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Action of nitric oxide on the physiological potential and biochemical mechanisms of pea seeds

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ABSTRACT: Nitric oxide (NO) can act in biochemical pathways of the germination process; however, there is little information about how it acts on the performance of pea seeds. The aim of this study was to evaluate the physiological and biochemical effects of NO on pea seed germination and vigor. Pea seeds cv. Itapuã 600 obtained from three seed lots with different levels of physiological quality were sown in a substrate moistened with water (control) or sodium nitroprusside (SNP) solution, a NO donor (50 µM), to assess germination, vigor, activity of antioxidant enzymes, reactive oxygen species, lipid peroxidation, and amylase activity. NO application does not alter pea seed germination, but it increases vigor. It is more effective in seeds with lower physiological potential. In addition, 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.

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

RESUMO: O óxido nítrico (ON) pode atuar em rotas bioquímicas do processo germinativo, no entanto, são poucas informações sobre a sua ação no desempenho de sementes de ervilha. O objetivo deste trabalho foi avaliar os efeitos fisiológicos e bioquímicos do ON na germinação e no vigor de sementes de ervilha. Sementes de ervilha cv. Itapuã 600, obtidas de três lotes com diferentes níveis de qualidade fisiológica, foram semeadas em substrato umedecido com água (controle) ou solução de nitroprussiato de sódio (SNP), doadora de NO (50 µM) para avaliação da germinação, vigor, atividade de enzimas antioxidantes, espécies reativas de oxigênio, peroxidação de lipídeos e atividade de amilases. A aplicação de ON não altera a germinação de sementes de ervilha, mas aumenta o vigor, sendo mais eficaz em sementes de menor potencial fisiológico. Além disso, o ON promove redução do estresse oxidativo, favorece a translocação de reservas para o embrião e tem potencial para ser utilizado no tratamento de sementes de ervilha visando o aumento do vigor das sementes.

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

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INTRODUCTION

Low seed vigor is associated with various cellular and metabolic changes, including loss of cell integrity, reduction in metabolic energy, and protein synthesis. These changes reduce seed performance under adverse environmental conditions and can be caused by natural deterioration and other factors that significantly increase the production of reactive oxygen species (ROS) (Ebone et al., 2019). The formation of ROS, above all the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), mainly occurs during the process of mitochondrial respiration and can be intensified by environmental stimuli to which the seeds are exposed (Zhang et al., 2021). In this context, oxidative stress can basically be defined as loss of seed capacity in maintaining ROS at base levels through antioxidant mechanisms (Mittler, 2017; Noctor et al., 2018).

The antioxidant enzyme system refers to the activity of various enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and others, that act by neutralizing the excess of ROS produced by the seed (Kapoor et al., 2019). The activity of these enzymes is directly related to the physiological potential of seeds, as well as seed responses to deterioration in diverse species (Yin et al., 2014; Mao et al., 2018; Nazari et al., 2020). Moreover, several factors, such as lipid peroxidation and the activity of enzymes related to the accumulation and transport of energy reserves, are related to the deterioration process and directly affect seed vigor (Ebone et al., 2019).

Recent studies have shown the effect of nitric oxide (NO) on diverse plant stress defense mechanisms (Hartman et al., 2019; Sharma et al., 2019; Ren et al., 2020; Oliveira et al., 2021). NO is classified as a free radical synthesized from L-arginine; it is colorless and inorganic (Kolbert et al., 2021). It clearly participates in numerous physiological processes in plants, such as cell division and expansion (Ötvös et al., 2005), senescence (Bruand and Meilhoc, 2019), photosynthesis (Rather et al., 2020), root development (Corpas and Barroso, 2015), fruit maturation (Rodríguez-Ruiz et al., 2017), and others.

In seed studies, NO is mainly known for stimulating germination in various species, such as arabidopsis (Albertos et al., 2015), mustard (Rather et al., 2020), quinoa (Hajihashemi et al., 2020), brachiaria grass (Oliveira et al., 2021), and others. Vidal et al. (2018) evaluated the effect of potassium nitrate (KNO_3) and of sodium nitroprusside (SNP; a NO donor) on the germination process and initial growth of pea seedlings and observed stimulation of seedling growth, as well as reduction in the abscisic acid (ABA) / gibberellic acid (GA) ratio and increase in antioxidant defenses.

Nevertheless, few studies have evaluated the effect of NO on the performance of peas seeds with different vigor levels, yet such studies may provide important responses for better understanding of the physiological and biochemical mechanisms involved. In light of the above, the aim of this study was to evaluate the physiological and biochemical effects of NO on germination and vigor of pea seeds.

MATERIALS AND METHODS

The study was conducted in the seed analysis laboratory of the *Departamento de Agronomia* at the *Universidade Federal de Viçosa*, Minas Gerais, Brazil. Three seed lots of the pea cv. Itapuã 600 (ISLA Sementes Ltda.) with different germination and vigor potentials were used. The following tests were performed on the seeds:

Germination: Four replications of 50 seeds were distributed on rolls of paper toweling (Germitest®) moistened with water (control) or with 50 µM SNP solution (concentration defined after performing a pre-test) at the proportion of 2.5 times the weight of the dry paper. The rolls were kept in a seed germinator at the temperature of 20 °C. The result consisted of the percentage of normal seedlings obtained on the eighth day after sowing (Brasil, 2009).

