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ANTIOXIDANT ENZYMES RESPONSES TO NaCl STRESS IN CALLI OF FOUR POTATO CULTIVARS

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Abstract

The internode cuttings of 4 potato cultivars viz., Agria, Diamant, Kennebec and Ajax grown in callus inducing media amended with 0, 50, 100 and 150 mM NaCl showed that callus growth of all the cultivars significantly decreased under salt stress. Superoxide dismutase (SOD) activity also decreased in all the cultivars when calli were grown in the presence of NaCl. However H_2O_2 detoxifying enzyme activities showed different results. Whereas ascorbate peroxidase (APOX) activity had decreasing pattern, peroxidase (POD) activity increased under salt stress. In high NaCl concentration these activities declined. Catalase (CAT) activity increased in potato cv. Agria and Diamant and declined in cv. Kennebec and Ajax. On the other hand, under salt stress, changes in SOD and POD isozyme patterns at 100 mM NaCl were found more in terms of increased or decreased in isozymes activity as compared to control. The results showed that antioxidant enzymes have an important role in plant defensive systems against oxidative stress. Presumably some of the antioxidant enzymes have more protective role than the others.

Introduction

Plants are exposed to a variety of abiotic stresses such as drought and salinity, that influence their growth and productivity. Salinity is one of the major abiotic stresses that affect plant productivity. Potatoes are classified as moderately salt-sensitive and very sensitive to water stress (Heuer & Nadler, 1998). On the other hand, in many countries potato is cultivated in arid and semi arid regions, where shortage or poor water quality are major factors limiting plant growth and yield (Ahmed & Abdullah, 1979). When plants are subjected to environmental stresses, such as salinity, the balance between the production of reactive O_2 species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and the quenching activity of antioxidants is upset, often resulting in oxidative damage of lipids (Fridovitch, 1986;Wise & Naylor, 1987), proteins (Halliwell & Gutteridge, 1985; Davies, 1987) and nucleic acids (Fridovitch, 1986; Imlay & Linn, 1988). Plants posses a number of antioxidant enzymes that protect them from these potential cytotoxic effects. 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 (APOX) (Chen & Asada, 1989) and a variety of general peroxidases (POD)(Chang *et al.*, 1984) catalyze the breakdown of H_2O_2. Therefore this enzyme system eliminates the damaging effects of toxic oxygen species. Since the basic resources for biotechnology are genetic determinants of salt tolerance and yield stability, then implementation of biotechnology strategies to achieve this goal requires the focus of substantial research program to identify salt tolerance effectors and the regulatory components that control these mechanisms during the stress episode (Hasegawa *et al.*, 2000). The objective of the present study was to determine the antioxidant enzymes (SOD, CAT, POD and APOX) responses to NaCl stress in callus tissue of four potato cultivars.

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Materials and Methods

Plant materials and treatments: Potato cultivars viz., Agria, Diamant, Kennebec and Ajax were maintained by subculture of nodal cuttings on sterile medium consisting of MS (Murashige & Skoog, 1962) salts and vitamins, 2 mg/l Ca-pantatonate, 2mg/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 callus induction. Callus induction medium consisted of MS salts and myo-inositol and (mg/l) of 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 treatments NaCl was added to this medium at 0, 50, 100, 150 mM. Internode segments were cultured in each Petri dish with 5 replicates per treatment. Culture conditions were in dark and 25°C. After one month the calli were subcultured in the same new medium. After another month the calli were harvested and their fresh weights determined and stored at -20°C for subsequent analysis.

Protein extraction: One g 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 and 1 mM ascorbic acid for ascorbate peroxidase only (Rout & Show, 2001). The homogenate was then centrifuged for 30 min., at 18000 g. A portion of eluent was analyzed immediately for catalase activity and the remainder was stored at -20°C for subsequent analysis of SOD, POD and APOX. Protein content was determined using Bradford's method (1976).

Enzyme determination: Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 0.3 ml of 3% H_2O_2, 2.5 ml of 0.05 M phosphate buffer (pH 7), and 0.2 ml extract (Aebi, 1983). APOX activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2 at 265 nm in a reaction mixture containing 2 ml of 0.05 M phosphate buffer (pH 6.5), 0.2 ml of 3% H_2O_2, 0.2 ml of 50 μM ascorbate. Reaction was started by adding 0.1 ml of extracted protein (Arrigoni *et al.*, 1992). 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 extracted protein (Abeles & Biles, 1991). SOD activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (Giannopolitis & Ries, 1977). 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 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 (Martinez *et al.*, 2001). For other enzymes (CAT, POD, APOX), the activity was defined as the changes in absorbance per minute for 1 mg protein (OD/min/mg protein).

