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CURSO DE DOUTORADO

ACCLIMATION OF SUNFLOWER PLANTS TO SALT STRESS WITH HYDROGEN PEROXIDE

Petterson Costa Conceição Silva

CRUZ DAS ALMAS - BAHIA
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ACCLIMATION OF SUNFLOWER PLANTS TO SALT STRESS WITH HYDROGEN PEROXIDE

Petterson Costa Conceição Silva
Engenheiro Agrônomo
Universidade Federal do Recôncavo da Bahia, 2012

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Orientador: Prof. Dr. André Dias de Azevedo Neto
Coorientador: Prof. Dr. Hans Raj Gheyi

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ACCLIMATION OF SUNFLOWER PLANTS TO SALT STRESS WITH HYDROGEN PEROXIDE

Comissão Examinadora da Defesa de Tese de
Petterson Costa Conceição Silva

Aprovada em: 10 de fevereiro de 2020

Prof. Dr. André Dias de Azevedo Neto
Universidade Federal do Recôncavo da Bahia
Orientador

Prof. Dr. Tales Miler Soares
Universidade Federal do Recôncavo da Bahia
Examinador Interno

Prof. Dr. Vital Pedro da Silva Paz
Universidade Federal do Recôncavo da Bahia
Examinador Interno

Prof. Dr. Rogério Ferreira Ribas
Universidade Federal do Recôncavo da Bahia
Examinador Externo

Prof(a). Dr(a). Manuela Oliveira de Souza
Universidade Federal do Recôncavo da Bahia
Examinador Externo

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Dedico esta conquista a Deus, aos meus pais (Adailton Santos da Silva e Aline Costa Conceição Silva), ao meu irmão (Jefferson Costa Conceição Silva), a minha querida esposa (Caroline Rastely dos Reis Silva), e em especial à minha avó (Elizabeth Costa Conceição).

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ACCLIMATION OF SUNFLOWER PLANTS TO SALT STRESS WITH HYDROGEN PEROXIDE

ABSTRACT: This study aimed to test the hypothesis that hydrogen peroxide (H_2O_2) can increases the tolerance of sunflower plants to salt stress, using different strategies of application. In the first experiment, a selection of the treatments of H_2O_2 priming with greater dry mass production was carried out. For this purpose, before of salt stress application, seed of sunflower were primed at four concentrations of H_2O_2 associated to three exposure periods for selection of the better treatments, during 35 days after soaking (DAS). The second experiment was performed using greater treatments selected from the experiment previous. In this experiment, every 7 days, the physiological and biochemical parameters of plants were monitored. In the third experiment, the selection of the treatments of H_2O_2 priming via leaf spraying with greater dry mass production was carried out. In this experiment, four concentrations of H_2O_2 (via leaf spraying) were associated at three numbers of application during 35 days after soaking (DAS). The fourth experiment was performed using greater treatments selected from the experiment previous. In this experiment, every 7 days, the physiological and biochemical parameters of plants were monitored. The fifth experiment aimed to evaluate the effect of different methods of applying hydrogen peroxide (H_2O_2) via seed and/or via leaf spraying in sunflower plants under salt stress. In this experiment, five treatments were tested: control (absence of NaCl and absence of H_2O_2); salt control (presence of 100 mM NaCl and absence of H_2O_2); 1 mM H_2O_2 via seed (in the presence of 100 mM NaCl); 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl); and 1 mM H_2O_2 via seed + 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl). At 20 DAS dry masses of plants were quantified. In general, for all experiments, the salt stress promoted a strong reduction of plant growth, in relation to control treatment. However, this reduction was less pronounced in some treatment H_2O_2-primed, confirming the hypothesis that H_2O_2 is able to attenuate the negative effect of salinity, increasing the tolerance of plants Our results showed that the priming of seed or plants with H_2O_2 can increase photosynthetic efficiency, reduce Na^+ and Cl^- transport to leaves and increase antioxidant enzyme activity, mainly catalase, contributing to increased salt tolerance. But we also verified that, application of H_2O_2 leaf spraying (after salt stress) is not able to attenuate the negative effect caused by salt in sunflower plants.

Key words: Antioxidative enzymes; Cross-tolerance; Ion transport; ROS; Salinity

ACLIMATAÇÃO DE PLANTAS DE GIRASSOL AO ESTRESSE SALINO COM PERÓXIDO DE HIDROGÊNIO

RESUMO: Este estudo teve como objetivo testar a hipótese de que o peróxido de hidrogênio (H_2O_2) pode aumentar a tolerância das plantas de girassol ao estresse salino, utilizando diferentes estratégias de aplicação. No primeiro experimento, foi realizada uma seleção dos tratamentos de H_2O_2 com maior produção de massa seca. Para isso, antes da aplicação do estresse salino, sementes de girassol foram preparadas em quatro concentrações de H_2O_2 associadas a três períodos de exposição para seleção dos melhores tratamentos, durante 35 dias após a embebição (DAE). O segundo experimento foi realizado utilizando os melhores tratamentos selecionados a partir do experimento anterior. Neste experimento, a cada 7 dias, foram monitorados os parâmetros fisiológicos e bioquímicos das plantas. No terceiro experimento, foi realizada a seleção dos tratamentos de condicionamento com H_2O_2 via pulverização foliar com maior produção de massa seca. Neste experimento, quatro concentrações de H_2O_2 (por pulverização foliar) foram associadas a três números de aplicações, durante 35 dias após a embebição (DAE). O quarto experimento foi realizado utilizando os melhores tratamentos selecionados a partir do experimento anterior. Neste experimento, a cada 7 dias, os parâmetros fisiológicos e bioquímicos das plantas foram monitorados. O quinto experimento teve como objetivo avaliar o efeito de diferentes métodos de aplicação de peróxido de hidrogênio (H_2O_2) via sementes e/ou pulverização foliar em plantas de girassol sob estresse salino. Neste experimento, cinco tratamentos foram testados: controle (ausência de NaCl e ausência de H_2O_2); controle de sal (presença de NaCl 100 mM e ausência de H_2O_2); 1 mM H_2O_2 via semente (na presença de NaCl 100 mM); 1 mM H_2O_2 via pulverização de foliar (na presença de NaCl 100 mM); e 1 mM H_2O_2 via semente + 1 mM H_2O_2 via pulverização foliar (na presença de NaCl 100 mM). Aos 20 DAS foram quantificadas massas secas de plantas. Em geral, para todos os experimentos, o estresse salino provocou uma forte redução do crescimento das plantas, em comparação ao tratamento controle. No entanto, essa redução foi menos pronunciada em alguns tratamentos condicionados com H_2O_2, confirmando a hipótese que o H_2O_2 é capaz de reduzir o efeito negativo da salinidade, aumentando a tolerância das plantas. Nossos resultados mostraram que, o condicionamento de sementes ou plantas com H_2O_2 pode aumentar a eficiência fotossintética, reduzir o transporte de Na^+ e Cl^- para as folhas e aumentar a atividade das enzimas antioxidantes, principalmente a catalase, contribuindo para o aumento da tolerância ao sal. Porém, verificamos também que a aplicação foliar de H_2O_2 (após o estresse salino) não é capaz de atenuar o efeito negativo provocado pelo sal em plantas de girassol.

Palavras chave: Enzimas antioxidantes; Tolerância cruzada; Transporte de íons; EROs; Salinidade;

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1. CHAPTER 1

1.1. GENERAL INTRODUCTION

Salinity is one of the environmental stresses that most affect crop growth and productivity in the world. Thus, it is one of the main challenges found in agriculture (PELEG et al., 2011).

In stress conditions occur several metabolic reactions in the plants. One of the metabolic process that occur in these conditions is the production of reactive oxygen species (ROS), formed during the plant metabolism, mainly in the chloroplasts, mitochondria and peroxisomes, from the reduction of oxygen (O_2) (MARQUES, 2013). ROS can also be used as signaling molecules in different processes of plant metabolism, for example, in growth and development, cell cycle, apoptosis, senescence and in responses to abiotic stresses (FINKEL, 2003 and MARQUES, 2013).

The process of acclimation is a biological mechanism that provides an alternative for the survival of plants to adverse conditions, mainly environmental stresses. This process involves morphological, physiological and biochemical changes, including gene expression, that are not transmitted to future generations (PRISCO and GOMES FILHO, 2010), allowing an individual to acquire greater tolerance to stress, compared to those who have not been acclimated. One of the techniques used to increase the efficiency in acclimation plants to abiotic stresses is the application of organic, inorganic or growth-regulating compounds, applied to the growing medium (root contact) or by spraying these compounds on the leaves (ASHRAF et al., 2008).

Under environmental stress conditions, the hydrogen peroxide that is an ROS has its concentration increased and can potentially cause several negative effects to plants. However, some studies on maize, tobacco, barley and rice plants have shown that the pretreatment with H_2O_2 in low concentration showed a significant improvement in the tolerance of plants to stress conditions such as salinity, heat and drought (PRASAD et al., 1994); UCHIDA et al. 2002; AZEVEDO NETO et al., 2005; GONDIM et al, 2012; SOUSA et al., 2016).

The physiological mechanisms of removal of ROS were developed by plants throughout evolution. In plant species, a variety of enzymes have stood out with the

ability to remove ROS, they are: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and others. Catalase has been listed as a key enzyme in the process of acclimation of pretreated plants with hydrogen peroxide acting as a plant protector to the salt stress condition (GONDIM et al., 2012), however, this mechanism has not yet been fully explained.

In Brazil, little has been done with the use of hydrogen peroxide in the pretreatment of agricultural crops to the acclimation of plants to abiotic stresses. In the word, most of the work in this line of research has been carried using maize, rice, barley and tobacco crops. Little is known yet about the capacity of acclimation to salinity by pretreatment with hydrogen peroxide in sunflower plants, since in Brazil, mainly in the northeast region which is the place where the greatest soil salinization problems occur.

The cultivation of sunflower is of great importance in the world market, mainly in the production of good quality oil used in human food. However, still little produced in the northeast region. Once the ability to acclimatize these plants to salinity through the application of H_2O_2 has been proven, this work can generate important data so that in the future there may be a greater incentive to the production of this culture in the region, mainly due to the fact that this species has a high yield of oil and with high added value.

1.2. LITERATURE REVIEW

1.2.1. Effect of salinity on plants

Approximately 40% of the world's irrigated soils are located in arid and semi-arid regions (FAO, 2006; BELTRÁN, 2010). Salinity is a limiting environmental problem for plant development and productivity (ALLAKHVERDIEV et al., 2000; ESTEVES and SUZUKI, 2008).

For the production of agricultural crops mainly in the northeast region of Brazil, it is essential to know about the effects that salts cause on plants, especially when using saline soil and/or saline water.

Excessive salts in the soil can cause several negative effects on plant growth. The soil normally contains a diluted concentration of salts, but when in excess, the presence of these salts causes a reduction in the osmotic potential of the soil solution,

providing a decrease in the free energy of the water, resulting in a decrease in the influx of water by the roots and transpiratory flow of plants (SALISBURRY and ROSS, 1992).

According to Aragão (2008), the effects of salinity on plant growth and development are numbered as: 1st - osmotic effect, due to the large amount of salts in the soil, retaining more and more water, and thus not making water available to plants; 2nd - toxic effect, characterized by the accumulation of specific ions in the plant, mainly Na^+ and Cl^-, affecting the respiratory chain, photophosphorylation, nitrogen assimilation and protein metabolism; 3rd - nutritional effect, in which the excess of one ion inhibits the absorption of another.

The gas exchange of plants is strongly affected by the presence of a high concentration of salts in the soil, reducing the permeability of water by the roots and providing stomatal closure, thus loss of water through transpiration, the consequence of which is the reduction of the photosynthesis and the impairment of the plant cycle (PEDROTTI et al., 2015). The reductions caused by salinity in photosynthesis are linked to indirect consequences induced by salt, as the presence of salt does not directly affect growth, however it has negative effects on cell turgor, on photosynthesis and enzymatic activities of plants (ARAGÃO, 2012).

It is known that the photochemical activity of plants presents a kind of resistance regarding exposure to short-term stress (NETONDO et al., 2004), without significant change in the potential quantum efficiency of photosystem II (Fv/Fm) (PRAXEDES et al., 2010). However, when exposed to prolonged stress, the decrease in the Fv/Fm ratio can be interpreted as photodamage, and may occur due to inactivation of the reaction center of photosystem II (PSII) and/or an excitation energy in favor of photosystem I (PSI), increasing the cyclical flow of electrons around the PSI (KRAMER et al., 2004; HILL and RALPH, 2005), especially when subjected to high density of photosynthetic photon flux (PPFDs) (BELKHODJA et al., 1999; LU et al., 2003).

An important factor in determining the photochemical capacity of PSII is the redox status of quinones (Qa). Often, the PSII efficiency factor (photochemical quenching - qP) represents the proportion of photon energy captured by the PSII reaction centers and dissipated via electron transport (JUNEAU et al., 2005), reflecting the degree of oxidation and reduction of the quinones. The qP represents the use of light energy for the photochemical processes of photosynthesis (donation of electrons

from the H_2O molecule to the $NADP^+$ acceptors. This energy generated by the breakdown of H_2O and the H^+ released in this breakdown is used in the formation of reducing power and ATP molecule, which are used in the biochemical phase of photosynthesis (SCHREIBER et al., 1986).

The excess of free energy can be the result of reduced photosynthetic efficiency due to environmental stress (LONG et al., 1994; LIMA NETO, 2012). A parameter that frequently increases as a function of salinity is the ETR/A ratio, which indicates the electron transport rate directed towards the assimilation of CO_2. The increase in this ratio indicates that more electrons are being directed to other drains (photorespiration, N metabolism, Mehler reaction, formation of ROS), suggesting a stress condition (MELONI et al., 2003; DRIEVER and BAKER, 2011; LIMA NETO, 2012). The various effects on the mechanisms that involve the ability to assimilate CO_2, photochemical activity and the existence of alternative electron drains to mitigate the damage caused by salinity are poorly studied and deserve more attention.

1.2.2. Oxigen reactive species on plants

ROS are by-products produced through biochemical events of aerobic cell metabolism (NOCTOR and FOYER, 1998). These species are produced through aerobic metabolism, during the electron flow in the mitochondria and chloroplast and/or when the reduced electron transporters react partially with O_2 in the absence of other electron acceptors (HALLIWELL and GUTTERIDGE, 1985; THOMPSON et al., 1987; GONDIM, 2012). In plant cells, ROS can also be produced in the peroxisomes, in the cytosol, in the plasma membrane and in the apoplast (GONDIM, 2012).

As O_2 absorbs radiant energy or is reduced by electron acceptor ROS are formed: singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radicals ($^{\bullet}O_2^-$) and hydroxyl ($HO^{\bullet}$) are the most commonly found ROS (APEL and HIRT, 2004; ABOGADALLAH, 2010; MARQUES, 2013).

Chloroplasts are considered the main sources of ROS production, since in this organelle there is the production of a large amount of $^{\bullet}O_2^-$, H_2O_2 and $^1O_2$, mainly when the plants are submitted to stress conditions (TAKAHASHI and MURATA, 2008; JASPERS and KANGASJARVI, 2010).

The redox state of the mitochondrial electron transport chain is an important indicator of the state of cellular energy and the second largest source of ROS

production, especially the $^{\bullet}O_2^-$ from complexes I and III, and the state of reduction of ubiquinone (TAYLOR et al., 2009).

The third largest source of intracellular ROS is peroxisomes. In these organelles is found the glycolate oxidase enzyme that produces H_2O_2 resulting from the conversion of glycolate to glyoxylate in the photorespiratory pathway (JASPERS and KANGASJARVI, 2010; MILLER et al., 2010). The activity of the glycolate oxidase enzyme plays an important role in plant metabolism, especially under abiotic stress conditions. In these cases, there is a reduction in the degree of stomatal opening accompanied by a reduction in the availability of CO_2 for the enzyme rubisco, intensifying the process of photorespiration and production of glycolate in chloroplasts (FOYER and NOCTOR, 2009). Glycolate is directed to peroxisomes where it is oxidized by the action of the glycolate oxidase enzyme leading to the production of H_2O_2 (GONDIM, 2012).

The $^1O_2$ is formed from the excitation of O_2 and arises when the latter absorbs enough energy to reverse the spin of one of its unpaired electrons. It is considered the most important reactive oxygen species responsible for the loss of PSII activity induced by excess light, which can result in cell death (MITTLER et al., 2002). $^1O_2$ can be neutralized by β-carotene, α-tocopherol or can react with PSII D1 protein (KRIEGER-LISZKAY, 2005).

The $^{\bullet}O_2^-$ is usually the first ROS formed in the cell. It is a moderately reactive ion that has both reducing and oxidizing properties and a short half-life of approximately 1 μs (HALLIWELL, 1977). It is formed from the reduction of O_2, with the transfer of only one electron. This process can occur with the use of several reducing agents. For example, under conditions of electron overload in the electron transport chain in chloroplasts, superoxide radical is formed by the oxidation of ferredoxin, through the Mehler's reaction(1) (MEHLER, 1951a; MEHLER, 1951b).

$$2\,O_2 + 2\,Fd_{(red)} \rightarrow 2\,{}^{\cdot}O_2^{-} + \,2\,Fd_{(oxi)} \quad (1)$$

The main place of production of $^{\bullet}O_2^-$ is the primary electron acceptor of the PSI linked to the thylakoid membrane (SINGH et al., 2014). Subsequently, $^{\bullet}O_2^-$ gives rise to secondary ROS from direct reactions or enzymatic processes (VALKO et al., 2005). The enzymatic process to reduce the superoxide radical(2), from the superoxide dismutase enzyme, gives rise to H_2O_2.

$$2\,{}^{\cdot}O_2^{-} + 2H^{+}\ \overrightarrow{SOD}\ O_2 + H_2O_2\ Fe^{3+}\ (2)$$

Hydrogen peroxide is moderately reactive and has a relatively long half-life of approximately 1 ms (MITTLER et al., 1991). H_2O_2 has particular characteristics that make it an important signaling molecule in biological processes involved in tolerance to various environmental stresses, when present in low concentrations (NEILL et al., 2002). For example, it is the only ROS that can diffuse through aquaporins in the membrane and travel long distances within the cell and is much more stable than other ROS (BIENERT et al., 2007). It is generated by the reduction of two O_2 electrons, catalyzed by some oxidases or indirectly via O_2 reduction (MITTLER et al., 2004).

The superoxide radical and hydrogen peroxide can give rise to the hydroxyl radical ($OH^{\bullet}$) through the Haber-Weiss's reaction(3). The formation of $OH^{\bullet}$ directly from H_2O_2 occurs through the Fenton's reaction(4) (BARBOSA et al., 2014).

$$^{\cdot}O_2^{-} + H_2O_2 \rightarrow OH^{-} + OH^{\cdot} + O_2\ (3)$$

$$^{\cdot}O_2^{-} + Fe^{3+} \rightarrow {}^{1}O_2 + Fe^{2+}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}\ (4)$$

The $OH^{\bullet}$ has short half-life and strongly positive redox potential. These characteristics make this ROS, different from H_2O_2, do not travel long distances and react with molecules nearby its formation site (ELSTNER, 1982).

Recently, several studies with a proteomic approach to the formation of ROS have been carried out for different species under conditions of environmental stress. The identified proteins belong to important physiological processes (metabolism of carbohydrates, amino acids, nitrogen and energy) and defense (ROS scavenging) (XU et al, 2015; AHMAD et al., 2016; MENG et al., 2016; LUO et al., 2017).

1.2.3. Antioxidative systems (Enzymatic and Non-enzymatic)

Under oxidative stress conditions, in order to eliminate ROS, plants increase the endogenous level of defense antioxidants (SHARMA et al. 2012). The term antioxidant refers to the function that enzymes, vitamins, secondary metabolites and

other phytochemicals perform against the harmful effects of high levels of ROS (MCDERMOTT 2000; ERASLAN et al., 2007; HONG-BO et al., 2008).

The defense mechanisms are essential for good cellular functioning, since they preserve the integrity of the cell membrane, protect the cell from redox enzymatic processes, DNA and proteins (GRENE, 2002). The processes of production and elimination of ROS can occur in several organelles, such as mitochondria, peroxisomes and chloroplasts (SHARMA et al., 2012). The defense mechanisms can be both enzymatic and non-enzymatic, as shown in Figure 1.1 (SOUSA, 2014).

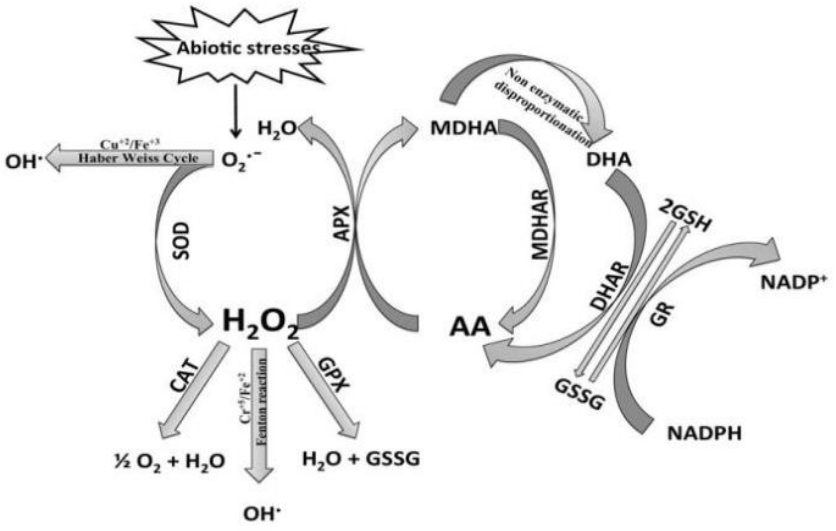

Figure 1.1 Antioxidant defense mechanism (GILL and TUTEJA, 2010).

Non-enzymatic antioxidants include the classes of antioxidants associated with a fat-soluble membrane (tocopherol and carotenoids) and that of water-soluble reducers (glutathione, ascorbate and phenolic compounds) (SOUSA, 2014).

Antioxidants such as ascorbic acid (ASA), glutathione (GSH) as well as tocopherols, flavonoids, alkaloids and carotenoids actively participate in the control of the pool of ROS in cells (APEL and HIRT, 2004). GSH is oxidized by ROS to form oxidized glutathione (GSSG), while ASA is oxidized to monodeshydroascorbate (MDA) and dehydroascorbate (DHA). In the glutathione-ascorbate cycle, GSSG, MDA and DHA can again be converted to GSH and ASA to maintain the ROS in concentrations that are supported by the cell (APEL and HIRT, 2004).

Several enzymes participate in maintaining the reduced status of ASA and GSH. Glutathione reductase participates in the reduction of GSSG to GSH, while monodeshydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) reduces MDA and DHA, respectively. All of these enzymes use NADPH as a reducing force (TSUGANE et al., 1999; APEL and HIRT, 2004). Both ASA and GSH are present in several cell compartments, mainly in chloroplasts, mitochondria and cytosol (POTTERS et al., 2002). Ascorbate participates in several cellular processes and, among these, the most important ones involve oxidative protection (APEL and HIRT, 2004), regulation of the transition from G1 to S phase in the cell cycle (POTTERS et al., 2002) and control growth by elongating cells (PASSARDI et al., 2004). For this purpose, the pathway of synthesis and degradation is distributed in mitochondria, cytosol (HOREMANS et al., 2000; GREEN and FRY, 2005) and apoplast (PASSARDI et al., 2004), indicating that the regulatory processes that ascorbate participates involve mechanisms located in several subcellular compartments.

