

ANTIOXIDANT CAPACITY IN LEAF AND ROOT TISSUES OF *PRUNUS* SPP UNDER FLOODING

ABSTRACT

The aim of this study was to evaluate the antioxidant responses and cellular damage in 'América' Japanese Plum and 'GxN-9' peach rootstock plants under soil flooding stress. Two-year-old plants propagated by cuttings were transplanted to pots (4 L) and two months after planting, they were submitted to different flood periods (2, 4 and 6 days), each period being composed of two treatments, control (irrigated daily to field capacity) and water stress (soil flooding). After each stress period, cells damage was evaluated by measuring lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2), and the specific activity of the antioxidant enzymes Superoxide Dismutase (SOD), Catalase (CAT) and Ascorbate Peroxidase (APX) from 200 mg of leaf and root tissues. Under stress conditions, an increase in the activity of the antioxidant enzymes and the levels of H_2O_2 and MDA in both cultivars and tissues were observed. Leaves showed the highest cellular damage when compared with roots, which suggests higher activity of photosynthetic and photorespiratory metabolism. The values of MDA and H_2O_2 were lower in America when compared to GxN-9, suggesting that the antioxidant system of the Japanese Plum cultivar is more efficient in relation to the peach rootstock under soil flood conditions.

Key-words: ROS, rootstocks, peach, plum, hypoxia.

1. INTRODUCTION

The peach tree (*Prunus persica*) is an important specie for Brazilian fruit production, and state of Rio Grande do Sul stands out as the main producer, with 127 thousand tons a year and represents 60% of the national production [1]. However, even if they hold the largest national production of peaches, their orchards have average productivity considered low ($\approx 10 \text{ ton ha}^{-1}$). One of the causes associated with low productivity is the mode of production of peach rootstocks that occurs in most cases using seeds of cultivar scions obtained from canned peach industries. This type of material does not have genetic and sanitary quality control, negatively influencing the production and yield of stone fruits [2].

Additionally, in the southern region of Rio Grande do Sul, another limiting cause for peach production is the climatic condition, where periods of drought or excessive rainfall can occur during the crop cycle, which combined with soils that are deficient in drainage can affect the production of this crop. Soil flooding is one of the most damaging environmental conditions in the region, especially for stone fruit trees. Such stress often occurs concurrently with one of the major plant development stages and critical to productivity, which are flowering and sprouting of buds [3]. This fact is intensified by the lack of definition of rootstocks that meet all the environmental needs of the region.

Abiotic stresses alone or in combination limit the growth, development and plant yield, mainly impairing the physiological and biochemical functioning of the cell by promoting oxidative stress [4,5]. In this way, flooding generates a stress condition for the plant. According to Oukarroum et al. [6], the adaptability of the plants under stress conditions were influenced by the duration and magnitude of the stress, in addition to the genetic variability of the plant.

Flooding causes restrictions on the cultivation of many species due to the low availability of oxygen to the root system [7], which can be harmful or lethal for plants. This situation blocks the transfer of oxygen and other gases between the soil and the atmosphere [8], activating in the roots metabolism of anaerobic respiration, significantly reducing energy production and the plant starts to obtain metabolic energy mainly through the associated glycolytic pathway to the fermentation processes [9,10].

The production of reactive oxygen species (ROS) in plants is common, occurring through several metabolic reactions and in multiple cell compartments [11]. ROS such as superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are also produced in response to various abiotic stresses and an accumulation of these leads to cellular imbalance, causing oxidative stress [12,13,14]. Higher levels of ROS can result in extensive damage to proteins,

DNA and lipids, which affects cellular functions, and may promote permanent metabolic dysfunction and plant death [4,5]. To minimize the stresses caused by ROS, the plants developed an antioxidant defense system, formed by enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) [15].