Table 1. Effect of NaCl treatment on callus fresh weight of four potato cultivars.

Potato cultivar	NaCl (mM)			
	0	50	100	150
	Callus fresh weight			
Agria	0.98 ± 0.17^{b}	0.56 ± 0.13^{cd}	0.35 ± 0.07^{efg}	0.218 ± 0.05^{ghij}
Diamant	0.525 ± 0.036^{cde}	0.3 ± 0.014^{fgh}	0.13 ± 0.017^{hij}	0.079 ± 0.009^{ij}
Kennebec	1.13 ± 0.12^{a}	0.965 ± 0.09^{b}	0.522 ± 0.07^{cde}	0.32 ± 0.026^{fg}
Ajax	0.667 ± 0.05^{c}	0.44 ± 0.027^{def}	0.317 ± 0.019^{fgh}	0.217 ± 0.012^{ghij}

The data are mean values of callus fresh weight (gr) ± S.E. Each value represent the mean of five replicates. Different letters within lines indicate significance (P≤<0.05) according to LSD test.

Electrophoresis and enzyme activity visualization: Electrophoresis was carried out using non-denaturating polyacrilamid gel electrophoresis (PAGE)(Hames & Rickwood, 1990) with 10% resolving gel at 4°C and 65 MA. POD isozymes were visualized by incubating the gels in a solution consisting of 80 ml of 0.2 M acetate buffer (pH 5), 8 ml of 3% H_2O_2, 4ml of 0.04 M benzidine. POD isozymes appeared with brown bands after 30-60 min., at 4°C (Van Loon, 1971). 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 (Wendel & Weeden, 1990).

Statistic: The data were statistically analyzed. Values in the text and tables indicate mean values ± S.E. and the least significant difference (LSD) between treatments at $P<0.05$ derived from an analysis of variance.

Results

Callus growth based on accumulation of fresh weight was significantly affected by salinity (Table 1). The growth decreased in all the potato cultivars with increasing NaCl level.

Antioxidant enzyme activities are presented in Fig. 1. Peroxidase activity (Fig. 1A) increased in all cultivars under salt stress. At 50 mM NaCl, POD activity in potato cv. Agria, Diamant and Ajax increased by 167, 50 and 143% respectively above the 0 mM NaCl level. More increase in NaCl level partially reduced POD activities. POD activity in Kennebec at 100 mM NaCl increased by 69% above the control level.

Catalase activity increased in Agria (Fig. 1B) with increasing NaCl concentrations. This increment was 78% above the control level when calli were grown in media containing 100 mM NaCl. Such increase was also observed in Diamant. At 150 mM NaCl, CAT activity in Diamant was 116% above the control level. CAT activity in Kennebec and Ajax partially decreased by NaCl treatment.

Ascorbate peroxidase and superoxide dismutase activities (Fig. 1C,D) decreased in all the potato cultivars under salt stress. SOD activity in cv., Agria and Kennebec was higher than cvs. Diamant and Ajax. On the other hand, the reduced rate of SOD activity in cv., Agria was less than the other cultivars at 50 mM NaCl.

The isozyme activities of POD and SOD at 100 mM NaCl were studied using 10% gels in PAGE. Electrophoretic patterns of POD isozymes were affected by salt stress (Fig. 2A). This alteration in isozymes pattern was mainly expressed as increased activity. On the other hand, there was a clear difference in POD isozymes among cultivars. For example, isozymes with Rm: 0.433 in Ajax, Rm: 0.413 in Kennebec and Rm: 0.5 in Diamant were specificity of these cultivars, so that we could identify these cultivars by POD isozyme patterns.

