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Antioxidant Isozymes Activities in Potato Plants (*Solanum tuberosum* L.) Under Salt Stress

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Abstract

To determine NaCl effects on growth and some antioxidant enzymes activity, the internode cuttings of four potato cultivars (*Solanum tuberosum* L.), Agria, Kennebec (relatively salt tolerant), Diamant and Ajax (relatively salt sensitive) were grown on callus inducing media amended with 0, 50, 100 and 150 mM NaCl at dark conditions. Callus growth of all cultivars was significantly reduced under salt stress. Total superoxide dismutase (SOD) activity decreased in all cultivars when calli were grown in the presence of NaCl. Peroxidase (POD), H_2O_2 detoxifying enzyme activity increased under salt stress, but at higher NaCl levels the activity was reduced. On the other hand, SOD and POD isozyme profiles at 100 mM NaCl were different from that of the control. These differences were quantitative and were expressed more in terms of increased or decreased isozymes activities. In dark conditions, callus tissue underwent oxidative stress in the presence of NaCl. In these conditions, more than any other SOD isozymes, Mn-SOD seems to play a major role in the scavenging of superoxide radicals during NaCl stress. Therefore, the increased Mn-SOD activity in salt treated-calli could reflect sustained O_2^- production in the mitochondria. On the other hand, in spite of increased activities of Mn-SOD and POD, reactive oxygen species (ROS) had damaging effects on the plant cells due to the SOD reduced total activity. Generally, there was no difference between relatively salt sensitive and tolerant cultivars in response to NaCl stress.

Keywords: Antioxidant enzymes; Salt stress; *Solanum tuberosum*

Introduction

Plants are exposed to a variety of abiotic stresses such as drought and salinity that influence their growth, development and productivity. Plants exposed to salt stress, undergo changes in their metabolism in order to cope with the changes taking place in their environment [1]. One of the biochemical changes occurring when plants are subjected to biotic or abiotic stresses is the production of reactive oxygen species (ROS). The main

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sites of ROS production in the plant cell during abiotic stress are the organelles with highly oxidizing metabolic activities or with sustained electron flow: chloroplasts, mitochondria and microbodies [2]. In plant cells, specially non-photosynthesizing cells, mitochondria is the major source of ROS [3]. The relative importance of mitochondria and chloroplasts in ROS production in light is not known.

ROS are highly reactive and when the capacity of plant for scavenging is less than ROS production they can seriously disrupt normal metabolism through oxidative damages of lipids, proteins and nucleic acids [4-7]. Plants posses a number of antioxidant systems that protect them from these potential cytotoxic effects. Antioxidant enzymes are the most important components in the scavenging system of ROS [7]. Superoxide dismutase (SOD) is a major scavenger of O_2^- and its enzymatic action results in the formation of H_2O_2. Catalase (CAT), ascorbate peroxidase (APX) [8] and a variety of general peroxidases (POD) [9] catalyze the breakdown of H_2O_2. Therefore, these enzymatic systems eliminate the damaging effects of toxic oxygen species.

Both SOD and POD exist in multiple isoforms in plant tissues. The SOD isozymes are generally classified according to their active site metal [10]: copper/zinc (Cu/Zn-SOD), iron (Fe-SOD) or manganese (Mn-SOD). Most plants have Fe-SOD and Cu/Zn-SOD in their chloroplasts. Chloroplastic Fe-SOD is generally the most abundant SOD in green leaves, while in germinating seedlings and in etiolated materials the cytoplasmic Cu/Zn-SOD and mitochonderial Mn-SOD are prevalent. The study of SOD isozyme activities can help in understanding salt effects on subcellular compartments [11].

Peroxidases (EC 1.11.1.7) are widely found in animals, plants and microbes and oxidize a vast array of compounds (electron donors) in the presence of hydrogen peroxide (H_2O_2). The plant peroxidase superfamily is divided into three classes based on differences in primary structure [12]. Class I plant peroxidases include intracellular enzymes in plants, bacteria, and yeast (*Saccharomyces cerevisiae*), such as microbial cytochrome *c* peroxidase (EC 1.11.1.5), bacterial catalase-peroxidase (EC 1.11.1.6), and ascorbate peroxidase (EC 1.11.1.11). Class II plant peroxidases are extracellular peroxidases from fungi, including lignin peroxidase (EC1.11.1.14) and Mn^{+2}-independent peroxidase (EC 1.11.1.13). Class III plant peroxidases (EC 1.11.1.7) were originally described as peroxidases and are secreted outside of the cells or transported into vacuoles.

