Effective Salt Criteria in Callus-Cultured Tomato Genotypes

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Na^+, Cl^-, K^+, Ca^{2+}, and proline contents, the rate of lipid peroxidation level in terms of malondialdehyde (MDA) and chlorophyll content, and the changes in the activity of antioxidant enzymes, such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), ascorbate peroxidase (APX: EC 1.11.1.11), and glutathione reductase (GR: EC 1.6.4.2), in tissues of five tomato cultivars in salt tolerance were investigated in a callus culture. The selection of effective parameters used in these tomato genotypes and to find out the use of *in vitro* tests in place of *in vivo* salt tolerance tests were investigated. As a material, five different tomato genotypes during a 10-day time period were used, and 150 mM NaCl was applied at callus plant tissue. The exposure to NaCl induced a significant increase in MDA content in both salt-resistant and salt-sensitive cultivars. But the MDA content was higher in salt-sensitive cultivars. The chlorophyll content was more decreased in salt-sensitive than in salt-resistant ones. The proline amount was more increased in salt-sensitive than in salt-resistant ones. It has been reported that salt-tolerant plants, besides being able to regulate the ion and water movements, also exhibit a strong antioxidative enzyme system for effective removal of ROS. The degree of damage depends on the balance between the formation of ROS and its removal by the antioxidative scavenging system that protects against them. Exclusion or inclusion of Na^+, Cl^-, K^+, and Ca^{2+} , antioxidant enzymes and MDA concentration play a key protective role against stress, and this feature at the callus plant tissue used as an identifier for tolerance to salt proved to be an effective criterion.

Key words: Antioxidant Enzyme, Salinity, Tomato

Introduction

Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes (Nemoto and Sasakuma, 2002). Salinity can cause hyperionic and hyperosmotic effects in plants leading to membrane disorganization, increase in reactive oxygen species (ROS) levels, and metabolic toxicity (Jaleel *et al.*, 2007). High-salt stress disrupts the homeostasis in water potential and ion distribution at both the cellular and the whole plant levels (Errabii *et al.*, 2007). Excess of Na^+ and Cl^- ions may lead to conformational changes in the protein structure, while osmotic stress leads to turgor loss and cell volume change (Errabii *et al.*, 2007). However, the precise mechanisms underlying these effects are not fully understood because the resistance to salt stress is a multigenic trait (Errabii *et al.*, 2007). To achieve salt tolerance, plant cells evolve several biochemical and physiological pathways. These processes are thought to operate additively to ensure plants' and cells' survival, and they include the exclusion of Na^+ ions and their compartmentation into vacuoles as well as accumulation of compatible solutes such as proline, glycinebetaine, and polyols (Errabii *et al.*, 2007). In order to survive under stress conditions, plants are equipped with oxygen radical-detoxifying enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR). Oxidative stress is the result of ROS, such as superoxide, H_2O_2, and hydroxyl radicals, and causes rapid cell damage by triggering off a chain reaction. ROS scavenging is one among the common defense responses against abiotic stresses. Changes in antioxidants and protective molecules reflect the impact of environmental stresses on plant metabolism (Jaleel *et al.*, 2007). Salt-tolerant plants, besides being

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able to regulate the ion and water movements, should also have a better antioxidative system for effective removal of ROS and higher activities of SOD, APX and glutathione transferase (GST) enzymes of chloroplasts which probably play a key role in defense against oxidative damage (Wang *et al.*, 2008).

The mechanisms of salt tolerance, not yet clear, can be, to some extent, explained by stress adaptation effectors that mediate the ion homeostasis, osmolytic biosynthesis, toxic radical scavenging, water transport, and long-distance response coordination (Jaleel *et al.*, 2007). Undoubtedly, plant breeders have made a significant achievement in the past few years, which improves the salinity tolerance in a number of potential crops using artificial selection and conventional breeding approaches. However, most of the selection procedures have been based on differences in agronomic characters (Ashraf and Harris, 2004). Agronomic characters represent the combined genetic and environmental effects on plant growth and include integration of the physiological mechanisms conferring salinity tolerance. Typical agronomic selection parameters for salinity tolerance are yield, survival, plant height, leaf area, leaf injury, relative growth rate, and relative growth reduction (Ashraf and Harris, 2004). Many scientists have suggested that selection is more convenient and practicable if the plant species possesses distinctive indicators of salt tolerance at the whole plant, tissue or cellular level (Ashraf and Harris, 2004).