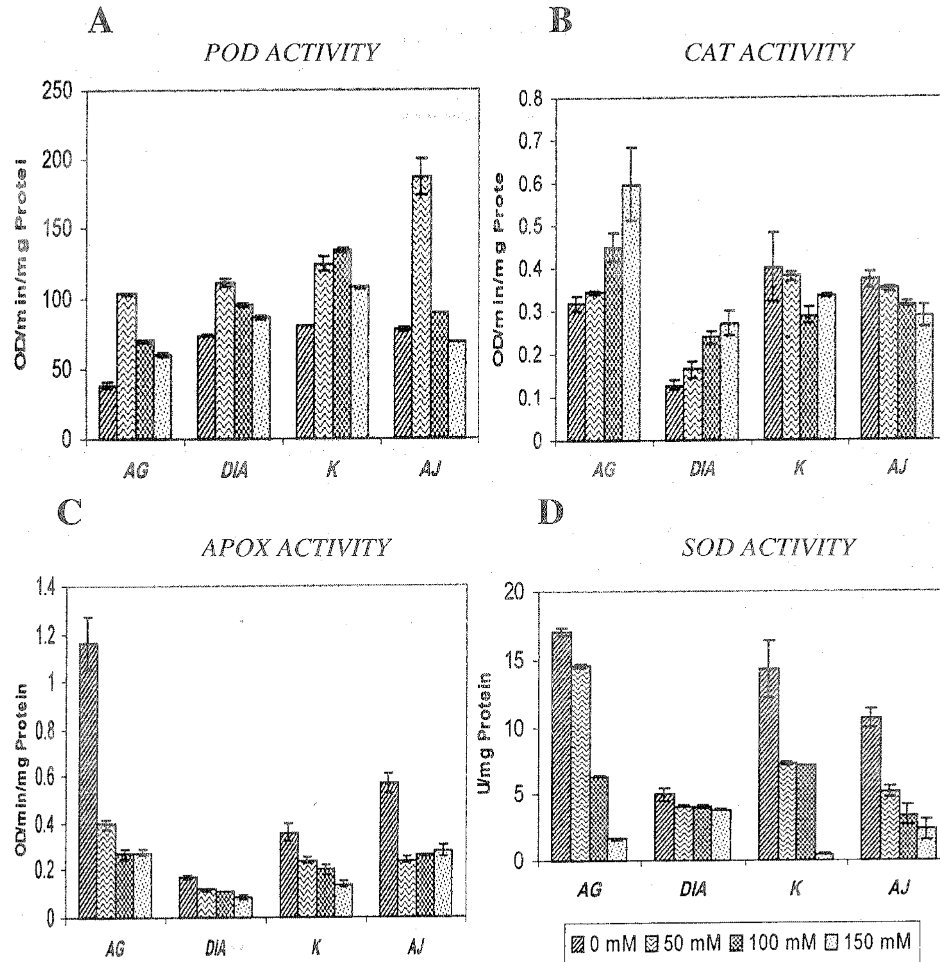

Fig. 1. Antioxidant enzyme response to NaCl treatments in calli of four potato cultivars. Bars indicate S.E. (AG) Agria; (DIA) Diamant; (K) Kennebec; (AJ) Ajax.

SOD isozyme activity was different among cultivars (Fig. 2B). There were 7-11 isozymes in different cultivars. Some of which 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 Kennebec were specific to these cultivars.

SOD isozyme activities at 100 mM NaCl in calli showed little difference with that in control. In more cases, there was a reduced activity in SOD isozymes. For example, this reduction was seen in isozymes with Rm: 0.186, 0.333, 0.373, 0.6 in Kennebec, Rm: 0.186, 0.573 in Ajax and Rm: 0.573, 0.613 in Agria.

