EFFECTS OF HYDROGEN PEROXIDE ON INITIAL GROWTH AND ENZYMATIC ANTIOXIDANT SYSTEM OF LACTUCA SATIVA L (ASTERACEAE)

RODRIGO MIRANDA MORAES, MARÍLIA CARVALHO, FERNANDA CARLOTA NERY, PLINIO RODRIGUES SANTOS FILHO, MARINA LIMA NOGUEIRA AND SANDRO BARBOSA

1Universidade Federal de Alfenas; Departamento de Ciências da Natureza, Alfenas – MG, Brazil
2Universidade Federal de Lavras; Departamento de Biologia, Lavras – MG, Brazil
3Universidade Federal de Alfenas; Departamento de Bioquímica, Alfenas – MG, Brazil
*Corresponding author’s email: sandro.barbosa@unifal-mg.edu.br

Abstract

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), can function as signaling molecules in basic cellular processes in plants, or cause oxidative damage to biomolecules when in excess. The objective of this study was to evaluate H_2O_2 phytotoxicity on germination, growth, initial development and cell cycle, in addition to the response of the enzymatic antioxidant system of L. sativa. For this purpose, L. sativa seeds were exposed to H_2O_2 solutions at the following concentrations: 0.0, 1.0, 2.5, 5.0, 7.5 and 10 mM for a period of 7 days. After 24 hours of exposure to H_2O_2, there was a significant reduction in germination percentage from 2.5 mM. With the increase in H_2O_2 concentration, there was a reduction in root length, and seedling fresh and dry biomass. However, dry biomass was more affected by H_2O_2 when compared to fresh biomass. H_2O_2 significantly reduced mitotic index, but did not induce significant chromosomal abnormalities. The treatment with 7.5 mM H_2O_2 led to a significant proline accumulation. On the other hand, the activity of superoxide dismutase and ascorbate peroxidase increased significantly only at a concentration of 2.5 and above 5.0 mM, respectively. There was no significant activity of the enzymes catalase and guaiacol peroxidase. Therefore, the exposure to H_2O_2 resulted in proline accumulation, and an increase in antioxidant enzyme activity, in response to H_2O_2 increase to maintain redox homeostasis inside the cell.

Keywords: Lettuce, Bioassay, Mitotic index, Reactive oxygen species, Plant metabolism.

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are metabolites constantly produced under normal conditions of the aerobic plant metabolism (Foyer & Noctor, 2009), and can function as signaling molecules in basic cellular processes (Sharma et al., 2012). These ROS may act as second messengers, mediating responses to different biotic and abiotic stimuli (Yi et al., 2014). However, they are detrimental when in excess, causing oxidative damage to biomolecules and, ultimately, cell death (Quan et al., 2008).

H_2O_2 is considered moderately reactive, with a relatively long intracellular half-life (1 ms) (Karuppanapandian et al., 2011), and therefore considered the most stable among ROS. Among its main physiological functions, the involvement in stomatal movement, root gravitropism, tolerance to oxygen deficiency, cell wall strengthening, senescence, phytoalexin production and cell cycle control can be highlighted (Yi et al., 2014; Pokora et al., 2017). However, since it has the ability to diffuse freely through the membranes, H_2O_2 can have a deleterious action, once it participates in the formation reaction of the hydroxyl radical (OH), the most reactive oxidant in the ROS family (Karuppanapandianet al., 2011). Thus, excess H_2O_2 can inactivate enzymes by oxidation and cause damage to nucleic acids, proteins, carbohydrates and lipids (Gill & Tuteja, 2010; Mittler et al., 2011).

For proper regulation of intra and extracellular ROS levels, plants have developed a complex antioxidant metabolism that includes both enzymatic and non-enzymatic activities (Sharma et al., 2012). The coordinated activity of these antioxidants regulates detoxification and/or elimination of ROS by reducing oxidative damage (Choudhury et al., 2013). Thus, there must be a balance between production and elimination of ROS, to regulate their metabolic signaling function, or to determine their role as cell-damaging molecules (Puthur, 2016).