The GSH in plant cells actively participates in sulfur metabolism, antioxidant defense and removal of xenobiotic compounds (POTTERS et al., 2002). The chemical reactivity of the thiol group of glutathione, its relative stability and its high solubility in water makes this molecule particularly suitable for receiving or donating electrons in physiological reactions (POTTERS et al., 2002). However, the evidence that the main pathway of GSH synthesis is located in chloroplasts (FOYER et al., 2001) makes this molecule a little explored target for oxidative metabolism in roots. On the other hand, its metabolic dynamics, including in the indirect action of DHA reduction, makes GSH essential for the control of ROS (POTTERS et al., 2002).

The other antioxidant molecules such as flavonoids and carotenoids, reported above, are still little explored in the literature and their effective role in the removal of ROS in subcellular compartments still deserves attention (APEL and HIRT, 2004). However, studies in *Arabidopsis* reveal that overexpression of a β-carotene hydrolase can cause an increase in the amount of xanthophylls in chloroplasts and result in an increase in tolerance to oxidative stress caused by excess light (DAVISON et al., 2002).

Enzymatic antioxidants are found in almost all cell compartments, which generally contain more than one enzyme, due to the joint action of enzymes with similar functions (MITTLER et al., 2004).

Among the main enzymes we can mention the superoxide dismutases (SOD; EC 1.15.1.1), ascorbate peroxidases (APX; EC 1.11.1.1), phenols (POX; EC 1.11.1.7) and glutathione (GPX; EC 1.11 .1.9) and catalases (CAT; 1.11.1.6) which, together with other regenerating enzymes of the active forms of antioxidants, promote the ROS scavenging (CAVALCANTI et al., 2004; CAVALCANTI et al., 2007).

The balance of SOD, APX, GPX and CAT activities is crucial in the suppression of toxic levels of ROS in the cell (APEL and HIRT, 2004). In recent years, a large number of studies have focused on the balance of these activities, on different cell organelles (MAIA, 2008).

It is not surprising, therefore, that SODs are present in practically all regions of the cell. In addition, the fact that biological membranes are impervious to charged O_2 molecules, makes the presence of SOD near $^{\bullet}O_2^-$ production sites necessary. SODs are classified, according to their metal cofactor, into three groups: Fe-SOD, present in chloroplasts; Mn-SOD, present in mitochondria and peroxisomes; and Cu/Zn-SODs, present in chloroplasts, cytosol and extracellular space (ALSCHER et al., 2002).

The APXs are considered the most important enzymes in the H_2O_2 scavenging in cytosol and chloroplasts (ASAI et al., 2004). APXs use ascorbate as their specific electron donor to reduce H_2O_2 to water. The APX isoenzymes are distributed in at least four distinct cellular compartments: in the stroma; bound to the chloroplast thylakoid membrane; membrane-bound (mAPX) in the micro-bodies, including glyoxysomes and peroxisomes; and cytosol (cAPX) (SHIGEOKA et al., 2002; D'ARCY-LAMETA et al., 2006). There are also isoforms of APX and other enzymes of the ascorbate-glutathione cycle in the mitochondria (mitAPX) (CHEW et al., 2003). All of these isoenzymes have a high specificity for ascorbate as an electron donor.

Phenol peroxidases (POX) from class III plants have been found in several cell compartments such as cytosol, vacuole, apoplast and cell wall (MAIA, 2004; MITTLER et al., 2004). However, even though there are several studies on its activity in plants under stress, its involvement in the ROS scavenging is still not completely clear, since this enzyme is also involved in the lignification process and growth arrest in stress conditions (CAVALCANTI et al., 2007).

Studies have shown that, in most cases, the expression levels of genes related to GPX activity are increased in response to stresses (NAVROT et al., 2006). Currently, GPX is defined as the general name for a family of multiple isoenzymes that catalyze

the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor (MARGIS et al., 2008).

Catalase (CAT) is an enzyme that contains a heme group in its structure that catalyzes the dismutation of H_2O_2 to water and O_2 (ZÁMOCKY and KOLLER, 1999) in peroxisomes and glyoxysomes by the oxidases involved in the β-oxidation of fatty acids, in reactions glyoxalate (photorespiration) and purine catabolism (VAN BREUSEGEM et al., 2001). Multiple forms of catalase have been described in many plant species. In maize, three isoforms (CAT-1, CAT-2 and CAT-3) were found whose genes are on separate chromosomes and are expressed distinctly with regulatory mechanisms and different locations: CAT-1 and CAT-2 located in peroxisomes, glyoxysomes and cytosol, while CAT-3 is probably located in the mitochondria (POLIDOROS and SCANDALIOS, 1997; VAN BREUSEGEM et al., 2001).

1.2.4. Hydrogen peroxide as a signaling molecule

Hydrogen peroxide is one of the ROS and a molecule well known for its toxic effect. However, currently several H_2O_2 signaling functions in plant physiology and biochemistry have been described in scientific studies (KUŹNIAK and URBANEK, 2000; NEILL et al., 2002; APEL and HIRT, 2004; HUNG et al., 2005).

Plants and other aerobic organisms have evolved different metabolic systems where ROS are used as signaling molecules in cellular processes. From this context, it is believed that H_2O_2, the most stable molecule of ROS, can act indirectly in the regulation of acclimation, defense processes and plant development (ŚLESAK et al., 2007).

Due to the long half-life of H_2O_2 when compared to the superoxide radical, it is likely that hydrogen peroxide acts as a signaling molecule over a long distance (VRANOVÁ et al., 2002). Transmebrane water channels known as aquoporins can facilitate the movement of H_2O_2 between cell membranes together with water (HENZLER and STEUDEL, 2000).

Currently, it is well documented that H_2O_2 plays a key role in plant responses to biotic and abiotic stresses. According to Ślesak et al., (2007), H_2O_2 has a key role in controlling a variety of responses to stress and physiological adjustment (Figure 1.2).

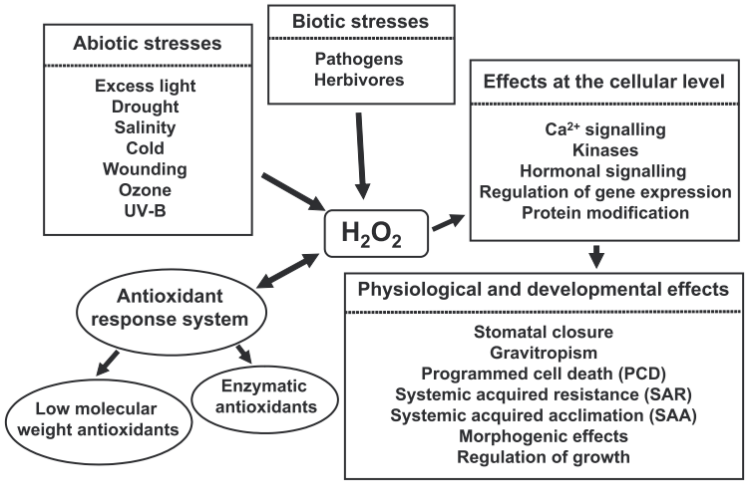

Figure 1.2. Central role of hydrogen peroxide in plants, in response to several environmental stresses (modified by DESIKAN et al., 2003).

Several studies have shown the effects of H_2O_2 as a signaling molecule associated with the response of abiotic stresses, such as drought (HAMEED and IQBAL, 2014; ASHRAF et al., 2015), salinity (SATHIYARAJ et al., 2014; MOHAMED et al. , 2015), cold (ORABI et al., 2015), high temperatures (WANG et al., 2014; WU et al., 2015), UV radiation (HE et al., 2005), ozone (OKSANEN et al., 2004) and heavy metals (WEN et al., 2013).

In the same way that H_2O_2 acts to improve the tolerance of plants to abiotic stresses, it can also modulate the expression of tolerance genes and the activity of antioxidant enzymes during abiotic stresses (NIU and LIAO, 2016). The H_2O_2 signaling process can interact with different signaling molecules, such as phytohormones (SHI et al., 2015), protein kinase (GONZÁLEZ et al, 2012) and many other small signaling molecules (LI et al. 2015). H_2O_2 and these other molecules can mutually influence different routes of positive and negative feedback. Thus, they regulate cell division and differentiation, antioxidant defense systems as well as the expression of genes of different physiological mechanisms (NIU and LIAO, 2016).

The components of signal transduction, including protein kinases, such as calcium dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPK) have been implicated in acting on plant tolerance to biotic and abiotic stress (WURZINGER et al., 2011). MAPK cascades are important pathways in abiotic stress responses and allow extracellular stimuli to be transduced into intracellular changes

(ZHOU et al., 2014). A series of cellular stimuli that induce the production of ROS (H_2O_2) can also activate MAPK pathways (TORRES and FORMAN, 2003; MCCUBREY et al., 2006), which can induce acclimation of plants to stress conditions.

1.2.5. The sunflower crop

The sunflower (*Helianthus annuus* L.) is an annual dicotyledon plant, order Asterales, family Asteraceae, subfamily Asteroideae and tribe Heliantheae. Original in North-America.

The sunflower plant has a root system with a pivoting main root and inflorescence known as a capitulum, whose shape can vary from concave to convex, and the stem can present different curvatures that are expressed in maturation (CASTIGLIONI et al., 1994).

It stands out worldwide as the fifth oilseed plant in production of raw material, second only to soybeans, rapeseed, cotton and peanuts, fourth oilseed in bran production after soybean, rapeseed and cotton and third in world oil production, after of soy and rapeseed. The largest grain producers are Russia, Ukraine, the European Union and Argentina (LAZZAROTTO et al., 2005).

Sunflower is highly important, as it produces oil of good quality and high nutritional value as a functional food for human consumption, as well as for ruminants, pigs and birds and, in addition, it can be used for silage as a forage option. Currently, it is arousing great interest worldwide, as it represents a new market alternative for the production of raw material for obtaining biofuels, due to the high oil content in the achenes and its wide adaptation to different edaphoclimatic regions.

In the past, sunflower was a crop considered moderately tolerant to salt stress (HARDWICK and FERGUSON, 1978; BLAMEY et al., 1986). However, there are currently results that show that there are several genotypes considered sensitive to salinity (MOTA, 2014).

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2. CHAPTER 2

Hydrogen peroxide seed priming increases the photosynthesis by improvement in the efficiency of PSII in sunflower plants under salt stress[2]

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Title Page

Hydrogen peroxide seed priming increases the photosynthesis by improvement of the efficiency of PSII in sunflower plants under salt stress

Petterson Costa Conceição Silva[a]*, André Dias de Azevedo Neto[a], Hans Raj Gheyi[a], Rogério Ferreira Ribas[a], Caroline Rastely dos Reis Silva[a], Alide Mitsue Watanabe Cova[a]

[a]Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380000, Bahia, Brazil

*Corresponding author:

Petterson Costa Conceição Silva

Universidade Federal do Recôncavo da Bahia

Cruz das Almas, 44380000, BA, Brazil

Tel: +5571991958112

E-mail address: petter.ufrb@gmail.com (P. C. C. Silva).

Petterson Costa Conceição Silva, ORCID - http://orcid.org/0000-0003-1350-1827

André Dias de Azevedo Neto, ORCID - http://orcid.org/0000-0002-4073-8726

Hans Raj Gheyi, ORCID - http://orcid.org/0000-0002-1066-0315

Rogério Ferreira Ribas, ORCID - http://orcid.org/0000-0003-0766-825X

Caroline Rastely dos Reis Silva, ORCID - http://orcid.org/0000-0002-6195-5084

Alide Mitsue Watanabe Cova, ORCID - http://orcid.org/0000-0003-0570-7008

ABSTRACT

Salinity commonly affects photosynthesis and crop production worldwide. However currently, some studies have shown that hydrogen peroxide (H_2O_2) priming can act in increase the tolerance of plants to salt stress. In this context, the aim of this study was tested the hypothesis that the H_2O_2 seed priming can contribute for improvement of photosynthetic efficiency in sunflower plants under salt stress. The experiment was conducted in completely randomized design, with 4 replicates. Six treatments were tested: control (deionized water); salt control (100 mM NaCl); 0.1 mM H_2O_2 (36 h) + 100 mM NaCl; 1 mM H_2O_2 (24 h) + 100 mM NaCl; 10 mM H_2O_2 (12 h) + 100 mM NaCl; and 100 mM H_2O_2 (12 h) + 100 mM NaCl. Plants in the salt stress showed a strong reduction of growth, compared to those of the control treatment. However, this effect was less pronounced in plants whose seeds were primed with H_2O_2. At the end of the experiment, H_2O_2 seed priming increased A, A/Ci, F_v/F_m, Y_{II}, ETR and the chlorophyll *b* content and reduced the non-photochemical quenching (Y_{NPQ} and Y_{NO}), compared to plants of the salt control treatment.

Keywords**:** *Helianthus annuus* L.; H_2O_2; salt tolerance; cross-talk; photosynthesis.

1. Introduction

Salinity is one of the environmental stresses that most affect crop growth and yield in the world, being one of the main challenges encountered in agriculture (Veeranagamallaiah et al. 2007).

Salinity reduces water availability to the roots, induces stomatal closure, and reduces photosynthetic rates and enzyme activities in plants, reducing their growth (Pedrotti et al. 2015). Under stress conditions, the reduction of photosynthetic efficiency may generate an excess of free energy in the photosynthetic apparatus and increase the production of reactive oxygen species (ROS) (Lima Neto et al. 2014).

ROS are produced through biochemical events of the aerobic metabolism (Noctor and Foyer 1998). These species arise mainly during the electron flow in mitochondria and chloroplasts when, in the absence of another acceptor, the electrons are transferred to O_2. In plant cells, ROS can also be produced in peroxisomes, cytosol, plasma membrane and apoplast (Caverzan et al. 2016).

Hydrogen peroxide (H_2O_2) is considered the main ROS found in plant tissue. Due to its electrochemical characteristics and small size, H_2O_2 is able to cross the membranes and diffuse between cell compartments, which facilitates its signaling function (Bienert et al. 2006).

Several scientific articles have evidenced the role of H_2O_2 as a signaling molecule with multiple functions in plants (Neill et al. 2002). Among these functions, H_2O_2 act with a key role in the plant response to abiotic stresses, such as salinity (Azevedo Neto et al. 2005), drought (Hossain and Fujita 2013), high temperatures (Wu et al. 2015), UV radiation (He et al. 2005), ozone (Oksanen et al. 2004) and heavy metals (Wen et al. 2013). H_2O_2 can modulate the expression of resistance genes and the activity of antioxidant enzymes during abiotic stresses, increasing the tolerance of plants (Niu and Liao 2016).

Although of recent reports have shown that the H_2O_2 can acclimate the plants to abiotic stress, very little is known about these physiological responses, especially regarding the contribution of H_2O_2 priming in photosynthesis impairment. Thus, the aim of this study was to tested the hypothesis that H_2O_2 seed priming increases the salt tolerance of sunflower plants under salt stress by minimizing photoinhibition and improving of photosynthetic efficiency of PSII.

2. Material and Methods

2.1 Experimental conditions

Two experiments were conducted in the Laboratory of Biochemistry and in the greenhouse of the Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, Brazil (12º40'19"S, 39º 6'23"W). The climate is hot and humid tropical (Af) according to Köppen's classification (Alvares et al. 2013), with annual means of rainfall, temperature and relative humidity of 1,224 mm, 24.5 °C and 80%, respectively.

Seeds of Agrobel 975 sunflower (AG 975) were used in both experiments. The seeds were kept for 5 minutes in 0.2% sodium hypochlorite (m/v) and subsequently 3-fold washed with distilled water for disinfection.

2.2 First experiment (selection of treatments)

Seeds were placed to germinate in Petri dishes (20 seeds per dish), on sheets of filter paper. Petri dishes containing the seeds were kept in a B.O.D. (Biochemical Oxygen Demand) germination chamber at temperature of 25 °C. Seeds on Petri dishes were soaked in distilled H_2O for 36 hours, or in H_2O_2 at 0.1, 1, 10 and 100 mM of H_2O_2 for 12; 24 and 36 hours.

Seedlings were transferred to polyethylene pots containing 15 L of full-strength nutrient solution (Furlani, 1997) + 100 mM NaCl, except for plants of the control treatment, totaling 14 treatments: T1 - control (absence of NaCl and absence of H_2O_2); T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 0.1 mM H_2O_2 (12 h); T4 - 1 mM H_2O_2 (12 h); T5 - 10 mM H_2O_2 (12 h); T6 - 100 mM H_2O_2 (12 h); T7 - 0.1 mM H_2O_2 (24 h); T8 - 1 mM H_2O_2 (24 h); T9 - 10 mM H_2O_2 (24 h); T10 - 100 mM H_2O_2 (24 h); T11 - 0.1 mM H_2O_2 (36 h); T12 - 1 mM H_2O_2 (36 h); T13 - 10 mM H_2O_2 (36 h); T14 - 100 mM H_2O_2 (36 h). The plants were harvested after 35 days under these conditions. The experiment was carried out in a completely randomized design with four replicates, containing one plant per replicate.

The harvested plants were partitioned into leaves, stems and roots, dried in an oven at 65 ºC for 72 h, and weighed on an analytical balance to quantify the dry masses of leaves (LDM), stem (SDM), and roots (RDM). With these data, total dry mass (TDM) was obtained.

2.3 Second experiment

In this experiment, the four treatments with H_2O_2 application which showed highest total dry mass accumulation in the previous experiment were selected. The treatments of H_2O_2 seed priming selected for this experiment were: T5 - 10 mM of H_2O_2 (12 h), T6 - 100 mM of H_2O_2 (12 h), T8 - 1 mM of H_2O_2 (24 h) and T11 - 0.1 mM of H_2O_2 (36 h). Two additional treatments were also included: T1 - control (absence of NaCl and absence of H_2O_2) and T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2), totaling 6 treatments, with four

replicates each. Plants were kept under the same experimental conditions imposed in the first experiment. A completely randomized design with four replicates was used, containing one plant per replicate. Three weekly harvests were performed at 21, 28 and 35 days after sowing (DAS) to analyze the behavior of the plants over time.

Gas exchange evaluations were carried out on the youngest fully expanded pair of leaves (Silveira et al. 2009), using an infrared gas analyzer - IRGA, Li-6400XT model (Li-Cor, Lincoln, NE, USA). CO_2 assimilation rate (A), transpiration (E), stomatal conductance (gs) and internal CO_2 partial pressure (Ci) were determined. These data were used to determine the instantaneous carboxylation efficiency (A/Ci).

Chlorophyll *a* fluorescence was measured by the saturation pulse method, at a photosynthetic photon flux density of 1,000 μmol m^{-2} s^{-1}, using the OS5-FL portable modulated fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire). Minimum fluorescence (F_0), maximum fluorescence (F_m) and the potential quantum yield of PSII (F_v/F_m) were measured on 30 minutes dark-adapted leaves. Light-adapted leaves were evaluated for the yields of the competitive pathways for the de-excitation of energy absorbed in PSII, the quantum yield of photochemical quenching in PSII (Y_{II}) and the quantum yield of regulated (Y_{NPQ}) and non-regulated (Y_{NO}) non-photochemical quenching in PSII. Electron transport rate (ETR) was estimated according to Bilger et al. (1995).

The same leaves used for photosynthetic evaluations were analyzed for the relative water content (RWC), electrolyte leakage (EL) (Silva, Silveira, Ribeiro, & Vieira, 2015), leaf succulence (SUC) and sclerophylly index (SI) (Cova et al. 2016). In addition, the contents of the photosynthetic pigments chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoids (Car) were extracted in ethanol (95%) and quantified by spectrophotometry at 664, 649 and 470 nm, using the equations proposed by Lichtenthaler and Buschmann (2001).

Another part of the plant material (leaves and stem) was dried in an oven at 65 °C for 72 h to measure the LDM, SDM, and ShDM.

2.4 Statistical analysis

All data from both experiments were subjected to analysis of variance (ANOVA) and the means were compared by Scott-Knott's test ($p \leq 0.05$), using the statistical program SPSS for Windows (SPSS, Chicago, IL).

3. Results

3.1 First experiment

The results showed a significant effect between the applied treatments ($p \leq 0.01$) for all variables analyzed (Table 2.1). As expected, in the salt control treatment (T2) there were reductions in the LDM, SDM, RDM and

TDM of approximately 73, 76, 71 and 73%, respectively, compared to plants in the control treatment (T1) (Table 2.1). However, in some treatments, the deleterious effects of salinity were significantly less pronounced due to the H_2O_2 seeds priming.

Table 2.1 Results of Fisher's test and Scott-Knott's test for the parameters analyzed in the first assay, in sunflower plants at 35 days of salt stress.

Sources of variation	LDM	SDM	RDM	TDM
Treatments	**	**	**	**
CV (%)	5.90	6.76	10.34	4.81
Treatments	Leaf	Stem	Root	Total
	Dry mass (% control)			
T1 - control	100	100	100	100
T2 - salt control	26.8 d	23.7 d	29.5 c	26.6 d
T3 - 0.1 mM H_2O_2 (12 h)	29.2 d	25.5 c	29.5 c	28.2 d
T4 - 1 mM H_2O_2 (12 h)	31.8 c	27.4 b	32.5 c	30.7 c
T5 - 10 mM H_2O_2 (12 h)	35.2 b	31.5 a	50.2 a	38.2 a
T6 - 100 mM H_2O_2 (12 h)	37.0 b	28.2 b	48.4 a	37.6 a
T7 - 0.1 mM H_2O_2 (24 h)	35.8 b	26.9 c	32.5 c	32.4 c
T8 - 1 mM H_2O_2 (24 h)	40.9 a	31.3 a	40.6 b	38.1 a
T9 - 10 mM H_2O_2 (24 h)	35.5 b	30.2 a	41.2 b	35.6 b
T10 - 100 mM H_2O_2 (24 h)	29.3 d	23.2 d	33.4 c	28.7 d
T11 - 0.1 mM H_2O_2 (36 h)	37.1 b	29.9 b	42.6 b	36.6 a
T12 - 1 mM H_2O_2 (36 h)	36.8 b	30.4 b	39.4 b	35.6 b
T13 - 10 mM H_2O_2 (36 h)	32.5 c	28.2 b	41.2 b	33.6 b
T14 - 100 mM H_2O_2 (36 h)	28.0 d	23.5 d	29.3 c	27.2 d

** Significant at $p \leq 0.01$. Means followed by the same letter, in column, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). LDM (leaf dry mass), SDM (stem dry mass), RDM (root dry mass) and TDM (total dry mass). Data in percentage of control.

The results of the Scott-Knott test showed that seeds priming with 1 mM of H_2O_2 (24 h) (T8) increased LDM by 53% in comparison to the salt control treatment (T2). For the stem, in eight out of the twelve treatments (T4, T5, T6, T8, T9, T11, T12 and T13) of the H_2O_2 seeds priming significantly increased the dry mass production (from 16 to 32%) compared to the T2 treatment. A similar behavior was observed in the roots and, in seven out of the twelve priming treatments (T5, T6, T8, T9, T11, T12 and T13), RDM was 43 to 71% higher than that of the salt control treatment (T2) (Table 2.1).

The selection of treatments for the second assay was based on the TDM of the plants. The treatments T5, T6, T8 and T11 were selected as the most effective in reducing the deleterious effects of salinity. In these treatments there was an increase in TDM, which changed between 38 and 43% when compared to plants of the salt control treatment (T2) (Table 2.1).

3.2 Second experiment

It can be seen in Table 2.2 that the tested treatments had significant effect on LDM, SDM and ShDM (at 21, 28 and 35 days), on RWC and SI (at 21 and 28 days), on SUC, Y_{II} and ETR (at 28 and 35 days), on the A/Ci ratio, Chl*b* and Car (at 21 and 35 days), Chl*a* (at 21 days) and on A, Ci, A/Ci, F_0, F_v/F_m, Y_{NPQ} and Y_{NO} (at 35 days). The other parameters analyzed were not affected significantly by the treatments applied.

Table 2.2 Results of Fisher's test for the parameters analyzed in the second assay, in sunflower plants at 21, 28 e 35 days of salt stress.