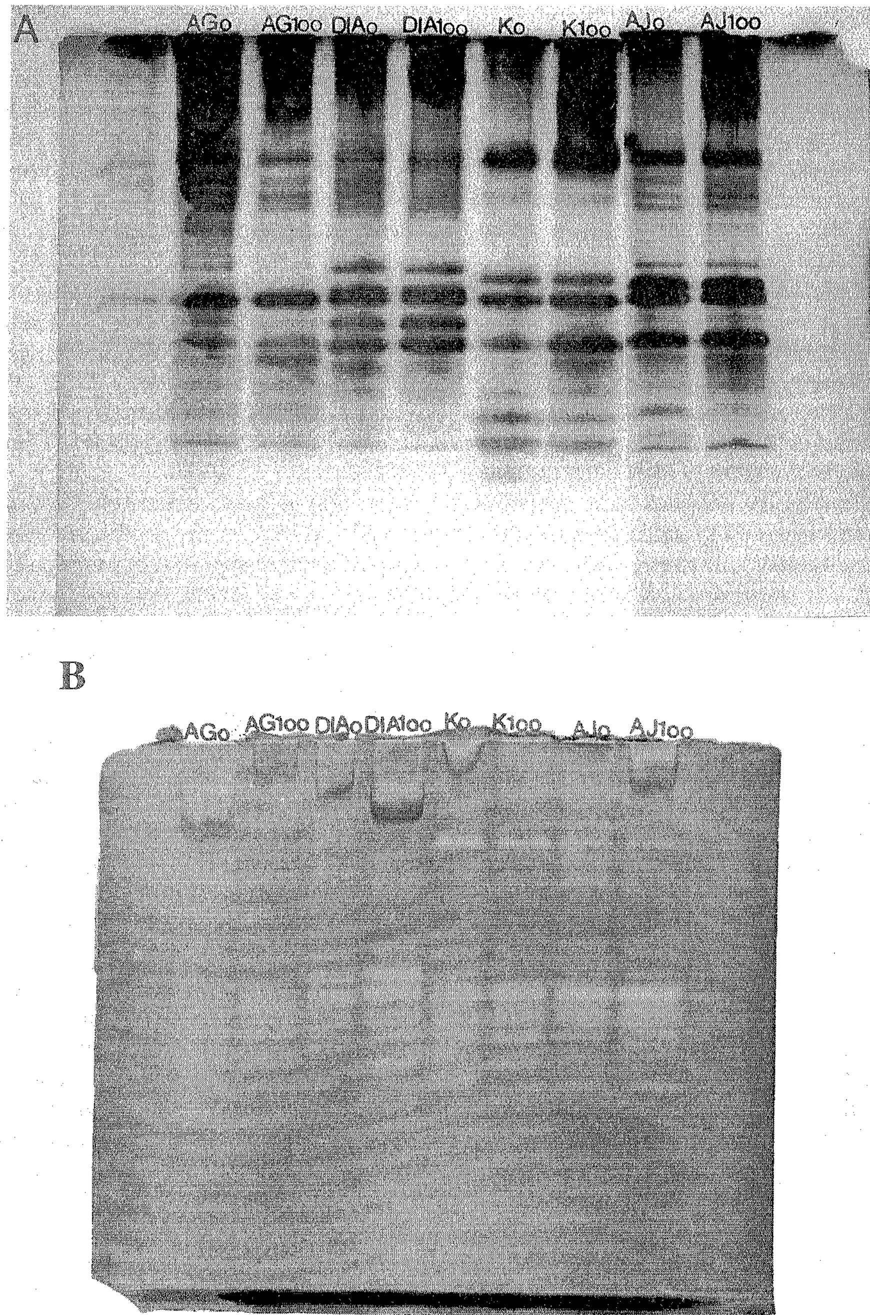

Fig. 2. Effects of 50 mM NaCl on POD (A) and SOD (B) isozymes activities in four potato cultivars. (AG) Agria; (DIA) Diamant; (K) Kennebec; AJ (Ajax); (0) 0 mM NaCl; (100) 100 mM NaCl.

Discussion

H_2O_2, OH and O_2^- radicals which are produced under environmental stress (such as salinity) are potentially harmful to plants. The plants have the capacity to cope with these reactive oxygen radicals by eliminating them with an efficient scavenging system (Breusegem *et al.*, 2001). Superoxide dismutases are considered as key players within the antioxidant defense system. They regulate the cellular concentration of O_2^- and H_2O_2 ($O_2^- \xrightarrow{SOD} H_2O_2 + O_2$). Their role in providing protection to plants against oxidative damage is well established.

In the present study, SOD activity in all potato cultivars decreased under salt stress. This is in contrast to the results of Benavides *et al.* (2000) who found that SOD activity increased significantly in the salt-sensitive colon of potato but remained unchanged in salt-tolerant colon when exposed to NaCl. Similar to our results Hernandez *et al.*, (1993) showed that under salt stress SOD activity increased significantly in the salt tolerant and decreased in the salt sensitive cultivars of pea. Gossett *et al.*, (1994) also observed similar changes in the activity of SOD in the callus of cottons.