In phytotoxicity studies, the use of bioassays with higher plants sensitive to the toxic agent is common. In this context, Lactuca sativa L. is widely used as a test organism (Reigosa et al., 2013; Moraes et al., 2015; Santos et al., 2017), due to its sensitivity to chemical agents, rapid germination, linear growth over a wide pH range and low sensitivity to osmotic potentials (Gomes et al., 2012). Its use is also justified by the wide cultivation around the world and its representativeness in human food. Considering the lack of studies relating the application of H_2O_2 to L. sativa, and aiming to understand the performance of this compound and stress tolerance mechanisms, the objective of this study was to evaluate H_2O_2-phytotoxicity on germination, growth, initial development and cell cycle, in addition to the enzymatic antioxidant activity of L. sativa.

Material and Methods

Plant material and treatments: For the evaluation of germination percentage (G %), root length (RL), fresh (FB) and dry biomass (DB), four replicates with 30 L. sativa seeds, cv. Grand Rapids were used. The seeds were randomly distributed in Petri dishes lined with a double layer of Whatman no. 2 paper, and moistened with 3 mL of H_2O_2 solution. The tested concentrations of H_2O_2 were 1.0, 2.5, 5.0, 7.5 and 10 mM, and distilled water was used as a negative control. For the quantification of proline, enzymatic antioxidant activity and lipid peroxidation, four
replicates with 50 seeds and 5 mL of H$_2$O$_2$ solution were used. For the cytogenetic characteristics: mitotic index (MI) and occurrence of chromosomal abnormalities (CA), three replicates with 30 seeds and 3 mL of H$_2$O$_2$ solution were used; 4,000 cells were analyzed per treatment. The plates containing the seeds were kept in a BOD germination chamber at 20 °C with a 12-hour photoperiod (Simões et al., 2013). Both pH and osmotic potential of the solutions were evaluated using, respectively, a digital pH meter (TecnopornPA 210 – Piracicaba - SP) and an automatic osmometer (Advanced Instruments - 3320 Micro-Osmometer – Norwood, MA).

**Evaluation of phytotoxicity and cytotoxicity:** G% was evaluated 24 and 48 hours after exposure to H$_2$O$_2$, counting the seeds that had a root protrusion of ± 0.1 cm. RL was obtained by the measurement of ten roots randomly selected at the end of the seventh day of cultivation, with the aid of a digital caliper. These plants were then weighed on an analytical scale (Marte, Shimadzu AY 220) for the determination of FB and placed in a forced air circulation oven (Nova Etica 400 ND) at 45°C until constant weight for the determination of DB. For the determination of MI and AC, L. sativa root tips were obtained 24 hours after exposure to each treatment. The roots were fixed in Carnoy solution, hydrolyzed in 5M HCl at 25°C and stained with Schiff's reagent. Cytogenetic preparations were made according to Pereira et al., (2013), and 4,000 cells were evaluated per treatment. MI consisted of counting the cells during the cell cycle, and was determined according to Moraes et al.,(2015).

**Enzymatic antioxidant activity and proline content:** For the extraction of antioxidant enzymes, 0.2 g fresh seedlings were macerated in liquid nitrogen, with 30% polyvinylpolypyrrolidone, and homogenized in 1.5 mL extraction buffer. The homogenates were centrifuged at 15,000 rpm for 10 minutes at 4°C, and the supernatants were collected for enzymatic analyses of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (POD) (Biemelt et al., 1998). The activity of SOD (EC 1.15.1.1) was determined by the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), proposed by Giannopolitis and Ries (1977). A unit of SOD was defined as the ability of the enzyme to reduce 50% NBT. The activity of APX (EC 1.11.1.11) was determined by decreasing the absorbance of ascorbate ($e = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) at 290 nm every 15 seconds for 3 minutes, according to Nakano and Asada (1981). The activity of CAT (EC 1.11.1.6) was determined by decreasing the absorbance at 240 nm, every 15 seconds for 3 minutes, monitored by the consumption of hydrogen peroxide, as described by Havir & MicHale (1987). The activity of POD (EC 1.11.1.7) was determined by the increase in absorbance at 470 nm, observed in the formation of tetraguaiacol for 3 minutes ($e = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$), according to Fang & Kao (2000). Proline content was determined at seven days of cultivation, according to the method described by Torello & Rice (1986). Color intensity was measured at 520 nm in a spectrophotometer (Halo SB-10 Dynamica Scientific) and the absorbances obtained were compared with the standard curve of proline.