In recent years, tissue culture has gained importance in the development of plants against various abiotic stresses as well as in elucidating mechanisms operating at the cellular level by which plants survive under various abiotic stresses including salinity (Jain *et al.*, 2001). Plant tissue culture allows to control the stress homogeneity and to characterize the cell behaviour under stress conditions, independently of the regulatory systems that take place at the whole plant level (Lutts *et al.*, 2004).

The objective of the present investigation was to study the effect of salinity stress on Na^+, Cl^-, K^+, Ca^{2+}, and proline contents, the rate of lipid peroxidation level in terms of malondialdehyde (MDA) and chlorophyll content, and the plant antioxidant systems (SOD, CAT, APX, and GR) in relatively salt-sensitive and -tolerant tomato cultivars in order to evaluate the relative significance of these parameters in imparting tolerance to NaCl oxidative stress.

Material and Methods

Growth conditions

Seeds of five tomato genotypes, four of which belonged to the local *Lycopersicum esculentum* species (TR-47815 *L. esculentum* Turkey; TR-47882 *L. esculentum* Turkey; TR-55711 *L. esculentum* Turkey; TR-68516 *L. esculentum* Turkey) and the other one belonged to the *L. peruvianum* wild species (PI-899-01 *L. peruvianum*), were used. The seeds were cleaned from bacteria and fungi by applying the superficial sterilization method of Ellis *et al.* (1988). Then six seeds of each species were put in magenta pots containing MS basal nourishment medium.

Explants collected from hypocotyls by cutting from the root neck of the seedlings after the formation of the first three leaves in a climate chamber were planted horizontally in Petri dishes containing MS medium supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. Petri dishes were placed in a climate chamber at (25 ± 2) °C under permanent dark conditions. When the root length of the seedlings reached 1 cm, magentas were held in a 16 h light/8 h dark photoperiod. Calli, formed approx. within 4 weeks, were placed in the subculture by being separated from the main explants. After 4 weeks, they were placed into nourishment medium containing 150 mM NaCl. Calluses, completing within 4 weeks in the subculture, were transferred into 15 Petri dishes as control. In each Petri dish, 8 callus tissues, each of 1 g, were placed. They were left to grow for 10 d in a medium containing 150 mM NaCl. At the end of the 10th day, the harvested leaves were kept in a deep freezer at −80 °C and homogenized in liquid nitrogen to do the enzyme analyses. Extracts were prepared from the calli of the control and salt-applied plants, and enzyme activities in the obtained supernatant were determined (Cakmak, 1994).

A randomized parcels experimental design was carried out with three replications in factorial order. Time, one of these three factors, has a replicated measurement quality. The factors were compared by the repeated measurement variance analysis from the view point of the properties concerned. The differences between the levels of the factors were examined by the least important

difference (L.I.D.) multiple comparison method. The calculations were made using MINITAB 13.0 statistical packet program. Statistica V.6.0 packet program was used for multiple comparisons of the properties.

Due to the homogeneous structure of the callus tissue and its providing convenience to study in a small area in a short time with a great number of plant materials, the impression of likelihood of preference of callus cultures in stress studies was got. Relying on the ideas above, the reactions of local genotypes to salt, TR-68516 and TR-55711 of *L. esculentum*, were determined to be similar to that of the *L. peruvianum* wild species, and these genotypes were classified as salt-tolerant. TR-47815 and TR-47882 belonging to *L. esculentum* were decided as salt-sensitive and were determined as the most different genotypes.

Determination of ion content

For ion measurements, calli were first rinsed for 5 min with cool distilled water in order to remove free ions from the apoplasm without substantial elimination of cytosolic solutes. Calli were oven-dried at 80 °C for 48 h and then were ground. The dry matter obtained was used for mineral analysis. The major cations were extracted after digestion of dry matter with HNO_3. The extract was filtered prior to analysis. Na^+ and K^+ contents were determined using a flame spectrophotometer. The Ca^{2+} concentration was quantified by an atomic absorption spectrophotometer (Shimadzu AA-6200, Kyoto, Japan) (Guerrier and Patolia, 1989). For Cl^- content estimation, ions were extracted with hot distilled water (80 °C during 2 h). Chloride was determined as described by Taleisnik *et al.* (1997).

Chlorophyll content

The total chlorophyll content was analysed according to Luna *et al.* (2000).

Lipid peroxidation

For measurement of the lipid peroxidation in leaves, the MDA content (red pigment) was determined according to Lutts *et al.* (1996).

Proline content

The amount of proline was determined according to the method of Bates *et al.* (1973).

