http://dx.doi.org/10.1590/2317-1545v38n1156663

Maintenance of quality of *Dalbergia nigra* Vell. all. ex. Benth seeds during storage by sodium nitroprusside[1]

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ABSTRACT - Nitric oxide (NO) is a compound that participates in vegetable physiologic processes. The purpose of this study was to investigate the efficiency of sodium nitroprusside (SNP), a NO donor, on the physiologic and biochemical responses of *Dalbergia nigra* (bahia rosewood) seeds under storage in different environmental conditions. Seeds recently collected were stored in cold chamber (5 ºC and 60% relative humidity, RH) or in two desiccators at 20 ºC: 55 and 93% RH for four months. Every month of storage seed samples were taken and imbibed in SNP solution at 10^{-4}M concentration for 48 hours or in water. The germination percentage, electric conductivity, lipid peroxidation and catalase enzyme activity were evaluated. Seeds of bahia rosewood stored in cold chamber at 55% RH maintained physiologic quality, while seeds imbibed in SNP reduced the membrane permeability, decreasing lipid peroxidation and increasing catalase activity. Seeds kept under 93% RH deteriorated quickly, and SNP was not efficient in revert the seed degradation. It was concluded that nitric oxide maintain the quality of seeds stored in cold chamber and at 20 ºC and 55% RH, by maintaining membrane integrity and stimulating catalase activity.

Index terms: bahia rosewood, deterioration, forest seeds, nitric oxide.

Manutenção da qualidade das sementes de *Dalbergia nigra* Vell. all. ex Benth durante o armazenamento por nitroprussiato de sódio

RESUMO- Óxido nítrico (NO) é um composto que participa de inúmeros processos fisiológicos vegetais. O objetivo deste estudo foi investigar a eficiência de nitroprussiato de sódio (SNP), um doador de NO, na resposta fisiológica e bioquímica de sementes de*Dalbergia nigra* (jacarandá-da-Bahia) sob armazenamento em diferentes condições ambientais. Sementes recém coletadas foram armazenadas em câmara fria (5 °C e 60% de umidade relativa, UR) ou em dois dessecadores a 20 °C: 55 e 93% UR por quatro meses. A cada 30 dias de armazenamento, foram embebidas em água ou em solução SNP 10^{-4} M por 48 horas. Foram avaliadas a porcentagem de germinação, a condutividade elétrica, a peroxidação lipídica e a atividade da enzima catalase. Sementes de jacarandá-da-Bahia armazenadas em câmara fria e a 20 ºC e 55% UR mantiveram sua qualidade fisiológica, e quando embebidas em SNP foi observada redução na permeabilidade da membrana, diminuindo a peroxidação lipídica e aumentando a atividade da catalase. Sementes mantidas sob 93% UR deterioraram rapidamente, e SNP não foi eficiente em reverter a degradação. Concluiu-se que o óxido nítrico manteve a qualidade das sementes armazenadas em câmara fria e a 20 ºC e 55% UR, mantendo a integridade da membrana e estimulando a catalase.

Termos para indexação: jacarandá-da-Bahia, deterioração, sementes florestais, óxido nítrico.

Introduction

The maintenance of plant biodiversity is a global concern and, according to Khoury et al. (2010), seed storage is the most efficient method of all *ex situ* conservation strategies. Storage consists in providing to seeds the maintenance of their physiologic potential for the longest period as possible,reducing its deterioration along time (Bewley et al., 2013). Inadequate environmental conditions can dramatically accelerate the deterioration process, causing considerable alterations.

Degenerative changes occur during storage period and results in alterations in seed quality, as well as loss of germinating capacity (Liu et al., 2011), lower resistance to

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[1] Submitted on 11/16/2015. Accepted for publication on 05/04/2016.

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stress conditions (Pereira et al., 2010), alterations in enzyme systems (Li et al., 2012), and changes of metabolism reserves (Gawrysiak-Witulskaet al., 2011) and membranes system (Corte et al., 2010). Besides, deterioration can cause oxidative stress in seeds, which can be detected by increase in lipid peroxidation products and changes in antioxidant system enzymes, such as catalase and superoxide dismutase (Toivonen, 2004).

