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Can priming with ascorbic acid or nitric oxide improve the germinability of stored sunflower seeds?

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ABSTRACT: Sunflower (*Helianthus annuus* L.) is a relevant oilseed species used as feed in human and animal nutrition and in multiple industrial applications. However, oilseeds need to deal with the loss of vigor when used as a propagule, due to loss of seed physiological quality (viability and vigor) caused by deterioration events. We have evaluated in the present study the effects of seeds priming techniques (water, ascorbic acid, and sodium nitroprusside) on vigor, germination, antioxidant enzymes and membrane integrity of stored sunflower hybrid HELIO 251 seeds. Germination parameters showed that non-primed seeds (control) delayed germination in almost 72 h while all primed seeds germinated 100% in 24 h. Electrolyte leakage and malondialdehyde levels were higher in non-primed seeds indicating higher degree of membrane damage. An increase in the activity of catalase and ascorbate peroxidase was also observed after 8 h of imbibition of primed seeds compared to non-primed seeds. Regarding superoxide dismutase, there was no significant differences between treatments after 8 h of imbibition, whereas the highest activity was after 24 h of imbibition in non-primed seeds. Therefore, it can be concluded priming with ascorbic acid or sodium nitroprusside exhibited better performance in germination of stored sunflower seeds, probably resulting from lower accumulation of reactive oxygen species and consequent reduced oxidative damage due to an efficient antioxidant enzyme system.

Index terms**:** aging, oilseed, oxidative damage, priming, seed vigor.

RESUMO: O girassol (*Helianthus annuus* L.) é uma espécie oleaginosa relevante utilizada na alimentação humana e animal e, em múltiplas aplicações industriais. No entanto, as oleaginosas precisam lidar com a perda de vigor quando utilizadas como propágulos, devido à perda da qualidade fisiológica da semente (viabilidade e vigor) causada por eventos de deterioração. Avaliamos no presente estudo os efeitos de técnicas de condicionamento de sementes (água, ácido ascórbico e nitroprussiato de sódio) no vigor, germinação, enzimas antioxidantes e integridade de membrana de sementes de girassol híbrida HELIO 251 armazenadas. Os parâmetros de germinação mostraram que as sementes não condicionadas (controle) atrasaram a germinação em quase 72 h enquanto todas as sementes condicionadas germinaram 100% em 24 h. O extravasamento de eletrólitos e os níveis de malondialdeído foram maiores nas sementes não condicionadas, indicando maior grau de dano à membrana. Um aumento na atividade da catalase e ascorbato peroxidase também foi observado após 8 h de embebição de sementes condicionadas em comparação com sementes não condicionadas. Em relação à superóxido dismutase, não houve diferenças significativas entre os tratamentos após 8 h de embebição, enquanto a maior atividade foi após 24 h de embebição em sementes não condicionadas. Portanto, pode-se concluir que o *priming* com ácido ascórbico ou nitroprussiato de sódio apresentou melhor desempenho na germinação de sementes de girassol armazenadas, provavelmente decorrente do menor acúmulo de espécies reativas de oxigênio e consequente redução do dano oxidativo devido a um sistema enzimático antioxidante eficiente.

Termos para indexação: envelhecimento, oleaginosa, dano oxidativo, priming, vigor da semente.

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INTRODUCTION

Sunflower (*Helianthus annuus* L., Asteraceae) is an annual cycle oilseed species (Simões et al., 2020) with broad agronomic and feed uses. The high oil content requires improved storage protocols to maintain the quality (germination/vigor) and promote better longevity of stores sunflower seeds (Abreu et al., 2013). Seed vigor encompass a set of attributes that ensures the seeds' potential to germinate faster and with better uniformity (synchronously) in shorter periods (Siadat et al., 2012). Whereas oil seeds generally show less chemical stability due to the percentage of lipids reserves, the increasing probability of lipid peroxidation and membrane damages intensifying the loss of vigor and ultimately germinability (Rajjou et al., 2012).

The reduction of seed vigor and geminability resulting from deterioration of its reserves during storage are associated with changes in cellular and metabolic events resulting in enzymatic inactivation and reduced energy metabolism, beyond overproduction of reactive oxygen species (ROS) (Hu et al., 2012; Xia et al., 2015). Under controlled conditions, the levels of ROS in seeds are regulated by superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) which act regulating ROS accumulation and minimizing oxidative damages (Kaur et al., 2019).