First germination count: This was performed together 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 on rolls of paper uniformly moistened and kept under the same conditions used in the germination test. Daily evaluations of normal seedlings were made, up to stabilization (Brasil, 2009). These data were used for calculation of the GSI, as proposed by Maguire (1962).

Cold Test: Four replications of 50 seeds were distributed on rolls of paper moistened under the same conditions used in the germination test. Rolls were made and kept in BOD at the temperature of 10 °C for seven days and then transferred to a seed germinator at 20 °C for better development of the seedlings, where they remained for five days. The number of normal seedlings was counted at the end of the period. The results were expressed in percentage of normal seedlings (Cícero and Vieira, 2020).

Hypocotyl and radicle length: Four replications of 10 seeds were distributed linearly and equally spaced on rolls of moistened paper toweling. The rolls were kept under the same conditions used for the germination test, for eight days. The hypocotyl and radicle length of the 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: This was performed together with 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 circulation oven at 70 °C until reaching constant weight. The structures were weighed and the results were expressed in mg of hypocotyl or radicle (Krzyzanowski et al., 2020).

Antioxidant enzyme activity: Four replications of 25 seeds were 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, 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 at pH 6.8, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% polyvinyl polypyrrolidone (PVPP) (w/v) (Peixoto et al., 1999). Then the extract was centrifuged and the supernatant removed. Activities of the enzymes catalase (CAT) (Anderson et al., 1995), peroxidase (POX) (Kar and Mishra, 1976), and ascorbate peroxidase (APX) were determined (Nakano and Asada, 1981). For calculation of enzymatic activity, the quantity of proteins was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Reactive oxygen species: Four replications of 25 seeds were placed to germinate as 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^-) was quantified through determination of the amount of accumulated adrenochrome, using the molar attenuation coefficient of $4.0 \times 10^3.M^{-1}$ (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: Four replications of 25 seeds were 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: Four replications of 25 seeds were 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 °C. 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 experimental design (CRD) was used in a dual factorial arrangement, consisting of 3 seed lots × 2 solutions (water and SNP), with four replications. 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 Tukey's test ($p \leq 0.05$). All the statistical analyses were performed with R software (R Core Team, 2020).

RESULTS AND DISCUSSION

The pea seeds from Lots 1, 2, and 3 had initial germination (control treatment) of 95%, 86%, and 70%, respectively. The NO did not lead to an increase in germination in any of the seed lots (Figure 1A). In contrast, the application of NO through SNP resulted in an increase of 16% for Lot 2 and 50% for Lot 3 in first germination count (Figure 1B); of 12% for Lot 1, 10% for Lot 2, and 26% for Lot 3 in the GSI (Figure 1C); and of 11% for Lots 1 and 2 and 20% for Lot 3 in the cold test (Figure 1D).

Unlike what was observed in this study with pea seeds, the application of NO was responsible for increasing the germination percentage in various species, such as arabidopsis (Albertos et al., 2015), carrot (Gama et al., 2018), mustard (Rather et al., 2020), quinoa (Hajihashemi et al., 2020), brachiaria grass (Oliveira et al., 2021), and others. Nevertheless, in this study, the application of NO led to an increase in seed vigor as determined by the first germination count test (Figure 1B), the GSI (Figure 1C), and the cold test (Figure 1D), especially in the seed lots of lower physiological potential (Lots 1 and 2).

There was no significant difference in hypocotyl (Figure 2A) and radicle (Figure 2B) growth between the treatments in Lot 1. However, the hypocotyl of the seedlings coming from Lots 2 and 3 were 8% and 46% greater in the seeds treated with SNP compared to the control, and the radicle of the seedlings coming from Lot 3 was 48% greater in the seeds treated with SNP. The seeds with application of SNP gave rise to seedlings with a greater amount of hypocotyl dry matter in all the seed lots. The average accumulation was 20% greater compared to the control (water). The amount of dry matter of the radicle in Lots 2 and 3 was also greater than the control, obtaining a greater accumulation of dry matter (14% and 41%, respectively) (Figures 2C and D).

