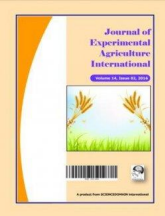

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Antioxidant Capacity in Leaf and Root Tissues of *Prunus* spp under Flooding

Elizete Beatriz Radmann[1], Elsa Kuhn Klumb[2*], Sidnei Deuner[2] and Valmor João Bianchi[2]

[1]Universidade Federal do Pampa – UNIPAMPA; Dom Pedrito – RS; Brasil.
[2]Universidade Federal de Pelotas – UFPel; Campus Capão do Leão; S/N; CEP: 96160-000 - RS; Brasil.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present study aims 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 stressed 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 the higher activity of photosynthetic and photorespiratory metabolism. The values of MDA and H_2O_2 were lower in America when compared to GxN-9; indicating that the antioxidant system of the Japanese Plum cultivar is more efficient as compared to the peach rootstock under soil flood conditions.

**Corresponding author: E-mail: elsakk91@yahoo.com.br;*

Keywords: ROS; rootstocks; peach; plum; hypoxia.

1. INTRODUCTION

The peach tree (*Prunus persica*) is an important species 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 of ≈10 ton ha^{-1}; which is considered as low. 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 as well as sanitary quality control; thereby; negatively influencing the production and yield of stone fruits [2].

Additionally; in the southern region of Rio Grande do Sul; another limitation 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; 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 limits 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 was influenced mainly by the duration and magnitude of the stress; along with the genetic variability of the plant.

Flooding causes restrictions on the cultivation of many species due to the low availability of oxygen in 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 ($O_2^{\bullet-}$); singlet oxygen ($^1O_2$); hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are also produced in response to various abiotic stresses. An accumulation of these leads to cellular imbalance; causing oxidative stress [12,13,14]. Higher levels of ROS can results in extensive damage to proteins; DNA and lipids; which affects cellular functions; and may promote permanent metabolic dysfunction and plant mortality [4,5]. To minimise the stresses caused by ROS; the plants developed an antioxidant defence 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 with 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 condition.

2. MATERIALS AND METHODS

Plants of 'GxN-9' almond-peach hybrid (*Prunus dulcis* Mill. Bastsch × *Prunus persica* L.) and 'America' Japanese Plum (*Prunus salicina* Lindl) of 2-years-old; propagated by cuttings; were transplanted to 4 litre pots; containing orchard soil (typical dystrophic yellow red argisol) as the substrate [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: (i) control (irrigated daily to the field capacity) and; (ii) 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 analyses.

For the antioxidant enzymes analysis; the plant material was grinded in liquid nitrogen with 20% insoluble PVP (polyvinylpolypyrrolidone) and homogenised 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 Bradford method [19]; with a frequency of 595 nm; and quantification of SOD; APX and CAT enzyme 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 measured 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 by the standard protocol of 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 H_2O_2.

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

Levels of cell damage were determined by lipid peroxidation through the quantification of thiobarbituric acid reactive species (TBARS); as described by Buege and Aust [23] and by quantification of the hydrogen peroxide (H_2O_2) content according to the methodology described by Sinha et al. [24].

The experimental design was completely randomised; for each cultivar; with two treatments (control and flooding); and three replicates per treatment; each replicate being composed of one plant. The results obtained were evaluated using 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 level.

3. RESULTS

For the SOD activity; in plant tissues and cultivars evaluated; there was a gradual increase in the values along with the days of stress. In both cultivars; the highest increment can be reported 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 exhibited 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).

Although APX activity in 'America' roots showed decreasing levels in stressed plants; it was not statistically significant; and on the fourth day; the leaves exhibited 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 in compared to the control (Fig. 2C). However; in 'GxN-9' leaves; APX activity presented less than half of the values when compared to the other tissues and cultivars evaluated (Fig. 2A).

Leaves and roots of ‘GxN-9’ recorded 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 a 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).

To evaluate the cellular damage levels; hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) were quantified. For both the quantifications; in the roots of the two cultivars evaluated; the values were stable throughout the stress condition 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 H_2O_2 levels in 'GxN-9' leaves (Figs. 4A and 5A). In ‘América’ leaves; MDA and H_2O_2 levels remained practically stable in stressed plants during the experiment; with a greater difference in values compared to each control at fourth and sixth days of stress (Figs. 4B and 5B).