Priming is known to recover and/or enhance the physiological quality by recovering from the aging effects that stored seeds usually suffers (Farooq et al., 2009). One of the most promisor priming methods to restore seed vigor is with signaling molecules as nitric oxide (NO) and ascorbic acid (AsA). The NO is a gaseous uncharged and lipophilic molecule able to act on some subcellular compartments (Takahashi and Shinozaki, 2019). Besides being the most abundant reactive nitrogen species in plants, NO perform an essential role in several physiological processes such as growth regulation and tolerance to various biotic or abiotic stressful conditions (Sánchez-Vicente et al., 2019). While AsA is a non-enzymatic antioxidant molecule with important role in germination as it may protect cells from the cytotoxic effects of ROS (Foyer and Noctor, 2005), therefore, reducing oxidative damage (Sharma et al., 2019) and acting on repair of damaged membranes (Tommasi et al., 2011) resulting from abiotic and biotic stresses.

Besides all efforts to evaluate the physiological responses of primed seeds, and vigor, there is a lack of information regarding this process on long-term stored seeds. Here we hypothesized that long-term stored seeds of the sunflower hybrid, HELIO 251, primed with NO or AsA can exhibit improvements in physiological quality, uniformity, and germination speed. Thereby, this work aimed to evaluate the germinability of stored sunflower hybrid HELIO 251 seeds submitted to priming with NO and AsA.

MATERIAL AND METHODS

Plant material and storage conditions

All experimental assays were performed at the Plant Growth and Development Lab and at the Seeds Central Lab at the *Universidade Federal de Lavras* (UFLA) located in the state of Minas Gerais. Seeds of the simple sunflower hybrid HELIO 251 were produced in 2016 by *Heliagro Agricultura e Pecuária*©. Seeds were harvested, processed, and stored in multilayered Kraft paper inside polyethylene bags and subsequently stored in a cold chamber (10 °C) during 42 months prior to use.

Priming, experimental conditions, and sampling

The priming experiment was carried out in a completely randomized design with 4 treatments and 4 replications each. Treatments were composed by control (non-primed seeds); water (hydropriming with deionized water); NO (priming with 100 mM sodium nitroprusside, a NO donor); and AsA (priming with 100 mM ascorbic acid). The priming processes consisted in imbibing the stored seeds in deionized water or in 100 mM AsA or in 100 mMNO during 4 hours (h) at 25 °C, followed by washing in running tap water and redrying at 35 °C for 12 h. The control treatment was not subjected to priming. Then, these seeds were placed into Petri dishes with germination paper moistened with distilled

water, in four replications with 25 seeds each, and incubated in a germination chamber at 12-hour photoperiod and average temperature of 25 °C and 60 % of relative humidity. Germination parameters were evaluated daily (24 h) for four days, by measuring the percentage of seed germination and germination speed index (GSI).

The biochemical analyses were carried on samples collected after 8 and 24 h of imbibition corresponding to phase II and phase III of the germinative process, respectively. Electrical conductivity was performed only with 8 h-imbibed seeds. Biochemical assays were performed with both 8h and 24h-imbibibed seeds and consisted of quantification of hydrogen peroxide (H_2O_2), malondialdehyde (MDA, an extension of lipid peroxidation) and antioxidant enzymes activities (catalase – CAT, ascorbate peroxidase – APX and superoxide dismutase – SOD) in a 2x4 factorial arrangement (two times of imbibition and three priming treatments plus control).

Germination parameters

Seed germinability was evaluated daily for 4 days. Germinated seed were considered at 2 mm radicle protrusion (Brasil, 2009). The percentage of seed germination was obtained through quantification of the mean of the daily-germinated seeds during the four days. The germination speed index was performed using the equation proposed by Maguire (1962), considering the sum of seeds germinated in each day of experiments

Electrical conductivity

The electrical conductivity test consisted in four replications with 25 seeds. Each replication was weighted before imbibition and placed in plastic recipients containing 75 mL of deionized water. Then, they were kept in a germination chamber for 24 h at constant temperature of 25 °C (Vieira and Carvalho, 1994). The samples were shaken, and the electrical conductivity was measured with a conductivity meter (MS TECNOPON®, mCA 150 model) and results expressed in $\mu S.cm.g^{-1}$ of seeds.