The nitric oxide (NO) is a colorless, inorganic free-radical, synthesized in plants fromenzy matic pathways, that include nitrate reductase or nitric oxide synthase or by non-enzymatic pathways, by production of NO from nitrification/denitrification processes (Scheler et al., 2013; Sidana et al., 2015). Its participation is evidenced in countless plant physiological processes, among which is the protective function against oxidative stress (Besson-Bard et al., 2008).

In seeds, NO stimulates germination both under normal and stress conditions (Zhang et al., 2011; He et al., 2014; Pires et al., 2016), favors overcoming dormancy (Wang et al., 2015; Ma et al., 2016), promotes elongation and formation of adventitious roots (Schlicht et al., 2013; Bai et al., 2014) and enhances desiccation tolerance of recalcitrant seeds (Bai et al., 2011).

Studies on the effects of NO on the physiology of seeds have been carried out, focusing on the exogenous application of NO donors (Beligni and Lamatina, 2001). Sodium nitroprusside (SNP) is the substance most used as donor of NO.

The protective of NO to recover the loss of seed viability and/or vigor role is based on its ability to regulate the level and toxicity of reactive oxygen species (ROS) (He et al., 2014). Application of exogenous NO in the form of SNP alleviates the adverse effects caused by heat, salt and drought stress, by decreasing hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents and modulating the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) (Lin et al., 2013; Filippou et al., 2013; Amooaghaie et al., 2015; Ma et al., 2016).

Thus, the use of this compound and their influence on the physiological and biochemical change caused by seed deterioration is a new study field of plant biology, potential to recover the loss of seed viability after storage.

The bahia rosewood (*Dalbergia nigra* Vell. All. ex. Benth - Fabaceae) is present in Minas Gerais, Rio de Janeiro, São Paulo and Mato Grosso do Sul, mainly in the Atlantic Forest and Cerrado biomes in Brazil (Lorenzi, 2009). In the ecological succession, it is considered a climax species, recommended for pasture or crop arborization. The wood produced is largely used for manufacturing of luxury furniture. At present, the species is included in the Brazilian list of threatened species by extinction (MMA, 2014).

D. nigra seeds have an orthodox behavior during storage, and the controlled seed deterioration studies was performed by Chaves and Usberti (2004), resulting that the lowest limits for viability is 65 ºC and 2.9% moisture content. The storage conditions used in this paper have been previously tested for *Melanoxylon brauna* (Borges et al., 2015), indicating decreases in seed quality after increases in environment water content.

In view of the relevance of studies on seeds conservation for valuable and marketable species, the present study aimed to investigate the efficiency of sodium nitroprusside (SNP) on the physiological and biochemical responses of *D. nigra* seeds during storage in controlled conditions.

Material and Methods

The present study was conducted from October 2011 to May 2012. *Dalbergia nigra* seeds were collected in Viçosa, MG, Brazil (20º45'14"S and 42º52'55"W) in September 2011. During the preparation phase, immature, spoiled or damaged seeds were removed. Selected seeds were conditioned in hermetic fiber containers and stored in cold chamber at 5 °C and 60% relative humidity (RH) conditions until the beginning of tests.

To evaluate the effects of sodium nitroprusside on the storage, seeds of *D. nigra*, with approximately 11.7% water content, were stored for four months. The seeds were kept under three controlled conditions: a) at 5º C in a cold chamber,with 60% RH, in hermetic fiber containers; b) at 20 ºC,containing calcium nitrate salt ($Ca(NO_3)_2$ solution that represents 55% RH, and c) at 20 ºC, containing ammonium monophosphate salt ($NH_4H_2PO_4$) solution, that represents 93% RH. The saturate salt solutions were employed in desiccators.

Freshly collected seeds (without storage) were used as control. Every thirty days, seed samples were taken from the three different controlled conditions. In each sampling period, seeds were imbibed in SNP solution at 10^{-4} M concentration or in water, according to Figure 1.