**Lipid peroxidation:** Lipid peroxidation was determined by the quantification of thiobarbituric acid reactive species (TBARS), as described by Buege&Aust (1978). Readings were determined in a spectrophotometer (Halo SB-10 Dynamica Scientific) at 540 nm. The concentration of the MDA/TBA complex was calculated by the following equation: $[\text{MDA}] = 540 / (\xi \cdot b)$, where: $\xi$ (extinction coefficient = 1.56 x 10$^3$ cm$^{-1}$); b (optical length = 1).

**Statistical analysis:** The experimental design was completely randomized, with six treatments (0.0, 1.0, 2.5, 5.0, 7.5 and 10 mM H$_2$O$_2$) and four replicates for phytotoxicity and biochemical parameters, and three replicates for cytological parameters. Data were submitted to analysis of variance and means were compared by the Scott-Knott test at 5% significance. The variables that did not meet the assumptions of normality and/or homogeneity of the residues were transformed by the square root.

**Results**

The pH values found in the solutions of the different treatments with H$_2$O$_2$ ranged from 5.36 to 5.57, while the values of osmotic potentials ranged from -0.09 to -1.30 MPa.

After 24 hours of exposure to H$_2$O$_2$, the concentrations 5.0, 7.5 and 10 mM significantly reduced seed germination percentage (G%) by 30%, 78% and 96%, respectively, in relation to the control treatment (Fig. 1A). However, at 48 hours, all treatments showed germination above 80%, not significantly different from each other; thus, the use of a positive control treatment was not required. Therefore, it was possible to observe that the germination of L. sativa seeds was more sensitive to H$_2$O$_2$ in the first 24 hours, when compared to the 48-hour exposure time (data not shown).

Root length (RL) of L. sativa seedlings was significantly reduced from the treatment with 1.0 mM H$_2$O$_2$ (Fig. 1B). Thus, the highest H$_2$O$_2$ concentrations (7.5 and 10 mM), resulted in a lower RL, with 3.36 and 2.02 mm, respectively.

The treatment with H$_2$O$_2$ reduced mitotic index (MI) at all tested concentrations (Fig. 1C). The reductions were 36.1%, 38.1%, 36.4%, 23.7% and 18.7% at the concentrations 1.0, 2.5, 5.0, 7.5 and 10 mM H$_2$O$_2$, respectively, when compared to the control treatment.

Seedling fresh biomass (FB) was significantly reduced only when exposed to concentrations of 7.5 and 10 mM H$_2$O$_2$ (Fig. 1D). However, concentrations from 1.0 mM H$_2$O$_2$ were sufficient to result in a decrease in dry biomass (DB) (Fig. 1D). Therefore, the DB of L. sativa seedlings was significantly more affected by the different H$_2$O$_2$ concentrations in relation to the FB, which was more sensitive to its phytotoxic action.
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Fig. 1. (A) Germination percentage (G%) in 24 hours; (B) Root length (mm); (C) Mitotic index (%); (D) Fresh and Dry biomass (g) of *L. sativa* in relation to H$_2$O$_2$ concentrations. Bar: Standard error of 4 replicates.

There was a greater proline accumulation at 7.5 and 10 mM H$_2$O$_2$ (Fig. 2). However, the H$_2$O$_2$ concentrations used were not sufficient to cause a significant increase in lipid peroxidation and malondialdehyde levels (data not shown).

After 7 days of exposure to H$_2$O$_2$, the activity of superoxide dismutase (SOD) in *L. sativa* seedlings exposed to the treatment with 2.5 mM H$_2$O$_2$ increased significantly by 20% in relation to the control, differing from the other concentrations tested (Fig. 3A). In addition, there was a significant increase in the activity of ascorbate peroxidase (APX) by 78% at 5.0 mM H$_2$O$_2$ in relation to the other treatments (Fig. 3B). Thus, APX and SOD significantly increased their activity at intermediate H$_2$O$_2$ concentrations (2.5 and 5.0 mM) but were inhibited at the highest concentrations (7.5 and 10 mM). However, there was no significant activity of the enzymes catalase and guaiacol peroxidase (POD) (data not shown).