The objective of the present study was to determine the role of SOD and POD isozymes in salt tolerance of potato plants in cellular level.

Materials and Methods

Plant Materials and Treatments

Four potato (*Solanum tuberosum* L.) cultivars, Agria, Kennebec (relatively salt tolerant) Diamant and Ajax (relatively salt sensitive) were used in this experiment. The plants were maintained by subculture of nodal cuttings on sterile medium consisting of MS [13] salts and vitamins, 2 mg/l Ca-pantatonate, 2 mg/l GA_3, 0.01 mg/l NAA, 1 mg/l STS, 30 g/l sucrose and 7 g/l agar. The internode segments of seedlings were used for the generation of callus in callus inducing medium. This medium consisted of MS salts and myo-inositol and (mgl^{-1}): thiamine-HCl (10), nicotinic acid (0.5), pyridoxine-HCl (0.5), glycine (2), kinetine (0.5), 2, 4-D (5), sucrose 3% and agar 0.75%. For salinity treatment, NaCl was added to this medium at four levels (0, 50, 100, 150 mM). Internode segments were cultured in each petridish with 5 replicates for any treatment. Cultures were grown in dark at 25°C. After one month the calli were subcultured in fresh media. In the end of second month the calli were harvested and their fresh weights were determined and were stored at −20°C for subsequent analysis.

Protein Extraction

One gram of frozen callus was homogenized in 1 ml of an ice cold solution containing 100 mM phosphate buffer (pH 7.4), 1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM ascorbic acid (for ascorbate peroxidase only) [14]. The homogenate was then centrifuged for 30 min at 18,000 g. The eluent was stored at −20°C for subsequent analysis of SOD, POD. Protein content was determined using Bradford method [15].

Enzyme Determination

POD activity was measured by the H_2O_2-dependent oxidation of benzidine at 530 nm, in a reaction mixture containing 2 ml of 0.2 M acetate buffer (pH 4.8), 0.2 ml of 3% H_2O_2, 0.2 ml of 0.04 M benzidine and 0.1 ml of the extracted protein [16]. SOD activity was measured by monitoring the inhibition of nitroblue tetrazolioum (NBT) reduction at 560 nm [17]. The reaction mixture contained 50 mM phosphate buffer (pH 7), 0.1 mM Na-EDTA, 75 µM riboflavin, 13 mM methionine and 10-25 µl enzyme extract. Reaction was carried out in test tubes at 25°C under the illumination of a fluorescent lamp

(40-W). The reaction was allowed to run for 8 min and stopped by switching the light off. Blanks and controls were run in the same manner but without illumination and enzyme, respectively. Under the experimental condition, the initial rate of reaction, as measured by the difference in increase of absorbance at 560 nm in the presence and absence of extract, was proportional to the amount of enzyme. The unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50%. SOD activity values are given in units per mg of protein [10]. For enzymes POD, the activity was defined as the changes in absorbance per minute for 1 mg protein (OD/min/mg protein).

Electrophoresis and Enzyme Activity Visualization

Non-denaturating polyacrilamid gel electrophoresis (PAGE) was carried out with 10% resolving gel at 4°C and 65 mA [18]. POD isozymes were visualized by incubating the gels in a solution consisting 80 ml of 0.2 M acetate buffer (pH 5), 8 ml of 3% H_2O_2, 4 ml of 0.04 M benzidine. POD isozymes appeared with brown bands after 30-60 min at 4°C [19]. SOD isozymes were visualized by incubating the gels for 30-45 min in the dark in a solution consisting of 100 ml of 0.2 M Tris-HCl (pH 8), 4 mg riboflavin, 4 mg Na-EDTA, 20 mg NBT and illuminating of the gels until the bands became apparent [20]. The three types of SOD, Mn-SOD, Fe-SOD and Cu/Zn-SOD were identified using specific inhibitors. Before staining, gels were incubated at 25°C for 30 min, separately, in solution of 5 mM H_2O_2, 2 mM KCN or both inhibitors. The sensitivity of Cu/Zn-SOD to cyanide (KCN) has been used as a diagnostic tool to distinguish Cu/Zn-SOD from Fe-SOD and Mn-SOD that are unaffected by cyanide. Likewise, Fe-SOD is irreversibly inactivated by H_2O_2, whereas Mn-SOD is resistant to both inhibitors [10].