Parameters	days		
	21	28	35
LDM (% of control)	**	**	**
SDM (% of control)	**	**	**
ShDM (% of control)	**	**	**
RWC (%)	**	**	ns
SUC (mg H_2O cm^{-2})	ns	**	*
SI (mg DM cm^{-2})	**	**	ns
EL (%)	ns	ns	ns
A (µmol CO_2 m^{-2} s^{-1})	ns	ns	**
E (mmol H_2O m^{-2} s^{-1})	ns	ns	ns
gs (mol H_2O m^{-2} s^{-1})	ns	ns	ns
Ci (Pa)	ns	ns	**
A/Ci [(µmol CO_2 m^{-2} s^{-1}) Pa^{-1}]	**	ns	**
A/E (µmol $mmol^{-1}$)	ns	ns	ns
F_0	ns	ns	**
F_v/F_m	ns	ns	**
Y_{II}	ns	*	**
ETR	ns	*	**
Y_{NPQ}	ns	ns	**
Y_{NO}	ns	ns	**
Chl*a* (mg g^{-1} FM)	**	ns	ns

Chl*b* (mg g^{-1} FM)	**	ns	**
Car (mg g^{-1} FM)	**	ns	*

*; ** Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; ns, not significant. LDM (leaf dry mass), SDM (stem dry mass), ShDM (shoot dry mass), RWC (relative water content), SUC (leaf succulence), SI (sclerophylly index) EL (electrolyte leakage), A (CO_2 assimilation rate), E (transpiration rate), gs (stomatal conductance), Ci (intercellular CO_2 partial pressure), A/Ci (instantaneous carboxylation efficiency), A/E (instantaneous water use efficiency), F_0 (minimal fluorescence), F_v/F_m (maximum yield quantum of PSII), Y_{II} (quantum yield of photochemical quenching in PSII), ETR (electron transport rate), Y_{NPQ} (quantum yield of regulated non-photochemical quenching in PSII), Y_{NO} (quantum yield of non-regulated non-photochemical quenching in PSII), Chl*a* (chlorophyll *a* content), Chl*b* (chlorophyll *b* content), Car (carotenoids content).

Salinity significantly reduced the relative biomass production of sunflower plants in all evaluated periods (Table 2.3). However, this effect was less pronounced in plants whose seeds were primed with H_2O_2. It is important to note in this table that, regardless of the harvest time, the relative productions of LDM, SDM and ShDM in T5 and T8 treatments were, on average, twice the value observed in T2 (salt control) (Table 2.3).

Table 2.3 Effect of salt stress and H_2O_2 seeds priming of on the dry mass (% control) of leaf (LDM), stem (SDM) and shoot (ShDM) of sunflowers plants in nutrient solution, at 21, 28 and 35 days of cultivation.

Dry mass	Treatments				
	T2	T5	T6	T8	T11
	% control (21 days)				
Leaf	27.8 b	51.0 a	32.3 b	47.5 a	46.5 a
Stem	29.0 c	64.0 a	43.0 b	41.0 b	44.0 b
Shoot	28.2 b	55.4 a	35.9 b	45.3 a	45.6 a
	% control (28 days)				
Leaf	10.3 b	25.5 a	26.8 a	27.9 a	21.0 a
Stem	11.1 b	18.1 a	12.8 b	22.4 a	18.1 a
Shoot	10.6 b	22.4 a	21.9 a	25.5 a	19.8 a
	% control (35 days)				
Leaf	18.4 b	35.1 a	30.5 a	31.0 a	27.2 a
Stem	18.2 c	35.0 a	28.3 b	32.8 a	26.0 b
Shoot	18.3 b	35.1 a	29.5 a	31.9 a	26.6 a

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Treatments: T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T5 - 10 mM H_2O_2

(12 h) + (100 mM NaCl); T6 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T8 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T11 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

Salt stress significantly reduced RWC at 21 (Table 2.4) and 28 (Table 2.5) days after sowing. It can be observed in these tables that the salt stress treatments did not differ, but the mean reduction of RWC was more pronounced at 28 days (22%) than at 21 days (12%).

In contrast, there was a trend of a salt induced SUC increase. At 28 days, in three of the five salt treatments (T2, T6 and T8) SUC was 19% higher when compared to the control treatment (Table 2.5) and, at 35 days, all salt treatments had higher values of SUC compared to the control treatment (Table 2.6). The SI increased by about 25.5% at 21 days, in all H_2O_2 seed priming treatments (Table 2.4). However, at 28 days, such increase was only observed in the treatments T2 (14%) and T8 (24%) (Table 2.5).

Table 2.4 Effect of salt stress and H_2O_2 seeds priming on the relative water content (RWC, %), sclerophylly index (SI, mg DM cm^{-2}), instantaneous carboxylation efficiency [(A/Ci, µmol CO_2 m^{-2} s^{-1}) Pa^{-1}], chlorophyll *a* content (Chl*a*, mg g^{-1} FM), chlorophyll *b* content (Chl*b*, mg g^{-1} FM) e carotenoids content (Car, mg g^{-1} FM) of sunflower plants at 21 days of salt stress.

Parameters	Treatments					
	T1	T2	T5	T6	T8	T11
RWC	65.0[a]	59.2[b] (-9)	56.6[b] (-13)	55.8[b] (-14)	55.8[b] (-14)	58.0[b] (-11)
SI	3.07[b]	2.86[b] (-7)	3.75[a] (+22)	3.88[a] (+26)	3.82[a] (+24)	4.00[a] (+30)
A/Ci	1.19[a]	1.04[b] (-13)	1.11[b] (-7)	1.09[b] (-8)	1.05[b] (-12)	1.07[b] (-10)
Chl*a*	1.21[b]	0.99[c] (-18)	1.30[a] (+7)	1.18[b] (-2)	1.32[a] (+9)	1.19[b] (-2)
Chl*b*	0.34[b]	0.29[c] (-15)	0.41[a] (+21)	0.35[b] (+3)	0.40[a] (+18)	0.36[b] (+6)
Car	0.35[a]	0.29[b] (-17)	0.36[a] (+3)	0.34[a] (-3)	0.38[a] (+9)	0.36[a] (+3)

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Values into parentheses represent the percentage increase (+) or decrease (-) compared to control. Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T5 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T6 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T8 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T11 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

CO_2 assimilation rate (A) did not differ between the control (T1) and salt control (T2) treatments at 35 days. However, in the priming treatments (T5, T6, T8 and T11), CO_2 assimilation rate was increased on average by 15 and 26%, compared to plants of T1 and T2 treatments, respectively (Table 2.6).

Table 2.5 Effect of salt stress and H_2O_2 seeds priming on the relative water content (RWC, %), sclerophylly index (SI, mg DM cm^{-2}), leaf succulence (SUC, mg H_2O cm^{-2}), quantum yield of photochemical quenching in PSII (YII), electron transport rate (ETR) of sunflower plants at 28 days of salt stress.

Parameters	Treatments					
	T1	T2	T5	T6	T8	T11
RWC	72.2[a]	59.7[b] (-17)	56.1[b] (-22)	53.4[b] (-26)	57.0[b] (-21)	56.3[b] (-22)
SI	3.50[b]	4.00[a] (+14)	3.76[b] (+7)	3.77[b] (+8)	4.33[a] (+24)	3.62[b] (+3)
SUC	18.4[b]	22.8[a] (+24)	19.8[b] (+8)	21.6[a] (+17)	21.6[a] (+17)	19.9[b] (+8)
Y_{II}	0.53[a]	0.54[a] (+2)	0.50[b] (-6)	0.56[a] (+6)	0.54[a] (+2)	0.49[b] (-8)
ETR	223[a]	228[a] (+2)	211[b] (-6)	233[a] (+5)	225[a] (+1)	205[b] (-8)

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Values into parentheses represent the percentage increase (+) or decrease (-) compared to control. Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T5 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T6 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T8 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T11 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

At 35 days, the salt stress increased the internal CO_2 concentration (Ci) and reduced the instantaneous carboxylation efficiency (A/Ci) only in the T2 treatment (Table 2.6). In contrast, H_2O_2 priming increased the A/Ci ratio by 17 and 38% compared to the treatments T1 and T2, respectively.

In the last harvest (35 days), salinity increased (11%) the values of F_0 in the T2 treatment, compared to the T1 (Table 2.6). On the other hand, compared to T1 and T2, H_2O_2 priming reduced the F_0 values by 14 and 23%, respectively. As opposed to F_0, reductions in F_v/F_m (8%), Y_{II} (22%) and ETR (23%) were observed only in plants of the T2 treatment (Table 2.6). In primed plants, F_v/F_m was not affected by salinity while Y_{II} and ETR increased on average by 11.5 and 43%, respectively, in comparison to T1 and T2 treatments.

Table 2.6 Effect of salt stress and H_2O_2 seeds priming on the leaf succulence (SUC, mg H_2O cm^{-2}), CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), intercellular CO2 partial pressure (Ci, Pa) instantaneous carboxylation efficiency [(A/Ci, µmol CO_2 m^{-2} s^{-1}) Pa^{-1}], minimal fluorescence (F_0), maximum yield quantum of PSII (F_v/F_m),

quantum yield of photochemical quenching in PSII (Y_{II}), electron transport rate (ETR), quantum yield of regulated (Y_{NPQ}) and non-regulated (Y_{NO}) non-photochemical quenching in PSII, chlorophyll *b* content (Chl*b*, mg g^{-1} FM) and carotenoids content (Car, mg g^{-1} FM) of sunflower plants at 35 days of salt stress.

Parameters	Treatments					
	T1	T2	T5	T6	T8	T11
SUC	15.0[b]	18.4[a] (+23)	17.8[a] (+19)	17.4[a] (+16)	18.4[a] (+23)	17.5[a] (+17)
A	30.1[b]	27.4[b] (-9)	33.4[a] (+11)	34.3[a] (+14)	36.2[a] (+20)	34.5[a] (+15)
Ci	29.3[b]	31.0[a] (+6)	28.7[b] (-2)	28.7[b] (-2)	28.7[b] (-2)	28.3[b] (-3)
A/Ci	1.03[b]	0.88[c] (-15)	1.18[a] (+15)	1.18[a] (+15)	1.27[a] (+23)	1.21[a] (+17)
F_0	247[b]	275[a] (+11)	203[c] (-18)	219[c] (-12)	214[c] (-14)	211[c] (-15)
F_v/F_m	0.73[a]	0.67[b] (-8)	0.79[a] (+8)	0.77[a] (+5)	0.77[a] (+5)	0.79[a] (+8)
Y_{II}	0.50[b]	0.39[c] (-22)	0.56[a] (+12)	0.56[a] (+12)	0.55[a] (+10)	0.56[a] (+12)
ETR	211[b]	163[c] (-23)	235[a] (+12)	235[a] (+12)	233[a] (+11)	236[a] (+12)
Y_{NPQ}	0.27[b]	0.33[a] (+22)	0.26[b] (-4)	0.25[b] (-7)	0.25[b] (-7)	0.26[b] (-4)
Y_{NO}	0.23[b]	0.28[a] (+22)	0.18[c] (-22)	0.19[c] (-17)	0.19[c] (-17)	0.18[c] (-22)
Chl*b*	0.87[a]	0.56[b] (-36)	0.95[a] (+9)	1.03[a] (+18)	0.87[a] (0)	0.98[a] (+13)
Car	0.38[b]	0.50[a] (+32)	0.31[b] (-18)	0.34[b] (-11)	0.36[b] (-5)	0.32[b] (-16)

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Values into parentheses represent the percentage increase (+) or decrease (-) compared to control. Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T5 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T6 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T8 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T11 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

At 35 days, the salt stress increased by 22% the quantum yield of regulated (Y_{NPQ}) and non-regulated (Y_{NO}) non-photochemical quenching in PSII, only in the T2 treatment (Table 2.6). By contrast, in primed plants, the Y_{NO} was on average 20 and 34% lower than that observed in the treatments T1 and T2, respectively.

At 21 days, the salt stress reduced the contents of Chl*a* (18%), Chl*b* (15%) and Car (17%) only in the T2 treatment (Table 2.4). On the other hand, in the treatments T5 and T8, the contents of Chl*a* and Chl*b* were higher than in the treatments T1 (8 and 19%) and T2 (32 and 39%), respectively. At 35 days, there was a reduction of 36% in Chl*b* and an increase of 32% in Car in the T2 treatment (Table 2.6).

4. Discussion

In both experiments the results showed that, under salt stress conditions, there is a reduction in the growth and dry mass production of plants, probably caused by disorders in the physiological and biochemical processes (Hasegawa 2013). However, H_2O_2 seed priming attenuated the deleterious effects of salinity, increasing salt tolerance and improving the growth of sunflower plants under stress conditions (Hossain et al. 2015).

In a more detailed manner, the second assay allowed to observe the parameters most affected by salt stress. The absence of stomatal limitation associated with the reduction in carboxylation efficiency (A/Ci) in the T2 treatment suggests that the reduction of growth is associated, at least in part, with a partial inactivation of the Rubisco activity and/or its degradation (Parry et al. 2008). The observation that plants of the priming treatments had higher A/Ci and higher growth than those of the T2 treatment supports this hypothesis. Silva et al. (2011) observed a significant reduction in the A/Ci of *Jatropha curcas* plants cultivated under 100 mM of NaCl and associated these results with a possible Rubisco inactivation. In a test with different cowpea genotypes, Andrade et al. (2018) also verified a strong salt-induced reduction in A/Ci ratio.

In plants that were stressed and not primed with H_2O_2 (T2), there were increases in the F_0, Y_{NPQ} and Y_{NO}, and reduction in the F_v/F_m, Y_{II} and ETR. Thus, the results of chlorophyll *a* fluorescence indicated the occurrence of damage to the photosynthetic apparatus (Li et al. 2010).

Plants of the T2 treatment also showed a decrease of chlorophylls (Chl*a* and Chl*b*) content and increase of carotenoids (Car) content. Chlorophylls are the main pigments responsible for the capture of light energy and, under salt stress, photosynthesis may be reduced due to inhibition of biosynthesis or increased degradation of these pigments (Pak et al. 2009). Carotenoids are integral constituents of the thylakoid membranes, acting as accessory pigments in the capture of light and as photoprotective agents in the dissipation of excess absorbed light (Baroli et al. 2003). Thus, while chlorophylls are related to the phenomena of capturing, transferring and converting light energy into chemical energy (evaluated by F_0, F_v/F_m, Y_{II} and ETR), carotenoids are mainly related to the dissipation of energy (evaluated by Y_{NPQ} and Y_{NO}). Taken together, the results of chlorophyll *a* fluorescence, the reduction in chlorophylls content and the increase in Car content support the occurrence of salt-induced damage to the photosynthetic apparatus of plants grown from H_2O_2 non-primed seeds (Klughammer and Schreiber 2008).

In contrast to the results observed in T2, the plants of all treatments whose seeds were primed with H_2O_2 showed normal values of chlorophyll fluorescence parameters and pigments content. These results suggest that the absence of damage to the photosynthetic apparatus may, at least partly, explain the greater salt tolerance observed in the H_2O_2 seed priming treatments.

These results also indicate that, for the induction of salt tolerance, the higher the concentration of H_2O_2, the shorter the period required for seeds imbibition. This result can be related to the fact that H_2O_2 plays a dual role in plants: at low concentrations, it acts as a signaling molecule triggering the responses against abiotic stresses (Niu and Liao 2016) and, at high concentrations, it can cause damage and even trigger programmed cell death (Bowler and Fluhr 2000).

Other studies also showed the beneficial effect of using different methods of H_2O_2 application in plants under salt conditions: via nutrient solution in maize (Azevedo Neto et al. 2005) and basil (Silva et al. 2019), via seeds in maize (Gondim et al. 2010), cotton (Santhy et al. 2014) and wheat (Wahid et al. 2007) and via leaf spraying in maize (Gondim et al. 2013) and onion (Semida 2016).

Studies carried out with plant tissues support the idea that H_2O_2, besides being a signaling molecule, also plays a pivotal role in the primary metabolism of the plants. Changes in the endogenous level of H_2O_2 may induce the expression of several genes, including those that encode enzymatic and non-enzymatic antioxidant agents (Neill et al. 2002; Niu and Liao 2016). Additionally, the application of H_2O_2 may increase the net CO_2 assimilation rate, quantum efficiency of PSII, photosynthetic pigments content, biomass accumulation and formation of adventitious roots (Gondim et al. 2013; Farouk and Abdul Qados 2018).

5. Conclusions

Our results allowed concluding that H_2O_2 seed priming mitigate the deleterious effects of salt stress on sunflower plants, especially at the concentration of 10 mM H_2O_2 during 12 h and 1 mM H_2O_2 during 24 h.

Taken together, the results of CO_2 assimilation, Chl*a* fluorescence, and photosynthetic pigments content indicate that the H_2O_2 seed priming promotes an increase in photosynthetic efficiency in stressed plants inducing an increase in salt tolerance.

Declaration of interest

The authors report no conflicts of interest. All authors are responsible for the content and writing of this manuscript.

Contributions

Petterson Costa Conceição Silva, André Dias de Azevedo Neto, Hans Raj Gheyi and Alide Mitsue Watanabe Cova contributed to write the manuscript. Petterson Costa Conceição Silva, André Dias de Azevedo

Neto, Hans Raj Gheyi designed the study and analyzed the data. Petterson Costa Conceição Silva, Rogério Ferreira Ribas and Caroline Rastely dos Reis Silva played a key role in conduction of the experiments and data acquisition. All authors have read and approved the final manuscript.

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415

3. CHAPTER 3

Hydrogen peroxide priming reduces Na^+ and Cl^- content in leaves and induces salt tolerance in sunflower plants[3]

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*Corresponding author:

Petterson Costa Conceição Silva

Universidade Federal do Recôncavo da Bahia

Cruz das Almas, 44380000, BA, Brazil

E-mail: petter.ufrb@gmail.com

Hydrogen peroxide seed priming reduces Na^+ and Cl^- contents in leaves and improves salt-tolerance in sunflower plants

Short running title:

H_2O_2 priming induces salt tolerance in sunflower

Petterson Costa Conceição Silva[1]*, André Dias de Azevedo Neto[2], Hans Raj Gheyi[1], Rogério Ferreira Ribas[1], Alide Mitsue Watanabe Cova[1], and Caroline Rastely dos Reis Silva[3]

[1] Center of Agrarian, Environmental and Biological Sciences, Federal University of Recôncavo of Bahia, 44380-000, Cruz das Almas, BA, Brazil.

[2] Center of Exact and Technological Sciences, Federal University of Recôncavo of Bahia, 44380-000, Cruz das Almas, BA, Brazil.

[3] Faculty of Administration, Federal University of Bahia, 40110-903, Salvador, BA, Brazil.

Key words**:** cross-tolerance / *Helianthus annuus* L. / H_2O_2 / ion homeostasis / salt stress / solutes accumulation.

Abstract:

Several studies show that priming with hydrogen peroxide (H_2O_2) can trigger a series of physiological mechanisms and increase plant tolerance to salt stress. However, we observed that many of these mechanisms have not yet been fully clarified. Thus, this study aimed to evaluate the changes in the balance of inorganic and organic solutes induced by seed priming with H_2O_2 in leaves and roots of sunflower plants grown under salt stress. In this study, six treatments were tested: T1 - control (absence of NaCl and absence of H_2O_2); T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl). The results showed that salt stress significantly restricted plant growth. However, priming with H_2O_2 was able to reduce the transport of Na^+ and Cl^- ions to the leaves, increase the absorption of K^+ and NO_3^- and consequently improve plant tolerance to salt stress.

1 Introduction

Soil salinization is considered one of the main causes of soil degradation, making it unfit for the cultivation of crops due to the reduction in crop growth and yield (Hossain, 2019). Current data show that around 1125 million hectares of soils in the world are affected by salts (Hossain, 2019).

High salt concentrations in irrigation water or soil solution can lead to a series of morphological, physiological and biochemical changes in plants (Parida and Das, 2005; Azevedo Neto et al., 2009). These effects of salinity can be divided into two phases: an osmotic phase, continuous, which reduces water absorption by plants due to reduced water potential in the rhizosphere; and an ionic phase, slower, resulting from the accumulation of specific ions over time, leading to ion toxicity and/or nutritional imbalance (Munns and Tester, 2008).

Under salt stress, plasma membrane components regulate the absorption and transport of toxic ions, playing an important role in maintaining ion homeostasis in the cytosol (Gupta and Huang, 2014). Under saline conditions, nutritional disorders related mainly to the increase in the absorption of Na^+ and Cl^- ions and decrease in K^+/Na^+ and/or NO_3^-/Cl^- ratios usually occur, which can lead to disturbances in cellular metabolism (Rodrigues et al., 2013; Li et al., 2017).

Under stress, plants can accumulate low-molar-mass organic molecules (carbohydrates, amino acids, betaines, etc.), in order to contribute to the maintenance of osmotic and ion homeostasis and stabilization of proteins and other macromolecules (Azevedo Neto et al., 2009). However, to ensure the accumulation of these solutes, the energy cost needed can compromise plant growth (Fricke, 2019).

In the last decade, some chemical primers, such as hydrogen peroxide (H_2O_2), have been used to induce plant acclimation to different stress conditions (Niu and Liao, 2016; Wang et al., 2018). H_2O_2 is known as a reactive oxygen species (ROS), potentially toxic and capable of causing damage to cellular structures. However, at low concentrations, H_2O_2 may play a key role as a signaling molecule, modulating the expression of genes involved in ROS control, signal transduction, transcriptional regulation and metabolism of proteins, carbohydrates and lipids. Additionally, H_2O_2 can assist in the flow of solutes and nutrients, contributing to the increase in plant tolerance to the stress condition (Li et al., 2011; Hossain et al., 2015; Niu and Liao, 2016).

Although there are many studies reporting the accumulation of solutes as a mechanism for plant acclimation to salt stress, there is still little effective evidence about the capacity of priming with H_2O_2 to induce plant tolerance through the synthesis and/or accumulation of organic and inorganic solutes in plants. In this context, our study aimed to test the hypothesis that the priming of sunflower seeds with H_2O_2 can cause changes in the accumulation of organic and inorganic solutes and increase plant tolerance to salt stress.

2 Material and methods

2.1 Growth conditions and treatments

The experiment was conducted at the Universidade Federal do Recôncavo da Bahia (UFRB) in Cruz das Almas – BA, Brazil, using seeds of sunflower (Agrobel 975 genotype) obtained from CEAPAR Representação S/C Ltda. The seeds were placed to germinate in Petri dishes (20 seeds/dish), placed in filter paper sheets and kept in a germination chamber at 25 °C.

From previous tests, using seeds soaked for 12, 24 and 36 hours at different concentrations of H_2O_2 (0.1, 1, 10 and 100 mM), the four combinations which promoted the best acclimation of plants to salt stress (data not shown) were selected. After the soaking period, the seedlings were transferred to polyethylene pots containing 15 L of nutrient solution (Furlani, 1997) + 100 mM NaCl, except in the control treatment.

For the present study, the established treatments were: T1 - control (absence of H_2O_2 and absence of NaCl); T2 - salt control (absence of H_2O_2 and presence of 100 mM of NaCl); T3 - 10 mM of H_2O_2 (12 h), T4 - 100 mM of H_2O_2 (12 h), T4 - 1 mM of H_2O_2 (24 h); and T5 - 0.1 mM of H_2O_2 (36 h). Harvests were performed out weekly at 21, 28 and 35 days.

In each harvest, approximately 1.0 g of samples of the first fully expanded leaf and the younger third of the root system were immediately frozen and freeze-dried to determine the contents of inorganic and organic solutes. Subsequently, the harvested plants were divided into leaves and stem, dried in an oven at 65 °C for 72 h and weighed on analytical scale to quantify leaf dry mass (LDM) and stem dry mass (SDM). These data were used to obtain shoot dry mass (ShDM).

