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Two-dimensional Electrophoresis of Soluble Proteins and Profile of Some Isozymes Isolated from Maize Plant in Response to Nacl

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Abstract: Maize genotype (*Zea mays* L) single cross 124 was grown in water culture in presence or absence of 150 mM NaCl for 15 days. Inhibition of plant growth and modification of plant morphology are the most sensitive responses of maize plant to salt stress. The growth of the 4th leaf 5,7,9,11 and 13 d after stress application of NaCl was strongly reduced in comparison to their corresponding control. Electerophoretic analysis of total soluble protein (2-D PAGE or SDS-PAGE) and isozymes profiles were carried out in order to evaluate the response of maize genotype to salt stress. SDS-PAGE analysis has reveled that plant grown under NaCl showed induction or repression in the synthesis of few polypeptide in shoots and roots .In addition, proteins extracted from roots were analyzed by two-dimensional electrophoresis reveled two unique polypeptide to salt stress. Electerophoretic profiles of SOD (superoxide dismutase), APX (peroxidase) CAT (catalase), AP (Acid phosphates), EST (esterase) isozymes showed differences under salt stress. This differences reflected the biochemical adjustment of the plant to cope with the saline conditions. These results can be translated into efforts aimed to develop salt tolerant genotypes and maximiz the use of saline soils.

Key words: isozymes ,maize, polypeptide level, two-dimensional polyacrylamide gel electrophoresis , and salinity

INTRODUCTION

Salinity stress is one of the major abiotic stresses affecting plant growth and productivity globally. In order to improve the performance of crops growing under salt stress, it is important to understand how plants cope under such conditions. Maize (*Zea mays* L.) is considered as a moderately salt-sensitive plant[1]. Salt tolerance of plants is a complex phenomenon that involves physiological, biochemical, and molecular processes as well as morphological changes[2]. Reduction in growth and yield are undoubtedly the most physiological conspicuous responses of plants to the excess of salt in the media. Alternatively, it can be due to specific effects of Na^+ and Cl^- ions that cause toxicity and nutritionally unbalance[3]. One approach to understanding the molecular basis of salinity tolerance is to identify stress induced changes in the levels of proteins. Among the studies done in the effect of salt stress on protein synthesis, Osmotin a 26 KDa "stress protein" isolated from potato plants adapted to NaCl were quoted[4].

Proteomics is an increasingly ambiguous term that is now being applied to almost any aspect of protein expression, structure and function. Furthermore, the analysis of the plant's proteome is an important amendment to analysis of the genome, because gene expression is altered under salinity stress. The proteome, in contrast to the genome, is not static but rather dependent on a number of responses influenced by internal and external factors[5]. In addition, the identification of differentially regulated proteins can lead to the identification of their corresponding genes, which are involved in the physiology of salt resistance[6]. Nowadays, 2-D polyacrylamide gel electrophoresis is a technique that used to study the molecular mechanisms of plant response to salinity. Moreover, 2-D system were used to detect differences in the protein patterns between NaCl-stressed and control plants of the two wheat genotypes differing in sensitivity to salt[7].

On the other hand, salt stress can increase the production of active oxygen species (AOS) such as hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Plants have evolved both enzymatic and non-enzymatic mechanisms for AOS scavenging[8].

Isoenzymatic profiles of several important enzymes can be used as useful tools to detect variable responses to stressing factor.In this concern**,** peroxidase isoform was used as a biochemical indicator to understand the mechanisms involved in the genetic expression to salt tolerance in four bean cultivars grown under saline

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stress[9]. However, when Stevens *et al*.,[10] studying isoenzymatic polymorphism of peroxidase under salt stress tolerance in *Brassica*, they mentioned that, it was not possible to observe the relationship between the salt level and banding pattern. On the other hand, results of Rashed *et al*.,[11] observed differences in the intensities in esterase bands between salt tolerant and salt sensitive genotypes of maize and wheat. Acid phoshphatase is widely disturbed in plant. Salt, water and osmotic stresses have been reported to increase AP activity[12]. In addition, seven SOD activity bands in *S. salsa* leaf extract were found including an Mn-SOD and several isoforms of Fe-SOD and CuZn-SOD[13]. It turned out that NaCl salinity and osmotic stress led to differential regulation of distinct SOD isoenzymes.

