Physiological and antioxidant changes in sunflower seeds under water restriction

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ABSTRACT: Seed vigor may be determinant for field performance, especially under water restriction conditions. Sunflower is a crop subject to these conditions in the field and, therefore, the aim of this study was to evaluate the physiological and antioxidant changes in sunflower seeds under water restriction. Two lots of sunflower seeds (cv. Hélio 253) with different vigor levels were used. After initial characterization, seeds were placed to germinate under water potentials of 0.0, -0.2, -0.4, -0.6 and -0.8 MPa and evaluated by tests of germination, first germination count, shoot length and primary root length. The activities of the antioxidant enzymes SOD, CAT, POX and APX were also evaluated at 0, 2, 4 and 6 days after sowing. Water restriction led to a decrease in germination and slower seedling growth, regardless of seed vigor level. SOD activity was similar in the two lots, with reduction in activity four days after sowing. CAT activity was affected differently during germination in the two lots, and it was generally higher in the most vigorous lot. In higher vigor seeds, there was lower POX activity in water restriction treatments compared to the control. In general, seeds of lower vigor have lower capacity for activation of antioxidant enzymes, especially peroxidases.

Index terms: enzyme analysis, water deficit, germination, Helianthus annuus L., vigor.

Alterações fisiológicas e antioxidativas em sementes de girassol submetidas à restrição hídrica

RESUMO: O vigor das sementes pode ser determinante para o seu desempenho em campo, especialmente sob condições de restrição hídrica. O girassol é uma cultura sujeita as essas condições no campo, e assim, o objetivo do trabalho foi avaliar as alterações fisiológicas e antioxidativas em sementes de girassol submetidas à restrição hídrica. Foram utilizados dois lotes da cv. Hélio 253 diferindo quanto ao vigor. Após a caracterização inicial, as sementes foram colocadas para germinar sob os potenciais de 0,0; -0,2; -0,4; -0,6 e -0,8 MPa e avaliadas quanto a germinação, primeira contagem e comprimento de parte aérea e raiz primária. Foram avaliadas também as atividades das enzimas antioxidativas SOD, CAT, POX e APX aos 0, 2, 4 e 6 dias após a semeadura. A restrição hídrica provocou decréscimo na germinação e menor crescimento das plântulas independentemente do nível de vigor das sementes. A atividade da SOD foi semelhante para os dois lotes, com redução aos quatro dias após a semeadura. A atividade da CAT foi afetada de modo diferente ao longo da germinação dos dois lotes sendo, em geral, mais alta no lote de maior vigor. Nas sementes de maior vigor, houve menor atividade da POX nos tratamentos de restrição hídrica em relação ao controle. Em geral, sementes de menor vigor possuem menor capacidade de ativação de enzimas antioxidativas, principalmente as peroxidases.

Termos para indexação: análise enzimática, déficit hídrico, germinação, Helianthus annuus L., vigor.
INTRODUCTION

The expressive increase in planted area of sunflower (*Helianthus annuus* L.) has led to an increase in the demand for high quality seeds. Sunflower is grown in most Brazilian states and is concentrated especially in the Cerrado region (Brazilian tropical savanna) between the soybean and maize crop seasons. It is of fundamental importance in rotation systems (CONAB, 2017). The crop is subject to variations in edaphic and climatic conditions, mainly in regard to soil water availability in the seedling emergence phase (Backes et al., 2008).

Water restriction in the soil at the time of sowing reduces the emergence and development of seedlings due to interference in the water uptake and cell elongation processes (Finch-Savage and Bassel, 2016; Marcos-Filho, 2015). Under water stress conditions, field emergence and initial seedling development depend on the level of seed vigor. Albuquerque and Carvalho (2003) found that the effect of seed vigor in sunflower on reduction of field emergence is associated with stress conditions at the time of sowing. These authors found that under water restriction at -1.1 MPa, obtained by moisture control in the soil, there was reduction in seedling emergence even for higher vigor seed lots.

In a study carried out with two sunflower cultivars under water stress, Carneiro et al. (2011) observed a reduction in germination, length, and dry matter of seedlings, above all at the water potential of -0.8 MPa with the use of polyethylene glycol (PEG 6000). Similar results were observed by Luan et al. (2014), using the osmotic agents PEG 6000 and sodium chloride (NaCl).