2.2 Determination of inorganic solutes

For determination of contents of sodium (Na^+), potassium (K^+), chloride (Cl^-), nitrate (NO_3^-) and ammonium (NH_4^+), extracts from leaf and root samples were prepared in deionized water following the methodology described by Gondim et al. (2011).

Na^+ and K^+ contents were determined in a Q498M2 flame photometer (Quimis Aparelhos Científicos Ltda, Diadema, SP, BR), as described by Faithfull (2002). Cl^-, NO_3^- and NH_4^+ contents were determined in a 2000 UV spectrophotometer (Bel Engineering, Piracicaba, SP, BR), following the methodologies described by Gaines et al. (1984), Cataldo et al. (1975), Weatherburn (1967), respectively.

2.3 Determination of organic solutes

For determination of contents of soluble carbohydrates, free amino acids, free proline and soluble proteins, extracts from leaf and root samples were prepared in buffer solution (100 mM potassium phosphate, pH 7.0, 0.1 mM EDTA) following the methodology described by Azevedo Neto et al. (2009).

Soluble carbohydrate content was determined at 490 nm, by the phenol-sulfuric acid method (Dubois et al., 1956). Free amino acids were determined at 570 nm by the ninhydrin method (Yemm and Cocking, 1995). Free proline content was determined by the acid ninhydrin method at 520 nm (Bates et al., 1973). Soluble proteins were determined at 595 nm by the protein-dye binding method (Bradford 1976), using bovine albumin as standard.

The experimental design was in randomized blocks, with four repetitions. The data were tested for normality (Shapiro-Wilk test) and then subjected to analysis of variance (ANOVA). The results of dry mass were transformed (Log_{10}) and subjected to principal component analysis (PCA) using the program SigmaPlot (SigmaPlot 14.0, Systat Software Inc. San Jose, CA, USA). The results of solute contents were normalized to create the graph of general representation and compared by the Scott-Knott test ($p \leq 0.05$), using the SigmaPlot program (SigmaPlot 14.0, Systat Software Inc. San Jose, CA, USA).

2.4 Experimental design and statistical analysis

The experimental design was in randomized blocks, with four repetitions. The data were tested for normality (Shapiro-Wilk test) and then subjected to analysis of variance (ANOVA). The results of dry mass were transformed (Log_{10}) and subjected to principal component analysis (PCA) using the program SigmaPlot (SigmaPlot 14.0, Systat Software Inc. San Jose, CA, USA). The results of solute contents were normalized to create the graph of general representation and compared by the Scott-Knott test ($p \leq 0.05$), using the SigmaPlot program (SigmaPlot 14.0, Systat Software Inc. San Jose, CA, USA).

3 Results

The radar graph (Fig. 3.1) shows an overview of the differences caused by salt stress and priming of seeds with H_2O_2 in the contents of organic and inorganic solutes in leaves and roots of sunflower plants grown under salt stress. In general, plants under salt stress (T2 to T6) showed lower NO_3^- content and higher contents of Na^+, Cl^-, NH_4^+, soluble carbohydrates, free amino acids and free proline in leaves and roots, compared to the plants of the control treatment (T1). By comparing the treatments of salt stress, it can be observed that the leaves and roots of

plants primed with H_2O_2 (T3 to T6) had higher concentrations of K^+ and NO_3^- when compared to those of the T2 treatment (salt control).

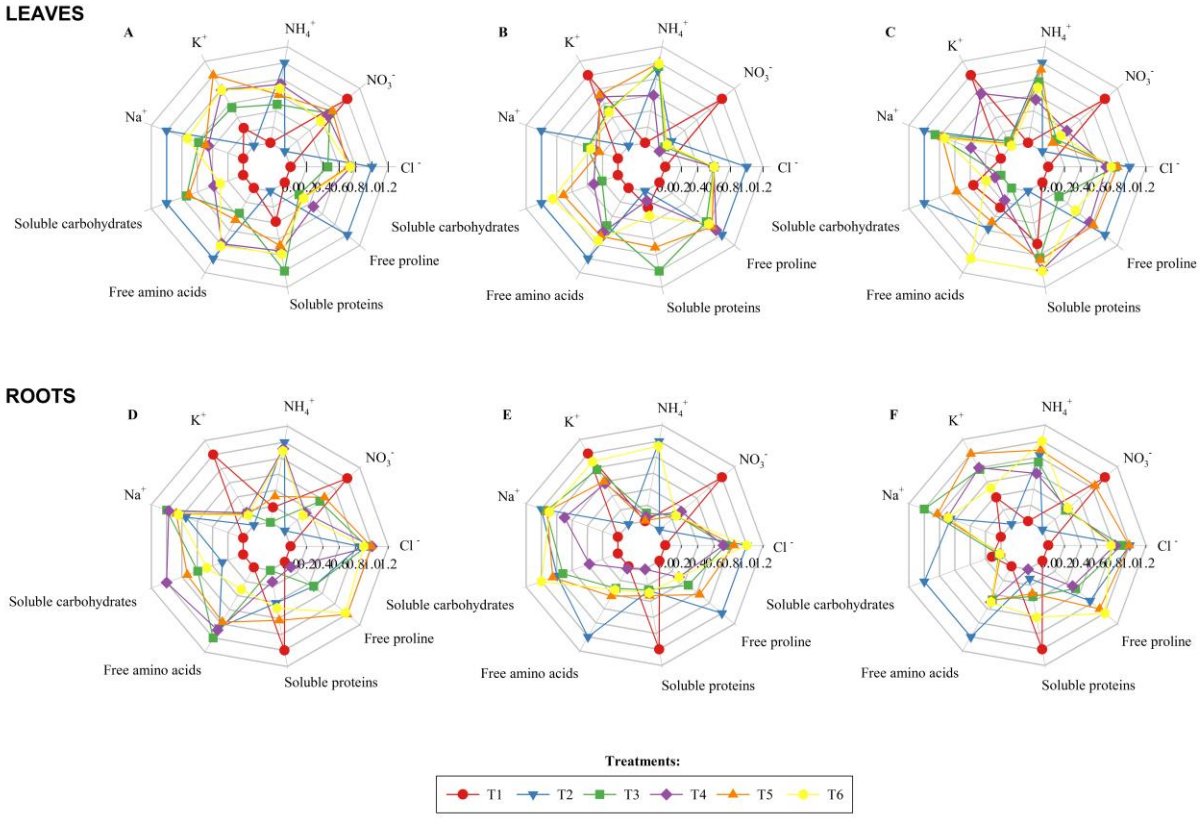


Fig. 3.1 Overall representation of the chemical and biochemical responses of leaves and roots of sunflower plants under salt stress and H_2O_2 seed priming, at 21 (A and D), 28 (B and E) and 35 (C and F) days of cultivation in nutrient solution. Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + 100 mM NaCl; T4 - 100 mM H_2O_2 (12 h) + 100 mM NaCl; T5 - 1 mM H_2O_2 (24 h) + 100 mM NaCl; T6 - 0.1 mM H_2O_2 (36 h) + 100 mM NaCl.

In Table 3.1, the results show that the leaf concentrations of Na^+ and Cl^- in plants of the T2 treatment were, respectively, 37-fold and 8-fold (21 days), 22-fold and 5-fold (28 days) and 19-fold and 2.5-fold (35 days) higher than those found in control plants (T1). However, under salt stress, the contents of Na^+ and Cl^- at 21, 28 and 35 days in the leaves of primed plants (T3 to T6) were, respectively, 66, 30 and 41% (Na^+) and 35, 17 and 20% (Cl^-) lower than in the T2 treatment.

At 21 and 28 days, the salt stress reduced the leaf content of NO_3^- by 44% and increased the NH_4^+ content by 141% in all salt treatments. At 35 days, salinity reduced the contents of NO_3^- by 38% and increased the contents of NH_4^+

158 by 372% in the T2 treatment. But, in treatments whose seeds were primed with H_2O_2, salinity did not affect NO_3^-
159 content, and the NH_4^+ concentrations were 30% lower than those observed in the T2 treatment.

160

161 **Table 3.1** Effect of salt stress and H_2O_2 priming of seed on contents of inorganic and organic in leaves of sunflower
162 plants cultivated in nutrient solution, at 21, 28 and 35 days.

163

Solutes	Treatments					
	T1	T2	T1	T4	T1	T6
	1st harvest (21 days)					
Cl^- (µmol g^{-1} DM)	7.41 c	58.97 a	38.14 b	38.17 b	38.36 b	38.36 b
NO_3^- (µmol g^{-1} DM)	101.2 a	61.76 b	56.53 b	52.41 b	58.04 b	58.46 b
NH_4^+ (µmol g^{-1} DM)	32.56 c	82.17 a	84.97 a	65.11 b	87.44 a	86.78 a
K^+ (mmol g^{-1} DM)	1.02 a	0.56 b	0.79 a	0.88 a	0.89 a	0.78 a
Na^+ (mmol g^{-1} DM)	0.03 d	1.11 a	0.46 b	0.34 c	0.29 c	0.41 b
Soluble carbohydrates (µmol g^{-1} DM)	97.6 b	122.6 a	102.8 b	105.5 b	115.4 a	118.9 a
Free amino acids (µmol g^{-1} DM)	12.58 c	67.93 a	42.18 b	47.15 b	50.40 b	53.99 b
Soluble proteins (mg g^{-1} DM)	3.56 b	2.58 b	7.31 a	3.16 b	5.91 a	4.05 b
Free proline (µmol g^{-1} DM)	0.40 b	1.16 a	0.97 a	1.09 a	1.04 a	1.00 a
	2nd harvest (28 days)					
Cl^- (µmol g^{-1} DM)	7.39 c	37.59 a	32.30 b	28.96 b	33.00 b	30.71 b
NO_3^- (µmol g^{-1} DM)	94.49 a	40.97 b	53.07 b	61.90 b	49.74 b	56.79 b
NH_4^+ (µmol g^{-1} DM)	24.90 c	67.08 a	57.04 b	47.68 b	63.55 a	54.01 b
K^+ (mmol g^{-1} DM)	0.90 a	0.61 c	0.61 c	0.82 b	0.60 c	0.59 c
Na^+ (mmol g^{-1} DM)	0.03 e	0.67 a	0.58 b	0.28 d	0.52 c	0.50 c
Soluble carbohydrates (µmol g^{-1} DM)	128.3 c	193.4 a	92.1 d	99.8 d	150.5 b	111.4 c
Free amino acids (µmol g^{-1} DM)	24.69 c	30.65 b	19.14 c	22.57 c	28.77 b	39.12 a
Soluble proteins (mg g^{-1} DM)	3.15 a	2.54 b	3.31 a	3.45 a	3.33 a	3.46 a
Free proline (µmol g^{-1} DM)	0.51 b	1.00 a	0.64 b	0.L88 a	0.91 a	0.77 a
	3rd harvest (35 days)					

Cl^- (µmol g^{-1} DM)	9.23 d	23.20 a	15.62 c	19.63 b	19.10 b	19.47 b
NO_3^- (µmol g^{-1} DM)	52.41 a	32.72 b	46.89 a	46.20 a	47.76 a	44.04 a
NH_4^+ (µmol g^{-1} DM)	11.70 c	55.25 a	32.63 b	43.56 b	37.87 b	41.22 b
K^+ (mmol g^{-1} DM)	0.55 b	0.49 b	0.62 a	0.68 a	0.73 a	0.68 a
Na^+ (mmol g^{-1} DM)	0.03 d	0.58 a	0.35 c	0.28 c	0.30 c	0.43 b
Soluble carbohydrates (µmol g^{-1} DM)	140.5 b	198.6 a	183.5 a	162.7 b	181.6 a	157.9 b
Free amino acids (µmol g^{-1} DM)	15.42 c	36.09 a	22.80 b	31.75 a	24.77 b	32.42 a
Soluble proteins (mg g^{-1} DM)	2.74 b	1.93 c	4.04 a	3.51 a	3.37 a	3.59 a
Free proline (µmol g^{-1} DM)	0.42 c	1.12 a	0.58 b	0.74 b	0.62 b	0.63 b

Mean of four repetitions. Means followed by the same letter, in lines do not differ statistically by Scott-Knott's test. ($p \leq 0.05$). Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

Considering the mean of the evaluated periods (21, 28 and 35 days), salinity in the T2 treatment increased the leaf contents of soluble carbohydrates, free amino acids and free proline by 40, 199 and 151%, respectively, and decreased the contents of soluble proteins by 26%, compared to the T1 treatment (Table 3.1). On the other hand, the priming of seeds with H_2O_2 (except T4 and T6 at 21 days) significantly increased the leaf contents of soluble proteins at 21 (156%), 28 (33%) and 35 days (88%) compared to the T2 treatment. It can also be observed in Table 2 that the free proline contents in the leaves of stressed plants (T2 to T6) at 21 and 28 days were similar. However, at 35 days, the free proline contents in the leaves of plants primed with H_2O_2 was 43% lower than in the T2 treatment.

For all periods evaluated (21, 28 and 35 days), salinity increased the contents of Na^+, Cl^- and NH_4^+ in the roots on average 367, 295 and 101%, compared to those of plants in the T1 treatment (Table 3.2). Substantial differences in the contents of these ions were not observed between salt treatments. In contrast, salt stress reduced the NO_3^- content in the roots, and this effect was more pronounced on unprimed plants. Thus, the NO_3^- contents in the roots of plants primed with H_2O_2 (T3 to T6) were about 58% higher than those observed in the roots of plants in T2 (Table 3.2). In all evaluations, K^+ content was reduced by about 48% in the T2 treatment. However, at 28 and 35 days, the priming of seeds with H_2O_2 increased the K^+ content in sunflower roots by about 71%, in comparison to the values observed in the T2 treatment.

185 **Table 3.2** Effect of salt stress and H_2O_2 priming of seed on inorganic and organic solutes content in roots of
186 sunflower plants cultivated in nutrient solution, at 21, 28 and 35 days.

Solutes	Treatments					
	T1	T2	T1	T4	T1	T6
	1st harvest (21 days)					
Cl^- (µmol g^{-1} DM)	14.08 b	54.07 a	57.99 a	61.12 a	60.67 a	56.35 a
NO_3^- (µmol g^{-1} DM)	114.17 a	40.24 d	81.72 b	64.71 c	87.16 b	61.85 c
NH_4^+ (µmol g^{-1} DM)	59.95 b	77.08 a	56.04 b	75.33 a	62.84 b	74.77 a
K^+ (mmol g^{-1} DM)	1.97 a	0.62 b	0.85 b	0.86 b	0.83 b	0.81 b
Na^+ (mmol g^{-1} DM)	0.23 b	1.00 a	1.26 a	1.23 a	1.13 a	1.10 a
Soluble carbohydrates (µmol g^{-1} DM)	281.3 c	333.2 c	394.4 b	472.2 a	420.4 b	371.5 b
Free amino acids (µmol g^{-1} DM)	75.80 b	99.63 a	103.8 a	100.7 a	97.46 a	84.54 b
Soluble proteins (mg g^{-1} DM)	4.24 a	3.12 b	2.35 b	2.62 b	3.53 a	3.24 b
Free proline (µmol g^{-1} DM)	3.88 c	4.84 b	4.84 b	4.08 c	5.96 a	5.90 a
	2nd harvest (28 days)					
Cl^- (µmol g^{-1} DM)	15.71 b	75.31 a	61.08 a	59.21 a	67.12 a	76.60 a
NO_3^- (µmol g^{-1} DM)	81.49 a	38.59 c	50.25 b	53.41 b	50.10 b	49.94 b
NH_4^+ (µmol g^{-1} DM)	38.76 b	63.38 a	41.25 b	40.23 b	38.99 b	61.86 a
K^+ (mmol g^{-1} DM)	1.43 a	0.72 b	1.27 a	1.13 a	1.15 a	1.35 a
Na^+ (mmol g^{-1} DM)	0.16 c	1.12 a	1.06 a	0.83 b	1.05 a	1.02 a
Soluble carbohydrates (µmol g^{-1} DM)	231.5 d	448.7 b	427.9 b	333.3 c	464.0 b	504.6 a
Free amino acids (µmol g^{-1} DM)	89.61 c	207.1 a	126.2 b	93.2 c	139.3 b	128.8 b
Soluble proteins (mg g^{-1} DM)	4.43 a	2.55 b	2.53 b	1.87 c	2.71 b	2.62 b
Free proline (µmol g^{-1} DM)	4.31 d	11.32 a	7.54 c	6.48 c	8.81 b	6.49 c
	3rd harvest (35 days)					
Cl^- (µmol g^{-1} DM)	15.52 b	57.22 a	53.15 a	49.76 a	56.94 a	47.69 a
NO_3^- (µmol g^{-1} DM)	58.2 a	26.49 c	38.16 b	39.13 b	53.10 a	39.39 b
NH_4^+ (µmol g^{-1} DM)	8.76 e	32.14 b	29.62 c	25.64 d	33.91 b	37.06 a
K^+ (mmol g^{-1} DM)	0.65 b	0.48 c	0.83 a	0.84 a	0.93 a	0.71 b

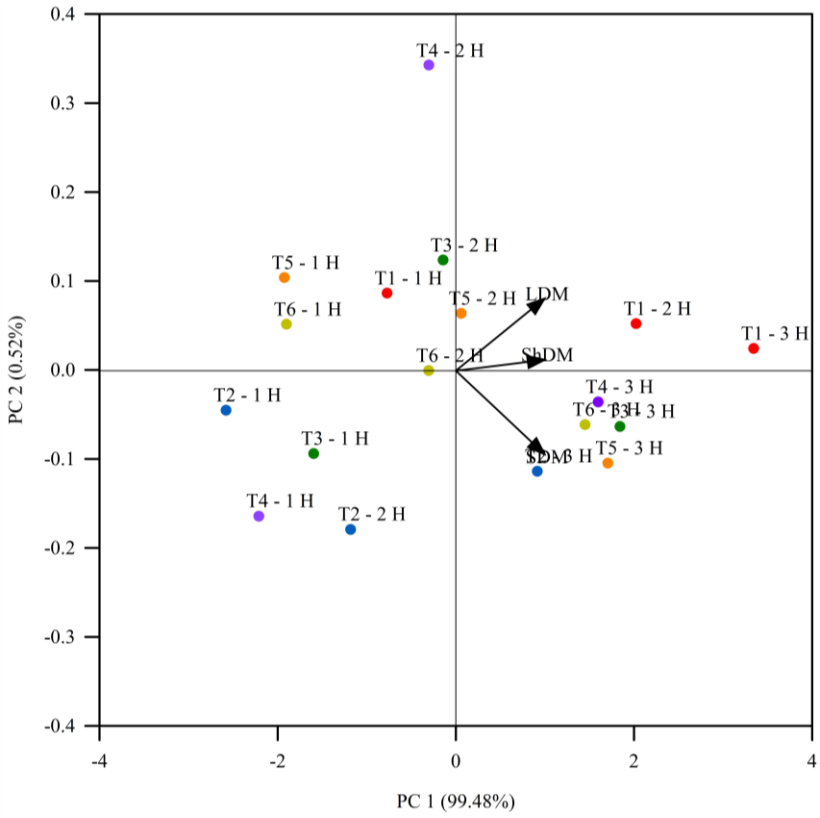
Na^+ (mmol g^{-1} DM)	0.43 c	1.04 b	1.36 a	1.08 b	1.20 a	1.07 b
Soluble carbohydrates (µmol g^{-1} DM)	307.4 b	775.0 a	259.5 c	254.0 c	245.3 c	252.6 c
Free amino acids (µmol g^{-1} DM)	45.27 b	170.9 a	104.8 b	109.1 b	106.2 b	108.8 b
Soluble proteins (mg g^{-1} DM)	3.52 a	2.24 c	2.56 c	2.07 c	2.51 c	2.94 b
Free proline (µmol g^{-1} DM)	3.66 d	9.51 b	7.71 c	7.36 c	10.69 a	11.33 a

Mean of four repetitions. Means followed by the same letter, in lines, do not differ statistically by Scott-Knott's test ($p \leq 0.05$). Treatments: T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

Among the organic solutes of the root, the most consistent responses were observed in the contents of soluble proteins, amino acids and free proline. At 21, 28 and 35 days, the contents of soluble proteins in salt treatments decreased by 30, 45 and 30% and the contents of free proline increased by 32, 89 and 155%, respectively, compared to those of the T1 treatment (Table 3.2). However, no consistent differences were observed between the seed priming treatments (T3 to T6) and the salt control (T2).

As observed for proline, salinity progressively increased the content of free amino acids in the roots, but this effect was smaller in treatments T3 to T6. Thus, the priming of seeds with H_2O_2 significantly reduced the contents of free amino acids in the roots at 28 (41%) and 35 (37%), compared to the values observed in the T2 treatment.

The metabolic alterations caused by the priming of seeds with H_2O_2 promoted a better equilibrium in the balance of solutes and induced an increase in plant tolerance to salts, verified by the increase in dry mass yield. Through PCA, the results showed that, for growth variables, the principal component 1 (PC1) was responsible for the largest variance observed in the data (99.48%), while the principal component 2 (PC2) was responsible for only 0.52% of this variance (Fig. 3.2).



206

207 **Fig. 3.2** Principal component analysis of the dry mass of leaves (LDM), stem (SDM) and shoot (ShDM) of
208 sunflower plants under effect of salt stress and H_2O_2 seed priming, at 21, 28 and 35 days of cultivation in nutrient
209 solution. Mean of four repetitions. T1 - control (absence of NaCl and absence of H_2O_2; T2 - salt control (presence
210 of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) +
211 (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl). 1 H - 1st
212 harvest (21 days), 2 H - 2nd harvest (28 days) and 3 H - 3rd harvest (35 days).

213

214 In Table 3.3, the loads of the principal components showed a strong relationship between PC1 and the
215 growth variables analyzed (LDM, SDM, ShDM). In Fig. 1, the position of the control treatment in PC1 in relation
216 to the other treatments confirms that salinity strongly reduced plant growth, regardless of harvest. However, the
217 results also show that, for each harvest, the treatments of seed priming with H_2O_2 showed higher values of LDM,
218 SDM and ShDM when compared to plants of the salt control treatment (T2), confirming the reduction of the
219 deleterious effect of the salt and increasing plant tolerance.

220

Table 3.3 Component loadings of principal component analysis (PCA) of the dry mass of leaves (LDM), stem (SDM) and shoot (ShDM) of sunflower plants under effect of salt stress and H_2O_2 seed priming, at 21, 28 and 35 days of cultivation in nutrient solution.

Component Loadings	**PC1**	**PC2**
LDM	0.997	0.081
SDM	0.996	-0.009
ShDM	1.000	0.012

4 Discussion

The presence of high Na^+ concentrations in the cytosol causes severe ion imbalance and can cause significant physiological disorders. Therefore, the physiological mechanisms of Na^+ exclusion from metabolically active tissues of the shoot may be responsible for increasing the tolerance of crops to salt stress (Azevedo Neto et al., 2000; Wu, 2018). Our results showed that, even with high levels of Na^+ in the roots, sunflower plants primed with H_2O_2 showed lower Na^+ content in the leaves. These results suggest that H_2O_2 may play a key role in inducing mechanisms of retention of Na^+ ions in the roots, reducing the transport of these ions to leaves, thus improving plant tolerance to salt stress.

Some authors affirm that H_2O_2 is able to induce increase in the expression and/or activity of HKT1 (high affinity K^+ transporter 1) Na^+-transporter membrane proteins (Zhu et al., 2017). These proteins, under stress conditions, are responsible for both Na^+ unloading from the xylem and Na^+ recirculation to the phloem, significantly reducing the content of Na^+ in the leaves, contributing to ion homeostasis and reducing negative impacts on photosynthetic organs (Rus et al., 2004; Zhu, 2016; Zhu et al., 2017).

