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ARTICLE

Nitric oxide in physiological potential and biochemical mechanisms of pea seeds under water deficit

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ABSTRACT: Nitric oxide (NO) has been standing out as a seed germination process stimulator, mainly under stress conditions. This work aimed to evaluate the physiological and biochemical effects of NO on germination and vigor of pea seeds submitted to water deficit. Seeds of cv. Itapuã 600 were sown in moistened substrate with water (control), PEG 6000 solution (-0.15 MPa) and PEG 6000 solution with sodium nitroprusside (SNP) (-0.15 MPa + 50 µM), an NO donor. The germination, vigor (germination speed and seedling development), activity of antioxidant enzymes, reactive oxygen species, lipid peroxidation, and amylase activity were evaluated. Germination and vigor of pea seeds were reduced under water deficit, but the application of SNP was able to improve the physiological and biochemical performance of the seeds under this condition. Water deficit promotes an increase in oxidative stress, but the application of NO promotes greater activity of antioxidative apparatus enzymes and amylases, softening the damage caused by water deficit during seed germination and growth of pea seedlings.

Index terms: antioxidant enzymes, germination, lipid peroxidation, sodium nitroprusside, water stress.

RESUMO: O óxido nítrico (ON) vem se destacando como estimulador do processo de germinação das sementes, principalmente sob condições de estresse. O objetivo deste trabalho foi avaliar os efeitos fisiológicos e bioquímicos do ON na germinação e no vigor de sementes de ervilha submetidas ao déficit hídrico. Sementes da cv. Itapuã 600 foram semeadas em substrato umedecido com água (controle), solução de PEG 6000 (-0,15 MPa) e em solução de PEG 6000 com nitroprussiato de sódio (SNP) (-0,15 MPa + 50 µM), um doador de ON. Foram avaliadas a germinação, vigor (velocidade de germinação e desenvolvimento de plântulas), atividade de enzimas antioxidantes, espécies reativas de oxigênio, peroxidação de lipídeos e atividade de amilases. A germinação e o vigor das sementes de ervilha foram reduzidos sob o déficit hídrico, mas a aplicação do SNP foi capaz de melhorar o desempenho fisiológico e bioquímico das sementes sob essa condição. O déficit hídrico promove um aumento do estresse oxidativo, mas a aplicação de ON promove maior atividade das enzimas do aparato antioxidativo e das amilases, amenizando os danos causados pelo déficit hídrico durante a germinação e crescimento de plântulas de ervilha.

Termos para indexação: enzimas antioxidantes, germinação, peroxidação de lipídeos, nitroprussiato de sódio, estresse hídrico.

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INTRODUCTION

Approximately 90% of all agricultural areas in the world are exposed to different environmental stresses (such as salinity, extreme temperatures, and water deficit), which compromise agricultural production (Waqas et al., 2019). Among these, the water deficit caused by drought is one of the main stresses that threaten large-scale plant production (Lau et al., 2021).

The seed germination depends directly on water absorption for metabolic activation and conversion/transport of energy reserves to the embryo (Jain et al., 2019). Therefore, as water availability is reduced, several cell changes occur in seeds, such as increased solute concentration in cells, changes in intracellular pH, protein denaturation, loss of membrane integrity, and others (Bewley et al., 2013; Marcos-Filho, 2015). In general, cell changes caused by water deficit result in a reduction in germination speed, delay in seedling development, and, at more drastic levels, seed death (Możdżeń et al., 2015; Pereira et al., 2020a).

The exposure of seeds to stressful conditions such as water deficit generates the formation of reactive oxygen species (ROS), mainly radical superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) (Zhang et al., 2021). In this context, oxidative stress occurs due to the loss of seed capacity to maintain cellular homeostasis of these ROS, causing several cellular damages that compromise the viability and physiological quality of seeds (Cechin et al., 2015; Noctor et al., 2018; Ebone et al., 2019). The neutralization of excess ROS at the cellular level occurs mainly through the action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and others (Kapoor et al., 2019). The performance of these and other antioxidant enzymes in seeds exposed to water deficit is essential for mitigating oxidative stress in species such as rice (Liu et al., 2019), cotton (Bai et al., 2020), sunflower (Morais et al., 2020) and others.

