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Development of Biochemical Markers for Salt Stress Tolerance in Cucumber Plants

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Abstract: The possible involvement of the ionic relations, proline accumulation, activity of peroxidase enzyme and proteins pattern in the tolerance to salt stress was studied in two cucumber cultivars, cv. Dott and alphabet. Cucumber seedlings were grown in a hydroponic solution for 2 weeks prior to salt treatment. NaCl was added to the solution at three level. Plant treatment with NaCl for 2 weeks resulted in the redistribution of Na^+, K^+ and Ca^{++} ions in the roots and leaves tissues. Na% in the dry matter of the roots and leaves were increased by increasing salt stress. Whereas Ca and K% of the roots and leaves were decreased. Proline concentration in leaves tissue increased significantly with increasing NaCl concentration in the growth media, but a high proline concentration was recorded in cv. Dott cultivar compared to the cv. Alphabet. Furthermore total peroxidase activity increased under NaCl salinity and the degree of elevation in the activity was salt concentration dependent. Nevertheless, the profile of isoperoxidase was modified during stress conditions as evident from the electrophoregram. Although, two isoforms were detected in both cultivars with different intensities. This study also found a subset of proteins induced by salt stress compared to control plant.

Key words: Salt stress, proline accumulation antioxidant enzyme, isoperoxidase protein synthesis

Introduction

Salinity stress is becoming one of the major problems resulting in losses of yield production in arid and semiarid regions. One way of solving this problem would be breeding tolerant varieties of crop plants that can be grown on saline soils, but these breeding programs are time consuming and remained elusive (Flowers and Yeo, 1995).

Therefore, the development of salt tolerant plants depend on the basis of physiological, biochemical and molecular markers are recommended and may provide mechanistic understanding the term of tolerance. Hence many metabolic changes are known to occur in plants subjected to salt stress, physiological parameters such as ionic relations (Ashraf and Waheed, 1993) have been suggested for use as tolerance indicators since they can be related to salt tolerance mechanisms.

The control of ions accumulation under salt stress in higher plants usually causes by either ions exclusion at the root cortex (Jeschke, 1984) or redistribution of excess ions to senescencing leaves (Yeo and Flowers, 1984).

Accumulation of free proline under abiotic stress (salt, drought and freezing stresses) have been reported for several plants (Potluri and Prasad, 1993).

Increased amount of proline is considered to be an indication of tolerance to salt stress because proline is thought to function either as an osmoregulator and /or a protector of certain enzymes (Aspinall and Paleg, 1981).

Active oxygen radicals (O^-_2, OH^-, H_2O_2) are commonly generated under salt stress which considered as a major peroxidative damaging factor and they need to be scavenged for maintenance of normal growth.

Plant cells posses different antioxidant enzymes such as catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) which eliminate these reactive free radicals or suppress their formation. Apparently the high value of antioxidant enzymes activity in NaCl tolerant plant could be related to salt adaptation process Piqueras *et al.* (1996).

Sreenivasulu *et al.* (1999) studied the activity of peroxidase and its isozymic profile in order to understand the role of peroxidase in conferring salt stress resistance. Salinity stress induced newly synthesized proteins in the leaf tissue of potato as found by Stephen *et al.* (1995). In addition two molecular weight of protein was found in roots and shoots of *Phaseouls vulgaries* which were unique to each tissue and their induction was clearly regulated during salinity stress (Hashim and Campbell, 1988).

Esaka and Hayakawa (1995) reported that six polypeptide bands were either new or of enhance intensity were observed after SDS-PAGE of the culture medium form suspension cultures of NaCl – adapted winged bean cells.

The objectives of this study were to determine ionic distribution in roots and leaves, proline accumulation in leaves, peroxidase activity in leaves and roots and its isoforms. We also determined the protein patterns induced by NaCl salinity in two cucumber cultivars, such

determinations if acceptable would be help to understand some of mechanisms of salt tolerant in cucumber plants if used for a large scale.

Materials and Methods

Pot experiment was carried out using water culture technique during 2001 in the green house of the Plant Nutrition Department, National Research Centre, Dokki, Cairo- Egypt.