Water restriction during the germination process can lead to oxidative stress in seeds and increase the production of reactive oxygen species (ROSs), such as the superoxide radical (O$_2^-$), the hydroxyl radical (OH$^-$), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (‘O$_2$) (Jaleel et al., 2007; Mouradi et al., 2016; Nguyen et al., 2019). The intensity of cell damage is determined by the capacity of seeds to eliminate these free radicals through defense systems, including the action of antioxidant enzymes, which promote control of the intracellular concentration of ROSs (Kapoor et al., 2015). The inner content of these compounds and the activation of the antioxidant defense system are associated with successful germination, especially in situations of abiotic stresses (Chen and Arora, 2013; Jisha et al., 2013; Savvides et al., 2016).

Among the main antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) are constantly regulated in the process of neutralization of excessive ROSs at the cell level (Bailly, 2004; Del Rio et al., 2018; Groß et al., 2013; Kibinza et al., 2011). However, this regulation may be inefficient if the stress is more accentuated, with an increase in free radical production. In sunflower seeds, antioxidant enzyme activity and seed physiological potential were not affected when under water stress and salt stress up to the water potential of -0.4 MPa, but under the potential of -0.8 MPa, antioxidant capacity during germination was reduced, affecting seedling growth (Carneiro et al., 2011).

Low vigor seeds are less efficient in neutralizing ROSs and, thus, oxidative stress increases ROS production and, consequently, reduces germination (Bailly, 2004). In oil seeds such as sunflower, the effects of oxidative stress are mainly related to peroxidation of lipids and oxidation of proteins and nucleic acids (Bailly et al., 2008; Xin et al., 2014; Yin et al., 2015). In general, studies on water stress in sunflower seeds do not consider the level of seed vigor and mainly evaluate aspects related to germination, emergence and seedling growth (Albuquerque and Carvalho, 2003; Carneiro et al., 2011; Luan et al., 2014). Therefore, the aim of this study was to evaluate the physiological potential and changes in the antioxidant enzyme system of sunflower seeds, with different vigor levels, when under water restriction.

MATERIAL AND METHODS

This study was conducted in the Seed Laboratory of the Plant Science Department of the *Universidade Federal de Viçosa*, Viçosa, MG, Brazil. Two seed lots of sunflower of the cultivar Hélio 253 were used, collected in the 2014 crop season and supplied by the HELIAGRO company. First, the seeds from each lot were evaluated in regard to physiological quality by the following tests:
Germination: This was conducted with eight replications of 25 seeds, following the method described in the Rules for Seed Testing (Brasil, 2009). The seeds were sown in paper moistened with water in the amount of 2.5 times the weight of the dry paper. Rolls were formed and they were kept in a seed germinator at 25 °C, with an eight-hour photoperiod. The number of normal seedlings were counted ten days after sowing, and results were expressed in percentage (Brasil, 2009).

First germination count: This was conducted together with the germination test, calculating the percentage of normal seedlings obtained on the fourth day after sowing (Brasil, 2009).

Shoot length and root length: Four replications of ten seeds were sown at an equal distance on a line drawn on the upper third of the rolls of paper towel moistened to 2.5 times the weight of the dry substrate and kept at 25 °C with an eight-hour photoperiod (Nakagawa et al., 1999). On the tenth day after sowing, shoot length and root length of the seedlings were measured through use of a ruler, and the results were expressed in cm.seedling^{-1}.

Seedling emergence: This was carried out in a greenhouse in trays containing a substrate of soil and sand in the proportion of 2:1. Four replications of fifty seeds were sown at a depth of 1 cm, and daily counts were made until stabilization of the number of seedlings to calculate the percentage of seedling emergence and the emergence speed index (ESI) (Maguire, 1962).

Accelerated aging: This was carried out with 250 seeds, distributed over a screen within a “Gerbox” plastic box containing 40 mL of distilled water at the bottom. The boxes were closed with a lid, enclosed in plastic bags, and kept in a BOD incubator at 41 °C for 48 hours. After that period, the seeds were placed to germinate as described for the germination test. The percentage of normal seedlings was evaluated at four days after sowing.