The low K^+ contents observed in the leaves and roots of plants in the T2 treatment can be attributed to the efflux of K^+. Salt stress can induce increase in the expression and activity of membrane transporters of the types KORCs (K^+ outward-rectifying channels) and NSCCs (non-selective cation channels), transporting K^+ out of the cytosol and causing deficiency of this ion in the tissues (Garcia-Mata et al., 2010 Demidchik et al., 2014). In contrast, the significant increase in K^+ content observed in plants primed with H_2O_2 reinforces the hypothesis that the priming of seeds with H_2O_2 can act indirectly in the regulation of ion uptake (Zhang et al., 2007; Hu et al., 2016). The negative regulation of the absorption and transport of Na^+ associated with the reduction of K^+ losses in plants under

salt stress are essential mechanisms for maintaining high K^+/Na^+ proportions in tissues, giving greater tolerance to salts (Shabala and Pottosin, 2014). Several studies have shown an increase in the H_2O_2-induced K^+/Na^+ ratio in plants under salt stress (Gondim et al., 2011; Christou et al., 2014; Silva et al., 2019).

As observed for Na^+, but through mechanisms not yet clarified, priming with H_2O_2 restricted the long-distance transport of Cl^-, reducing the Cl^- content in the leaves of salt-stressed sunflower plants and significantly increasing the NO_3^- contents, both in leaves and roots. Some studies show the antagonistic effect between NO_3^- and Cl^-. This effect occurs mainly because these two anions are monovalent, have similar ionic radius and are absorbed, in many cases, by the same carrier proteins (Parihar et al., 2015; Guo, 2017). Li et al. (2017) state that the NO_3^-/Cl^- ratio in shoot (similar to the K^+/Na^+ ratio) can be an important indicator of salt tolerance, since the reduction of growth is directly related to the decrease in NO_3^- content and/or increase in Cl^- content. Our results, corroborate with results obtained by Wahid et al. (2007) that the priming of wheat seeds with H_2O_2 significantly increased the contents of K^+ and NO_3^- and reduced the contents of Na^+ and Cl^- in the shoot of the plants.

Under stress conditions, the reduction of growth may lead to a reduction in N demand by plants, causing imbalance between the assimilation of NH_4^+ and synthesis of proteins and resulting in an accumulation of NH_4^+ and free amino acids (Silveira et al., 2012). In the cytosol, the NH_4^+ ion is potentially toxic due to its action in the dissipation of the proton electrochemical gradients, and its role in increasing ethylene synthesis, leading to plant senescence (Howitt and Udvardi, 2000; Jian et al., 2018). Additionally, the accumulation of NH_4^+ and amino acids can negatively regulate the absorption of NO_3^- by the roots through the feedback regulation mechanism (Silveira et al., 2012).

The strong increase in the contents of soluble carbohydrates, free amino acids and free proline observed in salt-stressed plants is a factor that may be associated with the capacity to adjust the osmotic potential in the cytosol as a tolerance mechanism (Ashraf and Foolad, 2007). The accumulation of such compounds during stress is important for osmoregulation and cell protection against salt stress (Molinari et al., 2007).

The reduction in the leaf content of soluble proteins associated with increased contents of free amino acids and NH_4^+ in plants of salt treatments not primed with H_2O_2 suggests the occurrence of proteolysis (Silveira et al., 2003). According to Puniran-Hartley et al. (2014), the accumulation of organic solutes in wheat and barley leaves may occur due to the increase in salt-induced oxidative stress. In our results, the mitigation of salt stress induced by priming with H_2O_2 contributed to the increase in the content of soluble proteins in salt-stressed plants. Such increase may be associated with the role of H_2O_2 as a metabolic signaling molecule, inducing the expression and

activity of antioxidative enzymes, reducing the negative effects caused by the salt (Hosssain et al., 2015; Niu and Liao, 2016).

The reduction in the dry mass of sunflower plants induced by salt stress, observed in the PCA (Fig. 3.2), can be associated both with the higher energy cost for synthesis and accumulation of organic solutes (Azevedo Neto et al., 2005; Fricke, 2019) and with the increase in the concentration of toxic ions, mainly Na^+ and Cl^- in the leaves (Munns and Tester, 2008).

5 Conclusions

Although several studies associate the use of H_2O_2 with increase in plant tolerance due to the increase in the content of organic solutes (Gondim et al., 2012; Hossain et al., 2015). Our results show that, the role of H_2O_2 in increase of the salt tolerance can be more associated with reduction in Na^+ and Cl^- contents in the leaves and positive regulation of K^+ and NO_3^- absorption, contributing to ion homeostasis.

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4. CHAPTER 4

Hydrogen peroxide seed priming improves the growth rates and antioxidative defense system of sunflower plants salt-stressed[4]

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Hydrogen peroxide seed priming improves the growth rates and antioxidative defense system of sunflower plants salt-stressed

Running title:

H_2O_2 priming alleviates salt stress in sunflower plants

Petterson Costa Conceição Silva[a]*, André Dias de Azevedo Neto[b], Hans Raj Gheyi[a], Rogério Ferreira Ribas[a], Alide Mitsue Watanabe Cova[a], Caroline Rastely dos Reis Silva[c]

[a] Center of Agricultural, Environmental and Biological Science, Federal University of Recôncavo of Bahia, Cruz das Almas, 44380-000, BA, Brazil.

[b] Center of Exact and Technological Science, Federal University of Recôncavo of Bahia, Cruz das Almas, 44380-000, BA, Brazil.

[c] Faculty of Administration, Federal University of Bahia, Salvador, 40110-903, BA, Brazil.

*Corresponding author:
Petterson Costa Conceição Silva
E-mail: petter.ufrb@gmail.com
Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380000, Bahia, Brazil

Abstract

Salt-induced redox imbalance may lead to increased oxidative stress and impair the plant growth. However, among the many reactive oxygen species (ROS) produced under stress, hydrogen peroxide (H_2O_2) plays a central role in several signal transduction pathways. Thus, the aim in this study was to test the hypothesis that seed priming with H_2O_2 can increase the tolerance of sunflower plants to salt stress by changes in the redox balance. For this, six treatments selected from previous trials were tested: T1 - control (absence of NaCl and absence of H_2O_2); T2 - saline control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + 100 mM NaCl; T4 - 100 mM H_2O_2 (12 h) + 100 mM NaCl; T5 - 1 mM H_2O_2 (24 h) + 100 mM NaCl; T6 - 0.1 mM H_2O_2 (36 h) + 100 mM NaCl. Salt stress reduced the growth rates of the sunflower plants. However, priming with H_2O_2 was able to enhance the plant tolerance to salt stress, mainly by increases the activity of catalase enzyme (CAT) in leaves and roots.

Keywords: *Helianthus annuus* L., H_2O_2, cross-talk, salt tolerance, oxidative stress

Introduction

Early in the 21st century is being marked by the global scarcity of water resources, environmental pollution and increased soil and water salinization. Associated with this, in arid and semiarid regions, low precipitation, high evaporation from the surface, weathering of rocks, irrigation with saline water and inadequate cultural practices have contributed to the increase in salinized areas at a rate of 10% per year (Shrivastava and Kumar, 2015). In this scenario, it is common that more than 50% of arable land will be salinized by the year 2050 (Jamil et al., 2011).

For agricultural production, salinity is one of the main agents responsible for reducing crop performance and yield. Salts affect plant growth mainly due to the reduction of soil osmotic potential, nutritional imbalance and ionic toxicity (Machado and Serralheiro, 2017). Salt stress, as well as other stresses, can also trigger the emergence of a secondary stress related to the imbalance between the production and removal of reactive oxygen species (ROS), leading to disturbances in the redox metabolism and damage to cellular structures in plants (Adem et al., 2014; Hossain et al., 2015; Cunha et al., 2019). Examples of ROS are hydrogen peroxide (H_2O_2), the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), the perhydroxyl radical ($OH_2^{\bullet}$) and singlet oxygen ($^1O_2$) (Halliwell, 2006; Khan et al., 2015).

Plant responses to stress involve a complex system mediated by different pathways of cell signal transduction, which seem to be induced by chemical conditioners such as H_2O_2 (Yeo 1998; Savvides et al., 2016). If on the one hand H_2O_2 is known as a ROS that can cause different types of damage to cells, on the other hand it is considered a secondary messenger associated with signaling cascades that can increase plant tolerance to various stresses (Hossain et al., 2015).

Therefore, the aim of this study was to test the hypothesis that H_2O_2 acts as a signaling molecule increasing the activity of the antioxidant system and reducing the deleterious effects caused by salt stress on sunflower plants.

Material and methods

The experiment was conducted at the Federal University of Recôncavo da Bahia (UFRB) in Cruz das Almas – BA, Brazil, using seeds of sunflower (Agrobel 975 genotype) obtained from CEAPAR Representação S/C Ltda.

The seeds were placed to germinate in Petri dishes (20 seeds/dish), wrapped in filter paper sheets and kept in a B.O.D. (Biochemical Oxygen Demand) germination chamber at 25 °C.

Based on previous trials, which used seeds soaked for different times (12; 24 and 36 hours) and H_2O_2 concentrations (0.1; 1; 10 and 100 mM), the four combinations that promoted the best acclimation of plants to salt stress were selected (data not shown). After the respective period of soaking, the seedlings were transferred to polyethylene pots, each of which containing 15 L of nutrient solution (Furlani, 1997) + 100 mM NaCl, except in the control treatment.

For the present study, the established treatments were: T1 - control (absence of H_2O_2 and absence of NaCl); T2 - saline control (absence of H_2O_2 and presence of 100 mM of NaCl); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl). To evaluate growth rates and activities of the antioxidant system, harvests were performed at 21, 28 and 35 days after sowing (DAS).

For determination of the activity of antioxidative enzymes and lipid peroxidation, the youngest pair of fully expanded leaves and the lower third of the root system were frozen in

liquid nitrogen, freeze-dried, ground to powder and kept in a freezer (Azevedo Neto et al., 2005).

The extracts were prepared using 0.15 g of dry mass (DM) in an icy extraction buffer (100 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA), as described by Azevedo Neto et al. (2005).

Lipid peroxidation (LP) was determined by measuring the content of malondialdehyde (MDA) produced by the reaction of thiobarbituric acid, as described by Heath and Packer (1968), and the result was expressed as μmol MDA g^{-1} DM.

The activity of the enzyme superoxide dismutase (SOD) (EC 1.15.1.1) was determined by measuring its capacity to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977), and the results were expressed in U g^{-1} DM. One SOD activity unit (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate. The activity of the catalase enzyme (CAT) (EC 1.11.1.6) was measured based on the decrease in H_2O_2 concentration following the method of Beers Jr and Sizer (1952), modified by Azevedo Neto et al. (2005), and expressed in μmol H_2O_2 min^{-1} g^{-1} DM. The activity of the ascorbate peroxidase enzyme (APX) was measured from the oxidation of the ascorbate, following the method described by Nakano and Asada (1981). For this enzyme, the results were expressed in μmol H_2O_2 min^{-1} g^{-1} DM, considering that 2 mols of ascorbate are required to reduce 1 mol of H_2O_2 (McKersie and Leshem, 1994).

Leaves and stems were dried in an oven at 65 °C for 72 h and weighed on analytical scale to quantify shoot dry mass (ShDM). Absolute and relative growth rates were evaluated as described by Hunt (1990).

The data were subjected to analysis of variance (ANOVA) and the means were compared by Scott-Knott test ($p \leq 0.05$), using the statistical program SISVAR 4.6 (Ferreira, 2011).

Results

Antioxidative enzymes activity and lipid peroxidation

In the first harvest, the activities of SOD, APX and CAT enzymes in the leaves of plants from the saline control treatment (T2) were respectively 2.1-fold, 1.9-fold and 2.3-fold higher than those of plants from the control treatment (T1) (Table 4.1). This result remained similar for the enzymes APX (1.6-fold and 1.9-fold) and CAT (2.0-fold and 1.8-fold), in the second and third harvest, respectively.

By comparing the enzymatic activities of seed priming treatments (T3 to T6) with those of the T2 treatment, the most significant results were an average reduction of 33% (1st harvest) in APX activity, increases of 67% (2nd harvest) and 31% (3rd harvest) in CAT activity, and a 24% increase (3rd harvest) in SOD activity (Table 4.1).

Also, during the 2nd and 3rd harvests, lipid peroxidation (LP) in the leaves of plants from the T2 treatment was 28 and 21% higher, respectively, than in the leaves of T1 plants. It can also be observed in Table 1 that, in the 3rd harvest, LP in the leaves of plants primed with H_2O_2 was 17% lower than that of plants from the T2 treatment (Table 4.1).

Table 4.1 Effect of salt stress and H_2O_2 seeds priming on enzymes activity: superoxide dismutase (SOD, U g^{-1} DM), ascorbate peroxidase (APX, µmol H_2O_2 min^{-1} g^{-1} DM), catalase (CAT, µmol H_2O_2 min^{-1} g^{-1} DM), and lipid peroxidation (LP, µmol MDA g^{-1}) in leaves of sunflower plants at 21, 28 and 35 days of salt stress.

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
	1st harvest (21 days)					
SOD	308.9 c	667.0 a	351.2 c	454.9 b	501.9 b	725.9 a

CAT	209.7 b	398.0 a	475.8 a	281.0 b	547.1 a	478.6 a
APX	2.24 c	5.15 a	3.28 b	3.67 b	3.43 b	3.46 b
LP	0.52 a	0.62 a	0.59 a	0.50 a	0.54 a	0.58 a
			2nd harvest (28 days)			
SOD	375.6 b	454.2 b	417.0 b	599.8 a	432.7 b	384.8 b
CAT	122.7 c	250.1 b	411.9 a	297.0 b	431.0 a	408.8 a
APX	1.70 b	2.78 a	1.93 b	2.33 a	2.71 a	2.33 a
LP	0.46 b	0.59 a	0.63 a	0.60 a	0.57 a	0.56 a
			3rd harvest (35 days)			
SOD	387.1 b	361.0 b	449.4 a	321.7 b	438.5 a	450.1 a
CAT	188.8 d	334.0 b	404.4 a	251.4 c	462.7 a	443.7 a
APX	1.62 b	3.06 a	2.32 b	1.80 b	2.81 a	3.37 a
LP	0.47 b	0.57 a	0.48 b	0.49 b	0.46 b	0.47 b

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Treatments: T1 - control (absence of NaCl and absence of H_2O_2); T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

In the roots, salt stress increased the enzymatic activity of SOD in the 1st and 2nd harvests. However, the activity of SOD in the 2nd harvest was 23% lower in plants primed with H_2O_2 (T3 to T6) than in plants from the T2 treatment (Table 4.2).

Table 4.2 Effect of salt stress and H_2O_2 seeds priming on enzymes activity: superoxide dismutase (SOD, U g^{-1} DM), ascorbate peroxidase (APX, µmol H_2O_2 min^{-1} g^{-1} DM), catalase

(CAT, μmol H_2O_2 min^{-1} g^{-1} DM), and lipid peroxidation (LP, μmol MDA g^{-1}) in roots of sunflower plants at 21, 28 and 35 days of salt stress.

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
	1st harvest (21 days)					
SOD	263.0 b	460.2 a	429.5 a	475.2 a	432.9 a	432.5 a
CAT	173.6 b	306.2 a	354.9 a	286.9 a	356.5 a	396.8 a
APX	1.86 c	4.21 a	2.61 b	4.36 a	3.03 b	3.69 a
LP	0.29 c	0.65 a	0.51 b	0.46 b	0.48 b	0.49 b
	2nd harvest (28 days)					
SOD	355.8 d	791.6 a	528.4 c	616.9 b	628.9 b	674.4 b
CAT	165.8 b	471.5 a	551.9 a	508.8 a	521.4 a	541.1 a
APX	3.86 c	6.96 a	1.73 d	1.89 d	2.71 d	5.16 b
LP	0.27 c	0.54 a	0.54 a	0.56 a	0.47 a	0.40 b
	3rd harvest (35 days)					
SOD	484.3 b	446.8 b	171.1 c	506.0 b	495.6 b	726.8 a
CAT	107.0 c	246.8 b	330.0 a	331.4 a	338.6 a	375.1 a
APX	1.98 b	2.88 b	2.77 b	1.93 b	3.46 a	4.01 a
LP	0.39 b	0.53 a	0.52 a	0.51 a	0.50 a	0.45 b

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Treatments: T1 - control (absence of NaCl and absence of H_2O_2); T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

Salinity increased CAT activity by 96% (1st harvest) and 213% (2nd harvest) in relation to T1 plants, but significant differences between saline treatments were not observed. In the 3rd harvest, salinity also increased CAT activity, but such increment was more pronounced in plants whose seeds were primed with H_2O_2 (Table 4.2).

In the 1st and 2nd harvests, the activity of APX in the roots of plants from the T2 treatment was 126 and 80% higher than in the control. On the other hand, APX activity in seed priming treatments in comparison to T1 and T2 varied substantially with the time of salt stress.

In a joint analysis of all data of CAT and APX enzyme activities, regardless of salt stress or priming with H_2O_2, it can be observed that the H_2O_2 removal capacity by CAT activity was about 117-fold higher than that by APX (Table 4.2).

In the 1st harvest, LP in sunflower roots increased in all treatments of salt stress, but such increment was less pronounced in plants primed with H_2O_2. Thus, seed priming with H_2O_2 reduced root LP by about 25% in the treatments T3 to T6, compared to T2. In the 2nd and 3rd harvests, salinity increased LP by 86 and 29%, respectively, but substantial differences between saline treatments were not observed (Table 4.2).

Absolute and relative growth rate of the shoot

Table 4.3 shows that salinity significantly reduced the absolute growth rate (AGR) and the relative growth rate (RGR) of sunflower plants. However, this reduction was less pronounced in treatments primed with H_2O_2. The AGR of plants from T2 (saline control) was 94 and 82% lower than those of control plants (T1), for the periods from 21 to 28 and from 21 to 35 days, respectively. In contrast, the AGR of plants primed with H_2O_2 was about 3.1-fold and 1.7-fold higher than that observed in the T2 treatment.

For the same periods evaluated (21 - 28 and 21 - 35 days), the salt stress reduced RGR by about 55 and 16%, respectively, compared to the T1 treatment.

Table 4.3 Effect of salt stress and H_2O_2 seeds priming on absolute growth rate (AGR, g day^{-1}), relative growth rate (RGR, g g^{-1} day^{-1}) of the shoot of sunflower plants cultivated under salt stress in periods at 21 - 28 or 21 - 35 days.

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
	Period (21 - 28 days)					
AGR	0.840 a	0.052 c	0.142 b	0.167 b	0.192 b	0.157 b
RGR	0.269 a	0.121 c	0.143 c	0.197 b	0.193.b	0.177 b
	Period (21 - 35 days)					
AGR	1.070 a	0.197 d	0.380 b	0.322 b	0.360 b	0.282 c
RGR	0.203 a	0.169 c	0.166 c	0.183 b	0.176 b	0.162 c

Mean of four replicates. Means followed by the same letter, in lines, are not statistically different by Scott-Knott's test. ($p \leq 0.05$). Treatments: T1 - control (absence of NaCl and absence of H_2O_2); T2 - salt control (presence of 100 mM NaCl and absence of H_2O_2); T3 - 10 mM H_2O_2 (12 h) + (100 mM NaCl); T4 - 100 mM H_2O_2 (12 h) + (100 mM NaCl); T5 - 1 mM H_2O_2 (24 h) + (100 mM NaCl); T6 - 0.1 mM H_2O_2 (36 h) + (100 mM NaCl).

Discussion

Oxidative stress may occur as a consequence of salt stress, from the imbalance between the production and removal of reactive oxygen species (ROS) and may be responsible for various types of damage caused to plant cells, such as the increase in LP (Azevedo Neto et al., 2005; Huang et al., 2019).

However, the results here showed that seed priming with H_2O_2 can increase plant tolerance to salt stress, confirmed by the increase in growth rates observed in primed plants, compared to plants from the salt control treatment (Table 4.3).

In maize plants, some authors found that priming with H_2O_2 via nutrient solution (Azevedo Neto et al., 2005) and via leaf spraying (Gondim et al., 2012) increased plant tolerance to salt stress. These results corroborate the hypothesis that H_2O_2 can act as a metabolic signaling agent, efficiently stimulating the antioxidant defense system and consequently promoting increased plant tolerance to salt (Hossain et al., 2015; Khan et al., 2018).

All changes observed in enzymatic activities suggest that the significant increase in CAT activity played a key role in eliminating excess H_2O_2 generated during salt stress. Such increase in CAT activity can promote the reduction of deleterious effects caused by salt stress, leading to an increase in sunflower tolerance to salt stress. Gondim et al. (2012) confirmed this hypothesis, studying the effect of pretreatment with H_2O_2 applied via leaf spraying on maize plants. In addition, these authors found that the increase in CAT activity promoted an H_2O_2 elimination rate on average 99-fold higher than that of APX, corroborating the results found in the present study, as the H_2O_2 removal capacity of CAT was about 117-fold higher than that of APX.

According to Silva et al. (2019), CAT and APX enzymes differ in affinity for the substrate, indicating that each of them has specialized functions for ROS removal. According to these authors, the high Km value of CAT seems to associate the response of this enzyme with a more general role in tolerance, while the lower Km value observed in APX may indicate a finer adjustment in H_2O_2 removal.

Our results suggest that the reduction of LP in plants primed with H_2O_2 may be associated with increased activity of the antioxidant system (Liang et al., 2018; Silva et al.,

2018), differing from the results found by Azevedo Neto et al. (2005), when studying the effect of H_2O_2 pretreatment via nutrient solution on salt-stressed maize plants.

Conclusions

Seed priming with H_2O_2 increases sunflower tolerance to salt stress by stimulating CAT activity in leaves and roots.

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5. CHAPTER 5

Salt-tolerance induced by leaf spraying with H_2O_2 in sunflower is related to the ion homeostasis balance and reduction of oxidative damage[5]

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Title Page

Salt-tolerance induced by leaf spraying with H_2O_2 in sunflower is related to the ion homeostasis balance and reduction of oxidative damage

Petterson Costa Conceição Silva[a]*, André Dias de Azevedo Neto[b], Hans Raj Gheyi[a], Rogério Ferreira Ribas[a], Caroline Rastely dos Reis Silva[c], Alide Mitsue Watanabe Cova[a]

[a] Centro de Ciências Agrárias Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380-000, BA, Brazil.

[b] Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380-000, BA, Brazil.

[c] Centro de Pesquisas e Análises Clínicas, Cruz das Almas, 44380-000, BA, Brazil.

*Corresponding author:

Petterson Costa Conceição Silva

Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380000, BA, Brazil

Tel: +5571991958112

E-mail address: petter.ufrb@gmail.com (P. C. C. Silva).

Abstract

Salinity is still one of the main factors that limit the growth and production of agricultural crops. However, currently, conditioning with hydrogen peroxide (H_2O_2) has become a promising technique to alleviate the deleterious effects caused by salt. Therefore, the aim of this study was to test different leaf spraying strategies with H_2O_2 to acclimation of sunflower plants to salt stress, identifying the main physiological and biochemical changes involved in this process. The experiment was conducted in a completely randomized design, with four replications. Initially, four concentrations of H_2O_2 were tested (0.1; 1; 10 and 100 mM) associated with different amounts of applications (1AP - one application (48 hours before exposure to NaCl); 2AP - two applications (1AP + 7 days after exposure to NaCl) and 3AP - three applications (1AP + 2AP + 14 days after exposure to NaCl) two reference treatments were also added: control (absence of NaCl and absence of H_2O_2) and salt control (presence of 100 mM of NaCl and absence of H_2O_2). Salt stress reduced the growth of sunflower plants, however the conditioning of plants through leaf spraying with H_2O_2 was able to reduce the deleterious effects caused by salt, especially in the 1 mM H_2O_2 treatment with one application. H_2O_2 acts as a metabolic signal assisting in the maintenance of ionic and redox homeostasis, and consequently increasing the tolerance of plants to salt stress.