The genus *Prunus* includes fruit-producing species (peach, plum, almond, apricot and cherry), but several are used as rootstocks [3]. The plums are represented by species with different degrees of ploidy, presenting the highest taxonomic diversity within the seedlings and exhibit higher levels of tolerance and adaptability to a wide range of climatic factors [3,16]. When compatible with the scion cultivars, the plum trees may appear as good alternatives for use as rootstock to produce stone fruits in the southern area of Rio Grande do Sul.

Since the information about the *Prunus* reaction to flood stress and the fact that the cultivars respond differently to the hypoxic conditions [17], the aim of this study was to evaluate the antioxidant activity of SOD, APX and CAT enzymes, as well as to quantify cellular damages in rootstock plants of 'GxN-9' almond-peach hybrid and 'America' Japanese Plum under soil flooding.

2. MATERIAL AND METHODS

Plants of 'GxN-9' almond-peach hybrid (*Prunus dulcis* Mill. Bastsch × *Prunus persica* L.) and 'America' Japanese Plum (*Prunus salicina* Lindl) with 2-years-old, propagated by cuttings, were transplanted to 4-liter pots, containing as substrate orchard soil (typical dystrophic yellow red argisol) [18]. Two months after planting, the plants were submitted to different periods of flooding (2, 4 and 6 days), each period being composed of two treatments: control (irrigated daily to the field capacity) and soil flooding (water blade until 5 cm above substrate level).

To determine the specific activity of the antioxidant enzymes and cellular damage, after each period of water stress, approximately 200 mg of leaf tissue (completely expanded leaves) and root (young roots) were collected and stored in ultra-freezer at -80 °C until the moment of the analyzes.

For the antioxidant enzymes analysis, the plant material was grinded in liquid nitrogen with 20% insoluble PVP (polyvinylpyrrolidone) and homogenized with 3.6 ml of extraction buffer (100 mM potassium phosphate pH 7.0), 0.1 EDTA mM and 10 mM ascorbic acid. After samples centrifugation, the supernatants were collected and desalted in sephadex column (GE Healthcare). The extracts were used for quantification of the proteins by the Bradford method [19], with readings at 595 nm, and quantification of SOD, APX and CAT enzymes activity.

The activity of SOD was based on the ability of the enzyme to inhibit the photoreduction of nitrotetrazolium blue (NBT) [20]. The readings were carried out at 560 nm. One unit of SOD corresponds to the amount of enzyme capable of 50% inhibition of NBT photoreduction under assay conditions.

The CAT activity was performed through the methodology established by Azevedo et al. [21], but with small modifications, which were estimated by decreasing the absorbance at 240 nm for 2 minutes in a reaction medium containing 100 mM potassium phosphate (pH 7.0) and 12.5 mM H₂O₂.

APX activity was determined according to Nakano & Asada [22], by monitoring the oxidation rate of ascorbate at 290nm. The decrease in absorbance for each reading was monitored for a period of two minutes.

Levels of cell damage were determined by lipid peroxidation through the quantification of thiobarbituric acid reactive species (TBARS), as described by Buege & Aust [23] and by quantification of the hydrogen peroxide (H₂O₂) content according to the methodology described by Sinha et al. [24].

The experimental design was completely randomized, for each cultivar, with two treatments (control and flooding), with three replicates per treatment, each replicate being composed of one plant. The results obtained were evaluated by means of the statistical program Sisvar (version 5.6), where analysis of variance was conducted, and the means compared within each day of the experiment and each cultivar through the Tukey test at 5% of probability.

3. RESULTS

For the SOD activity, in the plant tissues and cultivars evaluated, there was a gradual increase in the values a long with the days of stress. In both cultivars, the highest increment can be noticed at six days of stress, when compared to the control, both in leaves and roots. Peach leaves showed lower SOD activity values than those found in other tissues and evaluated cultivars, where it exhibits about 50% less activity in plants under normal growing conditions, as well as under stress conditions when compared to their roots or even with plum leaves (Fig. 1).