Statistic

Values in the text indicates mean values ± S.E, and the least significant difference (LSD) between treatments at $P \leq 0.05$ derived from an analysis of variance.

Results and Discussion

Growth

The effects of increasing concentrations of NaCl on the growth of potato calli are shown in Figure 1. Although, Agria and Kennebec were classified as relatively salt tolerant, their calli growth reduction was similar to that of NaCl sensitive cultivars under salt stress. Therefore NaCl tolerance mechanisms were not sufficiently active in the calli compared to that in whole plants. In whole plants, NaCl absorption mediated by specialized root cells. Non-differentiated callus tissue doesn't have endoderm layer, which controls ion absorption, and other controlling mechanisms that are present in the whole plant. Then, there isn't a difference between salt sensitive and tolerant cultivars growth in the presence of NaCl stress.

Enzyme Activity

As explained earlier, H_2O_2, OH and O_2^- radicals that produce under salt stress are potentially harmful to plants. Fortunately, plants have the capacity to cope with these reactive oxygen species by eliminating them with an efficient scavenging system [2]. Superoxide dismutase (SOD) is a major scavenger of superoxide (O_2^-) and its enzymatic action results in the formation of H_2O_2 and O_2. In the present study, SOD activity reduced in all cultivars under salt stress (Fig. 2A). However, the basic levels of SOD activity in Agria and Kennebec were higher than that in the sensitive cultivars. On the

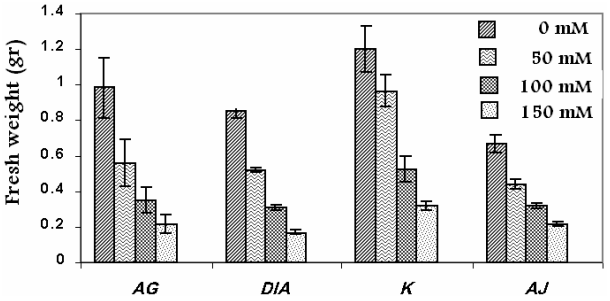

Figure 1. Effect of NaCl treatment on callus fresh weights of potato cultivars. Values are means±S.E of five replicates. (AG) Agria; (DIA) Diamant; (K) Kennebec; (AJ) Ajax.

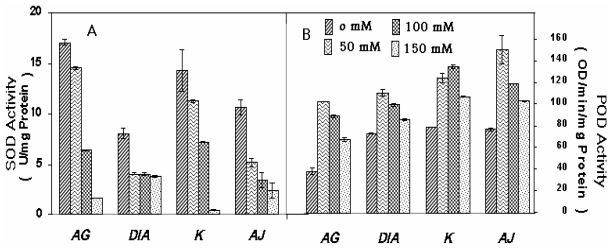

Figure 2. The effects of NaCl on antioxidant enzyme activities in potato calli. (A) SOD activity; (B) POD activity. Values are means±S.E of five replicates. (AG) Agria; (DIA) Diamant; (K) Kennebec; (AJ) Ajax.

other hand, at 50 mM NaCl, the reduced SOD activity in Agria and Kennebec was less than that in the sensitive cultivars. The present results are in contrast with the results of Benavides et al. [21]. They reported that SOD activity increased significantly in the salt sensitive colon of potato and remained unchanged in tolerant ones when exposed to NaCl stress. Other studies reported increased SOD activity in salt tolerant cultivars and reduced activity in sensitive ones [21-24].

Decomposition of O_2^-, whether enzymatic or non-enzymatic, is always accompanied by production of H_2O_2, therefore its removal by cells is essential in mitigating the oxidative stress. In plant this is achieved by a well organized functioning of catalase, ascorbate peroxidase and peroxidases. There are variable responses of H_2O_2 scavenging enzyme activities to NaCl stress. In cotton, it has been reported that, under salt stress, H_2O_2 scavenging enzyme activities are increased in salt tolerant cultivars and reduced or remained unchanged in non-tolerant ones [7,23,24]. These reports explain that, the increase in the enzyme activity could be associated with its salt tolerance character [24]. Rout and Shaw [25] suggested the active involvement of at least catalase and peroxidases among the H_2O_2 scavenging enzymes in salt tolerant plants. They concluded that APX probably did not have any role in salt tolerance. Benavides et al [20] reported that APX was probably more important than CAT in the H_2O_2 detoxification in potato which had been exposed to NaCl. In the present study POD activity was increased in all cultivars under salt stress. At 50 mM NaCl treatment, POD activity in Agria, Diamant, Kennebec and Ajax increased 167%, 50%, 55% and 100% respectively when compared with the 0 mM NaCl levels treated calli (Fig. 2B). At higher NaCl levels, POD activity was partially reduced.