Nitric oxide (NO) is classified as a free radical synthesized from L-arginine, colorless, and inorganic (Kolbert et al., 2021). In seeds, the action of NO has been reported as a germination stimulant in several species, such as *Arabidopsis thaliana* (Albertos et al., 2015), *Eucalyptus urophylla* (Pereira et al., 2020b), *Brassica juncea* (Rather et al., 2020), *Chenopodium quinoa* (Hajihashemi et al., 2020), *Brassica chinensis* (Ren et al., 2020) and *Urochloa brizantha* (Oliveira et al., 2021). In general, these authors report that the action of NO in seeds is mainly related to the greater protection of oxidative damage through the activation of enzymes and the greater hydrolysis of reserves to the embryonic axis, favoring the germination process, especially under stress conditions. In pea seeds, Vidal et al. (2018) observed that the application of sodium nitroprusside (SNP, an NO donor) stimulated germination, increased antioxidant activity, seedling growth, and reduction of abscisic acid/gibberellic acid (ABA/GA) ratio. In a previous study, Sekita et al. (2021) evaluated the physiological and biochemical effects of NO on germination and vigor of different lots of pea seeds. These authors concluded that NO application does not alter pea seed germination, but it increases vigor. Furthermore, they observed that it is more effective in seed lots with lower physiological potential (Sekita et al., 2021). However, studies evaluating the effect of NO on the performance of pea seeds exposed to water deficit are scarce and may bring important responses to a better understanding of the physiological and biochemical mechanisms involved.

In light of the above, this work aimed to evaluate the physiological and biochemical effects of NO on germination and vigor of pea seeds submitted to water deficit.

MATERIALS AND METHODS

The study was conducted at the Seed Analysis Laboratory of the Department of Agronomy at *Universidade Federal de Viçosa*, Minas Gerais, Brazil. Approximately 1,500 seeds from one lot of pea seeds cv. Itapuã 600 (ISLA Sementes Ltda.) were used. The seeds were submitted to the treatments and tests described below:

Germination: Four replications of 50 seeds were distributed in rolls of paper towels (Germitest®), moistened with water (control), PEG 6000 solution (-0.15 Mpa) and PEG 6000 solution with SNP (-0.15 Mpa + 50 µM) at the proportion of 2.5 times the weight of the dry paper. The osmotic potential and the SNP dose were defined according to pre-tests in a previous study (Sekita et al., 2021). The rolls were kept in a seed germinator at the temperature of 20 ºC. The results consisted of the percentage of normal seedlings obtained on the eighth day after sowing (Brasil, 2009).

First germination count: It was performed with the germination test. The result consisted of the percentage of normal seedlings obtained on the fifth day after sowing (Brasil, 2009).

Germination speed index (GSI): Four replications of 50 seeds were distributed in paper rolls uniformly moistened and kept under the same conditions used in the germination test. Daily evaluations of normal seedlings were performed until stabilization (Brasil, 2009). These data were used for calculation of the GSI, as proposed by Maguire (1962).

Hypocotyl and radicle length: Four replications of 10 seeds were linearly distributed and equidistant in moistened paper rolls. The rolls were kept under the same conditions used for the germination test, for eight days. The hypocotyl and radicle length of normal seedlings were measured with the aid of a ruler. The results were expressed in cm of hypocotyl or radicle (Krzyzanowski et al., 2020).

Hypocotyl and radicle dry matter: It were performed with the determination of hypocotyl and radicle length. The cotyledons of the normal seedlings used in determination of hypocotyl and radicle length were separated. The structures were deposited separately in paper bags and placed in a forced air oven at 70 ºC until reaching constant weight. The structures were weighed, and the results were expressed in mg of hypocotyl or radicle (Krzyzanowski et al., 2020).

Antioxidative enzyme activity: Four replications of 25 seeds were placed to germinate as performed in the germination test and removed at 0 (8 h of soaking); 1; 2; 3; 4; 5; 6 and 7 days after sowing. After that, 0.2 g of embryos (by removal of the seed coat) were macerated in liquid nitrogen and then, 2 mL of the following homogenization medium were added: 0.1 M potassium phosphate buffer 0.1 M at pH 6.8, ethylenediaminotetraacetic acid (EDTA) 0.1 mM, phenylmethylsulfonic fluoride (PMSF) 1 mM and polyvinylpolypyrrolidone (PVPP) 1% (w/v) (Peixoto et al., 1999). Then the extract was centrifuged, and the supernatant was removed. Activities of catalase enzymes (CAT) (Anderson et al., 1995); peroxidase (POX) (Kar and Mishra, 1976), and ascorbate peroxidase (APX) (Nakano and Asada, 1981) were determined. For the calculation of enzyme activity, the quantity of proteins was determined according to Bradford (1976), using bovine albumin (BSA) as a standard.