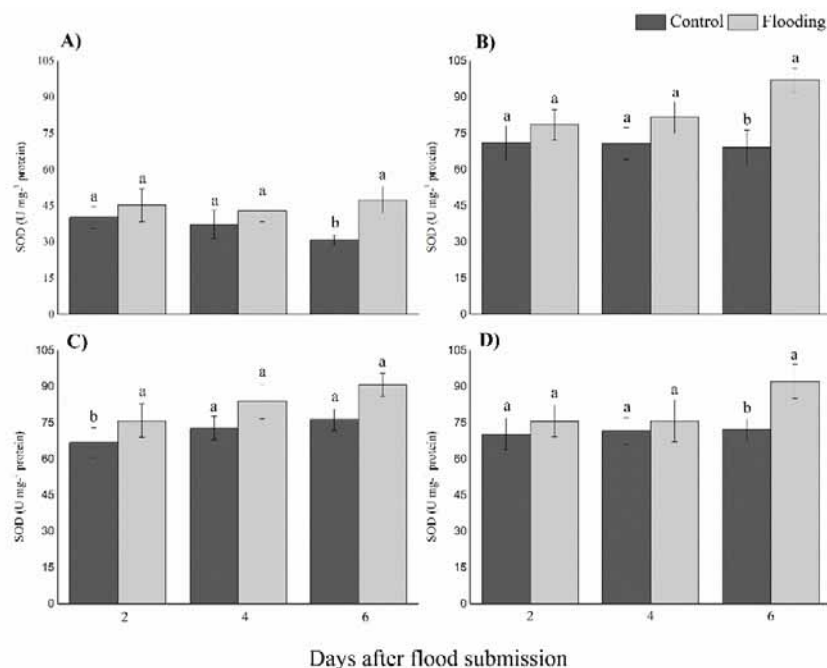


Fig. 1. SOD activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column).

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of three biological replicates.

Averages followed by the same letters do not differ between themselves by the Tukey test ($P = .05$).

Although APX activity in 'America' roots showed decreasing levels in stressed plants, it was not statistically significant, and on the fourth day the leaves showed the highest activity of this enzyme (Figs. 2D and 2B). In the 'GxN-9' roots, the APX activity increased over the days, and on the sixth day the stressed plants presented 25% more activity compared to the control (Fig. 2C). However, in 'GxN-9' leaves, APX activity presented less than half of the values found when compared to the other tissues and cultivars evaluated (Fig. 2A).

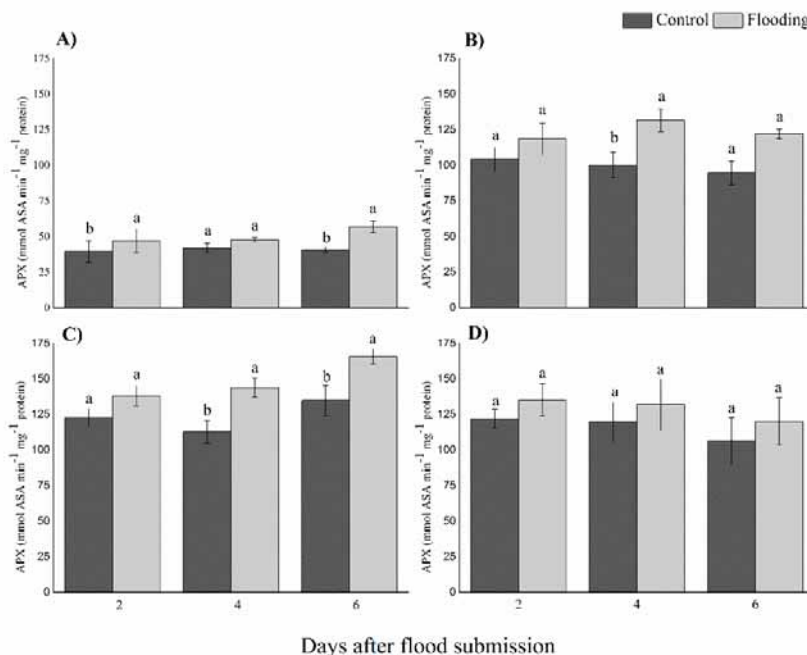


Fig. 2. APX activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column).