Electrical conductivity: Two hundred seeds, subdivided into four replications of fifty seeds from each lot and previously weighed, were placed in plastic cups containing 75 mL of distilled water and kept in a seed germinator at the temperature of 25 °C for 24 hours. After that period, the electrical conductivity of the solution was measured with a conductivity meter, and results were expressed in μS cm^{-1}.g^{-1}.

Seeds from each lot were subjected to water restriction. For that purpose, they were placed to germinate as described above for the germination test using paper moistened with PEG 6000 solutions at the following potentials: 0.0 (control), -0.2, -0.4, -0.6 and -0.8 MPa, obtained according to Villela et al. (1991). Germination was evaluated at four and ten days after sowing, and results were expressed in percentage of normal seedlings (Brasil, 2009). Root length and shoot length were measured as described for characterization of the lots.

For evaluation of antioxidant enzyme activity, seeds were used at 0 days (twelve hours of imbibition in paper towel moistened with water; control) and after 2, 4 and 6 days of germination for all treatments under water restriction. To obtain the extracts used in determinations of the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX), the plant material was frozen in liquid nitrogen and kept at -80 °C. After that, around 0.3 g of plant matter was macerated and 2 mL of extraction medium, potassium phosphate buffer (0.1M, pH 6.8) was added, containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% polyvinylpolypyrrolidone (PVPP) (w/v) (Peixoto et al., 1999). After that, the material was homogenized and then centrifuged twice at 15,000 xg for fifteen minutes at 4 °C to remove the oil layer from the supernatant.

Superoxide dismutase (SOD): The method proposed by Del Longo et al. (1993) was used, adjusted for sunflower seeds, through addition of 30 µL of crude enzyme extract to 2.97 mL of the reaction medium sodium phosphate buffer (50 mM, pH 7.8), containing 13 mM methionine, 75 µM nitroblue tetrazolium (NBT), 0.1 mM EDTA and 2 µM riboflavin. The reaction was conducted at 25 °C for five minutes in a reaction chamber lighted with 15 W fluorescent bulbs. The blank was obtained under the same conditions, however, in the absence of light. Thus, the photoreduction of the NBT was determined by measuring absorbance at 560 nm (Giannopolitis and Ries, 1977). A unit of SOD was defined as the amount of enzyme able to inhibit 50% of the photoreduction of the NBT (Beauchamp and Fridovich, 1971).

Catalase (CAT): This was determined according to the protocol proposed by Havir and McHale (1987) through addition of 30 µL of the crude enzyme extract in 2.97 mL of the reaction medium potassium phosphate buffer (50 mM,
pH 7.0 and 12.5 mM H₂O₂). Enzyme activity was obtained based on reading in a spectrophotometer at the wavelength of 240 nm during the first minute of the reaction at 25 °C, and then calculated using the molar extinction coefficient of 36 M⁻¹.cm⁻¹ (Anderson et al., 1995). The results were expressed in µmol.min⁻¹.mg⁻¹ of protein.

**Peroxidase (POX):** This was determined by the addition of 50 µL of the crude enzyme extract to 2.95 mL of the reaction medium potassium phosphate buffer (25 mM, pH 6.8), 20 mM pyrogallol and 20 mM hydrogen peroxide. During the first minute of reaction, the increase in absorbance was observed at the wavelength of 420 nm at 25 °C. Enzyme activity was calculated using the molar extinction coefficient of 2.47 mM.L⁻¹.cm⁻¹ (Chance and Maehly, 1955) and expressed in µmol.min⁻¹.mg⁻¹ of protein.

**Ascorbate peroxidase (APX):** This was determined through addition of 50 µL of the crude enzyme extract in 2.95 mL of the reaction medium potassium phosphate buffer (50 mM, pH 7.8), containing 0.25 mM ascorbic acid, 0.1 mM EDTA and 0.3 mM H₂O₂. The decrease in absorbance at 290 nm was observed during the first minute at 25 °C. Enzyme activity was calculated using the molar extinction coefficient 2.8 mM⁻¹.cm⁻¹ (Nakano and Asada, 1981), and the result was expressed in µmol.min⁻¹.mg⁻¹ of protein.