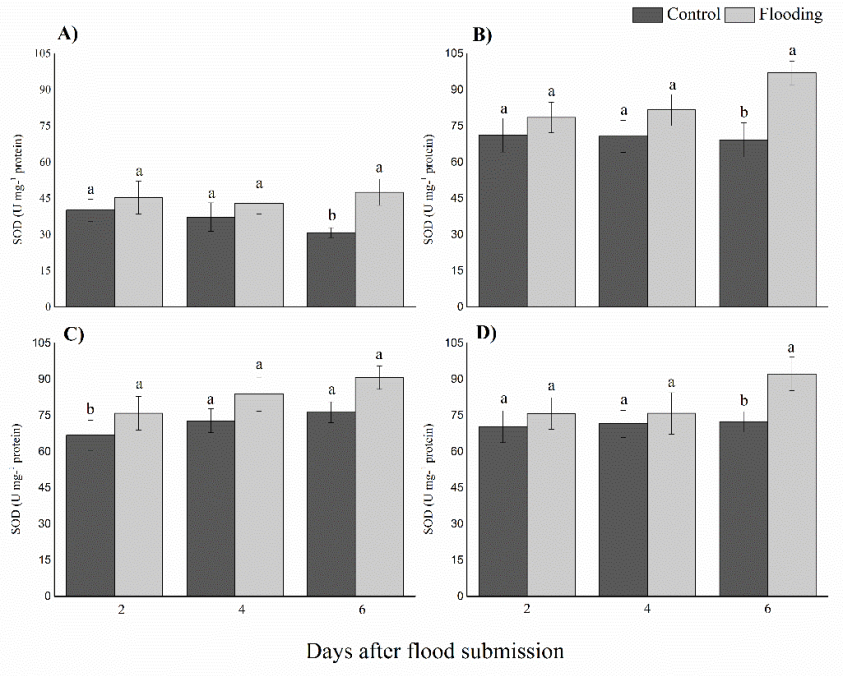

Fig. 1. SOD activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column)

Mean ± 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 = 0.05)

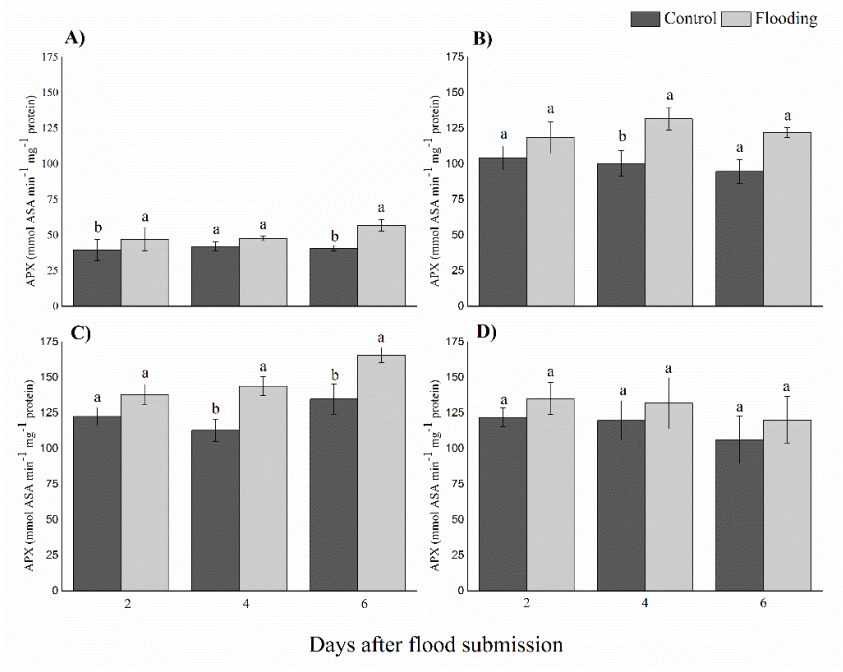

Fig. 2. APX activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column)

Mean ± 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 = 0.05)