The present work was done to elucidate how salt stress influence pattern of protein synthesis which was detected by both (SDS-PAGE and 2-D PAGE) together with the changes in isoenzymes profiles in maize genotype single cross 124.

MATERIALS AND METHODS

Greenhouse experiment: Seeds of Egyptian inbred line (*Zea mays* L); single cross 124 was imbibed in aerated 0.5 mM $CaSO_4$ for 1 day and then germinated at 26°C in the dark on filter paper moistened with 0.5mM $CaSO_4$. After 4 days seedling were transferred to the greenhouse into 2.8 L plastic containers (4 plants per container) on 1/4 concentrated nutrient solution, which was substituted by 1/2 concentrated solution after 2 days. Full strength nutrient solution was given after an additional 2 days and subsequently replaced every 3 days. The full concentration of the nutrient solution had the same composition as described elsewhere[14]. The experiment was carried out in the average temperature up to 30°C at day and 20°C at night and relative humidity was about 70–80%. After 1 week in the greenhouse, when the plants had reached the 4th leaf stage, the NaCl treatment was started by adding NaCl in 25 mM increments until a final concentration of 150 mM was achieved. Control plants were exposed to 1 mM NaCl. Each treatment was run in 4 replicates. During the NaCl treatment period the leaf length of the 4 th leaf, which developed during the stress application, was measured at 5,7,9,11 and 13days as described else were[15].

Protein preparation for SDS-PAGE: After 15 days of 150 mM NaCl treatment, plants were harvested, and then separated into root and shoot. Soluble protein were extracted by grinding one gram freeze dried sample with pestle and mortar in liquid nitrogen and 4ml buffer solution (250 mM sucrose, 25 mM Tris , pH 7.2). SDS-PAGE was performed by the method described previsouly[16].

Protein preparation for Isoelectric focusing (IEF): Proteins were prepared for IEF using DTT-TCA-Acetone precipitation method with several modification[17].

Two-dimensional gel electrophoresis was done with modifications[18]. IPG strips (11 cm, pH 3-10, Amersham Biosciences) were placed in the trays and 200 µl of the protein solution (150 µg protein) were applied. Strips were covered with paraffin oil. IEF was carried out in a IPG-phor chamber (Amersham Biosciences) applying the following conditions: 10 h rehydration; 100 V, 2 h; 500 V, 1 h ; 1000 V, 2 h; 8000 V, 2 h. Temperature was 20°C and current was 45 µA per strip. After running the first dimension, the strips were placed in equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea; 30% glycerol; 2% (w/v) SDS; bromophenol blue, 0.001% (w/v) containing 1% DTT (w/v) and carefully shaken for 10 min. Thereafter, the strips were incubated for additional 10 min in equilibration buffer with 4% (w/v) iodoacetamide without DTT under slow agitation. Strips were stored at -20°C. After thawing, the strips were rinsed several times with SDS-PAGE running buffer (25 mM Tris-base; 192 mM glycine; 0.1% (w/v) SDS).

The second dimension SDS gels contained 12.5% (v/v) acrylamide. Molecular weight standards in a range from 10 to 220 KDa were obtained from Invitrogen. The marker lane was positioned at the acidic side (pH 3) of the gel. Strips and marker dyes were mounted onto the gel surface and sealed with 1% (w/v) agarose containing 0.001% (w/v) bromophenol blue. The second dimension was run at 25°C and with constant current of 45 mA gel in a Hoefer (16 cm x 16 cm) vertical gel electrophoresis.

Coomassie staining was done according to a hot-staining protocol with Coomassie R 350 tablets. Molecular weight of the proteins was calculated using the standard marker.

Sample preparation for Isozyme profiles: Native PAGE was performed for Isozyme in vertical polyacrlymide gels with a discontinuous buffer system as determined previously[19].

The following enzymes systems were screened: SOD (superoxide dismutase), APX (peroxidase) CAT (catalase), APase (Acid phosphates), EST (esterase). Enzyme staining was performed according to the procedures described previously[20].