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of three biological replicates.

Averages followed by the same letters do not differ between themselves by the Tukey test ($P = .05$).

Leaves and roots of 'GxN-9' showed similar values of CAT activity, with a gradual increase over the days, and on the sixth day of stress the values increased by 20 and 25% in leaves and roots, respectively (Figs. 3A and 3C). The CAT activity in leaves of 'America' cultivar presented greater difference in relation to the control at the fourth and sixth days (Fig. 3B). However, in roots, CAT activity in control and flooded plants remained stable throughout the days (Fig. 3D).

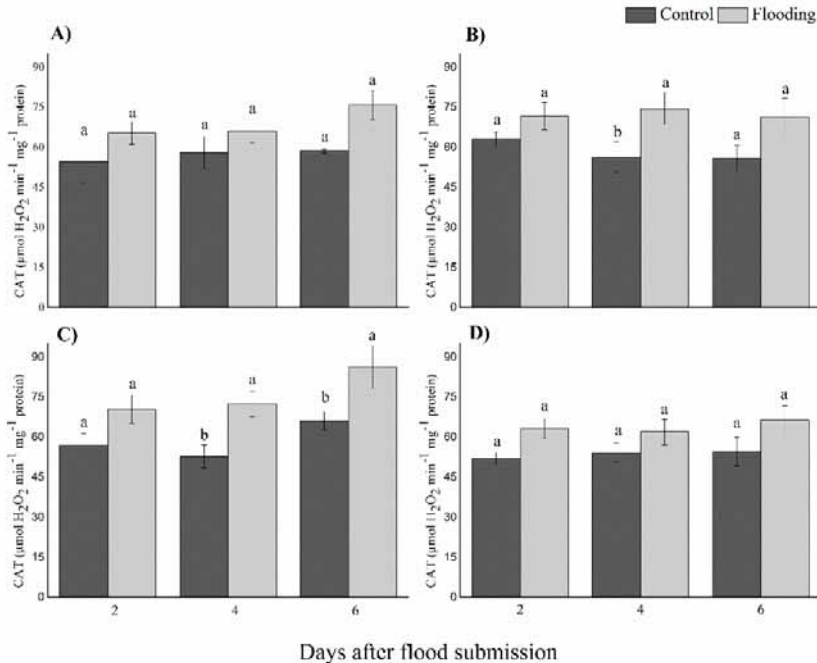


Fig. 3. CAT activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column).

Mean $\pm$ S.E.M = Mean values $\pm$ Standard error of means of three biological replicates.

Averages followed by the same letters do not differ between themselves by the Tukey test ($P = .05$).

To evaluate the cellular damage levels, hydrogen peroxide (H₂O₂) and lipid peroxidation (MDA) were quantified. For both quantifications, in the roots of the two cultivars evaluated, the values were stable throughout the stress time and below those found in leaves (Figs. 4C, 4D, 5C and 5D). Additionally, the fourth day of stress provided the highest increase in MDA and H₂O₂ levels in 'GxN-9' leaves (Figs. 4A and 5A). In 'América' leaves, MDA and H₂O₂ levels remained practically stable in stressed plants during the experiment, with a greater difference in values compared to each control at four and six days of stress (Figs. 4B and 5B).