Biochemical analyzes

Lipid peroxidation and hydrogen peroxide quantification

Lipid peroxidation was determined by malondialdehyde (MDA) quantification (Hodges et al., 1999). Samples of 100 mg of seeds were grinded in liquid nitrogen and homogenized in 1 mL of 80% ethanol, centrifuged, and the supernatant collected. This step was performed three times, totaling 3 mL. The reaction with 1ml of extract and 1 mL of 20% TCA, 0.65% tiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene (BHT) was carried. The estimate lipid peroxidation was calculated usingthe absorbance reading in a spectrophotometer at 440, 532, and 600 nm. Hydrogen peroxide (H_2O_2) was quantified as described by Velikova et al. (2000). Samples of 100 mg of seeds were grinded in liquid nitrogen and then homogenized with 1 mL of 0.1% trichloroacetic acid (TCA), and subsequently centrifuged and placed for reaction in 10 mM potassium phosphate (KH_2PO_4) buffer at pH 7.0 and 1M potassium iodide (KI). Analysis in spectrophotometer occurred by reading at 390 nm and then comparing it to a standard curve for H_2O_2 quantification.

Antioxidant system enzymes

The enzymatic antioxidant system activity (SOD, CAT, and APX) was evaluated in seeds after priming. The enzymatic extracts were obtained according to Biemelt et al. (1998) in which 200 mg of seeds were grinded in liquid nitrogen with PVPP (Polyvinylpyrrolidone) added to 1.5 mL of extraction buffer composed of: 400 mM potassium phosphate (pH 7.8), 10 mM EDTA, and 200 mM ascorbic acid. The solution was centrifuged at 13.000 *g* for 10 minutes at 4 °C and the supernatant collected to quantify enzymes activities.SOD activity was based on the enzyme capacity of inhibiting the photoreduction of nitrobluetetrazolium (NBT) (Giannopolitis and Ries, 1977). Absorbance readings were made at 560 nm. A unity of SOD corresponds to the number of enzymes able to inhibit 50% of NBT photoreduction in the assay conditions. The CAT activity was determined according to Havir and McHale (1987). This enzyme activity is calculated based on the absorbance decrease at 240 nm, every 15 seconds for 3 minutes, monitored by the hydrogen peroxide

consumption. The molar extinction coefficient used was 36 mM^{-1} .cm^{-1} (Azevedo et al., 1998). APX activity was analyzed as described by Nakano and Asada (1981) through ascorbate oxidation at 290 nm with a molar extinction coefficient of 2.8 mM^{-1} .cm^{-1}.

Statistical analysis

The experiment was carried out in a completely randomized design, considering one-way factor for germination and electrical conductivity and in a two-way 4×2 factorial arrangement for biochemical analysis, consisting of the four priming treatments and two imbibition times, with replications each. Data was subjected to ANOVA and, in case of normal distribution, Scott-Knot means test was selected at 5% significance using the Sisvar 5.6 software package.

RESULTS AND DISCUSSION

Changes in germination only occurred in control non-primed seeds. The cumulative germination (G%) showed that control seeds delayed germination in comparison to primed seeds regardless the priming solution. Primed seeds reached over 90% germination after 24 h imbibition (Figure 1A). The germination speed index (GSI) of control seeds was lower than of primed seeds (Figure 1B). G% and GSI did not show statistical difference among water, NO and AsA seeds.

All priming methods applied to the stored (aged) sunflower hybrid seeds resulted in better germination and vigor performance (speed and uniformity) in comparison to non-primed. Since germination is one of the most important processes in a plant's life cycle, its success depends on environmental conditions besides appropriate biochemical and physiological responses (Bewley et al., 2013). At cellular level, the seed aging is associated with a set of alterations including membrane integrity loss and the reduction of energetic metabolism that can lead to electrolyte leakage and decrease seed germination and vigor (Boniecka et al., 2019). The GSI and G% results for primed seeds indicate a reestablishment of ideal seed physiological capacity as seeds presented faster and uniform germination, i.e. better vigor.