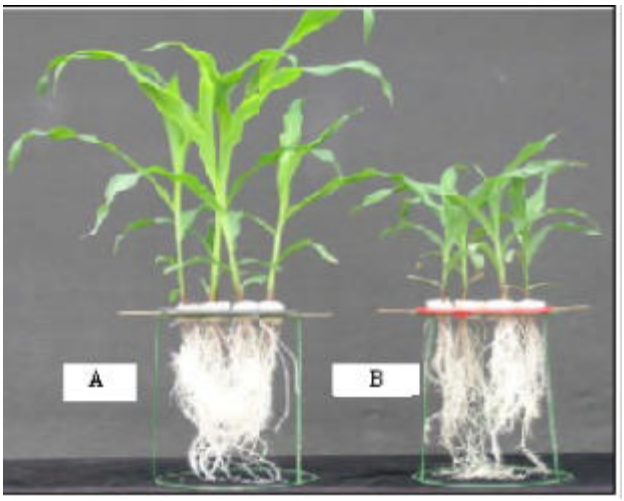

Fig. 1: Maize seedling after 15-days exposure to salt stress. A; control plant , B; 150 mM NaCl stress plant.

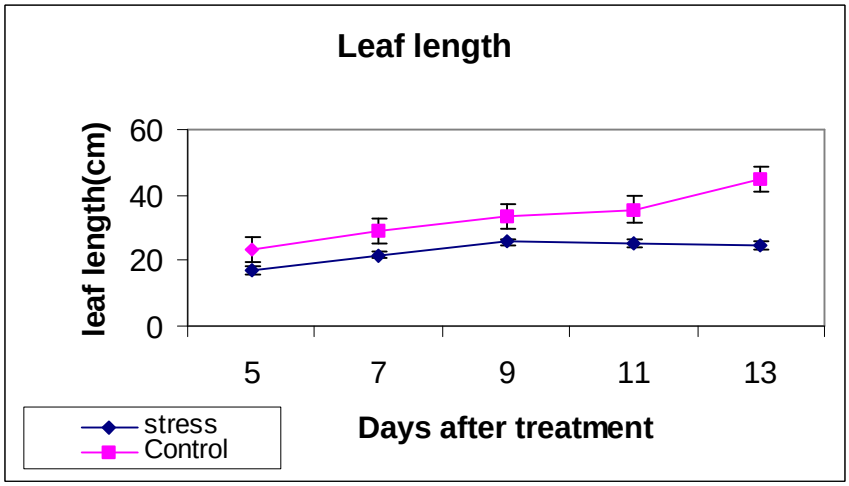

Fig. 2: Effect of 150 mM NaCl on the leaf length of the 4 th leaf of maize. Values are means of four replicate ± SD.

RESULTS AND DISCUSSION

Plant growth: Effects of salt stress on the growth of maize plant are shown in (Fig. 1) .In control (plants grown in 1 mM NaCl), no stress symptoms were observed in both roots and shoots .

After exposure of plants to 150 mM NaCl, morphological changes were observed. In addition to poor extension growth of shoots the roots become yellow. Although the salinity level used practically did not affect seeds germination, it strongly retarded seedling development. While under control conditions, no growth depression was observed.

This observation is in conformity with earlier reports[21]. The author reported that growth parameters were found to be the most important indicators for screening for salt tolerance.

Leaf length: The shoot length was more severely decreased under salt stress conditions. This decrease was more pronounced as the exposure time to salinity increased (Fig .2). After 13d exposure to NaCl stress, the reduction in shoot length due to salt stress was statistically significant. Reduced maize leaf growth under salinity is association with reactive oxygen species[22]. Similarly, Leaf area, which is essential for high rates of photosynthesis, was more impaired by NaCl[15].

SDS-PAGE: Salt stress caused an induction or inhibition in the synthesis of some polypeptides in the roots and shoots of maize plant. Protein patterns, which were examined by SDS-gel electrophoresis, were presented in

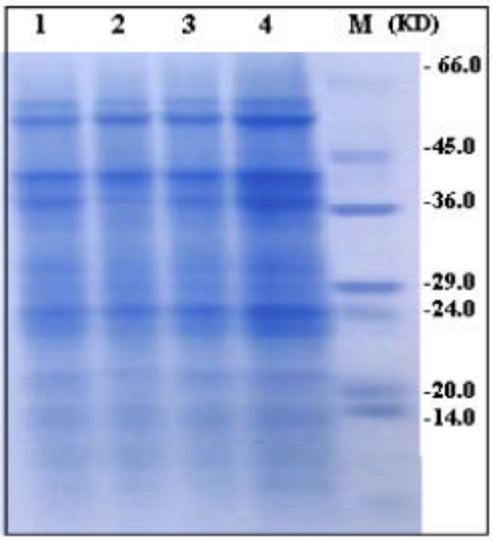

Fig. 3: Protein patterns of SDS-PAGE in maize plant. Lanes (1, 3) for control of shoots and roots and lanes (2, 4) for NaCl treatments in shoots and roots.