**Protein content:** This was determined by the method of Bradford (1976), using BSA as a standard. The quantity of 100 µL of the enzyme extract was used, adding 1 mL of the Bradford reagent, followed by shaking. After twenty minutes, absorbance of the sample was read in a spectrophotometer at 595 nm. The data were used for calculations of antioxidant enzyme activity.

**Experimental design and statistical analysis:** The experiment was set up in a completely randomized design with four replications. The data obtained in the tests of germination, first germination count, plant length and plant dry matter were analyzed in a 2 (lots) × 5 (osmotic potentials) factorial arrangement, and then by regression analysis. The data on enzyme activity, lipid peroxidation (MDA) and protein content were analyzed in a 2 (lots) × 5 (osmotic potentials) × 4 (days after sowing – DAS) factorial arrangement and were represented by the mean value, with the respective standard deviation.

**RESULTS AND DISCUSSION**

The two sunflower seed lots had similar germination percentages. Nevertheless, they differed in regard to vigor by the tests of first germination count, accelerated aging, seedling emergence, emergence speed index and electrical conductivity, with greater vigor for the seeds of lot 1 (Table 1).

There was reduction in germination and in first germination count in both seed lots with the decrease in osmotic potential (Figure 1). The increase in osmotic concentration of the PEG 6000 solution reduces water absorption by

| Lot | Germination (%) | First germination count (%) | Accelerated aging (%) | SL (cm.seedling⁻¹) | RL (cm.seedling⁻¹) |
|-----|-----------------|----------------------------|-----------------------|-------------------|-------------------|
| 1   | 95 A            | 91 A                       | 92 A                  | 11.9 A            | 13.7 A            |
| 2   | 91 A            | 80 B                       | 73 B                  | 10.1 A            | 12.4 A            |
| CV (%) | 3.0          | 5.5                       | 11.3                  | 18.0              | 24.3              |

| Lot | SDM (mg.seedling⁻¹) | RDM (mg.seedling⁻¹) | Emergence (%) | ESI | Electrical conductivity (μS.cm⁻¹.g⁻¹) |
|-----|---------------------|---------------------|---------------|-----|-------------------------------------|
| 1   | 45.0 A              | 15.9 A              | 100 A         | 11.1 A | 65.2 A                             |
| 2   | 44.8 A              | 12.4 A              | 86 B          | 8.9 B  | 75.7 B                             |
| CV (%) | 17.7            | 17.8               | 4.5           | 9.4  | 3.4                                |

SL = shoot length; RL = root length; SDM = shoot dry matter; RDM = root dry matter; ESI = emergence speed index. Mean values followed by the same letter in the row do not differ from each other by the F test (p < 0.05).
seeds, causing reduction in germination percentage (Lewandrowski et al., 2017). Generally, the germination percentage decreases along with the decrease in water potential, but for each species, there is a potential at which there is no germination (Ávila et al., 2007).

Results of the germination test showed that the two seed lots had linear reduction under water restriction. However, lot 1 (greater vigor) had higher germination than lot 2 (lower vigor), regardless of the potential tested (Figure 1A). Considering first germination count (Figure 1B), the most drastic water restrictions (-0.6 and -0.8 MPa) had a similar effect for the two seed lots tested, with a percentage of normal seedlings near 0% (Figure 1B). In a study carried out with sunflower genotypes, González-Belo et al. (2014) observed that reductions in germination at the potentials -0.3, -0.6, -0.9 and -1.2 MPa and different temperatures were correlated with linoleic acid contents in the seeds.

According to Barros and Rossetto (2009), reduction in sunflower seed germination occurs beginning at the water potential of -0.3 MPa, and total inhibition of germination occurs at -0.9 MPa. In the present study, the germination of lot 2 seeds reached levels near 0 at the potential of -0.8 MPa and of approximately 20% in lot 1 seeds (greater vigor) (Figure 1). It has already been observed that water stress in sunflower seeds brings about irregular germination and uneven establishment of seedlings (Albuquerque and Carvalho, 2003), and it reduces germination speed, as was observed in the first germination count test (Figure 1B).