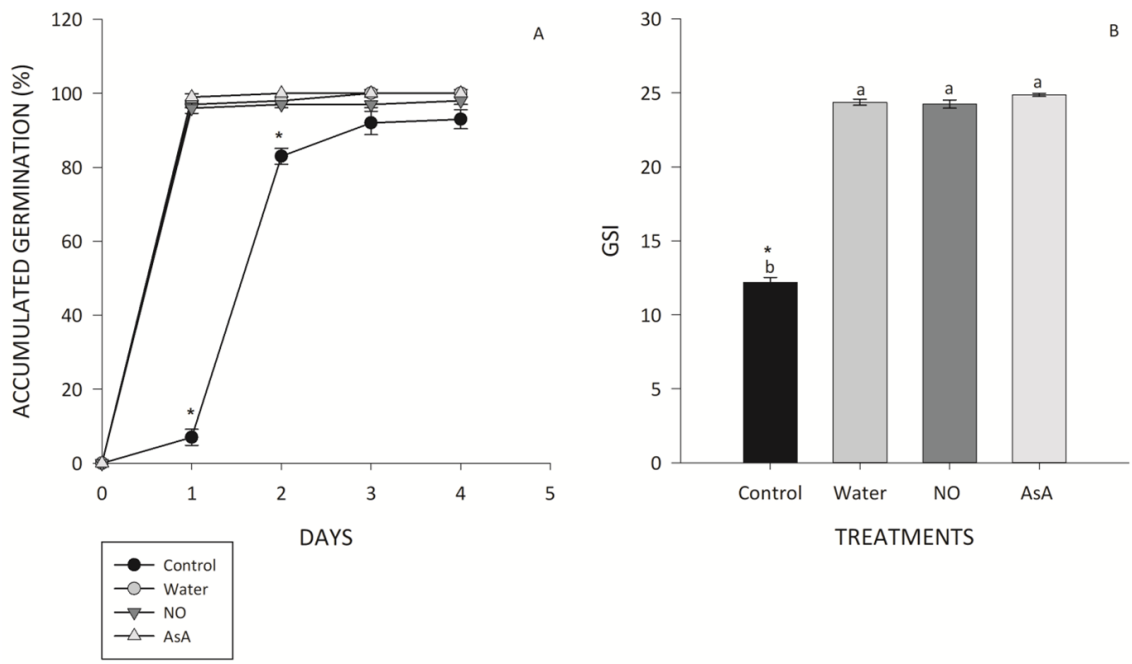

Figure 1. Cumulative germination (G%) (A) and Germination Speed Index (GSI) (B) of *Helianthus annus* seeds subjected to priming treatments Water, NO (100mM sodium nitroprusside) and AsA (100mM ascorbate), and the Control. Dots or bars are means ± standard error (n=4). Same letters do not differ each other on Scott-Knott test set at 5% of probability. (*) statistic differences.

The seeds' speed and capacity for repairing damages are directly involved to its performance during the germination and seedling establishment (Long et al., 2015). As previously reported, seeds that did not undergo any kind of priming (control) showed lower germination percentage and germination speed index. As practical results of this technique and the treatments established, there is an increase in the germination rate, germination speed, uniformity, and the restructuring of membranes. Once the imbibition process starts, there are a set of repairs that happens at cellular and structural level which are related to delay in germination of aged seeds (Xu et al., 2020). As seeds go through a post-harvest storage period, they undergo a gradual and continuous deterioration process which leads to reduction of their vigor (Sahu et al., 2017; Boniecka et al., 2019). As seeds age and lose vigor, more severe damage exceeds the capacity for self-repair (Bewley et al., 2013).

The levels of hydrogen peroxide (H_2O_2) (Figure 2A) were not show statistical differences between treatments after 8 h imbibition. However, it was possible to observe that the control non-primed seeds showed the lowest content of H_2O_2 when compared to any of the primed seeds. At 24 h, a higher concentration of H_2O_2 was observed in seeds subjected to imbibition in water. NO and AsA treatments did not show statistical difference during the evaluation at 24 h. The content of MDA was higher after 8 h imbibition in control seeds, while it was lower in NO primed seeds (Figure 2B), whereas water and AsA treatments showed similar contents. Similarly, the control seeds had higher content also after 24 h imbibition. Water, NO and AsA treatments did not present statistical differences. Electrical conductivity values were higher in non-primed control seeds, while the seeds subjected to all priming treatments exhibited the lower not significant values (Figure 2C).

The efficiency of seed priming with NO and AsA in improving both percentage and speed of germination correlated with ROS scavenging, thus limiting oxidative stress. The increase in ROS production was identified as one of the most important points that directly affects seed aging by promoting oxidative damage to lipids and proteins, loss of viability and electrolyte leakage (Bailly, 2004; Waszczak et al., 2018). The imbalance between the level of ROS and the consequent oxidative damage is associated with loss of seed viability (Morscher et al., 2015; Sano et al., 2016). Thereby, lipid peroxidation promoted by free radicals, membranes rupture and loss of cellular solutes have been reported as the main damage during deterioration (Fu et al., 2015).