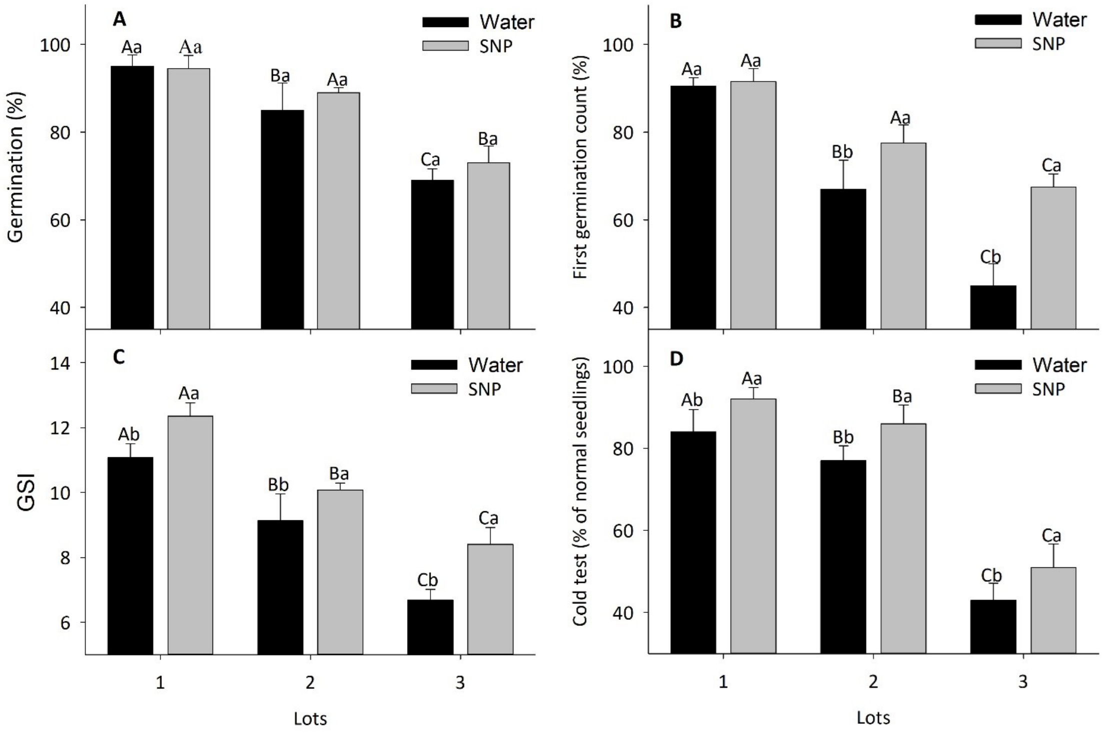

Figure 1. Germination (A), first germination count (B), germination speed index (C), and germination percentage in the cold test (D) of three lots of pea seed with or without application of 50 µM SNP. Mean values followed by the same uppercase letter do not differ among the lots (1, 2, and 3) by Tukey's test at 5% probability. Mean values followed by the same lowercase letter do not differ between the treatments [water (control) and SNP] by the F test at 5% probability. Bars: standard deviation.

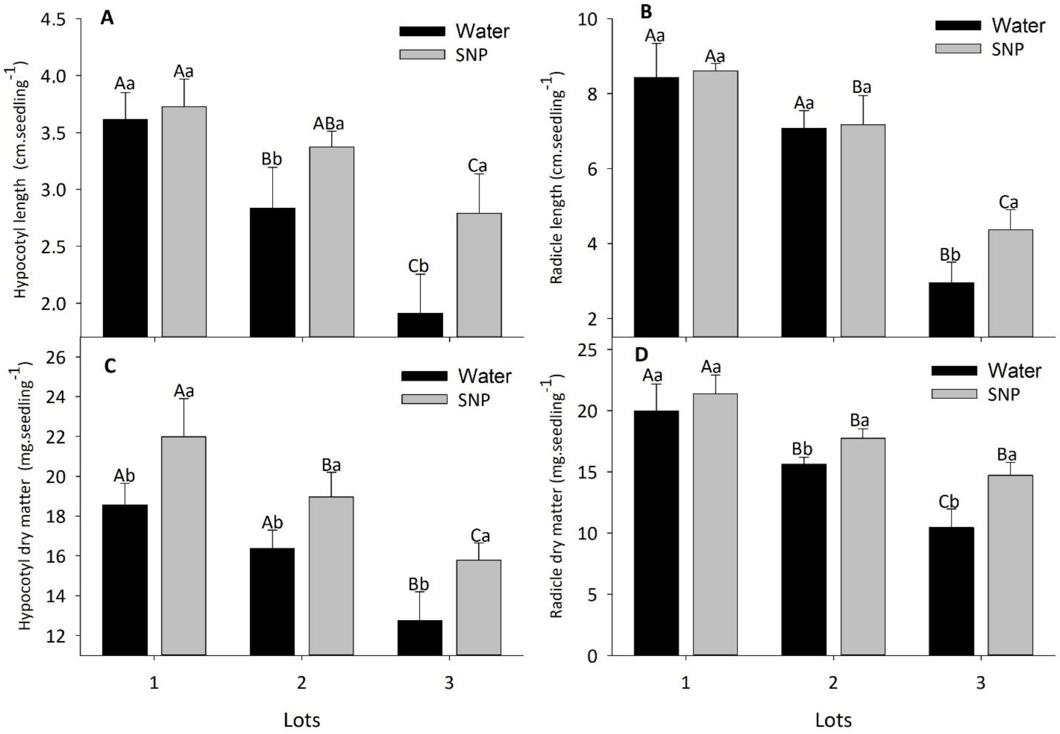

Figure 2. Hypocotyl length (A), radicle length (B), hypocotyl dry matter (C), and radicle dry matter (D) of seedlings coming from three lots of pea seeds with or without application of 50 µM SNP. Mean values followed by the same uppercase letter do not differ among the lots (1, 2, and 3) by Tukey's test at 5% probability. Mean values followed by the same lowercase letter do not differ between the treatments [water (control) and SNP] by the F test at 5% probability. Bars: standard deviation.