Reactive oxygen species: Four replications of 25 seeds were placed to germinate as performed in the germination test and removed at 1; 2; 3; 4; 5; 6 and 7 days after sowing. Samples of 0.2 g of embryos (by removal of the seed coat) were used. The superoxide anion ($O_2^{\cdot -}$) was quantified through determination of the amount of accumulated adrenochrome, using the molar attenuation coefficient of 4.0 x 10^3 M^{-1} (Boveris, 1984; Misra and Fridovich, 1971; Mohammadi and Karr, 2001). Hydrogen peroxide (H_2O_2) was quantified based on the calibration curve created with different concentrations of H_2O_2 (Gay and Gebicki, 2000; Kuo and Kao, 2003).

Lipid peroxidation: It was performed with four replications of 25 seeds placed to germinate as in the germination test and removed at 0 (8 h of soaking), 1, 2, 3, 4, 5, 6, and 7 days after sowing**.** After that, samples of 0.3 g of embryos (by removal of the seed coat) were homogenized with 0.1% TCA and incubated with 0.5% TBA and 20% TCA. Reading was made at 532 nm and 600 nm. The molar attenuation coefficient of 155 $mM^{-1}.cm^{-1}$ was used to quantify the concentration of lipid peroxides, and the results were expressed in nmol malondialdehyde ($MDA.g^{-1}$) (Heath and Packer, 1968).

Amylase activity: It was performed with four replications of 25 seeds placed to germinate as in the germination test and removed at 0 (8 h of soaking), 1, 2, 3, 4, 5, 6, and 7 days after sowing (DAS). Approximately 1 g of embryos (through removal of the seed coat) was macerated in 10 mL of cold water at 4 ºC. The extract was centrifuged at 15,000 x g at 4 °C for 30 minutes. The supernatant was collected and the α-amylase and β-amylase enzyme activities were quantified according to the methods proposed by Kishorekumar et al. (2007) and Tárrago and Nicolás (1976). The results were expressed in U mg^{-1}.$protein^{-1}$.

Experimental design and statistical analysis: A completely randomized design (CRD) was used with four replications for the control and for each treatment. Analysis of variance (ANOVA) was performed on the data. The normality of the data was tested by the Shapiro-Wilk test and homoscedasticity by the Bartlett test. The means were compared by the Tukey test ($p \leq 0.05$). All the statistical analyses were performed with the R Software (R Core Team, 2020).

RESULTS AND DISCUSSION

There was a reduction in the germination of pea seeds under water deficit at the -0.15 MPa potential, obtaining 63%, while in control (without water deficit) the germination was 98%. With the application of SNP, it was possible to observe an increase in 22 percentage points (p.p.), reaching 84% germination on the eighth day after sowing (Figure 1A). Studies with pea seeds using PEG 6000 indicate a reduction in germination under -0.2 MPa (Petrovic' et al., 2016) and -0.6 MPa (Pereira et al., 2020a) potentials. In fact, the water restriction promoted by the use of osmotic solutions reduces the pre-germination metabolic activity of the seeds, which have a prolonged imbibition phase II, with a consequent delay in the root protrusion (Bewley et al., 2013).

In the control treatment (without water deficit), the first germination count (FGC) (Figure 1B) and germination speed index (GSI) (Figure 1C) presented values of 96% and 12.9, respectively. In the treatment with water deficit (-0.15 MPa), these values were reduced to 54% and 4.47, respectively. The SNP application promoted a significant recovery in germination speed (GSI) in a situation of water deficit (Figures 1B and C). Therefore, as observed for germination (Figure 1A), the SNP application improved the performance of pea seeds under water deficit. Other studies also prove the positive effect of NO on the performance of lettuce seeds (Deng and Song, 2012), wheat seeds under osmotic stress (Zhang et al., 2003) and high salinity (Zheng et al., 2009). Seed germination is usually the most critical stage in seedling establishment, and this process is critically vulnerable to adverse environmental conditions. Water deficit has adverse effects on seed germination and seedling growth through physiological and biochemical changes such as oxidative damage. Thus, it is important to develop suitable measures to alleviate the negative effects of stress during seed germination and early seedling development. We verified that NO was a protective agent stimulating germination metabolic processes and improving seed performance under stress conditions. Therefore, our results show that exogenous NO can significantly enhance the germination of pea seeds under hydric stress probably because of the protective effect against oxidative molecules, as already related by some authors (Zheng et al., 2009; Lin et al., 2012).