Plant materials and growth conditions: Seeds of two cucumber (*Cucums sativus* L.) plant cultivars (cv. Dott) and (cv. Alphabet) were germinated on filter paper moistened with 0.2 mM $CaSO_4$. After 5 days in the dark at 22°C, seedlings were transferred to 2 L volume hydrobonic culture. (12 seedlings/vessel). The culture nutrient solution was previously described by Clark (1982) with the following composition: (mM) 0.7 K_2SO_4; 0.1 KCl; 2.0 Ca $(NO_3)_2$; 0.5 $MgSO_4$; 0.1 KH_2PO_4 ; (μM) 10 H_3BO_3; 0.5 $MnSO_4$; 0.5 $ZnSO_4$; 0.2 $CuSO_4$; 0.01 $(NH_4)_6$ Mo_7O_{24}. The pH was adjusted to 6.2.

Vessels were placed in controlled conditions at 16/8 h day/night regime with light intensity of 200 μmol m^{-2}. s^{-1} and relative humidity of 65-75%. The nutrient solution was exchanged every three days.

Salinity treatments and experimental design: After a growing period of two weeks in complete nutrient solution, salt stress treatments were initiated by adding 25 mmol NaCl to the culture medium for one week, increasing to desired NaCl (50 and 100 mmol)) at the second week, plants were harvested after salt stress treatments. Samples of each treatments (4 weeks old) were collected and separated into roots, fully expanded leaves. Thereafter, mixed thoroughly and representative samples were taken for analysis.

Minerals analysis: Roots and leaves tissues were prepared for the determination of minerals analysis according to Chapman and Pratt (1961) by rinse with distilled water, after drying at 70°C for 48 h, the tissues were ground to a fine powder and samples were digested by wet digestion method. The resulting solution was analyzed for Na, K and Ca with flame photometer (Genway PFP 7 model). Results were expressed as percentage of dry weight.

Proline content: Free proline content was estimated following the procedure of Bates *et al.* (1973). Fresh leaf tissue (0.5 g) were homogenized with 10 ml of 3% w/v sulphosalicylic acid and the homogenate was filtrated. The resulting solution was treated with acetic acid and acid ninhydrin and then shacked vigorously, boiled for 30 min and allowed to cool. The absorbency of the red colour was measured at 520 nm with a UV-VIS spectrophotometer (LKB-Ultsorpec II model). Proline content was expressed as μg/100mg f.wt of leaf tissue.

Protein extraction: All steps were performed according to the method used by Rothe (1997). Plant tissues (roots and leaves) were excised and homogenized using a mortar and pestle (tissue/ buffer ratio 1:4, w/v) extraction buffer pH 7.2 containing 250 mM sucrose, 0.2 mM DTT, 2% polyvinylpoly pyrolidine (w/v). The homogenate was filtered and centrifuged at 15000 g for 30 min. The resulting supernatant was used for analysis of the following determination:

Peroxidase activity: Peroxidase activity (EC 1. 11. 1.7) was determined by spectrophotometry method according to Amako *et al.* (1994). The reaction mixture contained 1.5 ml of 100 mM K-phosphate buffer (pH 6.8), 1 ml of 60 mM pyrogaloll, 0.48 ml of 0.6 mM hydrogen peroxide and 20 μl of the enzyme extract. Oxidation of pyrogaloll was followed by monitoring the increase in the absorbance at 430 nm. Proteins in the extracts were quantified by the method of Bradford (1976).

Peroxidase Electrophoresis: Non denaturing polyacrylamide gel electrophoresis (PAGE) was done in 10% (w/v) polyacrylamide. leaves protein extracts (30μg protein) were loaded onto the slots, vertical gel (Hoefer SE 600 14 x 16 cm x 0.75 cm) and subjected to 200 v for 3hr. After electrophoresis, the gels were stained with O-dianisidine for peroxidase isozymes as described by Amako *et al.* (1994).

Protein electrophoresis: Protein extract of leaves were identified by SDS polyacrylamide gel electrophoresis SDS-PAGE according to Laemmli (1970) using 12% (w/v) polyacrylamide and 0.1% (w/v) SDS. Each sample (30 μg protein) were loaded onto the slots. The gel was stained with coomassie blue R 250, and destained with an aqueous acetic acid and methanol solution. Molecular weight marker ranging from 14 to 70 KD to estimate molecular weight of the purified protein.

The gel was densitometrically scanned by using pro-analyzer scanner to measure the quantity of each band.

Statistics analysis: The data were statistically analyzed as randomized complete block design (RCBD) according to Sendecor and Cochran (1967). Comparisons among means of treatments were tested for significance against LSD values at 5% level of probabilities.

Results and Discussion

Hence roots are the primary organs exposed to salt stress, so the first defense presumably may be the selective exclusion of excess ions by roots and/ or transport of these ions into vacuole.