The results obtained with callus tissue were not in total agreement with those observed in seedling studies (data not shown). SOD activity in the seedlings of Agria increased under salt stress, where the fresh weight did not change, but in other cultivars the activity showed a decreasing pattern. With increasing salt levels, superoxide radicals have damaging effects to potato plants. In potato cv. Agria seedlings, increased SOD activity at 50 mM NaCl coped with injurious effects of O_2^-, but with increasing level of NaCl the protective role disappeared (data not shown).

As decomposition of O_2^- whether enzymatic or non-enzymatic, is always accompanied by production of H_2O_2, 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. The reports on the responses of the H_2O_2 scavenging enzymes to salt treatment are different. Gossett *et al.*, (1994, 1996) reported that the activity of these enzymes under salt stress increased in the callus tissue of the salt-tolerant cultivars of cotton and decreased or remained unchanged in non-tolerant cultivars. They believed that the increase in the activity of the enzymes in the callus of the salt-tolerant cultivar could be associated with its salt tolerance character (Gossett *et al.*, 1994). Rout & Shaw (2001) suggested the active involvement of at least catalase and peroxidases among the H_2O_2 scavenging enzymes in salt tolerance in plants. They observed that the activity of catalase and peroxidase increased significantly in salt-tolerant aquatic macrophytes in response to the salt treatment. On the other hand, they showed that the activity of ascorbate peroxidase in salt-tolerant aquatic macrophytes significantly decreased, but in salt-sensitive plants the activity of the enzyme increased. They explained that this enzyme probably did not have any role in salt tolerance. Benavides *et al.*, (2000) concluded that APOX was probably more important than CAT in the H_2O_2 detoxification in potato exposed to NaCl.

In the present study, POD activity increased in all potato cultivars under salt stress. This increment was seen in seedlings too. CAT activity increased significantly in potato cv. Agria and Diamant in response to increasing NaCl levels but partially decreased in cvs. Kennebec and Ajax. These results are in agreement with those found by Benavides *et al.*, (2000). In contrast to the report of Benavides *et al.*, (2000) ascorbate peroxidase activity decreased in all cultivars.

Based on the results observed with the enzymes responsible for H_2O_2 detoxification (CAT, POD, APOX) in seedlings and callus tissue, it can be concluded that POD and CAT probably have more important role in H_2O_2 detoxification than APOX in potatoes, although in potato cv. Ajax seedlings APOX together with CAT and POD played a detoxifying role in the plant. It could suggest that the importance of the enzyme in H_2O_2 detoxification depends on the cultivar. On the other hand, when SOD has increased activity under salt stress (similar to Agria seedlings), superoxide radicals can be efficiently reduced to hydrogen peroxide. The hydrogen peroxide can then be converted to H_2O and O_2 by CAT, APOX and POD. In callus tissue (similar to Diamant, Kennebec and Ajax seedlings) probably because of inefficient activity of SOD, superoxide radicals have more damaging effects on the growth. An elevated H_2O_2 detoxifying enzymes activities without an accompanying increase in the ability to scavenge superoxide radicals results in damaging effects in plants.

On the other hand because of different responses of antioxidant enzymes to salinity in salt sensitive plants presumably there are different mechanisms for increase in resistance to oxidative stress. In some plants, reduced SOD activity lead to oxidative stress, whereas in another plant the reduction in H_2O_2 detoxifying enzymes activity may result to the stress. In another case, the reduced activity of both SOD and H_2O_2 detoxifying enzymes may induce oxidative damages in plants.

The electrophoretic patterns of POD and SOD isozymes showed that the isozymes activities were affected by NaCl treatment. The effects of NaCl were expressed more on activity of constitutive enzyme pools. Salt stress has been reported to increase the synthesis of certain proteins in some plants (Ramagopal, 1987; Hurkman *et al.*, 1989). In the present study we observed a differential POD and SOD isozyme activities between cultivars. So we recommend these enzymes for the identification of potato cultivars. Under salt stress isozymes patterns at 100 mM NaCl showed significant differences as compared with control. We observed new SOD and POD isozyme activities in seedlings of these cultivars under salt stress. But in callus tissue the effects of salt treatment were expressed more with increase or decrease in isozyme activities.

There was a difference in the antioxidant response in seedlings and callus tissue, but these differences were not totally unexpected. 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. The present study confirms the defensive role of antioxidant system in potato cultivars under salt stress.

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