Considering the seedling development, it was observed that under water deficit there was a reduction in the length of the seedling hypocotyl, with values 78% lower when compared to those obtained under adequate conditions of water availability (Figure 1D). On the other hand, there was no significant difference between the control treatment and the water deficit in radicle length (Figure 1E). In addition, the application of NO was not efficient in improving the development of seedlings in length (Figures 1D and 1E). In general, there was a higher accumulation of dry matter in seedlings of the control treatment, which presented hypocotyl dry matter (HDM) values (Figure 1F) of 18.32 mg.seedling^{-1} and radicle dry matter (RDM) (Figure 1G) of 20.24 mg.seedling^{-1}. When submitted to water deficit, there was a reduction of 70 and 55% respectively. With the application of the NO, the HDM reduction under water deficit was from 70%, to 53%, and when observing the RDM/RL ratio, it was observed that the water deficit impaired the accumulation of radicle dry matter with values of 1.17 mg of DM.cm radicle^{-1} in seedlings under stress compared to 1.97 mg of DM.cm radicle^{-1} in control; in seedlings under stress and in the presence of NO, the radicle length was 1.81 mg of DM.cm radicle^{-1} which means a significant reduction of 41% and recovery of 33%. These results may be an indication that there is greater translocation and assimilation by the embryonic axis of the seeds, which is reinforced by the lower values of dry matter of cotyledons (CDM) in control and water deficit, with the presence of SNP (Figure 1H). The beneficial effect of NO is also reported for barley, where the application of SNP increased germination percentage, germination rate, radicle and shoot length, vigor index, and decreased mean germination time in water deficit situations (Karami and Sepehri, 2017). Therefore, we observed that the application of NO attenuated the hydric stress effects as indicated by enhancing the characteristics of seed germination and early seedling growth parameters.

