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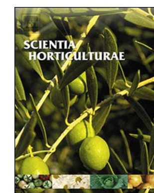

# Hydrogen peroxide promotes the tolerance of soybeans to waterlogging

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## ABSTRACT

We investigated the role of pretreatment of seeds with hydrogen peroxide ($H_2O_2$) in promoting the tolerance of seedlings of *Glycine max* to waterlogging. Soybean seeds were pretreated with a solution of $H_2O_2$ 70 mM or water for 24 h under aeration, and then were placed in plastic bags containing substrate. At 12 days after sowing, the seedlings were subjected to waterlogging for 32 days. There were three evaluations: 0, 16 days and 32 days of waterlogging. Biomass accumulation, root volume, stem diameter, gas exchanges, activity of antioxidant system and cell membrane damages were evaluated. The pretreatment of seeds with $H_2O_2$ generated seedlings with elevated activity of antioxidant enzymes, low $H_2O_2$ and $O_2^{-\cdot}$ content, and low cell membrane damages. Hydrogen peroxide pretreatment also resulted in increased content of photosynthetic pigments and net photosynthetic rate, high biomass accumulation, root volume and stem diameter. $H_2O_2$ pretreatment of soybeans seeds generated seedlings more tolerant to waterlogging. There is probably a stress memory involved in the process of induction of tolerance in soybeans mediated by hydrogen peroxide.

## 1. Introduction

Climate changes have changed significantly the volume of rainfall in different parts of the world. In this way, there are tendencies of excess water in certain regions and water deficit in other regions (Kreuzwieser and Gessler 2010). Increases in flooding events have been detected in parts of Asia, Peninsular India, Africa and America (Hirabayashi et al., 2013), causing a direct impact on productivity, since most crops are sensitive to flooding (Voesenek et al., 2006).

Excessive water in the soil leads to an excessive production of reactive oxygen species (ROS), culminating in oxidative stress and cellular damages such as lipid peroxidation, protein oxidation, damage to nucleic acids, enzyme inhibition and activation of programmed cell death (Noctor et al., 2002; Gill and Tuteja 2010; Sharma et al., 2012; Anjum et al., 2015). The impacts of the breakdown of cellular homeostasis by waterlogging can be observed by the reduction in the photosynthetic pigments and gas exchanges (Mutava et al., 2015). Commonly reductions in growth, especially in root elongation (Shi et al., 2008) and in dry weight accumulation (Fante et al., 2011; Lanza et al.2013) can also be observed in plants under waterlogging.

Despite the harmful effects of ROS to cell components, these reactive species can act as signaling molecules under stress conditions. Among the ROS, $H_2O_2$ is the most appropriate for the signaling function due to its longer half-life, greater stability and ability to diffuse freely through cell membranes. Currently it is known that the application of $H_2O_2$ in low concentrations can induce plant tolerance to stress (Hossain et al., 2015).

In the face of problems caused by biotic and abiotic stresses in crops, alternatives have been sought to increase plant tolerance. One rapid and effective alternative that has been used is the pretreatment of plants with agents that can increase the tolerance to different stresses (Jisha et al., 2013). Pretreatments consist of the application of a moderate stress condition that leads to the accumulation of dormant signals in different parts of the plant or seeds. Then, when there is a stress condition, the stored signals will drive molecular adjustments leading to the development of harder and more effective mechanisms of tolerance (Savvides et al., 2016). Pretreatment with $H_2O_2$ in several species protects plants from oxidative damage caused by high light intensity, low temperatures, salt stress, drought and exposure to heavy metals (Gechev et al., 2002; Chao et al., 2009; Ishibashi et al., 2011; Rajaeian and Ehsanpour 2015; Savvides et al., 2016).

Hydrogen peroxide pretreatments are related to higher activity of antioxidative enzymes, higher concentrations of non-enzymatic antioxidants (Hossain et al., 2015), increased stomatal conductance and photosynthetic rate, higher pigment content, biomass accumulation and formation of adventitious roots (Ishibashi et al., 2011; Liao et al., 2012; Gondim et al., 2013). All the physiological and morphological adjustments support the higher tolerance of pretreated plants (Savvides et al.,

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2016).