Sodium: Increasing salinity in the growth media from 50 to 100 mM resulted in an increase in root Na+ concentration (Table 1) the same trend also was found in leaves Na+ concentrations, in addition exclusion of Na+ from the roots to the leaves was probably minimal. Results also indicated that roots Na+ concentrations of (cv.Dott) was higher than in cv.alphabet. Salt tolerance in some plant species has negative correlations with Na content of leaves (Hampson and Simpson, 1990).

Calcium: Data showed that increasing salt treatment in the growth media gradually decreased Ca^{++} concentrations both in roots and leaves (Table 2). Whereas there were significant differences between both cultivars only in roots but not in leaves. The more pronounced effect of NaCl treatment on cucumber roots than leaves may be due to water loss from the roots during salt stress. Apparently the intact roots of cv. Dott was capable to increase the translocation of Ca^{++} ions to the leaves.

Potassium: Although root K^+ concentration of both cultivars decreased significantly with increasing NaCl treatment (Table 3). It is also clear that there were marked difference among both cultivars in their root K^+ concentrations, generally cv. Dott was always had lower concentration than cv. Alphabet, such observed differences were always significant. Meanwhile leaves K^+ concentration decreased when salinity treatments were increased from 50 to 100 mM but cv. Dott still had high concentration compared to cv. Alphabet.

These results indicated that salinity stress and specifically presence of Na^+ in the growth media had a dual effect on K^+ uptake. By other words low concentrations of Na^+ (50 mM) initially promoting a higher rate of K^+ uptake, but increasing Na^+ levels (100 mM) leading to a decrease in K^+ uptake this results in a good agreement with the result of (Reggiani *et al*., 1995 and Baalbaki *et al*., 2000) who reported that potassium represent the main cation in plant cells and is important component of cell osmotic potential, also this K^+ ions was selectively accumulated in leaf chloroplasts in response to increase salinity, in order to maintain photosynthetic activity.

In addition Ca^{++} and K^+% in the dray matter of the root and leaves were decreased while Na^+ % were increased with increasing nutrient solution salinity as had been found in cucumber (Al-Harbi and Burrage, 1999).

Plants can exhibit salt stress response when they are exposed to salt stress condition. One f the typical responses to salt stress is the control of ions accumulation. Abdullah, (1987) documented that the relationship between ions uptake and its accumulation may used as an indicator of salinity tolerance.

Proline accumulation:

The accumulation of proline in response to salt stress in cucumber plants presented in Fig. 1. Increasing concentration of NaCl from 50 to 100 mM progressively increased proline concentration in leaves tissue by a bout three fold over than control treatment. It is also interested to note that cv.Dott had significantly higher proline concentration compared to cv. Alphabet when grow at all salt treatment.

Among different parameters responding to NaCl, the most significant one is increasing proline accumulation which associated well with the salt stress tolerance (Potluri and Prasad, 1993). Previous work indicated that proline accumulation participate in the regulation of protein content. This conclusion was based on the observations that proline accumulation was probably due to new protein synthesis rather than the break down of proteins and resulting accumulation of amino acid pool and subsequent conversion to proline (Potluri and Prasad, 1993). It is obvious from the present results that accumulation of proline in high quantities in cv.Dott more than in cv. Alphabet could be related to salt stress tolerant.

Peroxidase activity: The activity of the antioxidative peroxidase enzyme in leaves of different cucumber cultivars was estimated. The data presented in (Table 4) revealed that the activity of peroxidase was significantly enhanced by NaCl treatment under both treatments. The activity of peroxidase in root tissue was more pronounced than in leaves. Assuming that salt stress produced excess superoxide radical in the roots.

Generally when salt was supplied to the growth media, peroxidase activity of cv. Dott was significantly much higher than in the cv. Alpha bet. This high activity may be correlated with the capability of this cultivar to quash oxygen free radicals, which can damage the cell compartment.