In general, the results observed for germination corroborate the results of Vidal et al. (2018), where the application of KNO_3 and SNP did not lead to an increase in the germination percentage of pea seeds, but increased the fresh weight of the seedlings. According to these authors, KNO_3 and SNP brought about an increase in the concentrations of GA_4 and reduced the concentration of ABA in pea plants, thus increasing the plant biomass. In a similar manner, other studies have shown the relationship of NO with an increase in GA_4 and reduction in ABA levels (Bethke et al., 2006; Matakiadis et al., 2009; Sanz et al., 2015). Pandey et al. (2019) evaluated chickpea seeds and observed an increase in germination and germination speed, which were related to greater production of NO and lower respiratory rates in the seeds. A study evaluating tomato germination reports that NO is involved with an increase in cell division and elongation, thus inducing seedling growth (Amooaghaie and Nikzad, 2013).

In general, treatment with exogenous NO resulted in a significant increase in the activity of the enzymes SOD (Figures 3A, C, and E) and CAT (Figures 3B, D, and F) during the time of germination of seeds from the three seed lots evaluated. On the seventh day of germination, the seeds treated with SNP showed greater activity of these enzymes compared to the control. This increase in SOD activity was 20%, 40%, and 37% (Figures 3A, C, and E) for Lots 1, 2, and 3, respectively; and CAT activity increased 19%, 28%, and 52% (Figures 3B, D, and F) for the same seed lots.

SOD acts in the dismutation of the superoxide radical in H_2O_2; this is a less reactive species with greater mobility in the cell. In contrast, CAT acts by converting excess H_2O_2 into water and oxygen. Thus, these enzymes are of extreme importance in attenuating the effects of oxidative stress, acting in the first lines of antioxidant defense (Choudhury et al., 2017). Therefore, exogenous NO clearly acted in enhancing the activity of these enzymes, above all after the fourth day of pea seed germination (Figure 3).

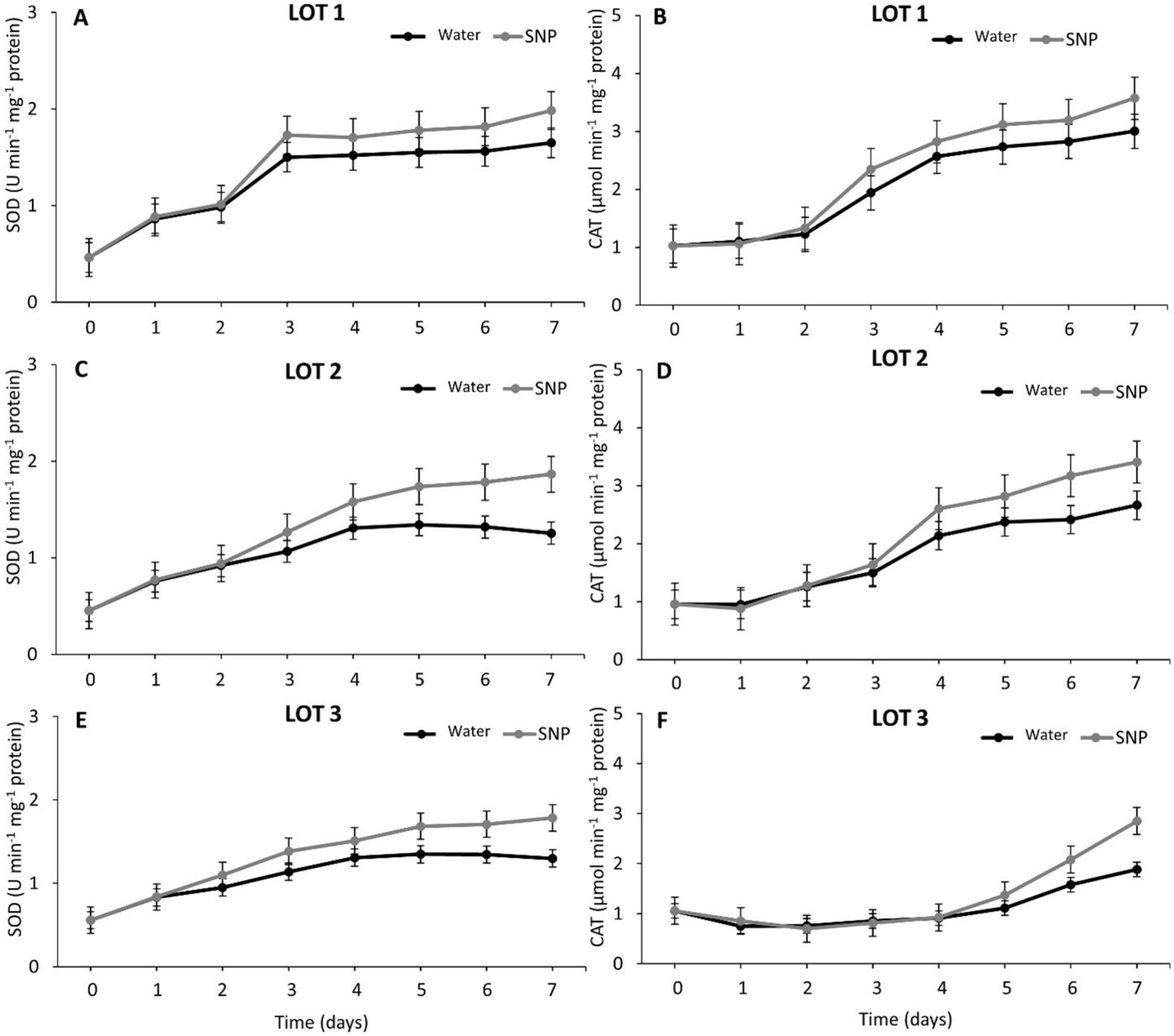

Figure 3. Activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) during germination of seeds from Lots 1 (A and B), 2 (C and D), and 3 (E and F) of pea with or without application of 50 µM SNP. Bars: standard deviation.