Isozyme Activity

It is known that enzymes that have multiple intracellular distributions are present as different isozyme forms in different cell compartments [26]. Therefore, the analysis of the activity of individual POD and SOD isozymes is important, because it can help to understand how each stress may affect the different subcellular compartments [11].

The electrophoretic profiles of POD and SOD isozymes showed that the isozyme activities were affected by NaCl treatment. The effects of NaCl were expressed more on activity of constitutive enzyme pools. In the present study we observed a differential POD and SOD isozyme activities among the cultivars (Fig. 3). For example, POD isozymes with relative mobility (Rm): 0.38, 0.42, 0.45, 0.51 in Ajax, Rm: 0.4, 0.45, 0.51 in Kennebec and Rm: 0.38, 0.45, 0.51 in Diamant and Rm: 0.45, 0.51 in Agria were specificity of these cultivars, so that we could identify these cultivars by POD isozymes pattern. In general, POD isozymes had increased activity under salt stress (Fig. 3A).

There were 7-11 SOD isozymes in different cultivars (Fig. 3B). Some of them were cultivar specific and some had higher activity in a cultivar. For example, isozymes with Rm: 0.606, 0.313 in Kennebec and Ajax, Rm: 0.346 in Kennebec and Rm: 0.36 in Ajax were unique. On the other hand, a very high activity in SOD isozymes with Rm: 0.413 in Diamant or Rm: 0.533 in Agria were specific to these cultivars. Therefore, we recommend that these enzymes could be used for the identifying of potato cultivars.

Contradictory results have been obtained in relation to the effect of salt and drought on the activity and protein levels of the various isozymes of SOD. Drought increased the activity and amount of cytosolic SOD in peas [27]. It has been reported that in NaCl tolerant pea cultivars, leaf mitochondrial Mn-SOD and chloroplastic Cu/Zn-SOD activities increased under salt stress, while the cytosolic Cu/Zn-SOD activity remained unchanged [22]. In the salt sensitive cultivar, chloroplastic SOD activity was not increased by salt, while the cytosolic and mithocondrial SOD activity was even decreased. In leaves of *Vigna unguiculata*, salt decreased Mn-SOD activity [22]. It is questionable whether one can integrate these contradictory data into a model and draw general conclusions. The experiments exploit different plant systems, with cultivars varying with respect to salt tolerance, and the effects were measured in different tissues and at various ages. All of these parameters should be taken into account when the level and activity of the enzymes are determined.

In the present study, SOD isoenzyme profile at 100 mM NaCl in calli showed less difference compared to that of the control. In most cases, there was a reduced activity in SOD isozymes in response to elevated levels of NaCl concentration. For example, this reduction was seen in isozymes with Rm: 0.17, 0.27, 0.29, 0.6 in Kennebec, Rm: 0.17, 0.56 in Ajax and Rm: 0.56, 0.6 in Agria.

There was no chloroplastic Fe-SOD and Cu/Zn-SOD, because the calli were grown in the dark conditions (Fig. 3C). Therefore, we concluded that the Fe-SOD activity in potato callus is related to peroxisomes. In the presence of H_2O_2 + KCN (Fig. 3D), it appeared that there were two SOD isoforms (Rm: 0.413, 0.45) in Diamant, two isoforms (Rm: 0.45, 0.48) in Agria, two isoforms (Rm: 0.017, 0.45) in Kennebec and Ajax, all of them belonging to Mn-SOD. In most cases, Mn-SOD

isozymes had higher activity than the others. SOD isozymes with Rm: 0.27, 0.29 were related to Fe-SOD. Other SOD isozyme activities with Rm: 0.56, 0.6, 0.65 which were Cu/Zn-SOD, generally reduced under salt stress. On the other hand, generally Mn-SOD activity increased in salt treated calli and this could reflect sustained O_2^- production in the mitochondria. Therefore, it is possible that an increase in enzyme activities under salt stress is indicative of increased production of ROS and a build up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants. However, because of reduction in total SOD activity this was not observed in potato calli.