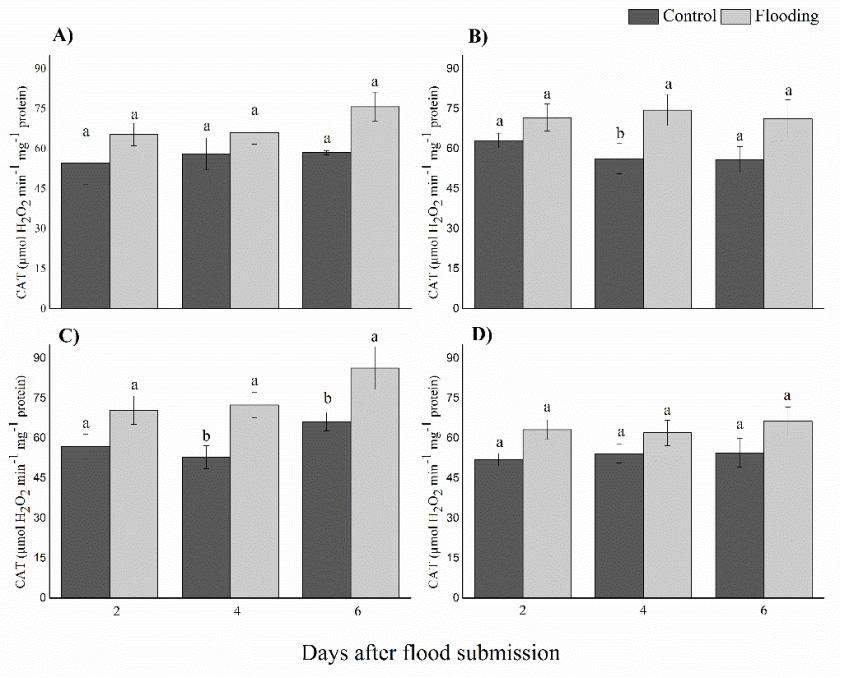

Fig. 3. CAT activity in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column)

Mean ± 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 = 0.05)

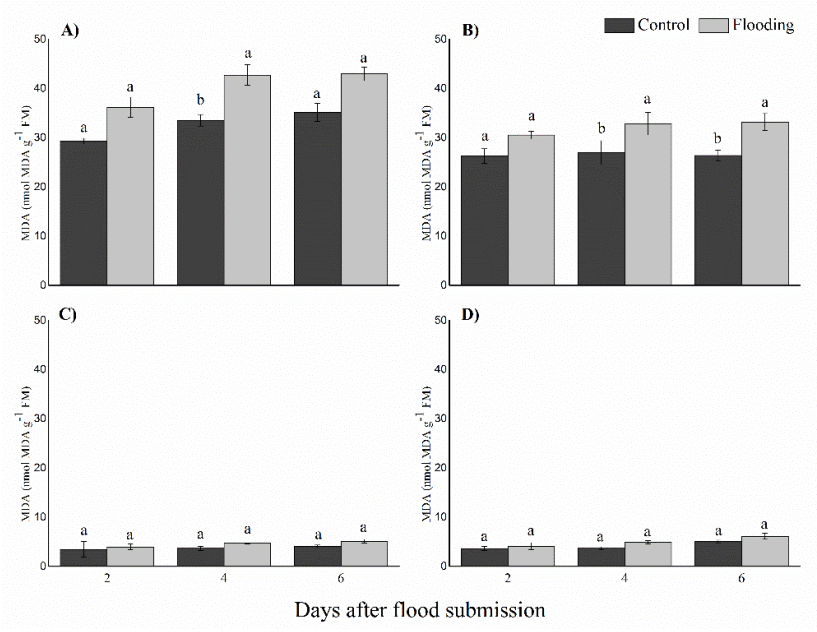

Fig. 4. MDA level in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column)

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 = 0.05)