The 10^{-4} M concentration is selected according Ataíde et al. (2015).The seeds were placed on two sheets of germitest paper in Petri dishes and pre-soaked in 14 mL of SNP solutions at 10^{-4}M concentration or in water for 48 hours, at 25 °C, under constant light. The plates were sealed with adhesive tape to prevent evaporation of solutions. At each month, after pre-soaking time, the seeds were removed of solution and the following quantifications were carried out:

Germination percentage – seeds were placed to germinate in Petri dishes, in germinator at 25 °C and under constant light, by four fluorescent lamps of 20W type light day, with radiant flux density at the height of the boxes of 15 mmol. m^{-2} s^{-1}. Germination was determined by daily counting of seeds that emitted radicle. Five replications of 20 seeds were used per treatment;

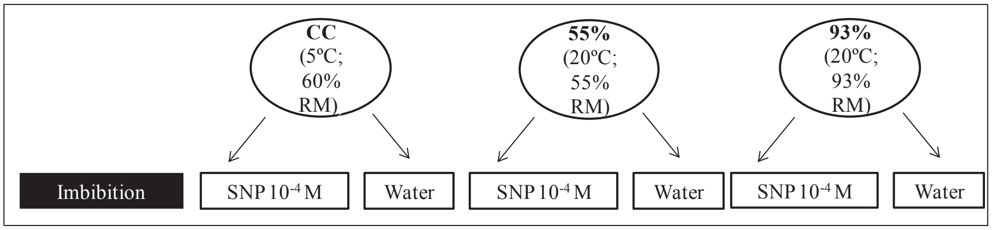

Figure 1. Demonstrative scheme of storage and embedding conditions in *Dalbergia nigra* seeds. CC = cold chamber, SNP 10^{-4} M = with imbibitions in SNP 10^{-4}M for 48 hours, Water = with imbibitions in water for 48 hours.

Electric conductivity – four samples of 50 seeds were weighed and placed in 75 mL of distilled water at 25 °C for 30 hours (Marques et al., 2002). Electric conductivity was determined using a conductivimeter. Results were expressed as $\mu s.cm^{-1}.g^{-1}$;

Lipid peroxidation – evaluated by TBARS (thiobarbituric acid reactive substances) determination according to Araujo (1995). Five replications of 20 seeds were used. The lipid peroxidation was determined by maceration of 0.2 g of seeds in ice, followed by the addition of 2.0 mL of trichloroacetic acid (TCA) and 3.0 mL of a solution with tiobarbituric acid (TBA) 0.5% (p/v) + TCA 20% (p/v) in 35 minutes. The results were expressed as mmol malonaldehyde.g^{-1}., after conversion of absorbance (532 and 600 nm) (Lehner et al., 2008);

Extraction and quantification of catalase enzyme (CAT) – the activity of catalase enzyme was determined according Peixoto et al. (1999) in five replications of 20 seeds. The enzyme extracts were obtained by maceration of 0.2 g of seeds in ice, followed by the addition of 2.0 mL of the following means of homogenizing: potassium phosphate buffer 0.1 M and pH 6.8, ethylene diaminetetra acetic acid (EDTA) 0.1 mm, phenylmethylsulfonyl fluoride (PMSF) 1 mm and polyvinylpolypyrrolidone (PVPP) 1% (p/v). After that, the extract was centrifuged at 15,000 g for 15 minutes at 4 ºC and the supernatant was collected, where determination were done. The enzyme activity was calculated using the molar extinction coefficient of 36 M cm^{-1} and the result expressed as mmol H_2O_2. min^{-1}. mg^{-1} protein. The protein content was assayed for each sample (Bradford, 1976).

The experimental delineation used was randomized. The results were submitted to ANOVA, followed by regression adjusted analyses for variables measured during seed storage. The estimated equations were compared by test of identity models, according to Regazzi (1993), to evaluate equality of tendencies, at 5% probability. To verify the degree of association between the variables, was used the correlation coefficient, at 5% probability. Softwares Statistica 8.0 and Excel 2010 were used.

Results and Discussion

The germination of stored *D. nigra* seeds in the three environmental conditions is shown in Figure 2. There was a decrease of 24% for germination of the seeds stored in cold chamber that were imbibed in water, and only 9% for seeds treated with SNP, after four months of storage (Figure 2A). The germination remained above 70% in this latter. In this case, SNP acted reducing, at least partially, the negative effects caused by storage on germination. Ataíde et al. (2015) concluded that the application of SNP increased seed germination and seed vigor of *Dalbergia nigra* seeds.