Keywords: *Helianthus annuus* L., H_2O_2, salinity, oxidative stress, physiological parameters, cross-talk

1. Introduction

Salinity is considered one of the main problems encountered in agriculture worldwide. The effects of salinity are more evident in arid and semi-arid regions, in these environments, limited rainfall, high evapotranspiration, high temperatures associated with inadequate water and soil management enhance the negative effects caused by salinity and directly impact agricultural production in these areas. regions (Azevedo Neto et al., 2006).

The excess of Na^+ and Cl^- ions in the root zone can alter the osmotic, ionic and nutritional homeostasis of plants (Wyn Jones and Gorham, 2002). These changes can lead to reduced growth and affect several physiological mechanisms. Under these conditions, both the photochemical phase and the biochemical phase of photosynthesis can be negatively affected (Dubey, 2005). In addition, salinity can increase lipid peroxidation and consequently reduce the integrity of cell membranes (Sairam et al., 2002). Saline stress also can cause an imbalance between the production and the scavenging of the reactive oxygen species (ROS), and this imbalance can cause various damages cell (Azevedo Neto and Silva, 2015). These species are very powerful oxidizers that can react with almost all components of living cells, producing severe damage to lipids, proteins and nucleic acids (oxidative stress situations) (del Río, 2015).

Therefore, understanding the mechanisms of plant tolerance for high concentrations of NaCl in soils can help improve yield and production in saline lands. Several studies have been carried out in an attempt to improve the tolerance of cultures to salt through conventional genetic improvement programs through the use of markers, however this technique is highly complex and expensive (Tavakkoli et al., 2011; Hoang et al., 2016).

As an alternative, plants can be prepared for future stress through priming. This technique also known as sensitization or hardening increases the plant tolerance to different types of stress. With the use of this technique, plants enter the priming state, which activates several protection mechanisms through different signaling pathway (Savvides et al., 2016).

Chemical priming is an emerging field in crop management under stress conditions. Plants treated with certain chemical agents (natural or synthetic) before stress events show increased tolerance when exposed to stress conditions (eg, salinity, drought, heat, heavy metals) (Hossain et al., 2015; Niu and Liao, 2016; Savvides et al. 2016).

Among several chemical agents used in this technique, is hydrogen peroxide (H_2O_2). Due to its electrochemical characteristics and small size, H_2O_2 is able to cross membranes and diffuse between cell compartments, which facilitates its signaling function (Bienert et al., 2006).

Several articles have stated that H_2O_2 can act as a key regulator in modulating the defense response of plants to various environmental stresses, such as salt stress (Azevedo Neto et al., 2005), drought (Hossain and Fujita, 2013), high temperatures (Wu et al., 2015) and heavy metals (Wen et al., 2013). However, few studies show how they establish the criteria for selecting concentrations for exogenous application and what are the main mechanisms responsible for the increase in plant tolerance induced by H_2O_2 priming.

Thus, the aim of this study was to test different leaf spraying strategies with H_2O_2 to acclimation of the sunflower plants to salt stress, identifying the main physiological and biochemical changes involved in this process.

2. Materials and methods

2.1 Experimental conditions

Para este estudo, dois experimentos foram conduzidos na casa de vegetação da Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, Brasil, utilizando sementes de girassol Agrobel 975 (AG 975) mantidas por 5 minutos em hipoclorito de sódio a 0,2% (m/v) e lavadas 3 vezes com água destilada.

2.2 First experiment (selection of treatments)

The seeds were placed to germinate in polyethylene trays containing washed sand and irrigated with Furlani's nutrient solution (1997) at medium strength. After the complete expansion of the first pair of leaves, the seedlings were transferred to polyethylene pots containing 15 L of total strength nutrient solution (Furlani, 1997) where the treatments were distributed.

The experimental design used was completely randomized, with four replications. The treatments consisted of four concentrations of H_2O_2 (0.1; 1; 10; 100 mM) associated with different amounts of applications (1AP - one application (48 hours before exposure to NaCl); 2AP - two applications (1AP + 7 days after exposure to NaCl) and 3AP - three applications (1AP + 2AP + 14 days after exposure to NaCl) two reference treatments were also added, submitted to three leaf spraying with deionized water: control (absence of NaCl and absence of H_2O_2) and salt control (presence of 100 mM NaCl and absence of H_2O_2), totaling 14 treatments.

All sprayed solutions were performed in the early evening (at 6:00 pm), using a manual sprayer with a pre-compression pump and a 1.5 L reservoir. The solutions used in the spraying were added with 0.025% Tween 20 (surfactant), in order to break the surface tension of the water and facilitate the penetration of the components applied in the leaves (Gondim et al., 2012).

Two days after the first leaf spraying, all nutrient solutions were renewed to establish salt stress (100 mM NaCl), except for the control treatment.

At 35 days after sowing (DAS) the plants were harvested and partitioned into leaves, stems and roots, dried in an oven at 65 °C for 72 h, and then weighed in an analytical balance to quantify the dry mass of leaves (LDM), stem (SDM) and roots (RDM). With these data the total dry mass of the plants (TDM) was determined.

2.3 Second experiment

For this experiment, one of the treatments with H_2O_2 application (from the previous experiment) was selected, which presented the highest dry mass in all partitions (leaves, stem and roots).

This experiment was carried out in random blocks with four replications. The tested treatments were: control (absence of NaCl and absence of H_2O_2), salt control (presence of 100 mM NaCl and absence of H_2O_2) and 1 mM H_2O_2 (1AP) + 100 mM NaCl, totaling three treatments. Two harvests were carried out at 21 and 35 DAS to evaluate the behavior of the plants over time.

2.4 Measurements of the gas exchange and photosynthetic pigments content

Gas exchange evaluations were performed on the youngest fully expanded leaf pair (Silveira et al., 2009), using the infrared gas analyzer - IRGA, model Li-6400XT (Li-Cor, Lincoln, NE, USA). The net CO_2 assimilation rate (P_N), transpiration (E) and stomatal conductance (gs) were determined. Additionally, the levels of photosynthetic pigments such as: chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), chlorophyll *a* + *b* (Chl *a* + *b*) and carotenoids (Car) were extracted in ethanol (95%) and quantified by spectrophotometry at 664, 649 and 470 nm, using the equations proposed by Lichtenthaler and Buschmann (2001).

2.5 Water status and electrolyte leakage

Relative water content (RWC), electrolyte leakage (EL) (Silva et al., 2015), leaf succulence (SUC) and the sclerophylly index (SI) (Cova et al., 2016) were also analyzed on the same leaves used for photosynthetic evaluations.

2.6 Inorganic solutes content

For the determination of the levels of sodium (Na^+), potassium (K^+), chloride (Cl^-), extracts from the samples of leaves and roots were prepared in deionized water following the methodology described by Gondim et al. (2011).

The levels of Na^+ and K^+ were determined by flame photometry model Q498M2 (Quimis, Diadema, SP, BR,), as described by Faithfull (2002). The Cl^- levels were determined in a spectrophotometer model 2000 UV (Bel Engineering, Piracicaba, SP, BR,), following the methodology described by Gaines et al. (1984).

2.7 Organic solutes content

For the determination of organic solutes, the youngest pair of fully expanded leaves and the youngest third of the root system were frozen in liquid nitrogen, lyophilized and maintained in a freezer (Azevedo Neto et al., 2005).

For the determination of the levels of soluble carbohydrates, free amino acids, free proline and soluble proteins, the extracts of the samples of leaves and roots were prepared in buffer solution (100 mM potassium phosphate, pH 7.0, 0.1 mM EDTA) following the methodology described by Azevedo Neto et al. (2009).

The determination of the content of soluble carbohydrates was carried out at 490 nm, using the phenol-sulfuric acid method (Dubois et al., 1956). Free amino acids were determined at 570 nm, using the ninhydrin method (Yemm and Cocking, 1995). To determine the free proline content, the 520 nm acid ninhydrin method was used (Bates et al., 1973). Soluble proteins were determined at 595 nm by the protein-dye binding method (Bradford 1976), using bovine albumin as a standard.

2.8 Antioxidant enzyme activity and lipid peroxidation

The extracts for determining the activity of antioxidant enzymes and lipid peroxidation were obtained similar to organic solutes.

The activity of the superoxide dismutase enzyme (SOD) (EC 1.15.1.1) was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977) and the results expressed in U g^{-1} of dry mass (DM), a unit of SOD activity (U) was defined as the amount of enzyme needed to cause 50% inhibition of the NBT photoreduction rate. The activity of the catalase enzyme (CAT) (EC 1.11.1.6) was measured from the decrease in the concentration of H_2O_2 following the method of Beers Jr and Sinzer (1952), modified by (Azevedo Neto et al., 2005) and expressed in expressed in µmol H_2O_2 min^{-1} g^{-1} DM. The evaluation of the activity of the enzyme ascorbate peroxidase (APX) was measured from the oxidation of ascorbate, following the method described by Nakano and Asada (1981). For this enzyme, the results were expressed in µmol H_2O_2 min^{-1} g^{-1} DM, considering that 2 moles of ascorbate are needed to reduce 1 mole of H_2O_2 (McKersie and Leshem, 1994).

Lipid peroxidation (LP) was determined by measuring the content of malondialdehyde (MDA) produced by the reaction of thiobarbituric acid, as described by Heath and Packer (1968), the result was expressed as µmol MDA g^{-1} DM.

2.9 Shoot dry mass quantification

The other part of the plant material (leaves and stem) was dried in an oven with forced air circulation at 65 °C for 72 h for measurements of the shoot dry mass (ShDM).

2.10 Statistical analysis

All data from both experiments were submitted to analysis of variance (ANOVA). In the first experiment, the means of the variables were compared using the Skott-Knott's test ($p \leq 0.05$). While for the second experiment, the means were compared using the Tukey's test ($p \leq 0.05$), using the Sisvar statistical program (Ferreira, 2011).

3. Results

3.1 First experiment

The results showed that there was a significant difference between the treatments applied ($p \leq 0.01$) for the growth variables analyzed (Table 5.1). Salinity reduced plant growth, but H_2O_2 priming via leaf spraying (in some treatments) was able to reduce the deleterious effects of salinity and improve plant tolerance to salt stress.

Table 5.1 Result of the analysis of variance and the Scott-Knott test for the parameters analyzed in the first experiment on sunflower plants, at 35 days of cultivation.

Sources of variations	LDM	SDM	RDM	TDM
Treatments	**	**	**	**
CV (%)	10.83	10.82	11.78	5.93
Treatments	Leaves	Stem	Roots	Total
	Dry mass (% of control)			
T1 - control	100	100	100	100
T2 - salt control	40.5 b	25.5 b	45.1 b	34.8 c
T3 - 0.1 mM H_2O_2 (AP1)	35.9 b	28.6 b	50.6 b	36.1 c
T4 - 1 mM H_2O_2 (AP1)	47.8 a	34.7 a	59.9 a	44.8 a
T5 - 10 mM H_2O_2 (AP1)	40.0 b	23.8 b	45.1 b	33.9 c
T6 - 100 mM H_2O_2 (AP1)	45.4 a	33.0 a	42.1 b	39.1 b
T7 - 0.1 mM H_2O_2 (AP2)	38.3 b	27.3 b	45.4 b	35.0 c

T8 - 1 mM H_2O_2 (AP2)	48.4 a	31.9 a	49.1 b	41.1 a
T9 - 10 mM H_2O_2 (AP2)	44.2 a	33.0 a	50.3 b	41.0 a
T10 - 100 mM H_2O_2 (AP2)	43.8 a	28.1 b	50.9 b	38.4 b
T11 - 0.1 mM H_2O_2 (AP3)	36.2 b	26.2 b	46.7 b	34.2 c
T12 - 1 mM H_2O_2 (AP3)	42.9 a	32.0 a	46.7 b	38.9 b
T13 - 10 mM H_2O_2 (AP3)	36.7 b	35.3 a	47.8 b	38.7 b
T14 - 100 mM H_2O_2 (AP3)	37.4 b	27.6 b	46.3 b	35.1 c

** Significant ($p \leq 0.01$). Means followed by the same letter, in the column, do not differ statistically from each other by the Scott-Knott's test ($p \leq 0.05$). LDM (leaves dry mass), SDM (stem dry mass), RDM (roots dry mass) and TDM (total dry mass).

The salt control treatment (T2) showed decreases in LDM, SDM, RDM and TDM of approximately 59.5; 74.5; 54.9 and 65.2%, respectively, when compared to plants of the control treatment (T1) (Table 5.1). In contrast, some treatments primed with H_2O_2 showed an increase of approximately 12% in LDM (T4, T6, T8, T9, T10 and T12), of 31% in SDM (T4, T6, T8, T9, T12 and T13) , 33% in the RDM (T4) and 23% in the MST (T4, T8 and T9) when compared with the plants of the T2 treatment.

The selection of treatments was carried out by the highest biomass production, considering all plant partitions (LDM, SDM, RDM and TDM). Considering all the variables collected, the T4 treatment (1 mM H_2O_2 1AP) was selected for its greater power reducing the negative effect of the salinity and, consequently increasing the tolerance of sunflower plants (Table 5.1).

3.2 Second experiment

The result of the analysis of variance showed that the treatments tested showed a significant difference for the production of dry mass, content of photosynthetic pigments, variables for assessing water status, leakage of electrolytes, content of inorganic and organic solutes and enzymatic activity in both leaves and roots (at 21 and 35 days). For CO_2

assimilation, the significant difference occurred only at 35 days, while stomatal conductance and perspiration were not significantly affected by the treatments applied (Table 5.2).

Table 5.2 Results of the Fisher's test for the parameters analyzed in the second experiment using sunflower plants, at 21 and 35 days of cultivation.

Parameters	21	35
	days	
ShDM (g plant^{-1})	**	**
P_N (µmol CO_2 m^{-2} s^{-1})	ns	**
E (mmol H_2O m^{-2} s^{-1})	ns	ns
gs (mol H_2O m^{-2} s^{-1})	ns	ns
Chl*a* (mg g^{-1} FM)	**	**
Chl*b* (mg g^{-1} FM)	**	**
Chl *a* + *b* (mg g^{-1} FM)	**	**
Car (mg g^{-1} FM)	**	**
RWC (%)	**	**
EL (%)	**	**
SUC (mg H_2O cm^{-2})	**	**
SI (mg DM cm^{-2})	**	**
Na^+ $_{(leaves)}$ (mmol g^{-1} DM)	**	**
K^+ $_{(leaves)}$ (mmol g^{-1} DM)	**	**
Cl^- $_{(leaves)}$ (mmol g^{-1} DM)	**	*
Na^+ $_{(roots)}$ (mmol g^{-1} DM)	**	**
K^+ $_{(roots)}$ (mmol g^{-1} DM)	**	**
Cl^- $_{(roots)}$ (mmol g^{-1} DM)	**	**
Soluble carbohydrates $_{(leaves)}$ (µmol g^{-1} DM)	**	**
Free amino acids $_{(leaves)}$ (µmol g^{-1} DM)	**	**
Free proline $_{(leaves)}$ (µmol g^{-1} DM)	**	**
Soluble proteins $_{(leaves)}$ (mg g^{-1} DM)	**	**
Soluble carbohydrates $_{(roots)}$ (µmol g^{-1} DM)	**	**
Free amino acids $_{(roots)}$ (µmol g^{-1} DM)	**	**

Free proline $_{(roots)}$ (µmol g^{-1} DM)	**	**
Soluble proteins $_{(roots)}$ (mg g^{-1} DM)	**	**
APX $_{(leaves)}$ (µmol H_2O_2 $min.^{-1}$ g^{-1} DM)	**	**
CAT $_{(leaves)}$ (µmol H_2O_2 $min.^{-1}$ g^{-1} DM)	**	**
SOD $_{(leaves)}$ (UA $min.^{-1}$ g^{-1} DM)	**	**
LP $_{(leaves)}$ (µmol MDA g^{-1} DM)	**	**
APX $_{(roots)}$ (µmol H_2O_2 $min.^{-1}$ g^{-1} DM)	**	**
CAT $_{(roots)}$ (µmol H_2O_2 $min.^{-1}$ g^{-1} DM)	**	**
SOD $_{(roots)}$ (UA $min.^{-1}$ g^{-1} DM)	**	**
LP $_{(roots)}$ (µmol MDA g^{-1} DM)	**	**

*; ** Significant at $p \leq 0,05$ and $p \leq 0,01$, respectively; ns, not significant.

Salt stress reduced the ShDM of sunflower plants by 73% (at 21 days). However, at 35 days, this reduction was less pronounced in plants treated with H_2O_2 (Fig. 1A). During this period, salt stress reduced ShDM by 68% in unprimed plants when compared to plants of the control treatment. On the other hand, in saline conditions, H_2O_2 priming increased ShDM by 29% when compared to unprimed plants (Fig. 5.1A).

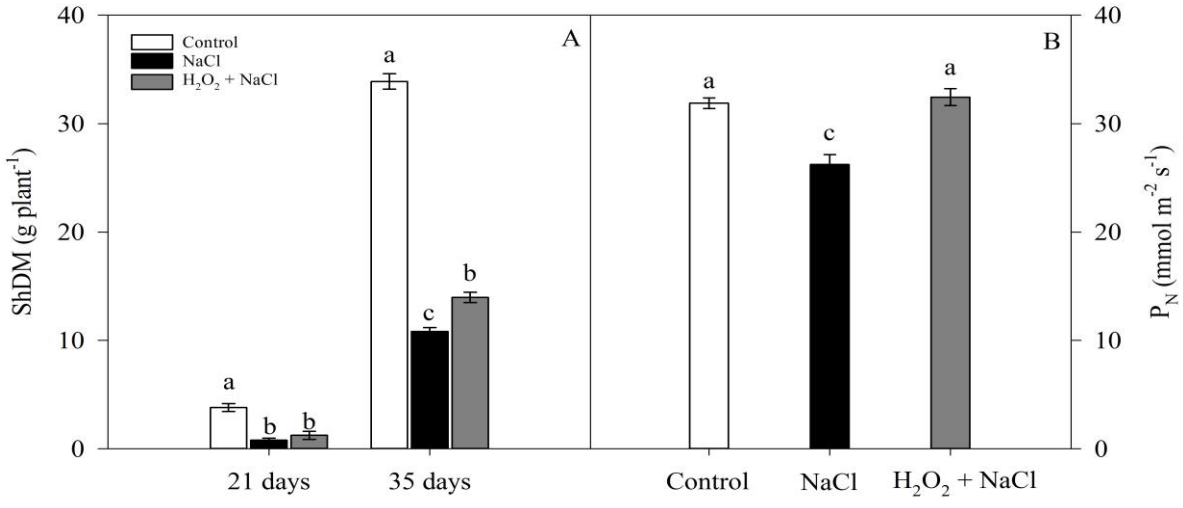

Fig. 5.1 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the shoot dry mass (ShDM) (at 21 and 35 days) (A) and on the net CO_2 assimilation rate (P_N) (at 35 days) (B) of sunflower plants grown in nutrient solution. Means of four repetitions ±

standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

Associated with this result, at 35 days, salinity also reduced P_N by 18% in plants unprimed with H_2O_2. In contrast, even under salt stress, H_2O_2 priming was able to maintain the P_N of sunflower plants at levels similar to the control treatment (Fig. 5.1B).

At 21 days, the levels of photosynthetic pigments (Chl*a*, Chl*b*, Chl *a* + *b* and Car) were, on average, 29, 26, 27 and 23% lower in plants under salt stress when compared to the plants in the control treatment, respectively (Fig. 5.2). However, at 35 days, the Chl*a* content was higher in plants under salt stress, especially when primed with H_2O_2, showing an increase by 37% in relation to the control treatment. H_2O_2 priming also increased the Chl*b* contents by 28% compared to the treatment plants under salt stress and unprimed. In addition, the contents of Chl *a* + *b* were also on average 15% higher in plants primed with H_2O_2 compared to plants in unprimed treatments (Fig. 5.2).

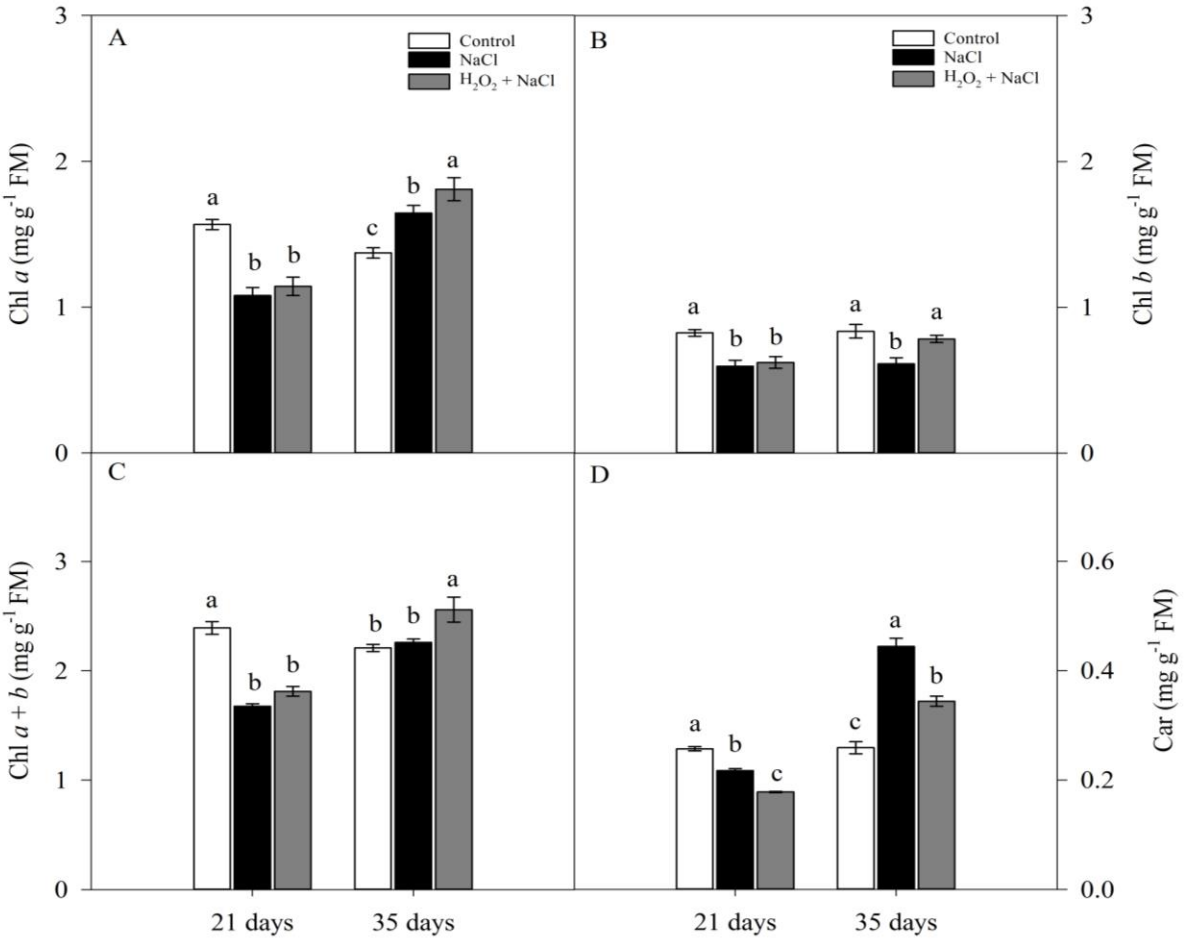

Fig. 5.2 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the content of chlorophyll a (Chl *a*) (A), chlorophyll b (Chl *b*) (B), chlorophyll *a* + *b* (Chl *a* + *b*) (C) and carotenoids (Car) (D) of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

Like the Chl*a* content, at 35 days, the Car content was higher in plants under salt stress. However, for this variable, the highlight was for plants unprimed with H_2O_2, presenting an increase of 71% in relation to the control treatment (Fig. 5.2D).