Enzyme assay

To determine the enzyme activities, 0.5 g of leaf tissues from control and treated plants were ground in liquid nitrogen and homogenized in 3 ml of buffer containing 50 mM KH_2PO_4 buffer (pH 7.0), 0.1 mM EDTA, and 1% PVPP (w/v). The homogenates were centrifuged at 15,000 × *g* for 15 min at 4 °C, and the resulting supernatants were freshly used for determination of SOD, CAT, GR, and APX activities.

The SOD (EC 1.15.1.1) activity was measured by the modified method of Beyer and Fridovich (1987). 3 ml of the reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 60 μM nitroblue tetrazolium (NBT), 0.1 mM EDTA, and 100 μl enzyme extract. The reaction was started by adding 60 μM riboflavine and placing the tubes under two 20-W cool white fluorescent lamps for 30 min. A complete reaction mixture without enzyme served as control. The reaction was stopped by switching off the light and putting the tubes into the dark. A non-irradiated completed reaction mixture served as a blank. The absorbance was recorded at 560 nm. One unit of SOD enzyme was defined as the amount that produces 50% inhibition of NBT reduction under the assay conditions and expressed as U SOD activity mg^{-1} protein.

For determination of the CAT (EC 1.11.1.6) activity, the reaction mixture contained 50 mM KH_2PO_4 (pH 7), 13 mM H_2O_2, and 30 μl enzyme extract. The decrease in absorbance of H_2O_2 was recorded at 240 nm for 3 min using a spectrophotometer (Shimadzu UV-VIS-1208). One unit of activity was defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 per min, calculated from the extinction coefficient (0.036 cm^2/μmol) for H_2O_2 at 240 nm (Öztürk and Demir, 2003).

The GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). The assay medium contained 0.025 mm sodium phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM $NADPHNa_4$, and 100 μl enzyme extract in a final assay volume of 1 ml. NADPH oxidation was determined at 340 nm. The activity was calculated using the extinction coefficient ε = 6.2 mM^{-1} cm^{-1} for GSSG. One unit of GR activity was defined as 1 mmol/ml GSSG reduced per min.

The activity of APX (EC 1.11.1.11) was measured according to the method of Karabal *et al.*

Table I. Na^+ (μg/mg dry weight) and Cl^- (μg/mg dry weight) contents in roots, stems, and leaves of the *L. esculentum* species in the presence of 150 mM NaCl. Results are expressed as means ± SE (n = 3).

Genotype		Roots		Stems		Leaves	
		Control	Salt	Control	Salt	Control	Salt
TR-68516	Na^+	3.6 ± 0.2	4.8 ± 0.3	3.5 ± 0.2	4.5 ± 0.3	3.3 ± 2	4.2 ± 0.3
	Cl^-	3 ± 0.1	4 ± 0.1	9 ± 0.1	12 ± 0.1	2 ± 0.1	3 ± 0.1
TR-55711	Na^+	3.6 ± 0.2	4.7 ± 0.2	3.4 ± 0.1	4.4 ± 0.2	3.2 ± 1	4 ± 0.2
	Cl^-	3 ± 0.1	5 ± 0.1	3 ± 0.1	5 ± 0.1	2 ± 0.1	3 ± 0.1
PI-899-01	Na^+	3.8 ± 0.4	5 ± 0.6	3.6 ± 0.3	4.5 ± 0.6	3.3 ± 3	4.2 ± 0.4
	Cl^-	2 ± 0.1	3 ± 0.1	20 ± 0.1	29 ± 0.1	12 ± 0.1	18 ± 0.1
TR-47815	Na^+	9.8 ± 0.6	15.8 ± 0.8	9.2 ± 0.5	14.8 ± 0.8	8.7 ± 5	13.9 ± 0.7
	Cl^-	2 ± 0.1	5 ± 0.1	2 ± 0.1	4 ± 0.1	2 ± 0.1	4 ± 0.1
TR-47882	Na^+	9.7 ± 0.1	15.5 ± 0.2	9.1 ± 0.1	14.6 ± 0.2	8.6 ± 1	13.7 ± 0.2
	Cl^-	3 ± 0.1	8 ± 0.1	3 ± 0.1	7 ± 0.1	3 ± 0.1	7 ± 0.1
	Na^+	LSD (genotype . NaCl treatment) ($P < 0.001$): 2.2		LSD (genotype. NaCl treatment) ($P < 0.001$): 2.3		LSD (genotype. NaCl treatment) ($P < 0.01$): 2.64	
	Cl^-	LSD (genotype . NaCl treatment) ($P < 0.001$): 2.5		LSD (genotype . NaCl treatment) ($P < 0.001$): 2.3		LSD (genotype . NaCl treatment) ($P < 0.01$): 1.8	

(2003). The reaction mixture consisted of 50 mM phosphate buffer (pH 6), 1.47 mM H_2O_2, 0.5 mM ascorbic acid, and 50 μl enzyme extract. The reaction was started by the addition of H_2O_2, and the oxidation of ascorbate was measured for 3 min at 290 nm. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient 2.8 mM^{-1} cm^{-1} at 290 nm for ascorbate.