Slight variation was noted in the germination percentage of seeds stored at 55% RH and 20 ºC, imbibed in SNP or in water, which maintained 88 and 85% germination rates, respectively, by the end of storage (Figure 2B). The results demonstrate that the 20 ºC temperature at low RH satisfactorily maintained *D. nigra* seeds quality within the short studied period. Aguiar et al. (2010) emphasized the importance of low temperatures for storage seeds of this species in maintaining viability for up to two years. In this case, the low RH also influenced to maintain seed quality.

Seeds stored at 93% RH and 20 ºC presented, approximately, 45% of germination decrease in second month of storage, losing completely viability at four months, irrespective of SNP application (Figure 2C). After adjustment of hygroscopic balance moisture curves for *D. nigra* seeds, Mesquita et al. (2001) noted that higher relative humidity of the environment implies in higher water absorption rate by seeds and higher balance values. Consequently, the environment with 93% relative humidity contributed to elevate the respiratory rate and metabolism of *D. nigra* seeds, resulting in loss of germinating potential, which was not reverted by SNP application in any of the evaluated months.

Despite NO being involved in the regulation of plant defense responses to biotic and abiotic stresses, it did not recover seed quality in 93% RH and 20 ºC. This condition is possibly associated to the intensity of reactions that characterize the seed physiologic activity when water content overcomes certain safety limits (Marcos-Filho, 2005). These alterations include reserves exhaustion, alteration on chemical composition, cellular membrane permeability, enzyme activity and nucleotide content.

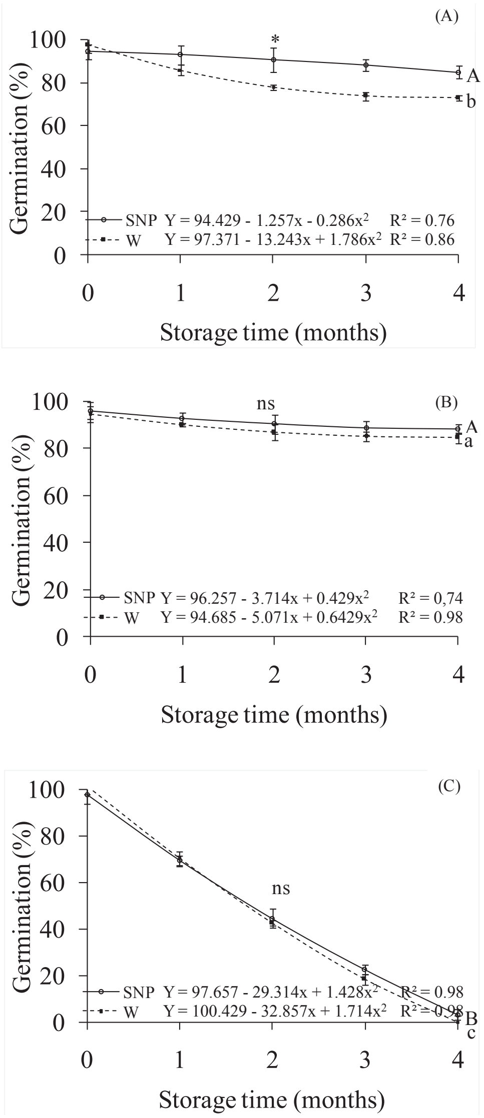

Figure 2. Percentage of germination of *Dalbergia nigra* seeds stored in cold chamber (A), 55% relative humidity (B) and 93% relative humidity (C), imbibed in SNP10^{-4}M (SNP - continuous line) or in water (W - dashed line) for 48 hours. Comparing storage conditions, tendencies SNP and W followed by same capital and small letter, respectively, do not differ at 5% probability. Within each storage condition, * represent significant difference and ns represents equality of equations, at 5% probability.

Seeds maintained in cold chamber at 60% RH and 55% relative humidity that were imbibed in SNP presented electric conductivity statistically different of the one that was imbibed in water (Figures 3A and 3B). The values of the imbibed in water varied from around 90 $\mu s.cm^{-1}.g^{-1}$ at time zero to 130 $\mu s.cm^{-1}.g^{-1}$ in four months.The seeds at 20 ºC and 93% RH imbibed in SNP or water presented significant increase in their electric conductivity values along storage, with significant statistical difference among them (Figure 3C), as consequence of cellular membrane permeability increase while the seed deteriorates.