Water restriction affected the initial development of the sunflower seedlings, with reduction in shoot length and root length as water restriction increased in the two seed lots (Figure 2). In general, water restriction affects germination and seedling development due to lower digestion and distribution of assimilates, and it limits diverse metabolic processes involved in the formation of new plant tissues and cell elongation (Bewley et al., 2013; Finch-Savage and Bassel, 2016).

Similar results were obtained by Carneiro et al. (2011), where the shoot length of sunflower seedlings declined in a linear manner as the PEG 6000 concentration increased, with the lowest values at the potential of -0.8 MPa. These authors reported that the root length at the potentials of -0.2 and 0.4 MPa was greater than the control, decreasing from this water potential on, which was also observed in the present study (Figure 2B). These results can be explained by the adaptation mechanisms of the seedlings when they were under water stress, such as directing photoassimilates to greater root growth in an attempt to increase water uptake.

Another possible effect on reduction of sunflower seedling growth under water restriction is lower chlorophyll production, as observed by Singh et al. (2015) and Manivannan et al. (2015). Such reductions were also observed by
Farjadeh et al. (2017) in different sunflower lines under water stress, and they associated these results to damage to the chloroplasts, caused by ROSs. In addition, the biosynthesis of chlorophyll precursors may be compromised under osmotic stress (Moharramnejad et al., 2015). Thus, reduction in osmotic potential led to a reduction in the germination percentage of the lots, regardless of seed vigor (Figure 1), associated with lower seedling growth, with reduction in shoot and root length (Figure 2).

In general, SOD activity was similar for the two seed lots tested, observing little change up to 4 days after sowing. In lot 1 (greater vigor), a slight reduction in activity was observed after four days for the potentials -0.2 and -0.8 MPa (Figure 3A). In lot 2 (lower vigor), this reduction was observed at all the osmotic potentials (Figure 3B). SOD constitutes a group of metalloenzymes that catalyze the dismutation or disproportionation of superoxide radicals (O$_2^-$) into molecular oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$) and act in the first line of antioxidant defense (Del Río et al., 2018; Wang et al., 2016). Consequently, the increase in SOD activity is known to confer tolerance to oxidative stress caused by adverse environmental conditions (Jaleel et al., 2007). In a different way than observed in the present study, Fernández-Ocaña et al. (2011) observed a significant increase in SOD activity in sunflower seedlings under stress from low temperatures, even without apparent oxidative stress. These authors associate these observations to a genetic expression that activates this enzyme to prevent potential oxidative stress.

In lot 2 (lower vigor), the increase in SOD activity regardless of the potential, in relation to the control, was not observed, and was greatest at the potential of -0.8 MPa in all the times analyzed (Figure 3B). According to Bailly (2004), low vigor seeds have lower efficiency in elimination of ROSs, generating oxidative stress. In this lot, reduction in SOD activity for all the potentials tested from the fourth day on confirms the lower capacity of activation of this enzyme in lower vigor seeds (Figure 3B).

CAT is present in glyoxysomes and peroxisomes and, together with the peroxidases, it is responsible for conversion of H$_2$O$_2$ into O$_2$ (Kibinza et al., 2011; Willekens et al., 1995). The activity of this enzyme was affected differently over the germination period in lots 1 and 2, and was generally higher in lot 1 (Figures 4A and B).

There was variation in CAT activity over the germination period in the two lots tested. In lot 1 (greater vigor), an increase was observed at the potential of -0.6 MPa up to four days, decreasing at six days. At the potentials of -0.2 and -0.4 MPa, the response was different, with reduction in activity from 0 to four days and increase at six days. Comparing the different potentials, at six days, differences among the potentials 0, -0.2 and -0.8 MPa were not observed, and they were significantly higher at the potentials -0.4 and -0.6 MPa (Figure 4A). In lot 2 (lower vigor), in general, a reduction was observed in activity.
up to two days, followed by an increase on the fourth day, and once more a decrease at six days (Figure 4B).

Reduction in CAT activity with the decrease in osmotic potential coincide with reduction in vigor observed by the tests of first germination count and shoot and root length (Figures 1 and 2). Carneiro et al. (2011) evaluated sunflower seedlings and found reduction in CAT activity at the water potential of -0.8 MPa, also induced by PEG 6000. In contrast, Naderi et al. (2014) observed an increase in CAT activity in wheat seedlings under the potentials of -0.4 and -0.8 MPa for five days.