Statistical analysis

Tables indicate mean values ± SE. Differences between the values for control and treated leaves were analysed by one-way ANOVA, taking $P <$ 0.001 as significance level, according to LSD multiple range tests.

Results and Discussion

In the absence of stress, in all tissues, the Na^+ level differed significantly ($P < 0.001$) among the cultivars and was lower in salt-resistant than in salt-sensitive cultivars, while no significant difference was recorded among cultivars in reffering to the Cl^- level. The exposure to NaCl induced a significant increase in Na^+ and Cl^- levels in both salt-resistant and salt-sensitive cultivars (Table I).

Table II. K^+ (μg/mg dry weight) and Ca^{2+} (μg/mg dry weight) contents in roots, stems, and leaves of the *L. esculentum* species in the presence of 150 mM NaCl. Results are expressed as means ± SE (n = 3).

Genotype		Roots		Stems		Leaves	
		Control	Salt	Control	Salt	Control	Salt
TR-68516	K^+	3.7 ± 0.1	5.9 ± 0.1	3.4 ± 0.1	5.5 ± 0.1	3.2 ± 0.1	5.2 ± 0.1
	Ca^{2+}	38 ± 0.7	66 ± 0.8	31 ± 0.9	57 ± 0.8	20 ± 0.8	33 ± 0.4
TR-55711	K^+	4.3 ± 0.1	6.9 ± 0.1	4.5 ± 0.1	6.5 ± 0.1	3.7 ± 0.1	6.1 ± 0.1
	Ca^{2+}	34 ± 0.8	59 ± 0.9	30 ± 0.6	55 ± 0.6	24 ± 0.9	39 ± 1.1
PI-899-01	K^+	4.2 ± 0.1	6.7 ± 0.1	4 ± 0.1	6.2 ± 0.1	3.8 ± 0.1	5.8 ± 0.1
	Ca^{2+}	37 ± 1.6	65 ± 0.7	35 ± 0.6	65 ± 0.6	23 ± 0.9	38 ± 1.2
TR-47815	K^+	3 ± 0.1	4.8 ± 0.1	2.8 ± 0.1	4.5 ± 0.1	2.6 ± 0.1	4.2 ± 0.1
	Ca^{2+}	35 ± 0.8	38 ± 2.4	29 ± 0.6	32 ± 0.6	17 ± 0.6	21 ± 1.2
TR-47882	K^+	2 ± 0.1	3.3 ± 0.1	1.9 ± 0.1	3.1 ± 0.1	1.8 ± 0.1	2.9 ± 0.1
	Ca^{2+}	56 ± 2.1	60 ± 1.9	49 ± 1.5	55 ± 0.5	41 ± 0.5	48 ± 0.6
	K^+	LSD (genotype . NaCl treatment) ($P < 0.001$): 0.6		LSD (genotype . NaCl treatment) ($P < 0.001$): 0.5		LSD (genotype . NaCl treatment) ($P < 0.01$): 0.5	
	Ca^{2+}	LSD (genotype . NaCl treatment): 1.7 $P < 1.2$		LSD (genotype . NaCl treatment): 0.95 $P < 0.6$		LSD (genotype . NaCl treatment): 0.8 $P < 0.7$	

Table III. Malondialdehyde (MDA) (μmol/g fresh weight), chlorophyll (μg/g fresh weight), and proline (μmol/g fresh weight) contents in leaves of the *L. esculentum* species in the presence of 150 mM NaCl. Results are expressed as means ± SE (n = 3).