The results obtained with callus tissue were not in total agreement with those found in potato seedlings. In opposite to callus tissue, in Agria and Kennebec seedlings, SOD activity was increased under salt stress, where the fresh weights were unchanged, but in relatively NaCl sensitive cultivars the activity showed reducing patterns [28]. H_2O_2 detoxifying enzymes in callus tissues showed similar changes as potato seedlings. Based on the present results, we concluded that in potato calli, POD probably had an important role in H_2O_2 detoxification. Therefore, we would suggest that the importance of the enzymes in ROS detoxification depends on the experimental tissue and the cultivar. Although, Mn-SOD activity increased under salt stress, total SOD showed reduced activity in the present study. Therefore, in callus tissue, probably because of inefficient activity of SOD, superoxide radicals had more damaging effects on the growth. It is therefore concluded that elevated H_2O_2 detoxifying enzyme activities without an accompanying increase in the ability to scavenge superoxide radicals, result in damaging effects in plants. The different responses of potato seedlings and calli to NaCl were not totally unexpected. Because gene regulation in undifferentiated callus tissue grown on phytohormone amended media is likely to be different from gene regulation in tissue that has undergone normal ontogeny.

Conclusion

In conclusion, more than any other SOD isozymes, Mn-SOD seems to play a major role in the scavenging of superoxide radicals during NaCl stress in potato calli. On the other hand, because of reduced total SOD activity, ROS had damaging effects on the plant cells and there was no difference between relatively salt sensitive and tolerant cultivars in response to NaCl stress.

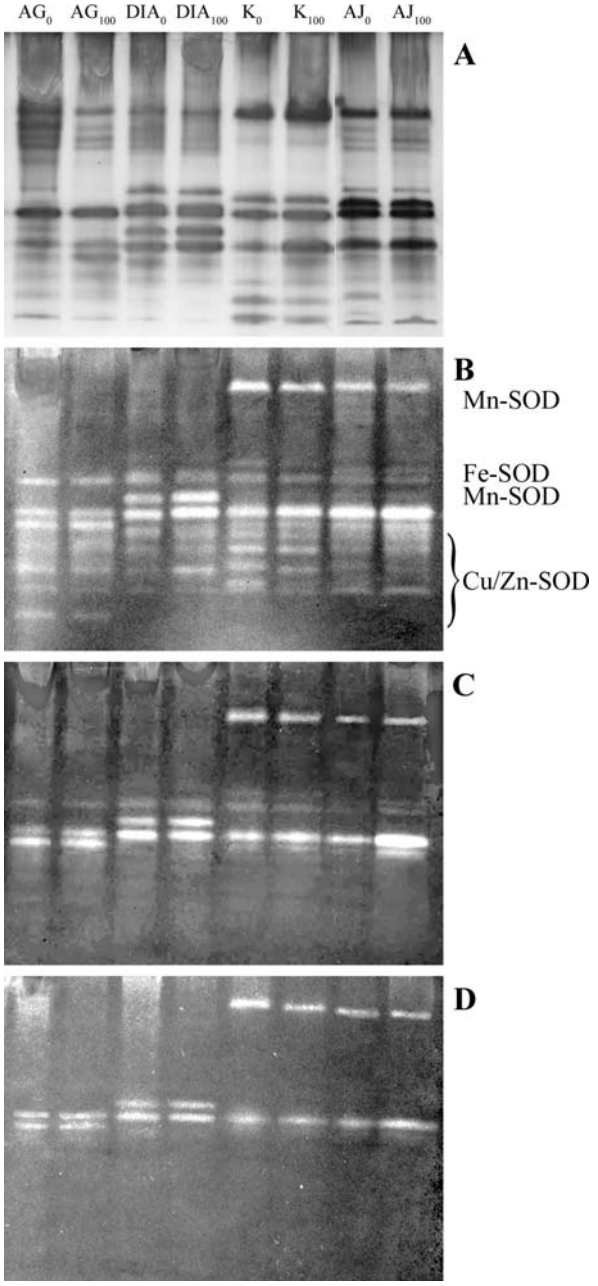

Figure 3. Effects of 100 mM NaCl on POD (A) and SOD (B, C, D) isozyme activities in potato calli. Inhibitor tests for SOD isozymes in the presence of KCN (C), H_2O_2 + KCN (D) and in absence of any treatment (Control; B). (AG) Agria; (DIA) Diamant; (K) Kennebec; (AJ) Ajax; (0) 0 mM NaCl; (100) 100 mM NaCl.

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