From the physiologic point of view, the sequence of events in seeds after maturity and before complete loss of viability begins with the disorganization of membranes and loss of permeability control (Marcos-Filho, 2005); these are the first structural signs of the deterioration process. Bewley et al. (2013) explain that, during water absorption, even in viable seeds, there is a quick efflux of organic and inorganic compounds to the imbibition medium, as the plasmatic membrane integrity is incomplete. In deteriorated seeds, there is loss of integrity of the cell membrane system, and the release rate of these electrolytes is elevated (Flavio and Paula, 2010).

The decreased values of electrical conductivity in seeds, an indicator of ion leakage, suggest that exogenous application of SNP reduced membrane permeability under the three experimental conditions. Therefore, SNP acts in the reorganization of seed cellular membranes, which return to a more stable configuration, reduced permeability and, consequently, electrolytes leakage. These results corroborate with those described in previous studies, which associate cellular membrane repair damaged after stresses to NO (Wang et al., 2011). It is emphasized that, even after embedded in SNP, seeds maintained at 93% RH presented large electrolytes release, especially from the third month of storage, indicating that their membranes were considerably altered. In this case, membranes can be so damaged, that the reversal by NO is less efficient.

Lipid peroxidation of stored *D. nigra* seeds are presented in Figure 4. There was peroxidation in all the storage conditions with seeds untreated with SNP, with higher increase in the 93% RH. Lipid peroxidation is the result of free radicals action with polyunsaturated fatty acid present in cellular membranes and in lipoproteins, causing membrane structure and permeability alterations (Verma and Dubey, 2003). Consequently, there is selectivity loss in ionic exchange and organelle content release, such as lysosomal hydrolytic enzymes, associated with formation of cytotoxic products, such as MDA, which may culminate in cellular death (Thérond et al., 2000). Lipid peroxidation increases has been used as indicator of cellular oxidative stress, and considered one of the probable reasons for viability loss of stored seeds (Sadeghipour and Aghaei, 2012).

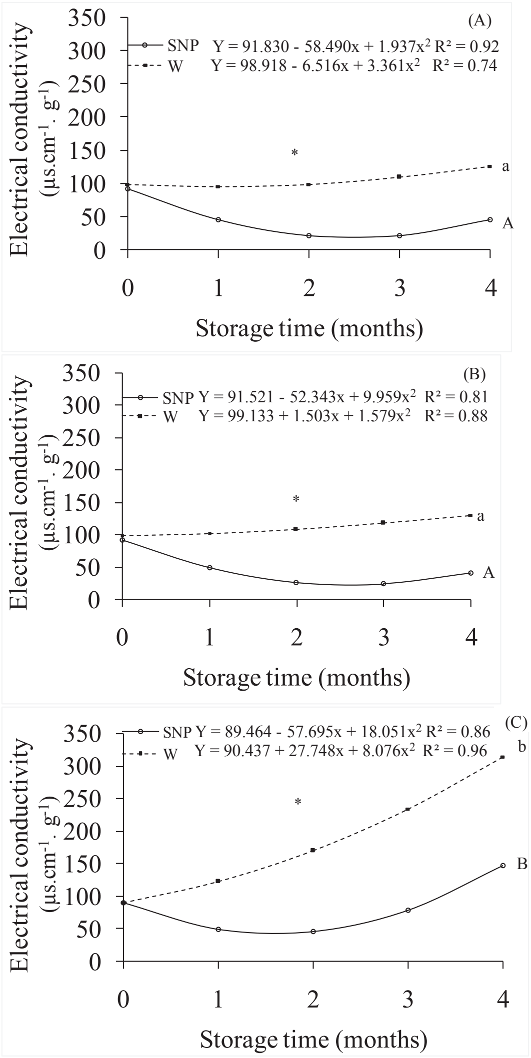

Figure 3. Electrical conductivity (µs.cm^{-1}.g^{-1}) of *Dalbergia nigra* seeds stored in cold chamber (A), 55% relative humidity (B) and 93% relative humidity (C), imbibed in SNP 10^{-4}M (SNP - continuous line) or in water (W - dashed line) for 48 hours. Comparing storage conditions, tendencies SNP and W followed by same capital and small letter, respectively, do not differ at 5% probability. Within each storage condition, * represent significant difference and ns represents equality of equations, at 5% probability.