(Fig. 3). Levels of proteins with molecular weight of 60, 40, 24, 22 and 14 KDa polypeptides for shoots (lane 1) and 60, 40, 36, and 21KDa polypeptides for roots (lane 3) of control plants respectively were increased. Application of NaCl stress caused changes in the levels of proteins with molecular weights of 45 KDa for shoots (lane 2) and 45, 40, 32 and 9 KDa for root tissues (lane 4).

These alterations ranged in molecular weight from as low as 9 KDa to as high as 45 KDa. From the general picture of stress proteins emerging from this work, one point is noteworthy, more protein alterations were scored in roots than shoots for stress tissue it is possible that this differential response in roots and shoots tissues reflect their relative sensitivities to stress condition

Maize plant respond to salt stress by induction and repression in the synthesis of few polypeptides .In the roots, the synthesis of 45, 40,32,and 9 KDa polypeptides was increased, which is generally in accordance with the results obtained previously[23]. One possible explanation for completely disappearance of some proteins under salt stress is that the gene (S) responsible for certain proteins had been completely suppressed as a r esult of stress. Therefore, the developed tissues had lost their ability to synthesize these proteins. It is also possible that the gene (S) had not been completely suppressed, but inhibited as the result of stress, and complete recovery of the inhibition was not achieved. This may apply to the protein that stained less densely under stress[24].

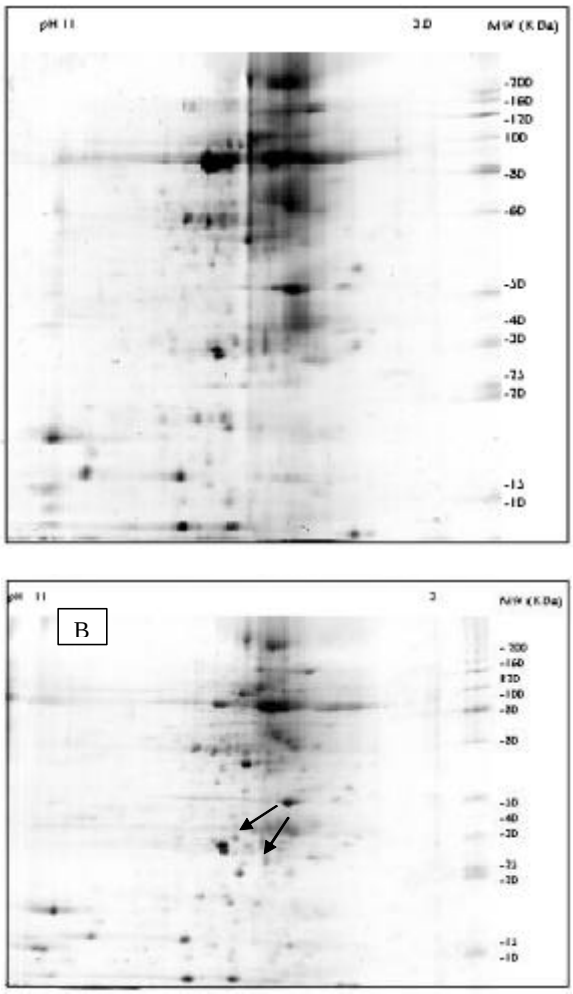

Fig. 4: Patterns of Two-dimensional electrophoresis of coomassie blue stained proteins extracted from roots of maize seedling grown (A) with 1mM NaCl and (B) with 1mM NaCl .

Root proteome: Since roots are the primary organs exposed to a saline environment. Roots thus make an important organ for studying molecular changes following exposure to salt stress. Fig (4) shows coomassie–stained 2-D gel images of 100µg of proteins extracted from control and 150 mM NaCl-treated maize roots.The quantity of differentially expressed proteins on the 2-D gels was reproducible. The higest number of spots was detected in the pH range of 4-8. More than 300 protein spots evenly distributed between pH 3 and 10 and molecular masses of 10 to 220 KDa (up-regulated, down regulated, newly appeared and disappeared).