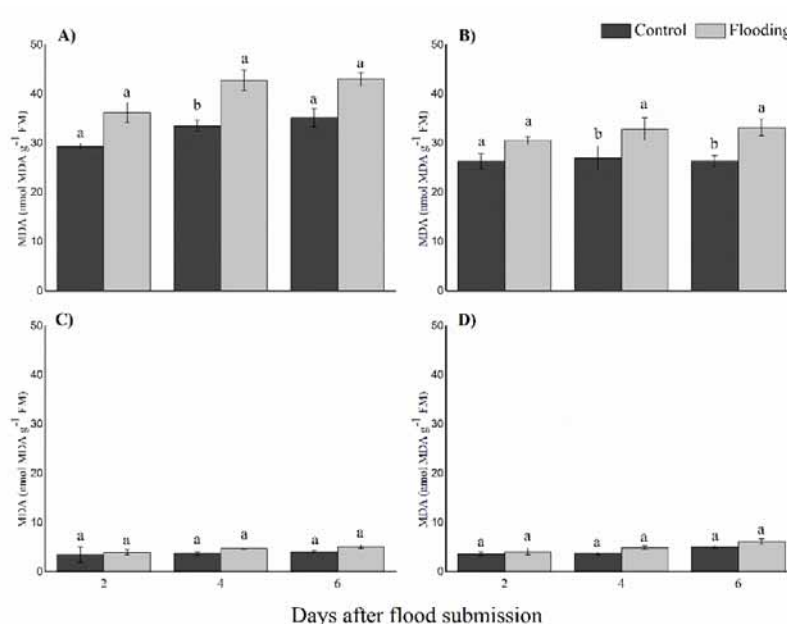


Fig. 4. MDA level in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column).

Mean ± S.E.M = Mean values ± Standard error of means of three biological replicates

Averages followed by the same letters do not differ between themselves by the Tukey test (P = .05)..

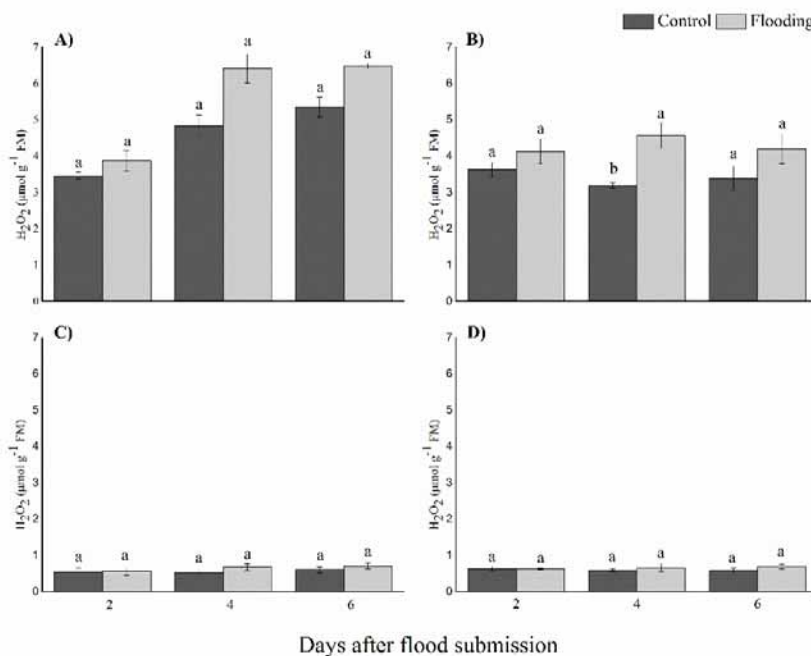


Figure 5. H₂O₂ level in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column).

Mean ± S.E.M = Mean values ± Standard error of means of three biological replicates.

Averages followed by the same letters do not differ between themselves by the Tukey test (P = .05)..

4. DISCUSSION

Plants are highly qualified in their redox control, using ROS and antioxidants to control various aspects of their biology, from gene expression to protein formation and metabolism regulation [25]. Additionally, under stressful conditions, plants use a highly regulated 'oxidative explosion' to alert metabolism, involving the production of superoxide and hydrogen peroxide, and thus modify the extracellular environment and increase oxidation in the cellular apoplast, while the cytosol remains in reduction [25,26].