In a way similar to that observed for SOD and CAT, exogenous NO through the application of SNP resulted in significant increases in the APX enzyme of 34%, 47%, and 47% (Figures 4A, C, and E) in Lots 1, 2, and 3, respectively, and in the POX enzyme of 35%, 27%, and 26% (Figures 4B, D, and F) in the same seed lots, compared to the control treatment (Figure 4).

Like CAT, APX and POX are part of a group of peroxidases that act to neutralize H_2O_2 by different routes of action (De Gara, 2004). The effect of NO/SNP on seeds has been reported in the activity of antioxidant enzymes in different species, such as in sesame seeds exposed to water stress, where SNP increased the activity of the antioxidant enzymes, proving to be an efficient system for elimination of ROS (Pires et al., 2016). In a similar manner, Pandey et al. (2019) observed the importance of NO in chickpea germination through control of respiration and of ROS accumulation. In pea seeds, SNP stimulated the activity of the SOD, APX, and AOX enzymes, and assisted in greater seedling development (Vidal et al., 2018).

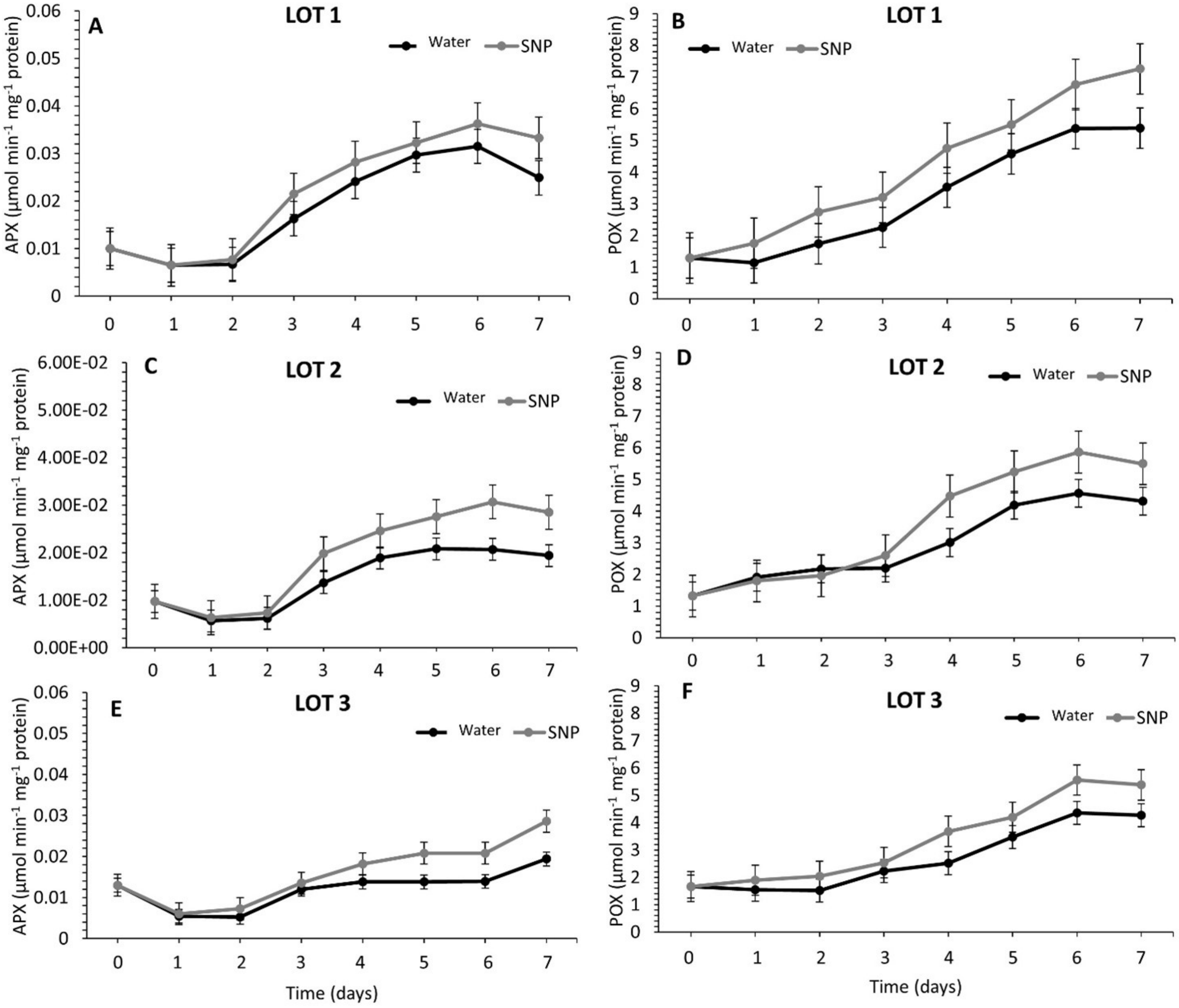

Figure 4. Activity of the enzymes ascorbate peroxidase (APX) and peroxidase (POX) during germination of seeds from Lots 1 (A and B), 2 (C and D), and 3 (E and F) of pea with or without application of 50 µM SNP. Bars: standard deviation.