The most striking change in roots of salt grown plants is an increase in the two new proteins synthesis of approximately 26 and 19 KDa polypeptides. The increase

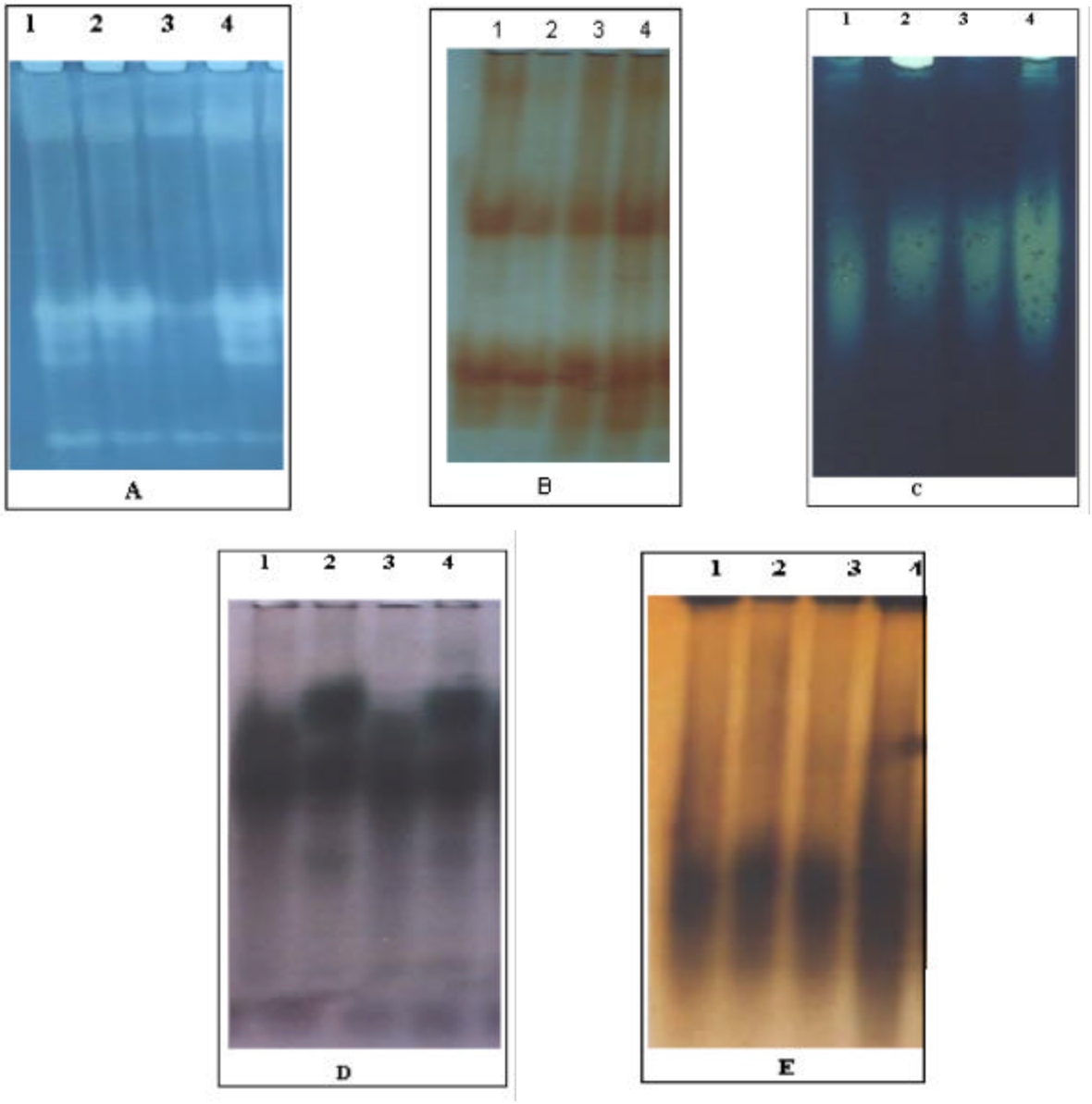

Fig. 5: Zymogram of five enzymes **,** SOD activity (A), APX activity (B) , CAT activity (C), AP activity (D) and EST activity (E)staining of shoots and roots extracts of maize plant .Lane 1,3 shoots and roots of control,, lane 2,4 shoots and roots of salt stress plant.

in the amount of these polypeptides during salt stress could be important in the adaptation of the plant to the saline conditions.