Fig. 2. Proline content in *L. sativa* seedlings exposed to different H$_2$O$_2$ concentrations. Bar: Standard error of 4 replicates.

Fig. 3. Activity of (A) superoxide dismutase (SOD) and (B) ascorbate peroxidase (APX) in *L. sativa* seedlings exposed to different H$_2$O$_2$ concentrations. Bar: Standard error of 4 replicates.
Discussion

According to Borella & Pastorini (2011), lettuce seeds tolerate a wide range of pH variation (from 4 to 10) for germination. According to Gatti et al. (2014), solutions with osmotic potential of up to -0.2 MPa do not significantly interfere in germination, growth and initial development of L. sativa. In plant bioassays, it is important to control the different variables, in addition to the requirement to standardize the procedures, to better understand the results obtained in this type of study (Souza Filho et al., 2010).

Seed germination is a parameter widely used in plant bioassays (Moraes et al., 2015), although it does not demonstrate other aspects of the germination process, such as delays, since it only involves final results (Silva & Aquila, 2006). Changes in the germination pattern may result in changes in membrane permeability, DNA transcription and translation, second messenger functionality, respiration due to oxygen sequestration, conformation of receptor proteins and enzymes, or the combination of these factors (Ferreira & Aquila, 2000). In this context, although H2O2 is a potentially toxic reactive oxygen species (ROS), it has aroused special interest due to its ability to lead to seed germination in different plant species (Ishibashi et al., 2012). In L. sativa seeds, it leads to ruptures in the endosperm (Zhang et al., 2014), which allows greater mobilization of reserves to the embryonic axis (Verma et al., 2015), resulting in germination. However, in this study, H2O2 had no effect on germination – on the contrary, it delayed this process in the first 24 hours, suggesting its phytotoxic effect at the highest concentrations tested.

Root length (RL) is another variable widely used in phytotoxicity bioassays (Simões et al., 2013; Moraes et al., 2015). Since the primary root is the first organ to emerge from seed germination, its growth and development are directly affected by environmental conditions such as temperature, humidity, gravity, availability of nutrients and other substances in the environment (Santisree et al., 2011). In this context, the inhibitory effect of H2O2 on the elongation of the Arabidopsis primary root was demonstrated by its exogenous delivery (Dunand et al., 2007). Thus, the reduction in L. sativa RL, verified in this study, may be related to the reduction in the percentage of dividing cells in the meristematic region, since this region is responsible for root growth (Adam & El-Ashy, 2010; Pereira et al., 2013). This inhibitory effect may occur due to an imbalance between the production of ROS and antioxidant activity, and may cause a rapid increase in intracellular ROS levels, called “oxidative burst” (Sharma et al., 2012), which results in oxidative damage to cell structures (Kim & Kwak, 2010).

According to Alvim et al., (2011), the increase or decrease in mitotic index (MI) can be used as cytotoxicity parameters. In this context, according to Pokora et al., (2017), changes in intracellular concentrations of H2O2 are responsible for modulating the expression of genes required for cell cycle progression. Thus, under normal conditions, H2O2 seems to coordinate growth and plant development, in addition to regulating the cell cycle (Slesak et al., 2007). However, according to Tsukagoshi (2012), the exogenous H2O2 supply may have a repressing effect on the expression of cell cycle-related genes, reducing the size of the root meristem. Thus, the increase in H2O2 concentrations had led to a cytotoxic action on the meristematic cells of L. sativa root tips, altering the cell division pattern, which directly reflected in the reduction in RL and plant biomass.

Cytogenetic analyses with detection and quantification of chromosomal abnormalities (CA) have been used to evaluate the mutagenic potential of contaminants (Alvim et al., 2011). Chromosomal breaks, c-metaphases, bridges, binucleate and micronucleated cells are the most frequently observed chromosomal changes (Matsumoto et al., 2006). However, for this study, there was no significant difference in the occurrence of CA, indicating that H2O2 did not have clastogenic potential.