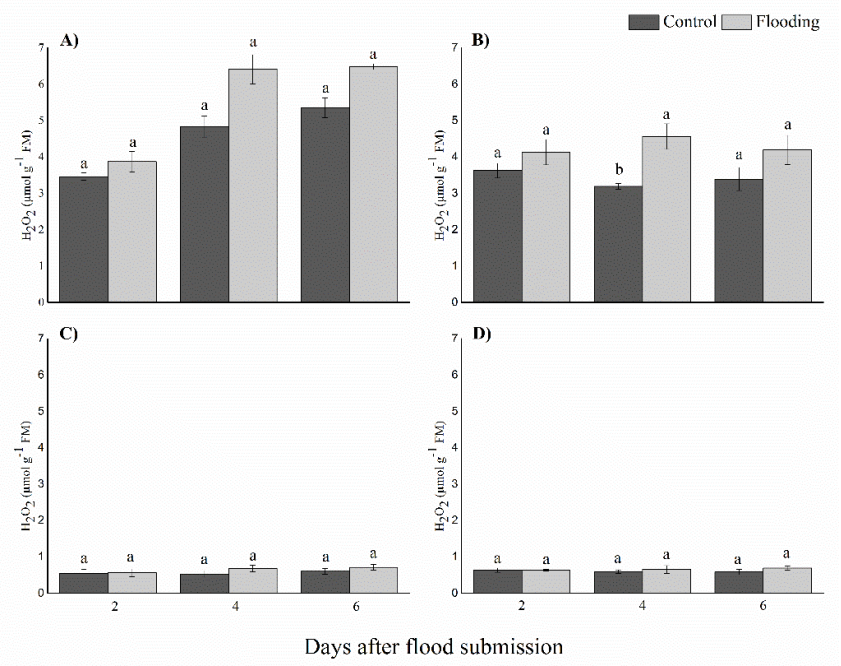

Fig. 5. H_2O_2 level in leaves (A and B) and roots (C and D) of 'GxN-9' (left column) and 'America' plants (right column)

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 = 0.05)

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 signalling molecules capable of detecting the decrease in soil oxygen levels [27]. Therefore; two models suggest either a reduction of ROS under conditions of oxygen deprivation or an increase in 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 the control plants. This situation occurs due to the increase in the formation of reactive oxygen species; evidencing with increase in the levels of MDA and H_2O_2 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 behaviour 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 defence against ROS; catalysing the dismutation of $O_2^{\bullet-}$; with the consequent formation of H_2O_2 and O_2; in mitochondria; chloroplasts; nucleus; peroxisomes; cytoplasm and apoplast [13,33].

It was revealed that the SOD activity was progressively increased in leaves as well as in roots for the two studied cultivars (Fig. 1). In 'GxN-9' leaves; SOD activity was much lower when compared to 'America' leaves; mainly on

the sixth day of stress (Figs. 1A and 1B). Such behaviour may indicate low O_2 production and high production of other ROS; suggesting the greater accumulation of H_2O_2 in 'GxN-9'; compared to 'America' (Figs. 5A and 5B). Gill et al. [33] endorsed 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 present study concerning SOD activity confirms what has been demonstrated in woody plants; such as *Citrus* spp. [35] and *Prunus* spp. [31]. The results 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 catalyses 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 H_2O_2 molecule [38].

Even though these enzymes perform similarly; the two exhibit distinctive features; ranging from subcellular location and biochemical properties; to the preferential metabolic utilisation 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 defence system; which influences the ability to control ROS metabolism during the stress conditions. The low Km in the APX reaction reflects its higher affinity to H_2O_2 in compared to CAT [40].

This was observed in roots of the two cultivars studied; demonstrating that in this organ there is a higher affinity of APX for H_2O_2; since a small accumulation of H_2O_2 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 H_2O_2 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] opined that APX plays an essential role in hypoxia conditions due to the ability to convert H_2O_2 to H_2O with $NAD(P)^+$ regeneration. In flooding conditions; 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] endorsed that increases in H_2O_2 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 H_2O_2 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 H_2O_2 considered normal in cultivated plants that are enough to act as signalling molecules; turn around 0.5 μM H_2O_2; while plants under stress conditions can show values between 5 and 15 μM H_2O_2 [44].

In 'GxN-9' leaves; this behaviour can be observed at four and six days after the flooding; where the plants under stress presented a high level of H_2O_2 and above the values considered normal (Fig. 5A). In 'America' leaves; even if the H_2O_2 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 to cause more significant damage to cells [44]. In roots of both cultivars (control and stressed plants); the H_2O_2 levels remained between 0.5 and 1.0 μmol; 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 similar trend of H_2O_2 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 stressed conditions; high concentrations of H_2O_2 may also be generated in the peroxisomes due to increased photorespiratory activity; which occurs by the decrease in the specificity of rubisco for CO_2 compared to O_2 and the lower relative solubility of CO_2 than O_2 [47].

Once the photorespiration begins; the displacement of H_2O_2 production from the chloroplasts to the peroxisomes is considered less dangerous; and the peroxisomes are more appropriate to deal with H_2O_2 abundance; mainly due to the high concentration of CAT in this compartment [46]. This justifies its greater accumulation compared to APX in leaves of 'GxN-9'; as 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 O_2 situations [7].

Additionally; few studies 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 behaviour 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 stressed 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 stressed condition and maintained low levels of H_2O_2; which were enough to promote cell signalling without causing major damage to membrane lipids; corroborating the low MDA values observed in this study.

In leaves of both cultivars; the major activities of APX and CAT were not able to control the oxidative explosion; since H_2O_2 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'; and is able to maintain H_2O_2 and MDA levels lower than the peach tree.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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