The application of SNP reduced the release of O_2^- by 34%, 39%, and 63% on the fourth day and by 60%, 65%, and 33% on the last day of evaluation for Lots 1, 2, and 3 respectively (Figures 5A, C, and E). Over the time of germination, the H_2O_2 content increased. However, the application of SNP made the content of this ROS decline compared to the control. On the seventh day of evaluation, H_2O_2 content declined by 32%, 34%, and 35% for Lots 1, 2, and 3, respectively (Figures 5B, D, and F). In contrast, an increase in release of O_2^- was observed up to the fourth day of germination, with reduction after that time (Figures 5A, C, and E).

O_2^- is considered a highly reactive molecule that has low stability and is damaging to cell membranes, and it is rapidly converted into H_2O_2 (Mittler, 2017). H_2O_2, in turn, is a more stable molecule that is synthesized naturally, especially in physiological processes such as photosynthesis and respiration. At base levels, H_2O_2 plays an important role in seed germination, flowering, root development, stomatal regulation, stress responses, and other processes (Carvalho and Silveira, 2020). Hajihashemi et al. (2020) reported the positive effect of the pre-treatment of quinoa seeds with SNP and H_2O_2, as these molecules lessened the effects of salinity on germination of this species. Therefore, this information helps to explain the response of ROS during the time of pea seed germination observed in this study, as well as the beneficial effect of NO on this response.

Thus, like H_2O_2 content, the MDA content increased over the germination period for all the seed lots; however, there was greater lipid peroxidation in the less vigorous lots, both in the treated seeds and the control seeds. The MDA content increased in untreated seeds for seed lots 1, 2, and 3 by 165%, 240%, and 306%, respectively, throughout the period evaluated. The treatment with SNP was able to lower lipid peroxidation in all the seed lots, which exhibited increasing values of 140%, 175%, and 211% for Lots 1, 2, and 3, respectively, in the same period evaluated. The application of SNP was more effective in the lower vigor seed lots, exhibiting differences of 25%, 65%, and 95% in seed lots 1, 2, and 3, respectively (Figures 6A, C, and E).