The reduction in dry biomass (DB) as a function of the increase in H2O2 concentration is possibly due to the stomatal closure caused by ROS signaling, resulting in a lower carbon fixation and a smaller DB increase (Schock et al., 2014). Important processes of cellular metabolism have been associated with H2O2, such as stomatal movement, whose closure causes a decrease in the carbon fixation rate via the Calvin cycle (Puthur, 2016). In addition, peroxidase associated with the polymerization of lignin precursors may restrict cell elongation due to the use of H2O2 as a substrate resulting cell wall thickening (Passardi et al., 2004). As a consequence, cell wall peroxidases may be involved in the regulation of root growth, involving the metabolism of H2O2 so that, at higher H2O2 concentrations (7.5 and 10 mM), there may have been an increase in lignin at the root tips, preventing water absorption. According to Jafariyan et al., (2016), the pretreatment of wheat seeds with H2O2 increased water absorption and content in seedlings, yielding higher biomass. However, the biomass differences in seedlings presented in this study can also be attributed to the germination delay observed during the first 24 hours of exposure to H2O2.

According to Yang et al., (2009), the exogenous application of H2O2 results in a series of signal transduction events, leading to a rapid proline accumulation. This amino acid is often associated with antioxidant metabolism, besides being involved in the inhibition of programmed cell death, since it acts as an osmoprotector, protein stabilizer, and as a lipid peroxidation inhibitor (Sharma et al., 2012; Puthur, 2016). In addition, the increase in proline content may be related to a plant stress response to avoid water loss, since this amino acid is important in osmotic adjustment in plant cells (Paixão et al., 2014). However, in this study, the increase in proline content as a function of the increase in H2O2 concentrations was not able to reduce biomass loss, especially at concentrations higher than 5.0 mM H2O2.

The growth restriction of L. sativa seedlings is possibly related to the phytotoxic effects of ROS, which can lead to changes in the structural conformation of proteins, instability and cell membrane permeability, with modifications that are detrimental to plant physiology (Lamhamdi et al., 2011). The toxic effect of H2O2 is linked to enzyme inactivation by the oxidation of their thiol groups, and damage to proteins, carbohydrates, lipids and nucleic acids (Gill & Tuteja, 2010; Mittler et
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In this context, since the cellular redox state is precisely regulated by processes of production and removal of ROS in different cell compartments (Kapoor et al., 2015), antioxidant enzymes were characterized by increasing their activity in plant tissues as a response to the oxidative stress induced by the high H₂O₂ concentration. Thus, not only are the biological effects of H₂O₂ dependent on its concentration, but also on its production site, plant developmental stage and the previous plant exposure to other types of stress, as discussed by Pokora et al., (2017).

Although H₂O₂ accumulation impairs the function and integrity of membranes due to the fact that they affect polyunsaturated fatty acids and cause lipid peroxidation (Farmer & Mueller, 2013), there was no significant increase in lipid peroxidation and malondialdehyde levels with increasing H₂O₂ concentrations. This indicates that none of the H₂O₂ concentrations tested affected the structure of the cell membranes. However, the maintenance of cell membrane integrity is possibly due to the increased enzyme activity of the antioxidant system. Thus, compartmentalization of antioxidant enzymes may have important consequences for the maintenance of adequate H₂O₂ levels (Gill & Tuteja, 2010; Kapoor et al., 2015). In addition, proline accumulation in the tissues is also a proof that the cell constantly tries to minimize oxidative stress. However, in multicellular organisms, increased expression of antioxidant enzymes is not the only cellular response to increased H₂O₂ concentration (Maia et al., 2012).

Conclusions

The exposure of L. sativa seeds to H₂O₂ was phytotoxic, resulting in a lower germination percentage, especially in the first 24 hours. In addition, higher H₂O₂ concentrations (7.5 and 10 mM) reduced root length, fresh and dry biomass, besides the activity of APX and SOD. This ROS was also cytotoxic; however, it was not clastogenic.

Therefore, exposure to H₂O₂ resulted in proline accumulation at the highest concentrations and also in an increase in the activities of antioxidant enzymes at intermediate concentrations, in response to the increase in H₂O₂ to maintain redox homeostasis inside the cell. This shows that L. sativa did not have an efficient antioxidant system to remove ROS generated mainly at concentrations of 7.5 and 10 mM H₂O₂.

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