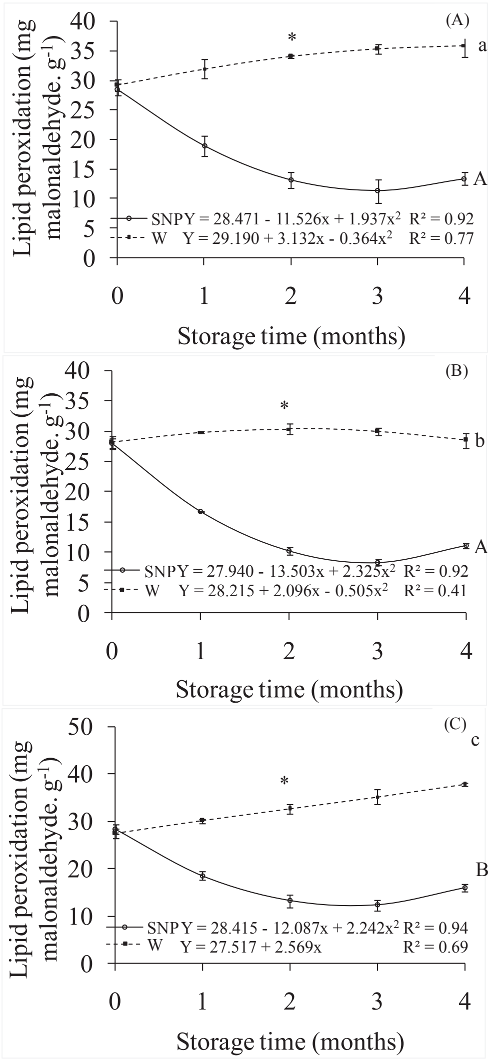

Figure 4. Lipid peroxidation (mg malonaldehyde.g^{-1}) of *Dalbergia nigra* seeds stored in cold chamber (A), 55% relative humidity (B) and 93% relative humidity (C), imbibed in SNP 10^{-4}M (SNP -continuous line) or in water (W - dashed line) for 48 hours. Comparing storage conditions, tendencies SNP and W followed by same capital and small letter, respectively, do not differ at 5% probability. Within each storage condition, * represent significant difference and ns represents equality of equations, at 5% probability.

The seeds imbibed in SNP presented lower averages of MDA than those observed in control seeds, demonstrating the association of nitric oxide in facing lipid peroxidation and in cellular protection. In the three storage conditions there were similar decrease of MDA content after SNP application. By associating these results to membrane permeability reduction of seeds with embedding (Figure 3), it is clear that the cytoprotective effects of SNP occur simultaneously in the defense of cellular membrane damages, acting both in membrane organization and in antioxidant defense activation.

Reductions in MDA after imbibition in SNP solution were observed in *Zea mays* (Wang et al., 2011) and *Brassica napus*

(Zhang et al., 2011) seeds, indicating that NO is involved in the lipid metabolism and its peroxidation under different conditions.

The activity of the enzyme catalase (CAT) reduced from the first storage month in the all environments conditions, in seeds imbided in SNP or water (Figure 5). In seeds imbibed in water, similar tendency was observed between the environmental conditions, with production of approximately 0.4 mmol.min^{-1}.mg protein^{-1}in the fourth storage month.