ROS has been considered as possible and important signaling molecules capable of detecting the decrease in soil oxygen levels [27]. Therefore, there are two models that suggest either a reduction of ROS under conditions of oxygen deprivation or an increase ROS concentration due to inhibition of the mitochondrial electron transport chain [28].

In all evaluations for antioxidant enzymatic activity, the values recorded were higher in plants that were subjected to flood stress compared to control plants. This situation occurs due to the increase of the formation of reactive oxygen species, evidencing with the increase in the levels of MDA and H₂O₂ found in this study. The ability to maintain SOD, CAT and APX activity at high levels under stress conditions becomes essential for the balance between the formation and removal of intracellular ROS to maintain cellular homeostasis [29]. This behavior has been reported in several previous studies with different species of *Prunus* in hypoxia [30,31], where the performance of each cultivar varies depending on the capacity to tolerate oxygen deficiency [17], proving that there are varying levels of tolerance to soil flooding within *Prunus* genus [3,17,31,32].

Considering the main enzymatic antioxidants, SOD is the first line of defense against ROS, catalyzing the dismutation of O₂^{•-}, with the consequent formation of H₂O₂ and O₂, in mitochondria, chloroplasts, nucleus, peroxisomes, cytoplasm and apoplast [13,33].

It was observed that the SOD activity was progressively increased in leaves as well as roots in the two cultivars studied (Fig. 1). In 'GxN-9' leaves, SOD activity was much lower when compared to 'America' leaves, mainly at the sixth day of stress (Figs. 1A and 1B). Such behavior may indicate low O₂ production and high production of other ROS, justifying the greater accumulation of H₂O₂ in 'GxN-9', compared to 'America' (Figs. 5A and 5B). Gill et al. [33] reported that species with lower tolerance to abiotic stress tend to have a smaller increase in SOD, in contrast to moderately tolerant species, where SOD activity levels are higher, even in control plants. However, there are contradictions about the role of SOD in the tolerance to soil flooding, where species may respond with decreasing or increasing activity of this enzyme [34].

The results of the present study concerning SOD activity confirm what has been demonstrated in woody plants, such as *Citrus* spp. [35] and *Prunus* spp. [31]. Also confirm previous reports on *Prunus* spp., where plums are generally considered more tolerant to soil flooding than peach and peach hybrids [31,33,36,37].

Peroxidases and catalases are the most important enzymes involved in the regulation of the intracellular concentration of hydrogen peroxide [34]. APX catalyzes the reduction of hydrogen peroxide in water, using ascorbate as an electron donor, and it is found in the chloroplast, cytosol, peroxisome, apoplast and mitochondria [38]. CAT is ubiquitous in peroxisome, where it promotes the formation of water and oxygen from a H₂O₂ molecule [38].

Even though these enzymes perform similarly, the two exhibit distinctive features, ranging from subcellular location and biochemical properties, to the preferential metabolic utilization of one enzyme to another by different species, resulting in a versatile and flexible antioxidant system capable to control the ROS accumulation [28,38-39]. Another significant factor in the antioxidant activity is the Km of enzymes involved in the defense system, which influences the ability to control ROS metabolism during stress conditions. The low Km in the APX reaction reflects its higher affinity to H₂O₂ compared to CAT [40].

This was observed in roots of the two cultivars studied, demonstrating that in this organ there is a greater affinity of APX for H₂O₂, since a small accumulation of H₂O₂ produced by the root cells of the stressed plants was enough to promote the activation of this enzyme (Figs. 2C, 2D, 5C and 5D). In 'America' leaves, the highest affinity of H₂O₂ is also for APX (Fig. 2B), but in 'GxN-9' the highest activity seems to be related to CAT (Fig. 3A). Jimenez et al. [41] and Blokhina et al. [28] reported that APX plays an important role in hypoxia conditions due to the ability to convert H₂O₂ to H₂O with NAD(P)⁺ regeneration. In flooding situations, microbial and vegetable activities rapidly consume oxygen. As a result, mitochondrial respiration can be reduced or absent, which leads to an accumulation of NADH, and the energy gain is through glycolysis, associated with the fermentation pathway [42].