Genotype	MDA		Chlorophyll		Proline	
	Control	Salt	Control	Salt	Control	Salt
TR-68516	32 ± 0.2	35 ± 0.3	326 ± 0.2	298 ± 0.2	30 ± 0.2	33 ± 0.3
TR-55711	26 ± 0.1	28 ± 0.1	328 ± 0.1	308 ± 0.4	25 ± 0.1	27 ± 0.1
PI-899-01	32 ± 0.2	33 ± 0.2	331 ± 0.2	312 ± 0.1	31 ± 0.2	34 ± 0.2
TR-47815	24 ± 0.2	44 ± 0.3	233 ± 1.3	123 ± 0.1	25 ± 0.2	39 ± 0.3
TR-47882	25 ± 0.1	45 ± 0.1	232 ± 0.1	140 ± 0.2	26 ± 0.1	43 ± 0.1
	LSD (genotype . NaCl treatment) ($P < 0.01$): 1.55		LSD (genotype . NaCl treatment) ($P < 0.01$): 1.33		LSD (genotype . NaCl treatment) ($P < 0.01$): 1.55	

But the Na^+ and Cl^- levels were higher in salt-sensitive cultivars.

In the absence of stress, in all tissues, the K^+ level differed significantly ($P < 0.001$) among the cultivars and was lower in salt-sensitive than in salt-resistant cultivars. The exposure to NaCl induced a significant increase in the K^+ levels in both salt-resistant and salt-sensitive cultivars (Table II). In this study, only the salt-tolerant cultivars maintained higher K^+ contents and lower Na^+ contents in all tissues of the plant (Tables I and II).

In the absence of stress, in all tissues, the Ca^{2+} level differed significantly ($P < 0.001$) among the cultivars and was lower in salt-sensitive than in salt-resistant cultivars. The exposure to NaCl induced a significant increase in the Ca^{2+} level in both salt-resistant and salt-sensitive cultivars (Table II). But the Ca^{2+} level was higher in salt-resistant cultivars.

The lipid peroxidation levels in leaves of the five tomato cultivars, measured as the content of MDA, are given in Table III. In the absence of stress, the MDA level differed significantly ($P < 0.01$) among the cultivars and was higher in salt-resistant than in salt-sensitive cultivars. The exposure to NaCl induced a significant increase in the MDA level in both salt-resistant and salt-sensitive cultivars. But the MDA level was higher in salt-sensitive cultivars. The better NaCl stress tolerance in salt-tolerant cultivars as compared to salt-sensitive cultivars observed during the present investigation may be due to the restriction of damage to cellular membranes with lower MDA and H_2O_2 content.

In the absence of stress, the chlorophyll level in leaf tissue differed significantly ($P < 0.01$) among the cultivars and was higher in salt-resistant than in salt-sensitive cultivars (Table III). The exposure to NaCl induced a significant decrease in the chlorophyll level in both salt-resistant and salt-sensitive cultivars. But the chlorophyll level was more decreased in salt-sensitive cultivars than in salt-resistant cultivars.

In the absence of stress, the proline level differed significantly ($P < 0.01$) among the cultivars and was higher in salt-resistant than in salt-sensitive cultivars (Table III). The exposure to NaCl induced a significant increase in the proline level in both salt-resistant and salt-sensitive cultivars.

Table IV. The differences between the superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) enzyme activities [μmol/(min mg fresh weight)] of the callus tissues of the different tomato genotypes according to the control. Values are given as the means of three repetitions ± standard deviation.

Genotype	SOD		CAT		GR		APX	
	Control	Salt	Control	Salt	Control	Salt	Control	Salt
TR-68516	144 ± 4	390 ± 12	43 ± 0.1	155 ± 0.1	125 ± 0.0	259 ± 0.1	111 ± 0.2	249 ± 0.1
TR-55711	184 ± 4	356 ± 23	52 ± 0.1	81 ± 0.1	136 ± 0.1	259 ± 0.1	124 ± 0.1	256 ± 0.1
PI-899-01	118 ± 6	357 ± 19	33 ± 0.1	311 ± 0.2	138 ± 0.2	288 ± 0.2	127 ± 0.1	273 ± 0.6
TR-47815	114 ± 8	138 ± 56	34 ± 0.1	39 ± 0.2	122 ± 0.1	215 ± 0.2	122 ± 0.0	198 ± 0.0
TR-47882	142 ± 4	167 ± 42	49 ± 0.1	52 ± 0.1	143 ± 0.2	216 ± 0.1	129 ± 0.3	154 ± 0.01
	LSD (genotype . NaCl treatment): 7.41 (SOD), 2.6 (CAT), 3.2 (GR), 2.6 (APX)							

But the proline level was more increased in salt-sensitive cultivars than in salt-resistant ones.

In the absence of stress, the SOD, CAT, GR, and APX activities differed significantly among the cultivars (Table IV). The exposure to NaCl induced a significant increase in the enzyme activities in both salt-resistant and salt-sensitive cultivars. But the enzyme activities were significantly higher in salt-resistant cultivars.

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