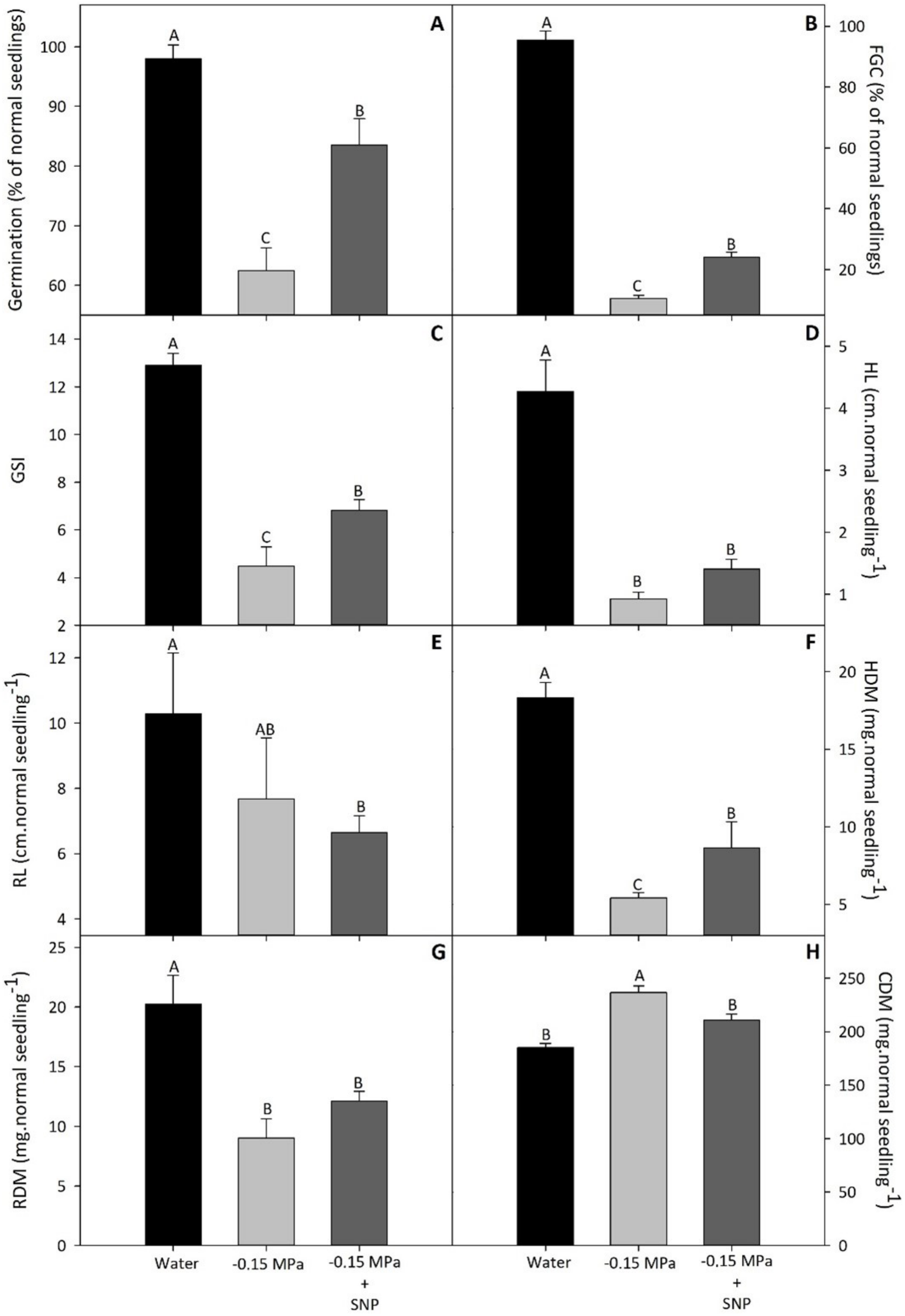

Figure 1. Germination percentage (A), first germination count (FGC) (B), germination speed index (GSI) (C), hypocotyl length (HL) (D), radicle length (RL) (E), hypocotyl dry matter (HDM) (F), radicle dry matter (RDM) (G), and cotyledon dry matter (CDM) (H) of pea seedlings from seeds subjected to soaking with water (control), PEG 6000 solution (-0.15 MPa) or PEG 6000 (-0,15 MPa) + SNP 50 µM solution. Means followed by the same letter do not differ among treatments by the Tukey test at 5% probability. Bars: Standard deviation.

Some studies corroborate the results observed in the germination and development of seedlings under water deficit. Pereira et al. (2020a) evaluated the germination and initial growth of pea seedlings (var. Aragon) under water deficit and observed a significant reduction in the physiological performance of the seeds and the development of seedlings, especially at -0.6 MPa potential. However, considering an osmotic potential of -0.2 MPa (similar to that evaluated in this work), these authors did not observe significant differences in germination and development of seedlings when compared to the control (0 MPa). This observation evidences the different responses of different genotypes when subject to water deficit (Nemeskéri and Helyes, 2019). Al-Quraan et al. (2021) observed that severe soil water stress (less than 40% of the water content of the retention capacity) should be avoided during the germination stage to ensure adequate growth and metabolism of pea seedlings.

Regarding the antioxidative apparatus during water deficit and with the application of the NO donor, it was possible to observe that antioxidant enzymes had increasing behavior during the germination of pea seeds in all treatments (seeds of the control treatment, submitted to water deficit and under water deficit in the presence of NO). The application of exogenous NO increased antioxidative enzymatic activity during germination of seeds under water deficit. Comparing only seeds under water deficit in the presence of NO or not, it is possible to observe that NO promoted an increase of 35, 22, 64, and 37%, in relation to the lowest value, of the activity of enzymes SOD, CAT, APX, and POX, respectively, on the seventh day of germination (Figure 2).

The action of antioxidant enzymes is one of the main mechanisms that plant cells use to combat the adverse effects of water stress (Lau et al., 2021). As already mentioned, the NO and the post-translational modifications mediated thereby play a fundamental role in plant responses to stresses. Nabi et al. (2019) point out that NO plays a crucial role in regulating plant responses under water deficit, including the potentiation of antioxidant mechanisms of plants under these situations. This information is reinforced by the higher activity of enzymes observed in the treatment -0.15 MPa + SNP (Figure 3).