Our results of 2-D gel indicate that a major protein accumulated of 26 KDa in salt stress roots. The present results of 2-Dgel were in harmony with those of SDS-PAGE. The prolonged adaptation of cells to salinity resulted in increase of 26 KDa protein that called "Osmotin"[25]. .Also the synthesis of new proteins with 26 and 27 KD has been reported in roots of rice plant grown under salt tress[26].

Isoenzyme: In addition to protein, we examined the response of some antioxidant isoenzymes (SOD, APX, CAT) and also other hydrolysis isozymes (AP and EST), which were detected in maize shoots and roots of maize plant, exposed to 150 mM NaCl.

When protein extracts were separated by native electrophoresis and monitored for activity, five different SOD isoenzyme in varying amount were observed in

control shoot (lane 1). After treatment with NaCl, however, only four isozymes were undetected (Lane 2). Although, roots exposed to NaCl (lane 4) showed two new bands compared to control one, this variation seems to have correlation with salt stress in the medium (Fig. 5A).

Electrophoretic profiles of peroxidase isozyme generally showed four activity zones among all samples (Fig. 5 B). In addition, a progressive increase in four new bands was detected in roots of NaCl stress tissue (lane 4). Moreover, roots exposed to NaCl stress caused enhancements in density of the APX isozyme bands.

Among this enzymatic system, CAT isoenzymes in both shoots and roots subjected to salt stress as presented in (Fig. 5C). One CAT isoenzyme band in varying density was observed in both shoots and roots. This band was not similar in its mobility in control and NaCl treated tissues and its density was increased under salt stress of root tissues.

Electrophoretic patterns of esterase isoenzymes showed differences in density and number of bands among control and treated sample (Fig. 5D). Under control condition, electrophoretic patterns were characterized by the appearance of four main groups of isozymes for both shoots and roots. Under salt stressed, 150 mM NaCl caused enhancement of the esterase isozyme bands in shoots similar pattern was observed in roots.

Generally four groups of acid phosphatase isozyme were electrophoretically detected in maize, shoots and roots (Fig. 5E). The zymogram showed that, four new bands exhibited in salt treated roots tissue (lane 4). The induction of new isoenzymes and the change in the isoenzyme profile is considered to play an important role in the cellular defense against oxidative stress, caused by salt stress.

Our focus was to observe the response of some antioxidant isoenzymes to increasing salinity. In order to clarify the protective mechanisms of the antioxidant enzymes against salt stress, the changes in the activation and inactivation of isozyme profiles in maize plant subjected to salt stress were detected. The activity and resolution of SOD, APX,CAT showed high differences, also Est and Ap showed strong activity and acceptable resolution .These results indicated that salt stress increased the accumulation of the AP enzyme. The qualitative and quantitative changes in the activity of several antioxidant enzymes isolated from plants subjected to salinity were observed. For example, seven isoform of SOD bands in *S. salsa* grown under 400 mM NaCl, this isoform suggested to play a major role in stress tolerance[13].

High peroxidase isozymic activity in the tissue of salt stress reflect the changed mechanical properties of the cell wall which, in turn could be related to salt adoption process. Similar results from the present study indicated that there is a greater activity of peroxidase isoform in tolerant variety under NaCl[27]. Also salinity increase EST isozyme,the highest numbers of esterases isoenzymes were detected under the highest NaCl concentration[28]. The intensities of AP were enhanced by NaCl, these results are in agreement with the findings of Shih and Kao[12] who reported that salt stress increased AP activity. In conclusion the 2-D system made it possible to detect differences in the protein pattern between NaCl stressed and control plants of maize plant. The 2-D technique can therefore be considered to be a valuable tool for testing the effect of salt on the polypeptide profiles of plants; moreover the evaluation of the protein decay due to salt stress could be a marker of the sensitivity of the concerning cultivar towards NaCl. In addition the activities of isozymes may be a good indicator for selecting salt tolerance.Further investigations into this role need to be done.

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