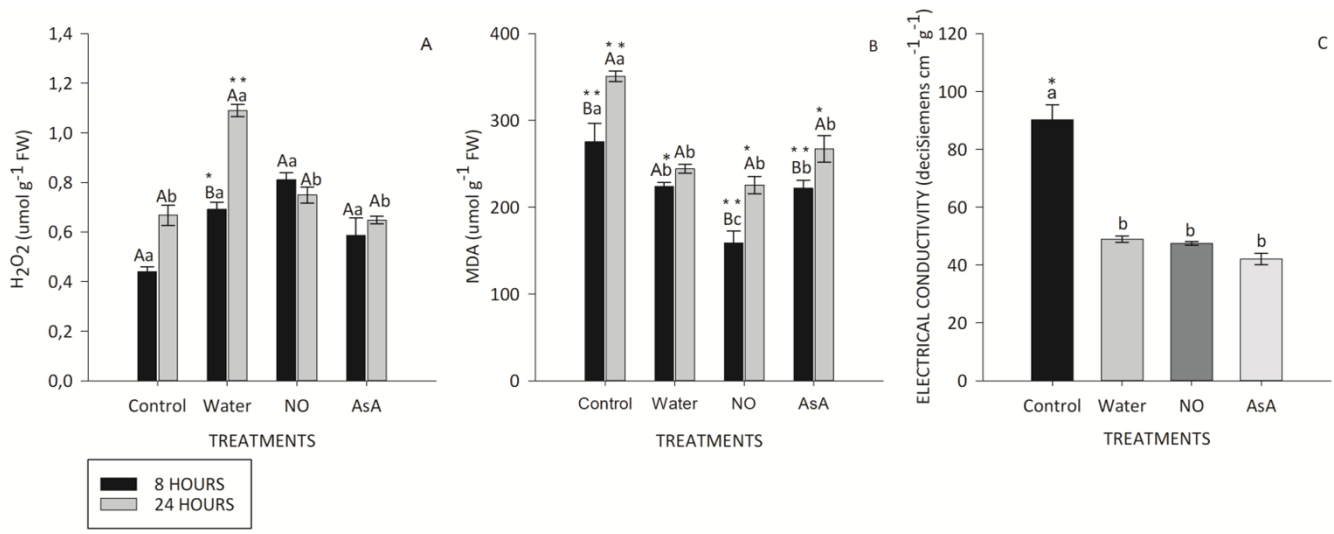

Figure 2. Hydrogen peroxide (H_2O_2) (A), malondialdehyde (MDA) content (B) and electrical conductivity (C) of *Helianthus annus* seeds subjected to priming treatments Water, NO (100mM sodium nitroprusside) and AsA (100mM ascorbate), and the Control. Bars are means ± standard error (n=4). Uppercase letters compare the levels of H_2O_2 or MDA between 8 h and 24 h in each treatment. Lowercase letters compare the levels of H_2O_2 or MDA between treatments of 8 h or 24 h. Equal letters do not differ from each other on Scott-Knott test set at 5% of probability. (*) statistic differences.

In oilseeds, the glyoxysomes produce a greater amount of hydrogen peroxide during the period that seeds mobilize its lipid reserves (Bailly et al., 2008; Gill and Tuteja, 2010). However, the results found demonstrate that, even the treatments being no different statistically regarding the H_2O_2 content, the control seeds exhibited the higher MDA rate and electrical conductivity after 8 h imbibition when compared to other treatments. The same can be seen after 24 h imbibition, where the non-primed control showed greater MDA content. Although the seeds have several mechanisms of repair and elimination of ROS, they may no longer be viable and may present delays in germination or even not germinate after a prolonged period of storage if the damage is greater than the repair capacity (Xu et al., 2020).