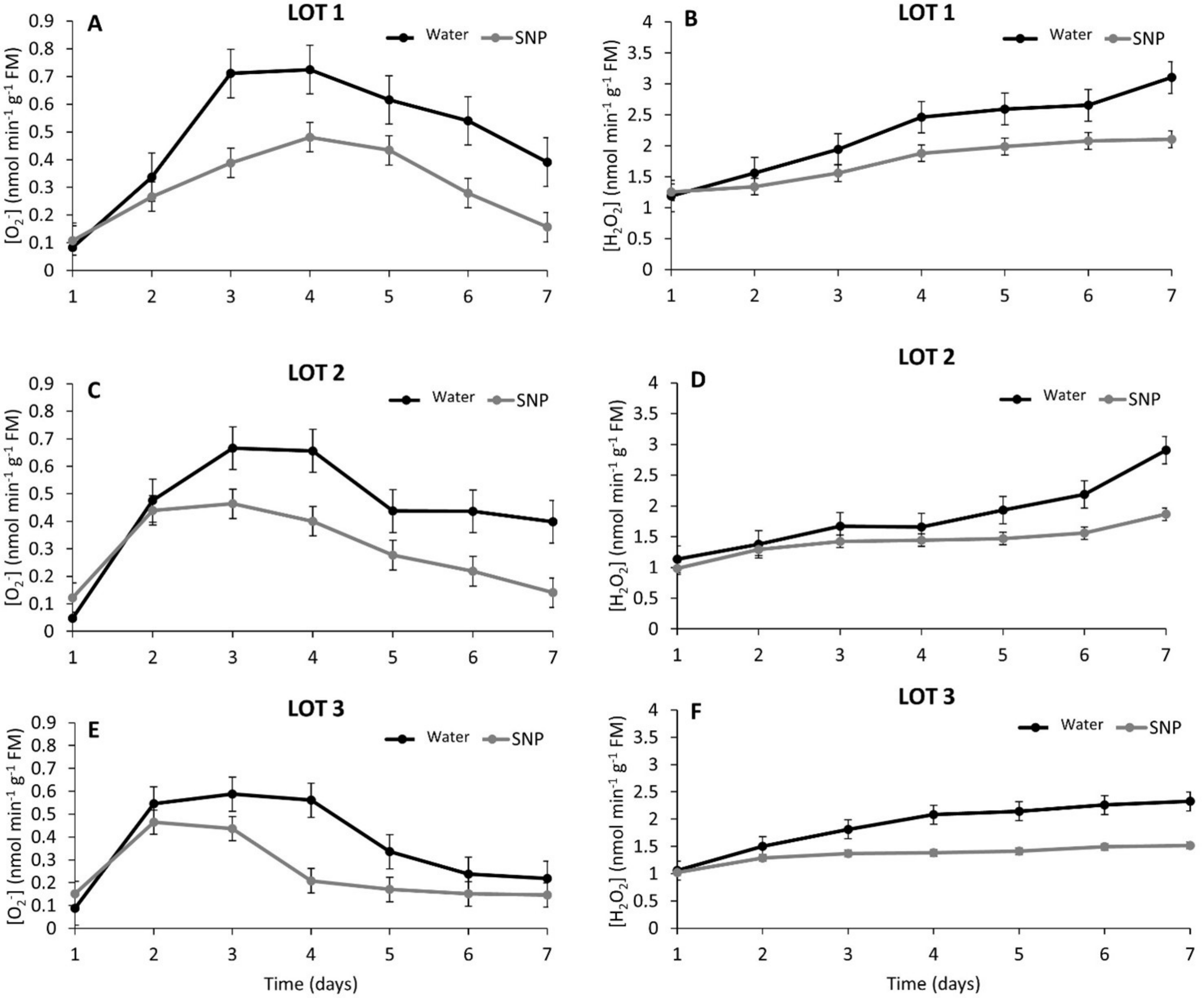

Figure 5. Content of the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) during germination of seeds from Lots 1 (A and B), 2 (C and D), and 3 (E and F) of pea with or without application of 50 µM SNP. Bars: standard deviation.

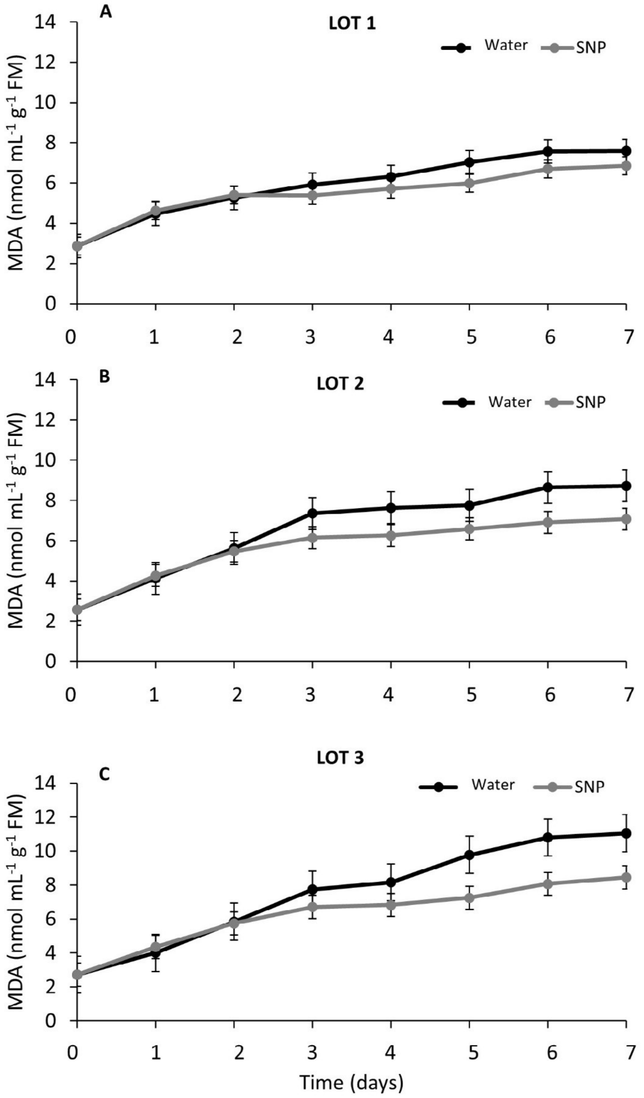

Figure 6. Content of malondialdehyde (MDA) during germination of seeds from Lots 1 (A), 2 (B), and 3 (C) of pea with or without application of 50 µM SNP. Bars: standard deviation.

Lipid peroxidation is considered one of the main effects of oxidative stress in seeds. Therefore, the lower MDA content observed in the treatment with SNP may be directly related to the beneficial effect of NO on mitigating oxidative stress through greater antioxidant activity (Figures 3 and 4) and lower concentration of ROS (Figure 5) during pea seed germination. Corroborating these results, Zheng et al. (2009) state that the application of NO donors is effective in protecting seeds in the germination process, due to greater protection against oxidative stress.

The activity of the enzymes α-amylase and β-amylase exhibited a similar response in all the seed lots and treatments. Nevertheless, Lots 1 and 2 had superior values both in the seeds of the control (water) and in the seeds treated with SNP compared to Lot 3. The application of SNP led to greater α-amylase activity in all the seed lots, showing an increase of 19% for Lots 1 and 3 and of 16% for Lot 2 on the last day of evaluation (Figures 7A, C, and E). The application of SNP was also effective in increasing the activity of β-amylase, adding 20%, 39%, and 32% on the seventh day of evaluation for Lots 1, 2, and 3, respectively (Figures 7B, D, and F).