In this concern Sreenivasulu *et al.* (1999) reported that tolerant cultivar had more peroxidase activity compared to the susceptible one. Also salt treatment enhanced the

Table 1: Sodium concentrations of roots and leaves of two cucumber cultivars grown in nutrient solution with different levels of NaCl

Cultivars / NaCl treatments	Na^+(%)					
	Roots			Leaves		
	cv. Dott	cv. Alphabet	Means	cv. Dott	cv. Alphabet	Means
Control	0.45	0.55	0.50	0.72	0.24	0.48
50 mM	3.87	4.07	3.97	4.25	4.30	4.28
100 mM	5.11	3.71	4.41	5.22	5.91	5.56
Means	3.14	2.77		3.39	3.48	
LSD (0.05) NaCl	0.359			0.457		
Cultivar	0.293			0.373		

Table 2: Calcium concentrations of roots and leaves of two cucumber cultivars grown in nutrient solution with different levels of NaCl

Cultivars / NaCl treatments	Ca^{++}(%)					
	Roots			Leaves		
	cv. Dott	cv. Alphabet	Means	cv. Dott	cv. Alphabet	Means
Control	1.03	2.25	1.64	2.34	2.61	2.47
50 mM	1.32	1.77	1.54	1.88	1.83	1.85
100 mM	1.22	1.56	1.39	1.97	1.53	1.75
Means	1.19	1.86		2.06	1.99	
LSD (0.05) NaCl	0.11			0.15		
Cultivar	0.09			0.12		

Table 3: Potassium concentrations of roots and leaves of two cucumber cultivars grown in nutrient solution with different levels of NaCl

Cultivars / NaCl treatments	K^+(%)					
	Roots			Leaves		
	cv. Dott	cv. Alphabet	Means	cv. Dott	cv. Alphabet	Means
Control	3.0	6.5	4.75	6.03	4.0	5.0
50 mM	2.2	5.4	3.80	5.0	3.6	4.3
100 mM	3.2	1.7	2.45	3.9	3.8	3.8
Means	2.8	4.5		4.96	3.8	
LSD (0.05) NaCl	0.59			0.92		
Cultivar	0.48			0.75		

Table 4: Peroxidase activity of roots and leaves of two cucumber cultivars grown in nutrient solution with different levels of NaCl

Cultivars / NaCl treatments	Roots			Leaves		
	cv. Dott	cv. Alphabet	Means	cv. Dott	cv. Alphabet	Means
Control	273.4	291.4	285.4	115.6	115.9	115.7
50 mmol	446.6	340.5	393.5	218.6	211.3	214.9
100 mmol	1161.8	614.1	887.9	250.4	226.4	238.4
Means	627.2	415.3		194.8	184.5	
LSD (0.05) NaCl	5.85			3.3		
Cultivar	4.77			2.7		

activities of antioxidative enzyme catalase and glutathione reductase in cucumber plants as found by (Lechno *et al*., 1997).

Peroxidase isozyme: Expression of the peroxidase enzyme was detected in leaf tissue extracted from the two cucumber cultivars treated with 50 and 100 mM NaCl using 10 % native PAGE (Fig. 2). The results showed that two bands were exhibited with different densities and intensities in untreated and salt treated plants of both cultivars. Looking at the first varieties, these bands were approximately similar in Rf values and their intensities, exceptionally, under 100 mM salt treated plants (lane 3) the band with Rf value 0.44 was high densities and intensities than in plants grown under control or 50 mM condition. These results indicated that salt stress increased the accumulation of the peroxidase enzyme and that the encoding gene (s) was accelerated in response to salt stress.

It is also interesting to note that the second cultivar cv. Alphabet showed similar pattern to the first cultivar especially in the very distinct band with Rf value 0.44 under 100 mM salt treatment. In general these banding patterns observed under control and stress treatment

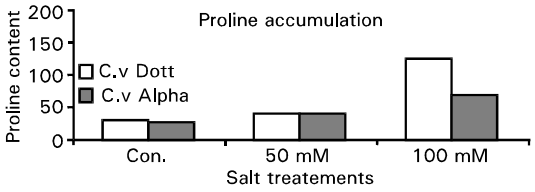

Fig. 1: Proline accumulation in leaves of two cucumber cultivars grown in nutrient solution with different levels of NaCl

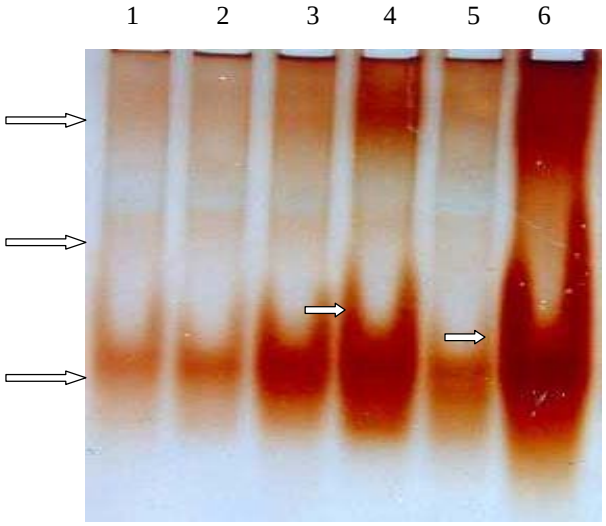

Fig. 2: Peroxide isozymes bands identification of cucumber cvs. Dott and Alphabet induced under salinity stress.
1- 0.0 mM NaCl for cv. Dott.
2- 50 mM NaCl for cv. Dott.
3-100 mM NaCl for cv. Dott.
4- 0.0 mM NaCl for cv. Alphabet.
5- 50 mM NaCl for cv. Alphabet.
6-100 mM NaCl for cv. Alphabet.