In relation to the cellular damage evaluated, Alves et al. [43] reported that increases in H₂O₂ and MDA concentrations are indicators of lipid peroxidation and, consequently, indicative of oxidative stress. Several types of environmental stresses can significantly increase ROS generation in plant

cells [33], and high levels of H₂O₂ may cause damage to membranes, since they accelerate the Haber-Weis reaction, resulting in hydroxyl radical formation and lipid peroxidation [27].

Lipid peroxidation is a metabolic process, occurring naturally in aerobic culture conditions, but it increases in anaerobic conditions, and is one of the most investigated consequences of ROS action on membrane structure and function [28]. Levels of H₂O₂ considered normal in cultivated plants that are enough to act as signaling molecules, turn around 0.5 µM H₂O₂, while plants under stress conditions can show values between 5 and 15 µM H₂O₂ [44].

In 'GxN-9' leaves, this behavior can be observed at four and six days after flooding, where the plants under stress presented a high level of H₂O₂ and above the values considered normal (Fig. 5A). In 'America' leaves, even if the H₂O₂ levels of the stressed plants were higher than the control, they were below those found in 'GxN-9' (Fig. 5B) and within a range where it does not consider enough for cause greater damage to cells [44]. In roots of both cultivars, in control and stressed plants, the H₂O₂ levels remained between 0.5 and 1.0µmol, values much lower than those found in leaves (Figs. 5C and 5D) but considered adequate for the metabolism occurs without major damage [44]. Consequently, the cellular damage evaluated by MDA followed the same trend of H₂O₂ levels, being high in leaves and low in roots (Figs. 4 and 5).

This difference can be explained by the fact that one of the main producers of plant ROS is the photosynthetic apparatus [45,46]. Additionally, under stress conditions, high concentrations of H₂O₂ may also be generated in the peroxisomes due to increased photorespiratory activity, which occurs by the decrease in the specificity of rubisco for CO₂ compared to O₂ and the lower relative solubility of CO₂ than O₂ [47].

Once the photorespiration begins, the displacement of H₂O₂ production from the chloroplasts to the peroxisomes is considered less dangerous, and the peroxisomes are more appropriate to deal with H₂O₂ abundance, mainly due to the high concentration of CAT in this compartment [46], which justifies its greater accumulation compared to APX in leaves of 'GxN-9' recorded in the present study (Figs. 2A and 3A).

Prunus species do not have anatomical adaptations as occurs in plants adapted to intermittent flooding [48,49]. As shown in the literature, plants can only survive a hypoxia/anoxia environment if they maintain ATP production and recycle NADH. Therefore, cells begin to limit processes that are highly energetic and begin to operate in anaerobic conditions in low O₂ situations [7].

Additionally, there are few studies that demonstrate the antioxidant activity in roots of woody plants under conditions of soil flooding. Therefore, the results of the present study, besides beginning to unravel the root behavior of *Prunus* spp. under conditions of soil flooding, indicate an activation of the antioxidant system in the species and tissues evaluated at different levels in this stress condition.

5. CONCLUSIONS

The higher APX activity along with CAT activity in roots of both cultivars evaluated suggests that the antioxidant system was effective during stress and maintained low levels of H₂O₂, which were enough to promote cell signaling without causing major damage to membrane lipids, corroborating the low MDA values found in this study.

In leaves of both cultivars, the major activities of APX and CAT were not able to control the oxidative explosion, since H₂O₂ and MDA levels remained high, probably due to the action of photosynthetic and photorespiratory metabolisms.

The Japanese Plum cultivar América has a more effective antioxidant system compared to 'GxN-9', able to maintain H₂O₂ and MDA levels lower than the peach tree.

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