In general, especially from two days of germination, the content of hydrogen peroxide (H_2O_2) and the release of superoxide anion ($O_2^{\cdot -}$) were lower throughout germination in seeds treated with NO (-0.15 MPa + SNP). The

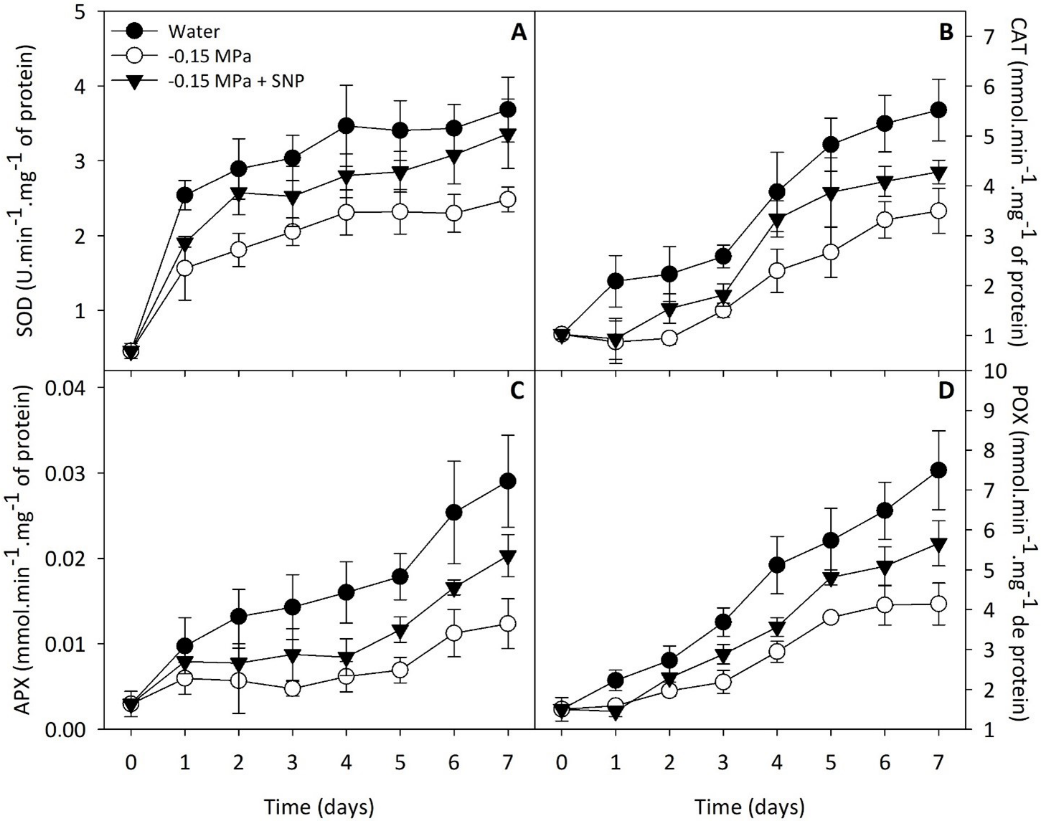

Figure 2. Activity of enzymes superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate peroxidase (APX) (C) (POX) (D) during germination of pea seeds subjected to soaking with water (control), PEG 6000 solution (-0.15 MPa) or PEG 6000 (-0,15 MPa) + SNP solution 50 µM. Bars: Standard deviation.

concentration of H_2O_2 increased throughout the evaluated period, however, the application of SNP promoted, on the seventh day of evaluation, a reduction of 22% in seeds treated with NO when compared to seeds under stress without the presence of NO (-0.15 MPa) (Figure 3A). Unlike H_2O_2, the release of $O_2^{\cdot -}$ increased until the third day of germination and then decreased from then on. On the seventh day of evaluation, the release of $O_2^{\cdot -}$ was 61% lower in seeds treated with NO when compared to seeds under stress without SNP (Figure 3B). Exogenous NO could significantly enhance the germination rate of wheat seeds and decreased the content of H_2O_2 and $O_2^{\cdot -}$ in the mitochondria under salt stress (Zheng et al., 2009).

Corroborating the results observed for ROS (H_2O_2 and O_2^-), MDA concentrations increased throughout germination and presented lower values in seeds treated with NO, which indicates that the application of SNP may be effective in protecting against oxidative stress during seed germination. On the seventh day of germination, the MDA concentration was 25% lower in seeds treated with NO when compared to seeds under stress without SNP (Figure 3C). MDA, a cytotoxic product of membrane lipid peroxidation, has been considered an indicator of oxidative damage. The stress hydric increased the MDA as well as H_2O_2 while NO reduce their content. These results suggested that NO with a suitable concentration can partially prevent oxidative damage as observed by Ren et al. (2020).