Salt stress significantly reduced the RWC of sunflower leaves, however, as with the ShDM results, this reduction was less pronounced in plants primed with H_2O_2 (Fig. 5.3A). In unprimed plants maintained under salt stress, the RWC was about 23% (21 days) and 12% (35

days) lower than in the control treatment plants. While in saline conditions, the RWC of plants primed with H_2O_2 was 17% (21 days) and 7% (35 days) higher than in unprimed plants (Fig. 5.3A).

The EL of plants not primed with H_2O_2 and maintained under salt stress was about 22% (21 days) and 35% (35 days) when compared with the other treatments. In contrast, the EL of plants primed with H_2O_2, even under salt stress, remained at levels similar to those of the plants of the control treatment (Fig. 5.3B).

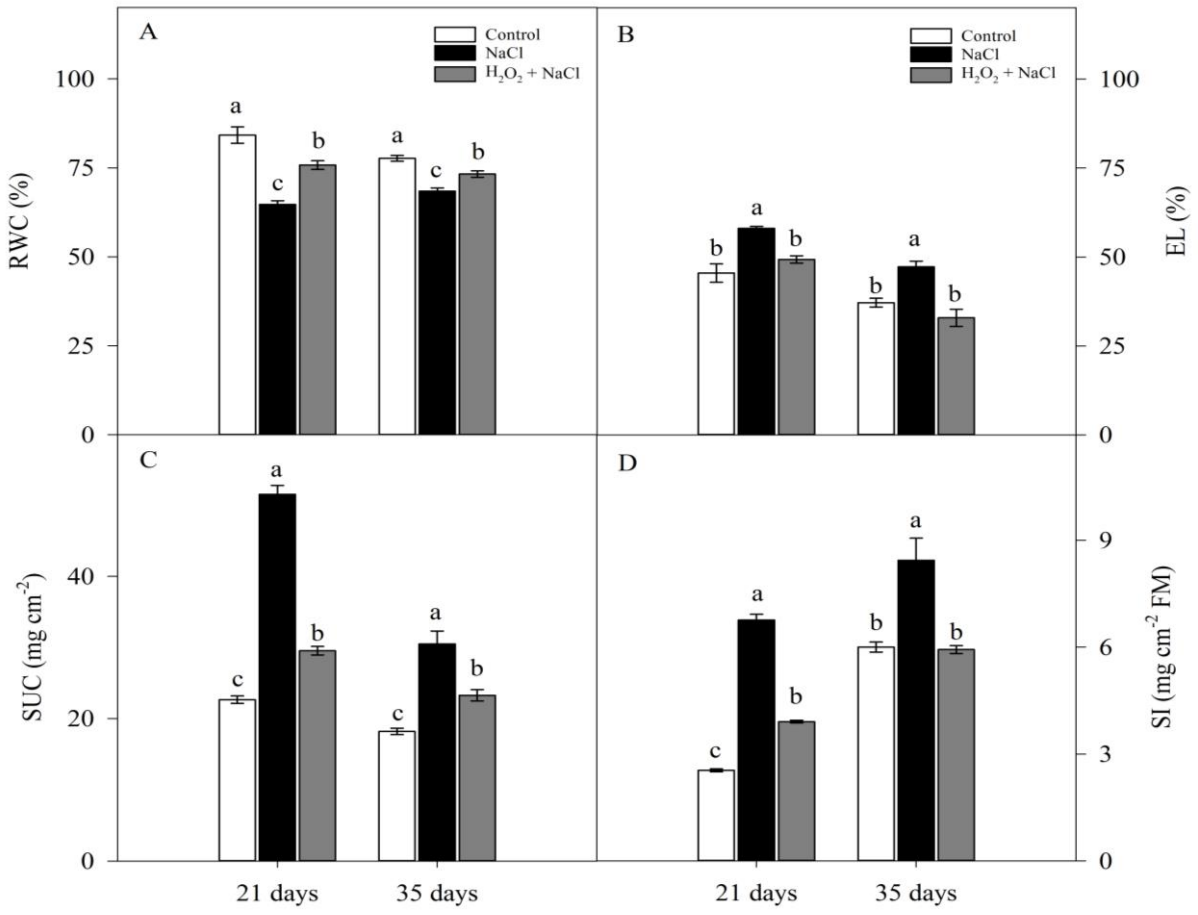

Fig. 5.3 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the relative water content (RWC) (A), electrolyte leakage (EL) (B), leaf succulence (SUC) (C) and sclerophylly index (SI) (D) of the leaves of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

The stress increased the SUC and the SI of the plants, except the SI in the treatment primed with H_2O_2 (at 35 days), however this increase was more expressive in plants unprimed (Figs. 5.3C and D). At 21 and 35 days, salinity in unprimed plants increased SUC (by 127 and 67%) and SI (by 166 and 41%), respectively when compared to control plants. In primed plants, for the same period, the SUC was 30 and 28% higher than in control treatment plants, while for SI, this increase was 53% (only at 21 days), with no significant difference at 35 days (Figs 5.3C and D).

The levels of Na^+ and Cl^- in sunflower leaves and roots also increased under conditions of salt stress in both periods of evaluation. However, H_2O_2 priming was able to significantly reduce the levels of Na^+ in the leaves and roots (at 21 and 35 days) and the levels of Cl^- in the leaves (21 days) and roots (35 days) (Fig. 5.4A, B, E and F). Analyzing the evaluation periods together (21 and 35 days), the salt stress in unprimed plants increased by an average of 14.1 and 10.3-fold (Na^+) and 5.9 and 3.4-fold (Cl^-) in the leaves and roots, respectively when compared to the plants of the control treatment. In contrast, H_2O_2 priming reduced the Na^+ content by an average of 40% (leaves) and 42% (roots) of sunflower plants under salt stress (Fig. 5.4A, B, E and F).

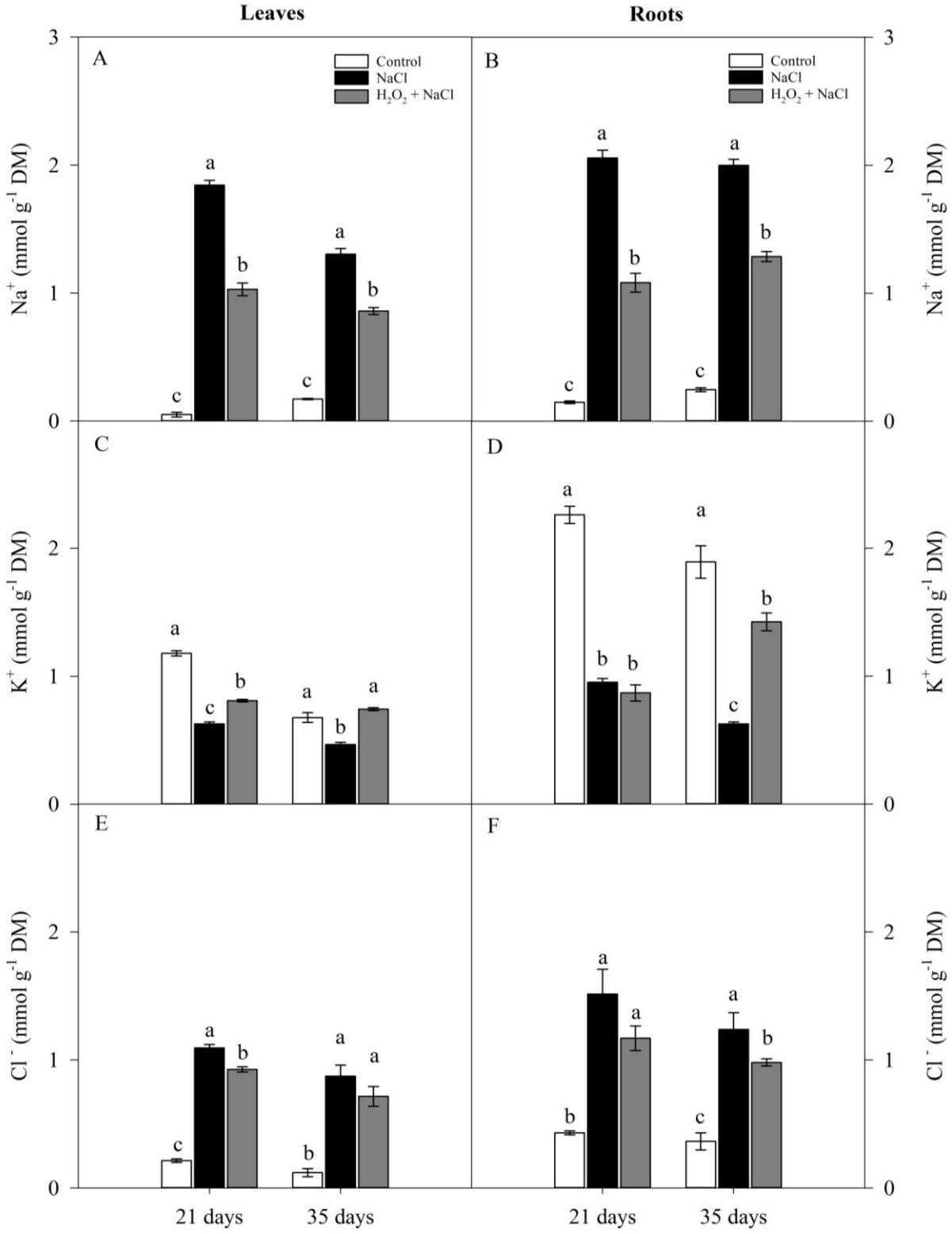

Fig. 5.4 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the contents of Na^+, K^+, Cl^- in leaves (A, C, E) and roots (B, D, F) of sunflower plants grown in nutritive solution, at 21 and 35 days. Means of four repetitions ± standard error. Means

followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

The K^+ contents were strongly reduced by the salt stress on the leaves (47 and 31%) and roots (58 and 67%) of the unprimed plants when compared with the plants of the control treatment, at 21 and 35 days, respectively (Figs. 5.4 C and D). In primed plants, with the exception of roots (at 21 days), the H_2O_2 increased potassium levels in the leaves by 29 and 59% (21 and 35 days), respectively, and in roots this increase was 128 %, at 35 days (Figs. 5.4C and D).

In both evaluated periods (21 and 35 days), in unprimed treatments, salinity significantly increased the levels of soluble carbohydrates, free amino acids and free proline, both in the leaves and in the roots of the sunflower plants compared to the plants of the control treatment (Figs. 5.5A, B and C and Figs. 5.6A, B and C). The joint evaluation of both periods (21 and 35 days) showed that the levels of soluble carbohydrates, free amino acids and free proline in this treatment were on average 1.9; 2.6 and 7.9-fold (leaves) and 2.1; 1.6 and 11-fold (roots) higher when compared to the plants of the control treatment. On the other hand, in the plants primed with H_2O_2 the average increase in the levels of soluble carbohydrates and free proline, in both periods, were 1.3 and 5.4-fold (leaves) and 1.3 and 7-fold (roots), respectively, compared to the control treatment (Figs. 5.5A, B and C and Figs. 5.6A, B and C).

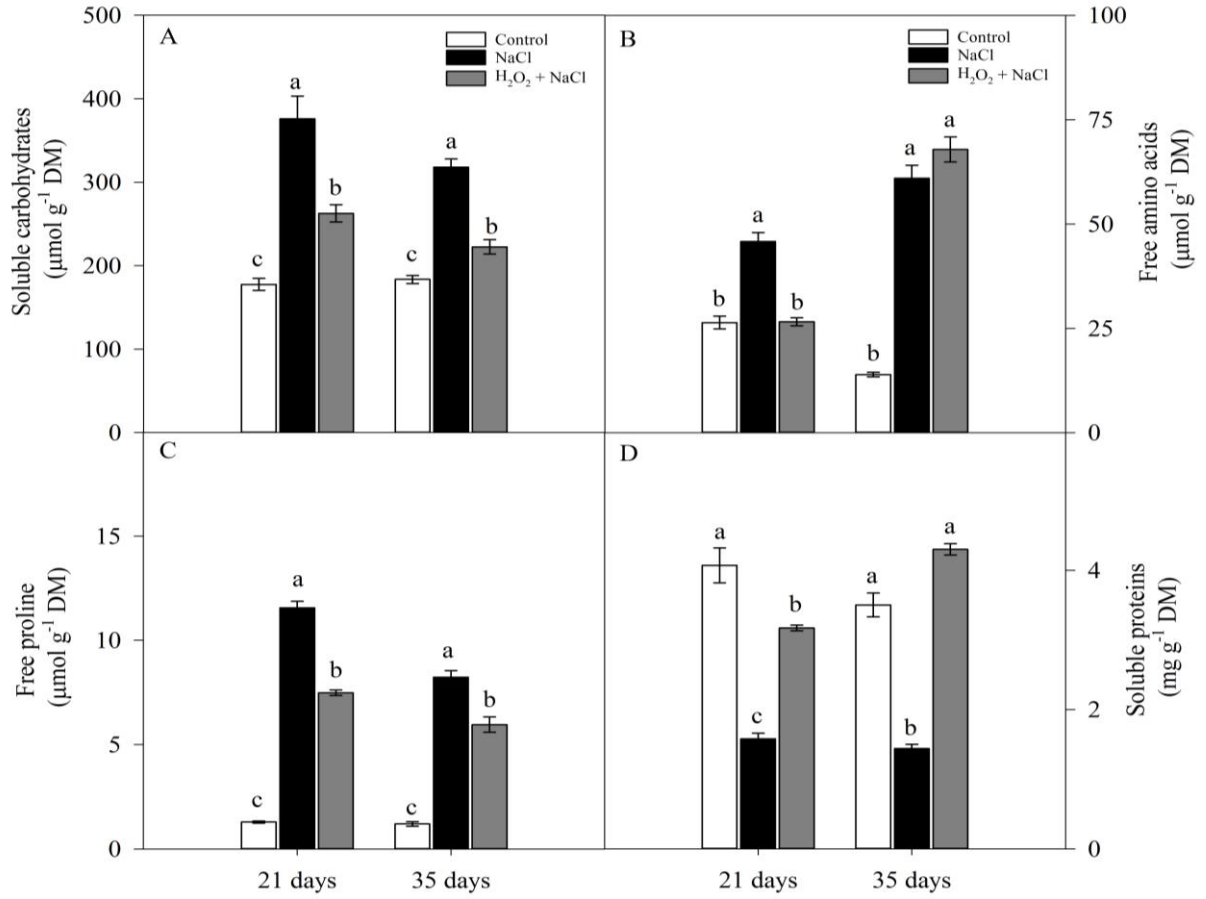

Fig. 5.5 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the levels of soluble carbohydrates (A), free amino acids (B), free proline (C), and soluble proteins (D) on leaves of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

At 21 days, H_2O_2 priming maintained the levels of free amino acids similar to those found in the plants of the control treatment, both in leaves and roots (Figs. 5.5B and 5.6B). In contrast, at 35 days, plants primed with H_2O_2 showed an increase in the content of free amino acids of 4.8-fold (leaves), compared to the control treatment, and of 2.7 and 1.7-fold (roots) in comparison to unprimed treatments under control conditions and under salt stress conditions, respectively (Figs. 5.5B and 5.6B).

Salt stress reduced the soluble protein content of leaves and roots by 61 and 40% (21 days) and 59 and 49% (35 days), respectively, in plants unprimed with H_2O_2 in relation to the control treatment (Figs. 5D and 6D). In contrast to these results, in primed plants, even under saline conditions, the soluble protein content was similar to plants of the control treatment, except in the leaves (at 21 days), where the increase in the soluble protein content was significant, but not enough to match the control treatment (Figs. 5.5D and 5.6D).

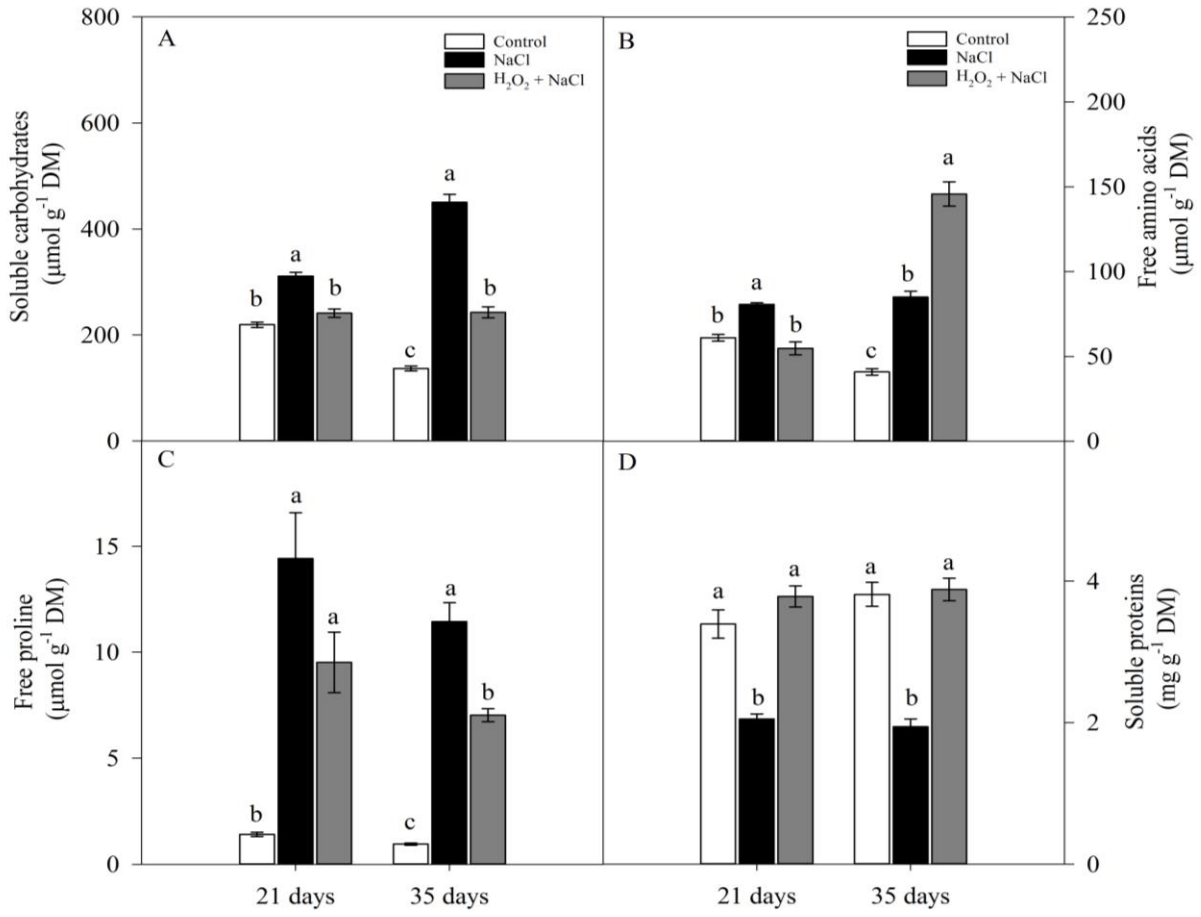

Fig. 5.6 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the levels of soluble carbohydrates (A), free amino acids (B), free proline (C), and soluble proteins (D) on roots of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

In general, salt stress increased the activity of antioxidant enzymes (APX, CAT SOD), except only of the SOD activity (in leaves) of unprimed plants, at 35 days (Figs. 5.7A, B and C). Regardless of H_2O_2 priming, salt stress increased the APX activity of leaves and roots by an average of 2.2 and 2.3-fold (21 days) and 2.3 and 3.1-fold (35 days), respectively, when compared to control treatment (Figs. 5.7A and 5.8A).

In the CAT activity, the increase was more expressive in plants primed with H_2O_2. At 21 and 35 days under salinity, CAT activity was 53 and 48% (leaves) and 110 and 60% (roots) higher in the plants submitted to H_2O_2 priming than in unprimed treatment (Figs. 5.7B and 5.8B). When compared with the plants of the control treatment, in the same period, this increase reaches 95 and 309% (leaves) and 381 and 255% (roots), respectively (Figs. 5.7B and 5.8B).

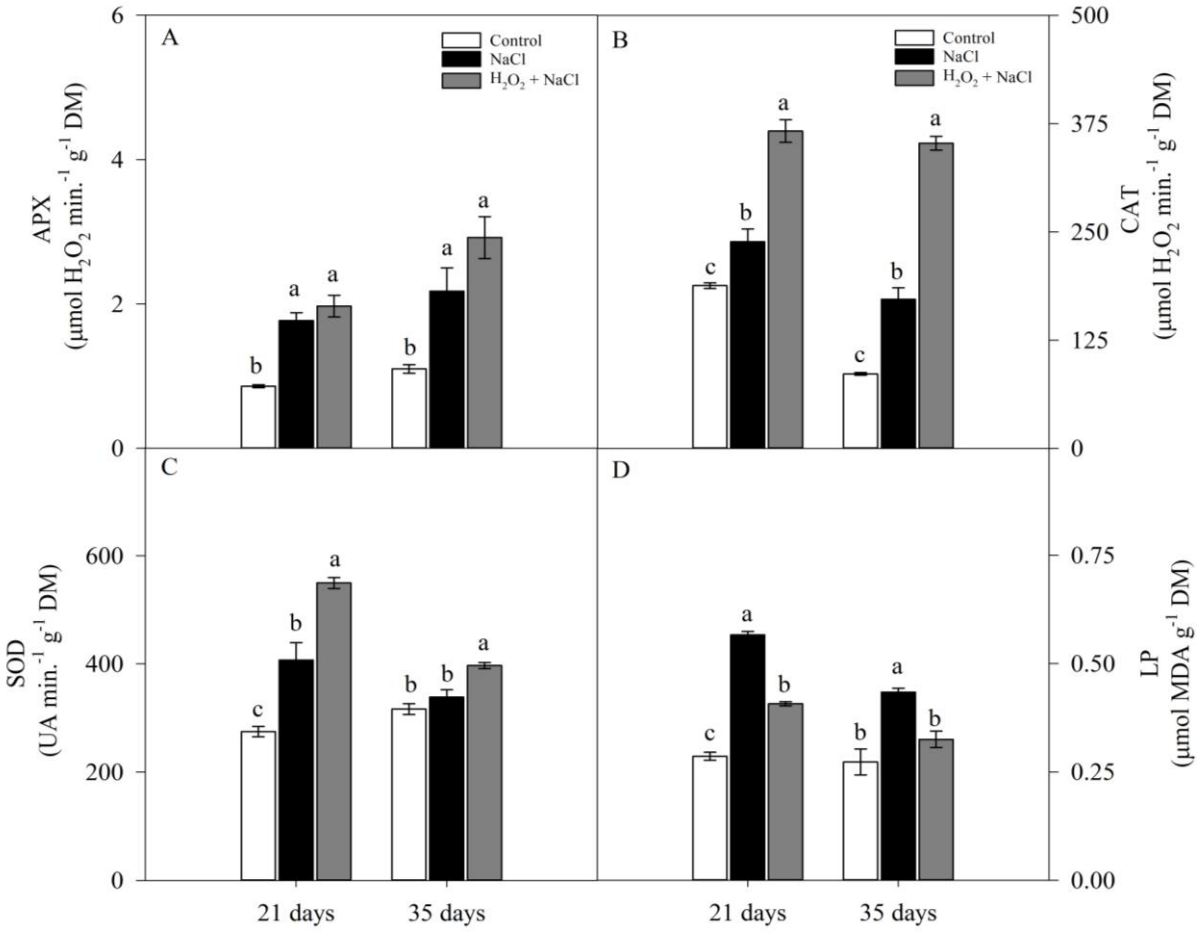

Fig. 5.7 Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the activity of ascorbate peroxidase (APX) (A), catalase (CAT) (B), superoxide dismutase (SOD) (C), and lipid peroxidation (LP) (D) in the leaves of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

At 21 days, SOD activity on leaves was increased under salt stress, but similar to CAT, this increase was more evident in plants primed with H_2O_2 (Fig. 5.7C). Compared to the control treatment plants, salt stress increased SOD activity by 48% in unprimed plants. While in the primed plants, this increase was 100% in relation to the plants of the control treatment (Fig. 5.7C).