Many studies about pretreatment with hydrogen peroxide or other chemical agents are focused on plants, however greater attention should be given to the pretreatment of seeds. The pretreatment of seeds is related to accumulation of late embryogenesis abundant proteins (LEA), heat shock proteins (HSPs) and chaperones to stabilize membranes and proteins, and preparation of the antioxidant system (Chen and Arora 2013). The imposition of stress before germination leaves a "stress memory" by the accumulation of dormant signals, so that the seedlings formed already have potential stress tolerance for coming of previously prepared seed (Perez and Brown 2014).

In the face of the role of $H_2O_2$ in increasing the tolerance of plant species to various types of stresses, we investigated the role of pretreatment of seeds with $H_2O_2$ in promoting the tolerance of seedlings of *Glycine max* to waterlogging.

## 2. Materials and methods

Seeds of soybean (*Glycine max* [L.] Merr.) cv. AN 8500, were pretreated with $H_2O_2$ 70 mM. The pretreatment was conducted by soaking the seeds in the $H_2O_2$ solution or in water, with constant aeration for 24 h. After this, seeds were washed with distilled water to remove residual solution. Seeds were sown in plastic bags (10 × 20 × 0.1 cm) containing a substrate composed of a mixture of soil, sand and manure in the proportion of 1:1:1. For germination, the seeds were kept in growth room with photoperiod of 12 h and 25 °C ± 3 °C for 12 days. Then, the seedlings were transferred to the greenhouse with an average temperature of 25 °C, where they were placed in pools of 1000 Liters (190 × 130 × 0.42 cm) for the imposition of waterlogging. Throughout the treatment period, the water level was constant for maintenance of a water slide two centimeters above the collar of the seedlings, without aeration.

Three evaluations were carried out: before the imposition of the treatments, after 16, and 32 days of waterlogging. In each time of evaluation plant material (leaves and roots) was harvested and appropriately stored for growth and chemical analyses.

Shoots and roots dry weight was obtained in plants dried in forced circulation oven at 70 °C until constant weight. The diameter of the stem was measured in the collar height using a pachymeter (Mitutoyo digital CD – CSX 8A – B).

To determine root volume, the roots were washed and stored in methanol (5%) at 4 °C until the analysis. The images were captured using HP Scanjet G2410 scanner, 1200dpi optical resolution coupled to an acrylic tub. Image processing occurred through the SAFIRA software – Fibers and Roots Analysis System (Jorge and Rodrigues 2008).

Chlorophyll and carotenoid content was quantified at the last fully expanded trefoil, always using median leaflet. One hundred milligrams of leaf tissue were macerated in 80% acetone. Then, the final volume was completed to 10 mL and the spectrophotometric readings were performed at 663.2, 646.3 and 470 nm (Lichtenthaler and Buschmann 2001).

Gas exchange evaluations were performed using an infrared gas analyzer (LI-6400XT Portable Photosynthesis System LI-COR, Lincoln, USA). We evaluated the central leaflet of fully expanded trifoliate for stomatal conductance (gs), transpiration (E), and net photosynthetic rate (A). The evaluations were performed between 9 and 10 am using artificial source of photosynthetically active radiation (PAR) set at 1500 μmol photons $m^{-2} s^{-1}$ (Blue + Red LED LI-6400-02B, LI-COR, Lincoln, USA).

The activities of enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were determined after enzymatic extraction (Biemelt et al., 1998). For this, 0.2 g of leaf fresh weight were grounded in liquid nitrogen and homogenized in 1.5 mL of extraction buffer containing: 0.1 M potassium phosphate buffer (pH 7.0), 0.1 mM disodium ethylenediamine tetraacetic acid (EDTA), and 10 mM ascorbic acid. The extract was centrifuged at 13,000*g* for 10 min at 4 °C. The supernatant was collected and stored at −20 °C during the analysis period.