Soluble sugars are readily available carbohydrates that serve as an energy source and carbon source to optimize the metabolic operation that occurs during seed imbibition, stimulating seed germination and initial seedling development. For there to be adequate provision of soluble sugars for the embryonic axis, there must be hydrolysis of the starch

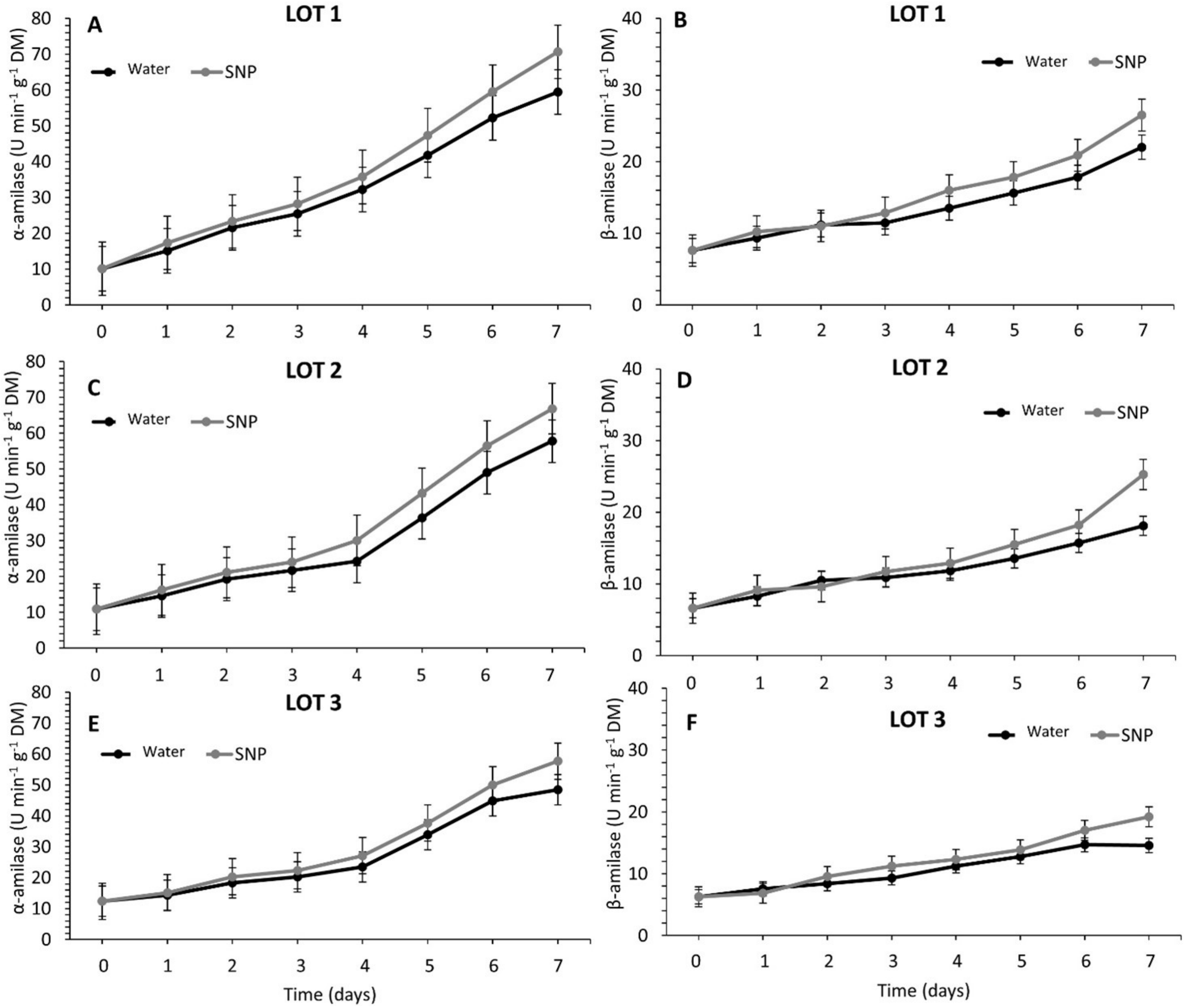

Figure 7. Activity of the enzymes α-amylase and β-amylase during germination of seeds from Lots 1 (A and B), 2 (C and D), and 3 (E and F) of pea with or without application of 50 µM SNP. Bars: standard deviation.

through amylase activity (Kumari et al., 2010; Sfaxi-Bousbih et al., 2010). The greater activity of the amylases over the time of germination confirms this information, and, in addition, the NO released through SNP clearly stimulated the greater activity of these enzymes (Figure 7). Similar to what was observed in this study, in chickpea seeds, it was shown that expression of the α-amylase gene increased 750 times at 30 minutes after application of SNP (Pandey et al., 2019). In wheat seeds, Zhang et al. (2005) observed that the addition of SNP was able to induce a rapid increase in β-amylase activity without affecting α-amylase. Thus, NO clearly acted in facilitating the conversion of the starch into soluble sugars in the pea seeds, mainly favoring seed vigor.

CONCLUSIONS

Nitric oxide (NO) through application of 50 µM sodium nitroprusside (SNP) does not alter the germination of pea seeds, but increases their vigor.

The application of SNP is more effective in pea seeds of lower physiological potential.

The NO leads to reduction in oxidative stress, favoring translocation of reserves to the embryo, and has potential for use in treatment of pea seeds aiming at increasing seed vigor.

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