In general, excessive accumulation of ROS is highly toxic to cells, as they can react with biomolecules (such as membrane lipids) and result in oxidative stress, which is one of the main causes for seed deterioration (Ebone et al., 2019). Similar to the considerations in this study, Al-Quraan et al. (2021) found that the MDA content in pea seedlings increased significantly after seven days of water restriction. On the other hand, in sunflower plants submitted

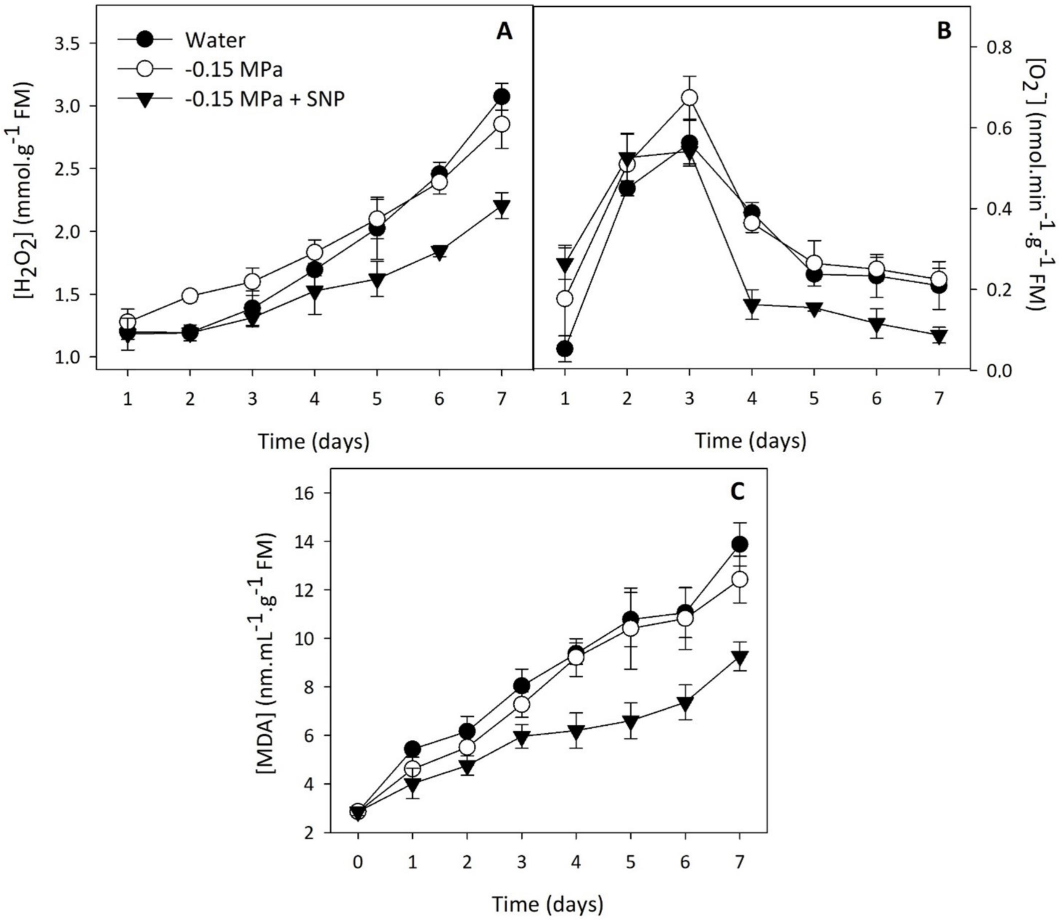

Figure 3. Hydrogen peroxide content (H_2O_2) (A), Superoxide anion content ($O_2^{\cdot -}$) (B) and malondialdehyde (MDA) (C) content during germination of pea seeds subjected to soaking with water (control), PEG 6000 solution (-0.15 MPa) or PEG 6000 (-0,15 MPa) + SNP 50 µM solution. Bars: Standard deviation.

to water stress and treated with SNP (10 uM), there was an increase in antioxidant activity and reduction of the level of MDA, relieving the negative effects of stress on membrane integrity (Cechin et al., 2015). In *Eucalyptus urophylla* seeds submitted to saline stress, the supply of NO through conditioning with SNP was beneficial, acting as a cell protector and assisting in the maintenance of germination, vigor, and development of seedlings (Pereira et al., 2020b). Farooq et al. (2009) mention that NO induces genes that encode antioxidant enzymes, increasing their activity and reducing lipid peroxidation in water stress situations. Therefore, in this study, the NO application (-0.15 MPa + SNP) contributed to reducing oxidative stress and, consequently, lipid peroxidation during pea seed germination.