SOD activity was measured by the ability of enzyme to inhibit the photoreduction of nitroblue tetrazolium (NBT) (Giannopolitis and Ries 1977). Aliquots of enzymatic extract were added to incubation medium containing 50 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 μM EDTA, 75 μM of NBT, and 2 μM riboflavin. Then, tubes containing the reaction medium and 10 μL of sample were illuminated for 7 min with a fluorescent lamp of 20 W. The same reaction medium without a sample was illuminated as a control. Readings were taken at 560 nm. One unit of SOD is able to inhibit 50% of the photoreduction of NBT under the assay conditions.

CAT was evaluated by adding aliquots of enzymatic extract were added to incubation medium containing 50 mM potassium phosphate (pH 7.0), and 12.5 mM hydrogen peroxide, previously incubated at 28 °C. Enzyme activity was determined by the decrease in absorbance at 240 nm every 15 s for 3 min, monitored by the consumption of hydrogen peroxide. The molar extinction coefficient used was 36 $mM^{-1} cm^{-1}$ (Havir and McHale 1987).

APX activity was determined by monitoring of the rate of oxidation of ascorbate at 290 nm every 15 s for 3 min. Aliquots of enzymatic extract were added to incubation buffer, consisting of 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM hydrogen peroxide (Nakano and Asada 1981). The molar extinction coefficient was 2.8 $mM^{-1} cm^{-1}$.

For $H_2O_2$ quantification, 200 mg of leaf fresh weight were macerated in liquid nitrogen and homogenized in 1500 μL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000*g* for 15 min at 4 °C. $H_2O_2$ was determined by measuring the absorbance at 390 nm in a reaction medium containing the extract, 2.5 mM potassium phosphate (pH 7.) and 0.5 M potassium iodide (Velikova et al., 2000).

To determine the $O_2^{-\cdot}$ radical levels, fresh material was extracted in 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.3% Triton-X. The extract was centrifuged at 12000*g* for 20 min. An aliquot of the supernatant was added to incubation buffer consisting of 50 mM potassium phosphate buffer (pH 7.8) and 1 mM hydroxylamine hydrochloride, incubating the mixture at 25 °C for one hour. Then it was added 17 mM aminobenzene sulphonic acid, and 7 mM α-naphthylamine, proceeding incubation at 25 °C for 20 min. A standard curve of $NO_2$ was constructed using the same method described above. Determination of $O_2^{-\cdot}$ content was performed by measuring the absorbance at 530 nm (Wang and Luo 1990).

Lipid peroxidation was determined by quantification of thiobarbituric acid reactive species (Buege and Aust 1978). Two hundred milligrams of leaf fresh weight were macerated in liquid nitrogen, and homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. Aliquots of the supernatant were added to the reaction medium containing thiobarbituric acid (TBA) 0.5% (m/v) and TCA 10% (m/v), and then incubated at 95 °C for 30 min. The reaction was stopped by rapid cooling on ice and readings were determined in a spectrophotometer at 535 nm and 600 nm. Concentration of MDA/TBA complex was calculated by the following equation: $[MDA] = (A_{535} - A_{600})/(\xi.b),A_{600})/(\xi \cdot b)$, where: ξ (extinction coefficient = $1.56 \times 10^{-5} cm^{-1}$); b (optical length = 1).

Membrane permeability was measured by electrolyte leakage. Six root segments of equal size (2 cm) were washed with distilled water and dipped in 6 mL of distilled water for 12 h. Then measured was the conductivity of the solution (EC1) with the help of a conductivity meter (Model 0405 M; Quimis®). Samples were then boiled for 20 min and cooled to ambient temperature. The conductivity of tissue (EC2) was measured again. The electrolyte leakage was calculated using the formula (EC1/EC2) × 100 and the result is expressed in% of extravasation (Blum and Eberco 1981).

The experiment was conducted in completely randomized design, in a factorial arrangement (2 × 3) with two treatments and three times of

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