NO can act both in the production and in the degradation of ROS, presenting a complex feedback regulation between these two signaling molecules. The action of NO in the elimination of ROS can occur directly or in the action of this reactive nitrogen species (RSN) in inducing the action of the antioxidant system (Singh and Bhatla, 2017). Priming with NO or AsA resulted in seeds with greater capacity to repair the damages and consequently presented less oxidative damages, which resulted in the better percentage and speed of germination. As observed in electrical conductivity results, the non-primed control seeds showed the highest rate of electrolyte leakage when compared to the priming seeds. The electrolyte leakage is an indicator of altered cell membrane permeability, consequence of structural modifications induced by lipid peroxidation and damage to the phospholipid layer during seed deterioration and vigor decrease (Mira et al., 2010; Sahu et al., 2017). The reduction of electrolyte leakage by hydropriming, AsA and NO is strong evidence of the potential of these molecules to induce the retention or restructuration of the cell membrane integrity during germination. In addition, NO can act as a chain-breaking pathway during the lipid peroxidation process, being able to react directly with membranes or with reactive molecules such as superoxide anion, thus reducing damage to the system (Mazid et al., 2011).

The difference in cell membrane permeability assessed after 8 h imbibition (phase II of germination) suggests changes in the extent of damage caused by ROS to the cell membrane (Nabi et al., 2019). The content of MDA in both treatments is proportional to the activity of the antioxidant systems that act in scavenging of ROS, which affects the integrity of membranes and electrolyte leakage. The proposed priming methods seemed to effectively recover and improve membrane dysfunction while reducing lipid peroxidation. A common fact of aged seeds is the increase of leachates when seeds are immersed in water (López-Fernández et al., 2018). However, the evaluation of MDA as a marker of oxidative stress in plants in this work allowed the observation of a correlation between the evaluated imbibition stages, the H_2O_2 accumulation and the membrane damage. Variations in the ROS amounts and its production time have also been reported in the work developed by Leymarie et al. (2012), where the authors suggest that ROS assumes different functions during germination.

The values of superoxide dismutase (SOD) activity (Figure 3A) were higher after 8 h imbibition in all treatments, although they were not statistical different during this period. The evaluation at 24 h imbibition showed the highest activity in non-primed control seeds. Lower activity of catalase (CAT) and Ascorbate peroxidase (APX) (Figures 3B and 3C) was only perceived after 8 h imbibition in non-primed control seeds. None of the treatments showed significant differences after 24 h imbibition. Whereas NO and AsA treatments did not present differences after 8- and 24-h imbibition.

Considering the interaction between time factors and types of conditioning factors (priming), it was observed that only SOD and APX presented significant values. Regarding SOD, the activity after 8 h imbibition was greater than after 24 h. In addition, the non-primed control differed from the primed seeds which presented higher average in relation to the other treatments after 8 h. Within 24 h, all treatments, except the control, significantly reduced SOD activity over time. Regarding APX, its activity increased over time in all treatments, being statistically different in control and hydropriming. The non-primed control seeds showed less activity of APX after 8 h imbibition compared to the other treatments. On the other hand, CAT presented greatest activity after 24 h than after 8 h in all treatments. At 8 h, the control treatment differed significantly from the others, presenting the lowest activity of this enzyme in this time interval. Within 24 h, there was no statistical difference between treatments.

It is reported that the reduction in the activity of the antioxidant system, mainly the enzymatic action, may be responsible for the accumulation of ROS, thus leading to loss of seed vigor (Yin et al., 2014). SOD showed a high activity