These results indicated that efficient antioxidant systems were activated by NO, which effectively increased the activities of SOD, CAT, POX, and APX, and reduced contents of MDA and H_2O_2. Thus, our results demonstrated that NO application on pea seeds may be a great option to improve seed germination and seedling growth under hydric stress by modulating the physiological responses resulting in better seed germination and seedling growth. The application of NO had a protective effect, demonstrated by an improvement in seed performance and increased H_2O_2 scavenging ability in mitochondria, reducing the oxidative stress caused by water deficit. This beneficial effect may be related to a reduction of oxidative stress in seeds treated with NO, resulting in less damage to embryo cells and, consequently, their development until seedling formation.

The activity of α enzymes and β-amylase increased over time (Figures 4A and B). However, seeds under water deficit showed a reduction of 42 and 45% in activity for α and β-amylase, respectively, when compared to control. The SNP application partially reversed reduction in the activity of these enzymes, showing a recovery of 9% of the activity of α-amylase and 22% of β-amylase (Figure 4).

The activity of amylases is essential for the starch hydrolysis and a supply of soluble sugars to the embryonic axis during seed germination (Kumari et al., 2010; Sfaxi-Bousbih et al., 2010). The effect of NO on the increased activity of amylases is also reported in species such as wheat (Zheng et al., 2009) and tomato (Amooaghaie and Nikzad, 2013), corroborating the hypothesis that NO increases the conversion of starch into soluble sugars through the higher activity of these enzymes. In chickpea seeds, the SNP application caused the genetic expression of α-amylase 750 times (Pandey et al., 2019). Basahi (2021) states that oxidative and metabolic disorders are among the main causes of the successful germination of pea seeds. Based on this information, one can verify the direct relationships between higher germination and vigor (Figure 1), higher enzymatic activity (Figure 2), lower contents of H_2O_2 and $O_2^{\cdot -}$ and MDA (Figure 3) and higher activity of amylases (Figure 4) observed in seeds submitted to -0.15 MPa + SNP treatment when compared to water deficit (-0.15 MPa). In general, these results are similar to those observed by Sekita et al. (2021),

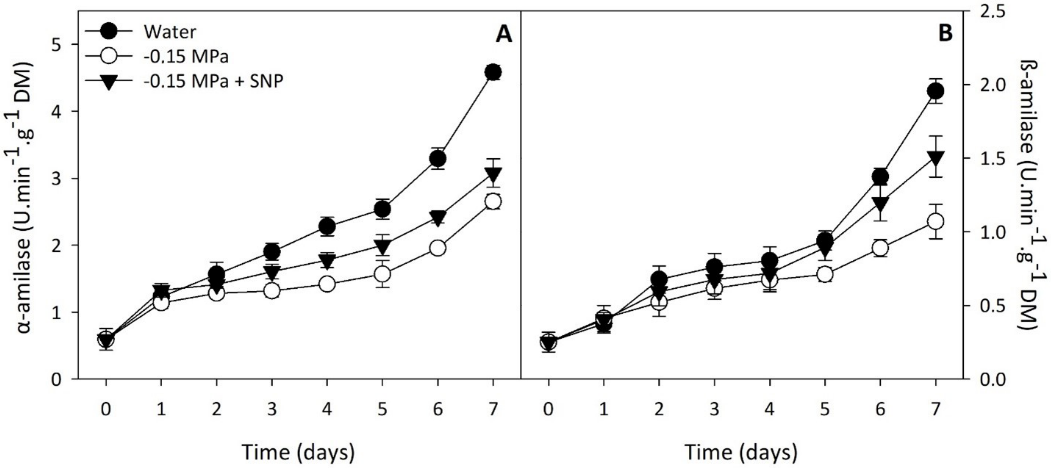

Figure 4. Enzymatic activity of α (A) and β-amylase (B) during germination of pea seeds submitted to soaking with water (control), PEG 6000 solution (-0.15 MPa) or PEG 6000 (-0.15 MPa) + SNP solution (-0.15 MPa and 50 µM). Bars: Standard deviation.

who concluded that NO leads to reduction in oxidative stress, favors the translocation of reserves to the embryo, and has potential for use in the treatment of pea seeds to increase seed vigor. Therefore, all these observations show the beneficial effect of NO on pea seeds in water deficit situations.

CONCLUSIONS

The application of sodium nitroprusside (SNP) 50 µM promotes improvement of physiological and biochemical performance of pea seeds under water deficit. Nitric oxide (NO) promotes greater activity of antioxidative apparatus enzymes and amylases, softening the damage caused by water deficit during seed germination and growth of pea seedlings.

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