{Ba}	655 ^{Aa}

Means followed by same lowercase letter in each column and the same uppercase letter in each row do not significantly differ according to the Newman Keuls test at 5% probability.

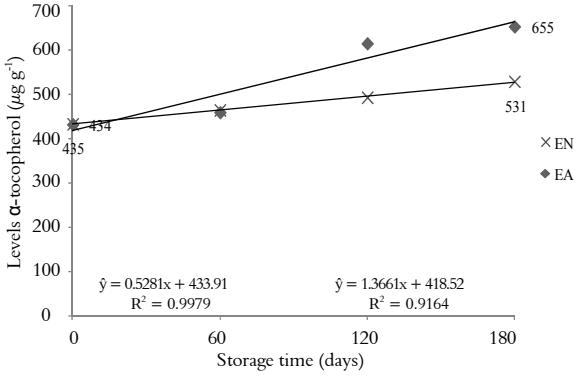

Figure 2. Contents of α-tocopherol (μg g^{-1}) in soybean seeds as a function of four storage times, natural aging (EN), and artificial aging (EA). Dourados, Mato Grosso do Sul State, 2009.

According to Sattler et al. (2004), lipid oxidation has been proposed as a significant factor for seed longevity. Some studies have shown an inverse correlation between various lipid oxidation products and the longevity of seeds during natural and accelerated aging. However, other studies have not noticed such a relationship, which suggests that other processes such as membrane integrity, levels of other antioxidants, damage to nucleic acids and proteins, and activities of ROS scavenging enzymes (e.g., catalase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase) also determine seed longevity.

Conclusion

Levels of α-tocopherol in soybean seeds showed a linear correlation with increasing storage time. The artificially aged seeds have higher levels of α-tocopherol than those naturally aged.

References

CARRÃO-PANIZZI, M. C.; ERHAN, S. Z. Environmental and genetic variation of soybean tocopherol content under Brazilian growing conditions. **Journal of the American Oil Chemists Society**, v. 84, n. 10, p. 921-928, 2007.

LIM, H.; WOO, S.; KIM, H. S.; JONG, S. K.; LEE, J. Comparison of extraction methods for determining tocopherols in soybeans. **European Journal of Lipid Science and Technology**, v. 109, n. 11, p. 1124-1127, 2007.

MARCOS FILHO, J. **Fisiologia de sementes de plantas cultivadas**. Piracicaba: Fealq, 2005.

MUNNEÉ-BOSCH, S. The role of α-tocopherol in plant stress tolerance. **Journal of Plant Physiology**, v. 162, n. 7, p. 743-748, 2005.

PARRISH, D. J.; LEOPOLD, A. C. Soybean seed aging. **Plant Physiology**, v. 61, n. 3, p. 365-368, 1978.

RIBEIRO JUNIOR, J. I.; MELO, A. L. P. **Guia prático para utilização do SAEG**. Viçosa: Folhas Artes Gráficas Ltda., 2008. v. 1.

SATTLER, S. E.; GILLILAND, L. U.; MAGALLANES-LUNDBACK, M.; POLLARD, M.; DELLAPENNA, D. Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. **The Plant Cell**, v. 16, n. 6, p. 1419-1432, 2004.

SIMONTACCHI, M.; CARO, A.; FRAGA, C. G.; PUNTARULO, S. Oxidative stress affects alpha tocopherol content in soybean embryonic axes upon imbibition and following germination. **Plant Physiology**, v. 103, n. 3, p. 949-953, 1993.

TORRES, S. B.; MARCOS FILHO, J. Teste de envelhecimento acelerado em sementes de maxixe (*Cucumis anguria* L.). **Revista Brasileira de Sementes**, v. 23, n. 2, p. 108-112, 2001.

UJIIE, A.; YAMADA, T.; FUJIMOTO, K.; ENDO, Y.; KITAMURA, K. Identification of soybean varieties with high α-tocopherol content. **Breed Science,** v. 55, n. 2, p. 123-125, 2005.

Received on March 1, 2011.
Accepted on October 14, 2011.