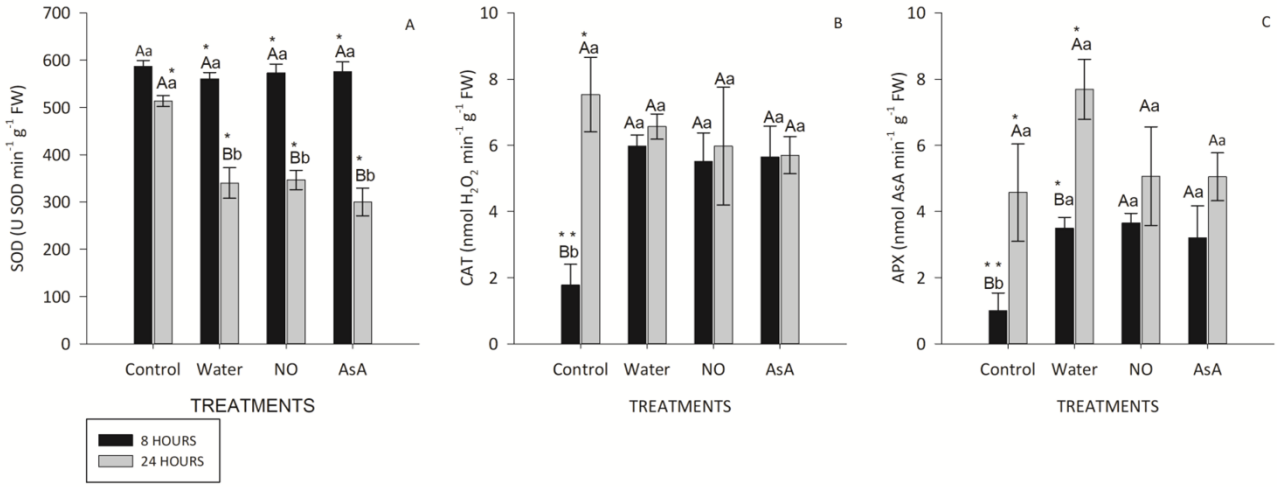

Figure 3. Superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) activity of *Helianthus annus* seeds subjected to priming treatments water, NO (100mM sodium nitroprusside) and AsA (100mM ascorbate), and the Control. Bars are means ± standard error (n=4). Uppercase letters compare the activity of SOD, CAT or APX between 8 h and 24 h in each treatment. Lowercase letters compare the activity of SOD, CAT or APX between treatments of 8 h or 24 h. Equal letters do not differ from each other on Scott-Knott test set at 5% of probability. (*) statistic differences.

in the first evaluation, followed by a reduction at 24 h evaluation in all treatments except the control. The activity of CAT, responsible for reducing H_2O_2 in water (Das and Roychoudhury, 2014), differed statistically only at the 8 h period in the control treatment, where the lowest activity of this enzyme was observed. Interestingly, the H_2O_2 level in the 8-hour control treatment was lower than other treatments, although its oxidative damage assessed by the MDA marker was greater. The increase CAT and APX activities over time showed here played a key role in the elimination of ROS and damage decrease. Similar results have been identified in studies with aged soybean seeds (Xin et al., 2014). Thus, it can be considered that the membrane damage is the main event that occurs in the aging of seeds (Ratajczak et al., 2019).

The main mechanism of repair of the priming with AsA and NO in naturally aged seeds was the increase of enzymatic activity in relation to control and the reduction of damage by lipid peroxidation. As AsA is water-soluble, it has advantages over other constraints when used in seed pre-treatment, being involved in distinct cell processes, including the cell division, membrane restructuring, and vigor increase (De Gara et al., 2013). The action of AsA on seed germination was observed in studies with sunflower (El-Saidy et al., 2011). Regarding NO, there is also a relationship between the involvement of the signaling pathways by ROS and reactive nitrogen species (RNS), where it is assumed that these molecules interact with enzymes self-regulating mechanisms (Begara-Morales et al., 2018). The results of the enzymatic antioxidant activity in the treatment with seeds primed NO show that this molecule acts positively on the germination of aged seeds.

One of the main characteristics of the SNP, a widely used NO donor in the nitrosyl-iron complex category, deals with the formation of the inorganic iron-NO complex, where iron is in its ferrous state (Fe^{2+}) and not bound to this as NO^+ (Singh and Bhatla, 2017). This fact may be correlated with the release of Fe^{2+} by the donor, thus increasing their concentration in the medium and promoting the occurrence of Fenton reactions. However, in response to exogenous SNP application, Keisham et al. (2019) reported an abundance of 117 altered proteins. Among these proteins, functional categories are involved in stress responses, signal translation, proteolysis, among others. Furthermore, these authors also reported that NO plays a protective role during the proteolytic degradation process in oilseeds, thus minimizing the reduction of oxidative damage like was observed.

CONCLUSIONS

It can be concluded that stored sunflower seeds, when subjected to physiological priming with water (hydropriming), 100 mM NO and, 100 mM AsA show better performance in terms of germination speed, cumulative germination, ROS-scavenging system, and their cytotoxic effects, as well as electrolyte leakage and enzymatic antioxidant system. Since the vigor of seeds is essentially dependent on the ability to restructure the damage caused by aging and maintain essential metabolic activities, the NO acted as the primed that maintained the antioxidant enzymatic activity reducing the ROS produced and presented less oxidative damage, that is, favored its repair. Thus, the use of this seed's technologies is suggested to achieve uniform germination and seedling establishment and to promote the reuse of these seeds, minimizing costs and obtaining quality input.

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