In the roots, the results showed that the H_2O_2 priming did not alter the SOD activity, but on the other hand, the salt stress was able to increase the SOD activity by an average of 80% (21 days) and 81% (35 days) compared to the control treatment plants (Fig. 5.8C).

At 21 days, the LP of the leaves and roots were significantly increased by salt stress, but these increases were less pronounced in plants primed with H_2O_2 (Figs. 5.7D and 5.8D). The LP of the leaves and roots of the plants under salt stress and unprimed were about 98 and 95% higher than in the plants of the control treatment. However, in the same conditions, H_2O_2 priming was able to reduce the LP of the leaves and roots by 28 and 31%, respectively, when compared to unprimed plants (Figs. 5.7D and 5.8D).

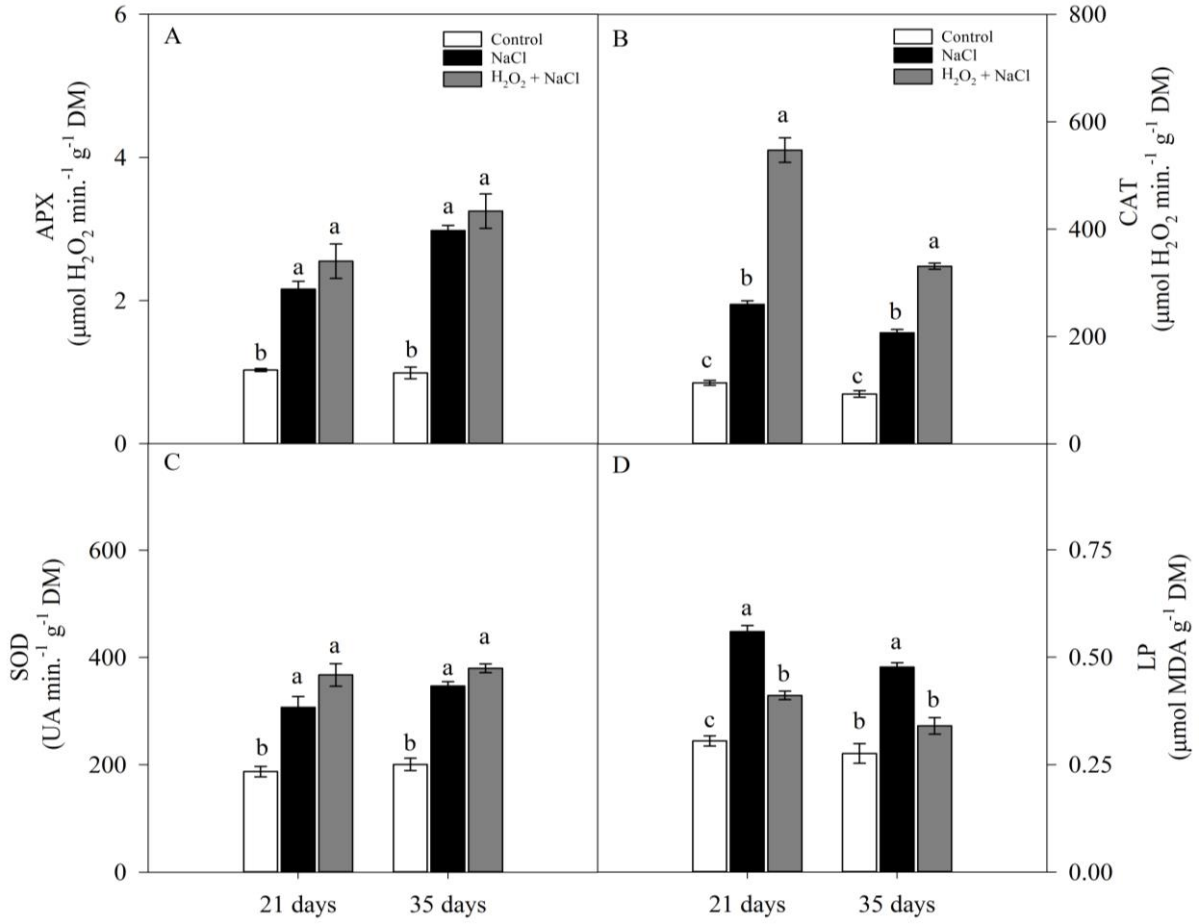

Fig. 5.8. Effect of salt stress (100 mM NaCl) and leaf spraying with H_2O_2 (1 mM H_2O_2 1AP) on the activity of ascorbate peroxidase (APX) (A), catalase (CAT) (B), superoxide dismutase (SOD) (C), and lipid peroxidation (LP) (D) in the roots of sunflower plants grown in nutrient solution, at 21 and 35 days. Means of four repetitions ± standard error. Means followed by the same letters, on each date, do not differ statistically from each other, using the Tukey's test ($p \leq 0.05$).

At 35 days, the salt stress increased the LP of the leaves and roots only in the unprimed plants (59 and 73%, respectively) in relation to the plants of the control treatment. In this period, H_2O_2 priming reduced the LP while maintaining levels similar to those of the control plants (Figs. 5.7D and 5.8D).

4. Discussion

As expected, the results of the first experiment showed that salt stress reduced the growth of sunflower plants. However, in some treatments, in especially, leaf spraying with 1 mM H_2O_2 applied 48 h before exposure to salt stress was able to increase dry mass production in all partitions of sunflower, improving plant tolerance to salt stress. In this experiment, the results also showed that the H_2O_2 application strategy via leaf spraying (concentration and number of applications) was different from those found in other studies. These results indicate that it is necessary to carry out preliminary tests to identify the best application strategy for each crop. Gondim et al., 2012 stated that from tests performed (data not shown) the best concentration used for leaf spraying was 10 mM H_2O_2 applied only to corn plants 48 h before exposure to salt. Already Semida (2016), using another application strategy, stated that the 1 mM dose of H_2O_2, applied at 20, 40 and 60 days after transplantation was the one that improved the responses of onion plants to salt stress.

In the second experiment, the analysis of the results showed that the reduction of growth and of the net CO_2 assimilation rate in sunflower plants cultivated with salt stress did not occur due to stomatal limitation. However, the increase in the concentration of toxic ions (Na^+ and Cl^-) may have been the main factor for the reduction of growth and photosynthesis. Studies affirm that the reduction of the RuBisCo carboxylation efficiency can be directly related to the accumulation of Na^+ and Cl^- in photosynthetic tissues (Silva et al., 2011).

The increase in ShDM and P_N in primed plants (Fig. 5.1A) can be directly associated with the signaling role of H_2O_2. The H_2O_2-induced cross-tolerance mechanism is based on the triggering of several highly complex reactions that are mainly related to the pathways of mitogen-activated protein kinases (MAPKs route) and the route of calcium-dependent protein kinases (CDPKs) (Hossain et al., 2015; Kurusu et al., 2015). The MAPK signaling cascade is one of the signaling pathways most studied by plant biologists. It consists of three groups of

proteins (MAPKKK, MAPKK and MAPK), responsible for signaling and signal transduction in response to various types of stress (Knight and Knight, 2001; Hossain et al., 2015). The CDPKs route, on the other hand, are signaling-related pathway regulated by $Ca^{2+,}$ and can also be modulated by H_2O_2. Ca^{2+} is considered to be one of the main secondary messengers related to several metabolic responses including increased plant tolerance to various environmental stresses (Hossain et al., 2015; Kurusu et al., 2015).

Several studies have shown that H_2O_2 is capable of increasing the tolerance of maize plants (Azevedo Neto et al., 2005; Gondim et al., 2012; Gondim et al., 2013), strawberry (Christou et al., 2014) , onion (Semida, 2016), tomato (Ezzat Mohamed et al., 2015) pistachio (Bagheri et al., 2019) and basil (Silva et al., 2019).

The reduction in pigment content observed at 21 days (Fig. 5.2) can be attributed to the increase in chlorophyllase activity, which is the main enzyme responsible for the degradation of chlorophylls (Santos, 2004; Taïbi et al., 2016). In contrast, the increase observed at 35 days may be due to the fact that the chloroplast protein associated with chlorophyll is unexcited, facilitating the process of chlorophyll extraction under salt stress conditions (Liang et al., 2018). Cova et al. (2019) and Silva et al. (2019) also found that salinity increased the Chl a content in noni (*Morinda citrifolia*) and basil (*Ocimum basilicum*) plants, respectively.

The increase observed at 35 days in the carotenoid content in plants stressed by salt (Fig. 5.2D) occurred as a plant protection mechanism, dissipating the excess energy accumulated by stress in the form of heat through the xanthophyll cycle. Salt stress disrupts the balance between photosynthetic electron transport and Calvin-Benson's cycle reactions, leading to over-reduction and excess energy within of the thylakoids (Cerqueira et al., 2019). Carotenoids are integral constituents of thylakoid membranes acting as accessory pigments in the capture of light and as photoprotective agents in the dissipation of excess absorbed light (Baroli et al., 2003). In primed plants, on the other hand, the reduction of the deleterious effects of salt stress

associated with the increase in PN may have contributed to the reduction of excess energy in thylakoids and maintaining the balance of carotenoid production.

Our results also showed that, for the unprimed treatment with H_2O_2, the salt stress reduced the WRC and increased the SUC and IE in the leaves (Figs. 5.3C and D). In contrast, conditioning with H_2O_2 was able to improve the water status of the plants. High values of EI indicate an increase in leaf thickness, some authors claim that an increase in EI is a mechanism developed to increase the resistance to the diffusion of water in the leaf and, consequently, to minimize water losses (Cunningham and Strain, 1969). As a consequence of this mechanism, there is also an increase in SUC, which is a variable that indicates the amount of water per unit leaf area. Some authors claim that the increase in SUC, besides maintaining water storage, can be an important mechanism for diluting toxic ions (Cova et al., 2016; Silva et al., 2019).

The increase in the levels of Na^+ and Cl^- and reduction of K^+ in the cytosol, verified in plants under salt stress can cause several physiological disturbances and cause an ion imbalance (Fig. 4). However, in plants primed with H_2O_2, the decrease in Na^+ and Cl^- levels and the increase in K^+ content in both leaves and roots indicate that H_2O_2 was able to trigger physiological mechanisms of Na^+ and Cl- exclusion and reduction of efflux of K^+ from tissues, promoting an improvement in ion homeostasis and increasing the sunflower's tolerance to salt stress. H_2O_2 can activate Ca^{2+} input channels to the cytosol and this, in turn, is one of the main responsible for the activation of the SOS (salt overly sensitive) pathway, formed by the SOS1, SOS2 and SOS3 proteins and responsible for the extrusion of Na^+ do cytosol (Niu and Liao, 2016; Kong et al., 2016).

Some studies have stated that H_2O_2 can induce an increase in the K^+/Na^+ ratio and reduce the Cl^- content in plants under salt stress (Gondim et al., 2011; Christou et al., 2014; Silva et al., 2019).

The expressive increase in the content of soluble carbohydrates, free amino acids and free salt-induced proline in the leaves and roots of unprimed plants can be considered a mechanism to protect plants from salt stress (Reddy et al., 2017). Under salt stress, plants accumulate organic compounds of low molecular mass, whose main functions are to help maintain hydration, protect the cell against oxidative damage, and act as signaling agents during stress (Wahid et al., 2007; Azevedo Neto et al., 2009).

The increase in the content of soluble proteins in primed plants may be related to the signaling role of H_2O_2 in the expression of specialized proteins that respond to salt, including antioxidant enzymes (Hosssain et al., 2015; Niu and Liao, 2016; Černý et al., 2018).

The excess of free energy verified in unprimed plants associated with the increase in the concentration of toxic ions induced by salt stress may have contributed to the increase in the production of ROS and, consequently, increasing the damage caused to the plasma membrane, indicated by the increase in EL and of LP. In contrast, the plants primed with H_2O_2 were similar to the plants of the control treatment, indicating once again a significant reduction in the negative effects induced by NaCl (Figs. 5.3B, 5.7D and 8D). H_2O_2 priming can increase the activity of antioxidant enzymes and consequently maintain redox homeostasis. H_2O_2 is capable of activating genes that encode antioxidant enzymes and its function has profound effects in controlling the excessive accumulation of ROS (Hossain et al., 2015).

Under stress conditions there is an increase in the production of ROS such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) (Azevedo Neto et al., 2008). The increase in the production of ROS can lead to an imbalance of redox homeostasis, characterizing oxidative stress, and consequently causing disturbances in cell structure and metabolism (Zhu, 2016).

H_2O_2 priming induces an increase in antioxidant activity by increasing the level of transcripts and expression of antioxidant enzyme genes, such as superoxide dismutase, catalase,

ascorbate peroxidase, guaiacol peroxidase and others (Azevedo Neto et al., 2005; Gondim et al., 2012; Hossain et al., 2015; Savvides et al., 2016). The increased expression of these enzymes can significantly contribute to the maintenance of redox homeostasis, acting as one of the key mechanisms to mitigate the deleterious effect of salt stress (Azevedo Neto et al., 2005).

Among the antioxidant enzymes, catalase stands out for the significant increase in its activity in both leaves and roots for the two evaluation periods. The results observed by the present study confirm the hypothesis that suggested by Yang and Poovaiach (2002), Azevedo Neto et al. (2005) and Gondim et al. (2012) that the overexpression of catalase activity induced by conditioning with H_2O_2 is crucial in detoxification, increasing the tolerance of plants to salt stress.

5. Conclusions

In conclusion, our results show that the conditioning of plants with H_2O_2 via leaf spraying is able to increase the tolerance of plants to salt stress, mainly by the balance of ion homeostasis (by reducing the levels of Na^+ and Cl^- and increasing the levels of K^+) and homeostasis redox (due to increased antioxidant activity, mainly catalase).

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6. CHAPTER 6

Evaluation of methods of application of H_2O_2 for salt acclimation of sunflower plants[6]

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Evaluation of methods of application of H_2O_2 for salt acclimation of sunflower plants

Petterson Costa Conceição Silva[1]*, André Dias de Azevedo Neto[2], Hans Raj Gheyi[1], Rogério Ferreira Ribas[1], Alide Mitsue Watanabe Cova[1], Caroline Rastely dos Reis Silva[3]

[1] Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia. E-mail: petter.ufrb@gmail.com

[2] Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia.

[3] Faculdade de Administração, Universidade Federal da Bahia. E-mail

Abstract: The objective of this study was to evaluate the effect of different methods of application of hydrogen peroxide (H_2O_2) via seed and/or via foliar in sunflower (*Helianthus annuus* L.) plants under salt stress. The experiment was conducted in a greenhouse, in the experimental area of the Núcleo de Engenharia de Água e Solo - UFRB. Five treatments were tested: control (absence of NaCl); salt control (presence of 100 mM NaCl); 1 mM H_2O_2 via seed (in presence of 100 mM NaCl); 1 mM H_2O_2 via foliar (in presence of 100 mM NaCl); 1 mM H_2O_2 via seed + 1 mM H_2O_2 via foliar (in presence of 100 mM NaCl). The assay was conducted in a completely randomized design with 4 replicates. The plants were maintained during a period of 20 days in a floating type hydroponic system. Salt stress affected negatively the production of leaves, stem, roots and total dry mass. Pretreatment with H_2O_2 application via seed and the combination via seed + foliar via were able to reduce the deleterious effects of salinity, providing higher relative biomass yields.

Key words: *Helianthus annuus* L., brackish water, hydrogen peroxide.

Avaliação de métodos de aplicação de H_2O_2 para aclimatação de plantas de girassol à salinidade

Resumo: Objetivou-se com o presente estudo avaliar o efeito de diferentes métodos de aplicação de peróxido de hidrogênio (H_2O_2) via semente e/ou via foliar em plantas de girassol (*Helianthus annuus* L.) sob estresse salino. O experimento foi conduzido em casa de vegetação, no campo experimental do Núcleo de Engenharia de Água e Solo, UFRB. Foram testados cinco tratamentos: controle (ausência de NaCl); controle salino (presença de 100 mM NaCl); 1 mM H_2O_2 via semente (na presença de 100 mM NaCl); 1 mM H_2O_2 via foliar (na presença de 100

mM NaCl); 1 mM H_2O_2 via semente + 1 mM H_2O_2 via foliar (na presença de 100 mM NaCl). O ensaio foi realizado em delineamento inteiramente casualizado, com 4 repetições. As plantas foram mantidas durante um período de 20 dias em sistema hidropônico do tipo *floating*. O estresse salino reduziu significativamente a produção de massa seca das folhas, caule e raízes. O pré-tratamento com aplicação de H_2O_2 via semente e a combinação via semente + via foliar foram capazes de reduzir os efeitos deletérios da salinidade, proporcionando maiores produções relativas da biomassa.

Palavras-chave: *Helianthus annuus* L., água salobra, peróxido de hidrogênio.

Introduction

Salinity is one of the abiotic stresses that most affects crop growth and productivity in worldwide (Veeranagamallaiah et al., 2007), therefore, it is one of the main challenges encountered in agriculture (Peleg et al., 2011). Many efforts have been made in order to develop strategies that aim to improve the plant tolerance to abiotic stresses, such as, for example, the use of chemical signals that perform metabolic functions.

Hydrogen peroxide (H_2O_2) is considered the main reactive oxygen species (ROS) found in plant tissue. Due to the electrochemical properties of the molecule, H_2O_2 diffuses between cell compartments, which facilitates its signaling function (Bienert et al., 2006). Several studies have shown the role of H_2O_2 as a signaling molecule with multiple functions in plants (Neill et al., 2002; Hung et al., 2005). Although these results prove the effective role of H_2O_2 in increasing the tolerance of plants to abiotic stresses, little is known about its best applications.

Thus, this study aimed to evaluate the effect of different methods of applying hydrogen peroxide (H_2O_2) via seed and/or leaf in sunflower (*Helianthus annuus* L.) plants under salt stress.

Material and Methods

The experiment was carried out in a greenhouse, on the campus of the Universidade Federal do Recôncavo da Bahia, in Cruz das Almas, Bahia. Sunflower seeds (*Helianthus annuus* L.), genotype AG 975 were used, chosen from previous experiments because of their sensitivity to salt stress. Based on the results of previous tests, the best treatments were selected for the present study using H_2O_2 applied via seed and leaf spraying. Thus, the treatments were: control (absence of NaCl); salt control (presence of 100 mM NaCl); 1 mM H_2O_2 via seed (in

the presence of 100 mM NaCl); 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl); 1 mM H_2O_2 via seed + 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl).

The H_2O_2 concentration used was 1 mM H_2O_2, both in the application via seed (24 h), and in the treatment of leaf spraying, which was performed only once, on the abaxial and adaxial surfaces of the leaves at 7 days after the beginning of cultivation. The experimental design used was completely randomized, with four replications. The plants were transferred to polyethylene pots, containing 15 L of nutrient solution (SN) by Furlani (1997) + 100 mM NaCl, except in the control treatment.

The SN was aerated every 3 h with the aid of an air compressor, each event lasting 15 min. After 20 days of cultivation, the plants were harvested and partitioned into leaves, stems and roots. Subsequently, they were taken to an air circulation oven (65 ºC) to determine the dry mass of leaves (LDM), stem (SDM), roots (RDM) and total (TDM), using an analytical balance. The data were submitted to ANOVA using the F test ($p \leq 0.05$) and the means compared by the Tukey test ($p \leq 0.05$).

Results and Discussion

Salinity significantly reduced plant biomass production. However, this reduction was less pronounced when the plants were pretreated with H_2O_2 (Table 6.1). In the salt control treatment (presence of 100 mM NaCl and absence of e H_2O_2), the yields of LDM, SDM, RDM and TDM were approximately 80, 86, 72 and 80% lower, respectively, when compared to the control treatment (Table 6.1).

According to Hasegawa (2013), salt stress causes several disturbances in the physiological and biochemical processes, such as photosynthesis, consequently reduces the growth and productivity of plants. For all the variables analyzed, the application of 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl) did not show any significant difference when compared to the plants of the salt control treatment. This absence of significant effect may have occurred due to the management adopted in the application of H_2O_2 via leaf, which in this case occurred after saline stress.

Table 6.1 Relative production (%) of the dry masses of leaves (LDM), stem (SDM), roots (RDM) e total (TDM) of the sunflower plants cultivated in nutrient solution with or without 100 mM NaCl and treated with different methods of applications of H_2O_2, Cruz das Almas BA, 2019.

Treatments	MSF	MSC	MSR	MST
Control (absence NaCl)	100	100	100	100
Salt control (100 mM NaCl)	19.6 b	13.6 b	28.1 b	20.0 b
H_2O_2 via leaf spraying + 100 mM NaCl	20. 8 b	13.3 b	30.0 b	20.1 b
H_2O_2 via seed + 100 mM NaCl	30.0 a	26.2 a	45.0 a	33.5 a
H_2O_2 via seed + leaf spraying + 100 mM NaCl	32.0 a	23.6 a	42.3 a	31.2 a
CV (%)	12.49	10.70	8.36	8.88

Means followed by the same letter in the column, do not differ statistically from each other by the Tukey test, at the 0.05 probability level.

These results suggest that the application of H_2O_2 after salt stress is not able to mitigate the negative effects caused by salt stress. The relative production of sunflower biomass in treatments with applications of 1 mM H_2O_2 via seed (in the presence of 100 mM NaCl) and 1 mM H_2O_2 via seed + 1 mM H_2O_2 via leaf spraying (in the presence of 100 mM NaCl) does not differ statistically from each other. However, they were 50% higher when compared to the salt control plants (in the presence of 100 mM NaCl and absence of H_2O_2).

The analysis of these results indicates that the application of H_2O_2 via seed (before salt stress) is able to significantly reduce the negative effect of salt stress, without the need for further application (via leaf spraying). Several authors found that H_2O_2 pretreatment reduced the deleterious effects of salt stress on plant growth. The results obtained by Azevedo Neto et al. (2005) showed that the H_2O_2 added to the nutrient solution two days before the NaCl additions, led to a process of acclimation to salt stress in maize plants.

Similar results were found by Wahid et al. (2007) in wheat plants from seeds pretreated with H_2O_2 and grown under salt conditions. In this study, the authors state that the H_2O_2 pretreatment gave plants an increase in tolerance to salt stress.

Conclusions

Even with the reduction in dry mass production, the application of H_2O_2 via seed (24 h) can be recommended to increase the tolerance of sunflower plants to salt stress.

The leaf application of H_2O_2 after salt stress was not able to mitigate the negative effect caused by salt in sunflower plants.

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FINAL CONSIDERATIONS

Currently, salinity is still a limiting factor in agriculture, especially with regard to regional development in the semiarid region. With this, the search for alternatives that contribute to improve the tolerance of plants to salt stress has been increasing.

With the present study, we were able to prove that the application of hydrogen peroxide (via leaf spraying or via seed) can be a viable technique for increasing the tolerance of plants to salinity, contributing to the increase in the production of agricultural crops in regions already salinized, as the northeast region of Brazil, providing an increase in the farmers' source of income.

We can confirm that many studies related to this research topic are still needed, since the H_2O_2 signaling mechanism is considered extremely complex and involves increasing tolerance to various types of stress. For each species, different H_2O_2 application strategies should be tested, varying the application site, doses, exposure time, number of applications to then identify which technique is most recommended to increase the tolerance to stress in each specific culture.