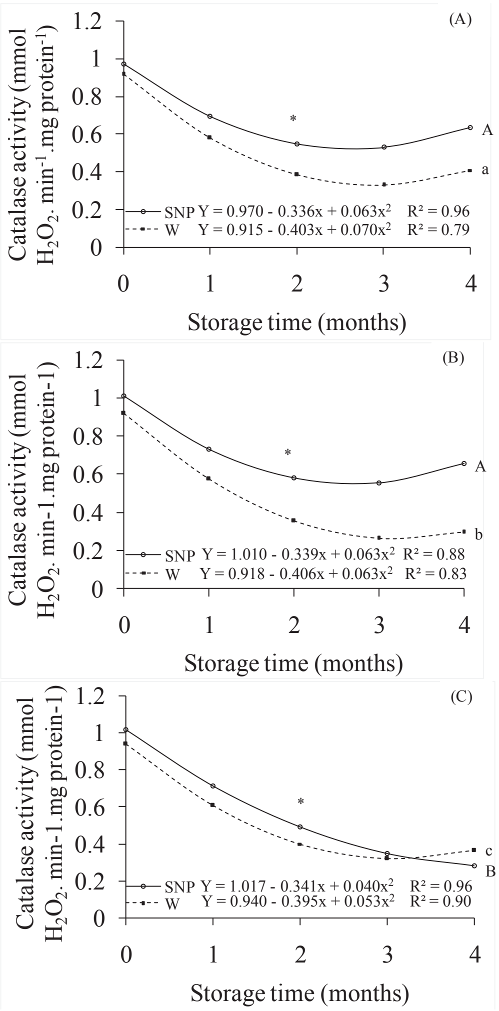

Figure 5. Catalase activity (mmol H_2O_2. min^{-1}. mg protein^{-1}) of *Dalbergia nigra* seeds stored in cold chamber (A), 55% relative humidity (B) and 93% relative humidity (C), imbibed in SNP 10^{-4}M (SNP -continuous line) or in water (W - dashed line) for 48 hours. Comparing storage conditions, tendencies SNP and W followed by same capital and small letter, respectively, do not differ at 5% probability. Within each storage condition, * represent significant difference and ns represents equality of equations, at 5% probability.

CAT is responsible for removing hydrogen peroxide present in high concentrations in peroxisomes, protecting cells from oxidative damages (Mittler, 2002). Reduction in the enzyme activity implies loss of the cell capacity to eliminate H_2O_2, which may result in metabolic disorder, especially at the membrane level. In seeds, reduction of the catalase enzyme activity has been associated to significant reduction of the germinating potential during artificial aging (Ataíde et al., 2012), saline stress (Zhang et al., 2011) and storage (Corte et al., 2010).

Seeds imbibed in SNP solution presented higher CAT values comparing with seeds imbibed in water, however this activity was reduced significantly in both treatments during storage. At the 93% RH and 20 ºC environment, the highest decreases were observed. NO application in this environment did not allow intense lixiviation (Figure 3), which would result in the exit of peroxide from the cell, causing possible substrate reduction and maintaining the enzyme in low activity. The highest catalase activity in treatments with SNP might be attributed, at least partially, to the reduction of available H_2O_2 to associate to cell free fatty acids and cause lipid peroxidation.

Table 1 illustrates the association between the variables measured during the storage of *D.nigra* seeds, by the correlation coefficient. An inverse relationship between the electrical conductivity and germination of the seeds was confirmed by the significant coefficient. One of the first signs of deterioration of seeds is the change or loss of integrity of cell membranes, measured by electrical conductivity, which influences the germination seed capacity (Ribeiro et al., 2009).

The electrical conductivity positively correlated with lipid peroxidation, indicating that the membrane damage during deterioration may result from the occurrence of lipid peroxidation and consequent increase in conductivity (Coolbear, 1995). Catalase activity correlated positively with germination, but not with the other parameters, such as lipid peroxidation. This may occur because hydroxyl and superoxide radicals, responsible for lipid peroxidation, are not eliminated by catalase.

Although SNP is widely used as NO donor, it has many limitations. Used in aqueous solutions, NO donors can lead to nitrogen oxides production. For instance, the photolysis of SNP was proved to release more cyanide than NO (Arc et al., 2013). Indeed, cyanide may actually be the active compound when applying SNP to seeds (Bethke et al., 2006). Thus, in this study, the effect of SNP can be NO dependent or independent, to explain the effect in *Dalbergia nigra* seeds. Therefore, the characterization of SNP in seed physiology context will undoubtedly reveal new area of research to explore for understanding the control of germination and deterioration processes.

Table 1. Correlation coefficients between the variables germination, electrical conductivity, lipid peroxidation and catalase activity during storage of *Dalbergia nigra* seeds.

	Germination	Eletrical conductivity	Lipid peroxidation	Catalase
Germination	1.0	-0.63*	-0.02	0.56*
Eletrical condutivity		1.0	0.66*	-0.01
Lipid peroxidation			1.0	0.06
Catalase				1.0

* - values significant at 5%, by t test.

Conclusions

The loss of quality of stored seeds of *Dalbergia nigra* is reversed, partially, by the sodium nitroprusside;

In cold chamber at 60% RH,the increase of membrane permeability during storage is reverted by SNP;

In the 93% RH environment, seeds deteriorated quickly, and SNP is not efficient to revert the degradation condition.

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