could be paralleled with the regulation of their salt stress tolerance.

These results are in agreement with the finding of Sreenivasulu *et al.* (1999) who reported that high peroxidase isozymic activity was found in tolerant cultivar compared to salt susceptible cultivar of Fax- tail millet which related to the salt adaptation process.

However, Rashed *et al.* (1994) reported that the occurrence of differential response in the decreases of intensity rather than in the isoforms of peroxidase in favor of salt tolerant genotypes under stress.

Protein SDS-PAGE patterns: The protein patterns of the two cucumber cultivars grown at different level of salt

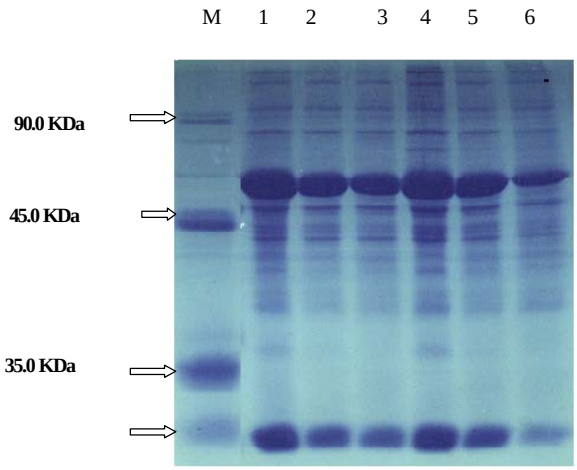

Fig. 3: Protein patters of total soluble protein from cucumber cvs. Dott and Alphabet induced under salinity stress.
1- 0.0 mM NaCl for cv. Dott.
2- 50 mM NaCl for cv. Dott.
3-100 mM NaCl for cv. Dott.
4- 0.0 mM NaCl for cv. Alphabet
5-50 mM NaCl for cv. Alphabet
6-100 mM NaCl for cv. Alphabet M-Protein marker

stress were analyzed by SDS-PAGE method. The separated bands of protein subunit were photographed and presented in Fig. 3.

In general both cultivars under all treatment contained two major distinct bands of 60.0 and 25 KDa exhibited in high intensity.

In contrast, several protein bands were appeared in one variety and disappeared in other. For example, major bands with MW 47, 36 and 25 KDa were disappeared from cv. Dott under 50 and 100 mM compared to control treatment, these bands differ in their intensity. Meanwhile, in case of cv. Alphabet salt stress led to disappearance of several protein bands with Mw 92, 80, 61, 40 and 36 KDa compared to control treatment.

In addition, several new protein bands were induced by salt stress in cv. Dott for example two bands with Mw 56 and 42 KDa were detected only under 50 and 100 mM salt treatment compared to control treatment, whereas the second cultivar cv. alphabet showed also a new band with MW 42 KDa under both treatments.

Consequently, this band can be considered as a biomarker to characterize salt stress, this newly synthesized protein indicated that salt stress induced a salt related gene to produce this salt inducible protein. Generally the results of SDS-PAGE analysis could be reveled two different genetic mechanisms i.e., that salt stress resulted in the

over expression of some gene and / or de novo induction of gene expression. The present resulted are in agreement with the finding of Ericson and Alfinito, (1984) where two different protein bands with molecular weight of 32 and 20 KDa were exhibited in high intensity in the salt adapted tobacco cells. This is in addition to the occurrence of a new protein band with molecular weight of 26 KDa which was unique to the salt exposed cells.

El-Farash *et al.* (1993) studied the expression of 12 different proteins, which were induced in salt stressed tomato plants. They reported that the expression of these proteins was genetically regulated, depending on the salt concentrations well as the genetic differences

The study demonstrated that, the biochemical parameters associated with salinity tolerance in higher plants. It offers a simple and fast method which can be used to investigate the salinity tolerance of cucumber plant.

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