Just like CAT, POX oxidizes organic substrates, with H$_2$O$_2$ as the electron receptor molecule, resulting in release of H$_2$O and O$_2$ (Mittler, 2002). POX activity was lower in the treatments with water stress in relation to the control (0 MPa) for the two lots evaluated, and it was more evident in lot 1 (higher vigor) (Figure 5). For the two lots, the potentials of
0 and -0.2 MPa resulted in higher POX activity, regardless of the exposure time. In lot 1 (higher vigor), this increase was more accentuated from the second day on, with an even more significant increase from the fourth day on (Figure 5A).

In lot 2 (lower vigor), this increase was less accentuated, especially for the potentials of -0.4, -0.6 and -0.8 MPa (Figure 5B). These results may be due to the delay in seedling development and to the sensitivity of the less vigorous lots to stress. However, as CAT activity was greater (Figures 4A and B) and these enzymes exercise similar functions, such observations may also be associated with equilibrium of activity of these enzymes, especially in relation to lot 2. Similar to the present study, Manivannan et al. (2014) reported increases in POX activity in roots, stems and leaves of five sunflower cultivars under water stress.

The APX enzyme participates in conversion of $\text{H}_2\text{O}_2$ into $\text{O}_2$ through a series of oxidations in the glutathione/ascorbate cycle (Caverzan et al., 2012). Similar to POX, APX activity was more accentuated for the higher vigor lot (Figures 6A and B). These results are in agreement with germination percentage and shoot and root length, which

![Figure 5. Activity of the enzyme peroxidase (POX) determined at 0, 2, 4 and 6 days after sowing for seedlings from lots 1 (A) and 2 (B) of sunflower, cultivar Hélio 253, under water restriction in PEG 6000 solutions.](image)

![Figure 6. Activity of the enzyme ascorbate peroxidase (APX) determined at 0, 2, 4 and 6 days after sowing for seedlings from lots 1 (A) and 2 (B) of sunflower, cultivar Hélio 253, under water restriction in PEG 6000 solutions.](image)
were lower in the lower quality lot when the seeds were placed under water restriction (Figures 1 and 2).

In lot 1, there was an increase in APX activity on the second day in all the treatments, and the greatest activity was found at the potential of -0.6 MPa and the lowest activity in the control treatment. From the fourth day on, APX activity at the water potential of -0.8 MPa declined significantly, while in the treatments with a lower level of stress, activity declined especially from the second day on (Figure 6A).

In a way similar to lot 1, lot 2 (lower vigor) generally had greater APX activity in relation to the control in all the periods evaluated, especially from the second day on (Figure 6B). Chakraborty and Pradhan (2012) found an increase in the activity of this enzyme in wheat seedlings on the third day of germination under water stress. Baloğlu et al. (2012) conducted a study on the effect of water stress in sunflower seedlings and concluded that APX is important for protection of root tissues in this species under more severe stress conditions. Locato et al. (2010) affirm that some enzymes of the peroxidase family are expressed in a constitutive manner. Others may be induced by environmental stresses, such that under more intense stress situations, there is greater activity of the APX enzyme.

It is important to emphasize that the ROSs are predominantly beneficial to plant metabolism, performing diverse functions, from germination to cell signaling (Mittler, 2017). In addition, Manivannan et al. (2014) state that the level of enzyme activity of the antioxidant system depends not only on the species and vigor, but also on the duration and intensity of the stress, which was generally observed in this study. In general, considering the enzymes that act as peroxidases (CAT, POX, and APX), an increase can be seen in the activity with the longer time of exposure to water restriction, especially for the seeds with highest vigor (lot 1).

**CONCLUSIONS**

Water restriction led to a decrease in germination and lower growth of seedlings, regardless of the vigor level of the seeds.

Water restriction affects antioxidant enzyme activity in sunflower seeds, especially beginning at two days of exposure to stress.

Lower vigor seeds have lower capacity for activation of antioxidant enzymes, especially the peroxidases.

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