EFFECT OF RELATIVELY HIGH CONCENTRATIONS OF MANNITOL AND SODIUM CHLORIDE ON REGENERATION AND GENE EXPRESSION OF STRESS TOLERANT (*ALHAGI GRAECORUM*) AND STRESS SENSITIVE (*LYCOPERSICON ESCULENTUM L.*) PLANT SPECIES

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Summary. Regeneration and gene expression of salt/drought stress tolerant *Alhagi graecrum* and stress sensitive *Lycopersicon esculentum* under different concentrations of NaCl and mannitol were studied. Mannitol and NaCl applied at concentrations of 86, 172, 256 and 43, 86, 172 mM, respectively, in a shoot regeneration medium, caused similar water potentials. In *L. esculentum,* regeneration was influenced negatively by NaCl. Indirect regeneration pathway was chosen for it is more sensitive to stress than that of the direct one. Growth adaptation of calli under salt the stress by gradual increase in NaCl concentration improves the regeneration capacity of stress tolerant species under high concentration of NaCl. Under stress conditions, water content in calli decreased. The decrease in stress sensitive species was higher than that of the stress tolerant species. Free amino acids and proline contents as well as relative peroxidase activity increased under stress conditions. These values in stress tolerant species were higher than those of stress sensitive species. Studying native PAGE indicated that protein expression in stress tolerant plant species was more stable than that of stress sensitive plant species. In addition, while SDS PAGE profile under salt stress was the same as the profile under drought stress in *A. graecorum*, expression of different polypeptides were detected in *L. esculentum*. In *A. graecorum*, glutamate oxaloacetate transaminase showed extra isoenzyme forms under stress condition although malate dehyderogenase expressed extra bands in *L.*

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esculentum. In both plant species, new isoenzyme forms of peroxidase were detected under stress conditions.

Keywords: Tissue culture, hormones, salinity, drought, protein, isoenzymes.

INTRODUCTION

Water deficit such as drought and salinity is a major component of environmental stress. The effects of these two stress factors show similarities on many physiological and molecular processes, e.g. increasing stomatal closure, decreasing water content, inhibiting the rate of photosynthesis, decreasing the rate of respiration, altering the gene expression, decreasing the protein synthesis and/or increasing protein hydrolysis. This could lead to the accumulation of free amino acids especially proline, enhancing the formation of oxygen free radicals, reducing DNA replication and cell division, and consequently reducing the plant growth and yield (Shaddad, 1990; Holland et al., 1993; Lewis et al., 1997; Hassanein, 1999; Mohamed et al., 2000; Demir & Kocacaliskan, 2001; Cherian & Reddy, 2003).

The exact mechanism by which plant cells regulate the expression of macromolecules during their natural development or under stress conditions, are not known. Most probably they are either at gene level, or in the pathway between gene and functional enzymes (Scandalios, 1974). Stress tolerance in plants has long been accepted as a multigenetic trait dependent on the coordinated expression of certain genes and the silencing of others (Hare et al., 1996). The expression of these genes is influenced by multifarious environmental factors (Foolad, 2004). Changes in their expression can be detected by studying the protein pattern of expression. Appearance or disappearance of isoenzyme bands marks if there is a change in the isoenzyme expression. Moreover, an increase in the staining intensity of isoenzyme band represents the increase in enzyme activity (Khavkin & Zabrodina, 1994; Hassanein et al., 1999). Such change in isoenzyme expression suggests that the gene involved in the synthesis of this isoenzyme form is differentially activated under specific conditions (Chawla, 1991; Hassanein et al., 1999; El-Tayeb & Hassanein, 2000).

There are many plants (such as *A. graecorum*), which are tolerant to drought and salinity stresses (Zahran, 1976; El-Katib et al., 1999), unlike others, sensitive for both types of stresses, e.g. *L. esculentum* (Foolad, 2004). Many important questions still have no answers. Are the similarities in physiological responses during drought and salinity due to water deprivation caused by these types of abiotic stress? Hassan and Wilkins (1988) reported that growth of the selected line under relatively high concentrations of NaCl was not due to osmotic stress tolerance because its growth in mannitol was similar to that of the non-selected line. In addition, examination of the abiotic stress commonalities is more appropriate than the study of individual stress responses in isolation, and will provide us with useful information, which can be

used to improve plant stress tolerance. We aimed to determine whether drought and salinity have the same effect on direct and indirect regeneration capacities of the cultured tissues under equal water potential, and, on the other hand, protein and isoenzyme expressions of stress tolerant and sensitive plant species. Consequently, direct regeneration from explants, and indirect regeneration from calli after sudden exposure to salt stress or after adaptation of calli to salt stress by gradual increase in NaCl concentration were studied. Native and SDS PAGEs, and isoenzymes were investigated. Proline and free amino acids that increased in general and played certain role during stress conditions in both resistant and sensitive plant species were also studied.

MATERIAL AND METHODS

Establishment of shoot culture

Seeds of *Lycopersicon esculentum* L. or *Alhagi graecorum* were surface-sterilized in 5% commercial bleach solution for 10 min followed by 5 min treatment in 75 (v/v) ethanol. After three successive rinses in sterile distilled water for 5 min each, seeds were placed in MS (Murashige & Skoog 1962) agar medium supplanted by 30 g/l sucrose. The medium was solidified with 8 g/l agar. The seeds were germinated at 25 ± 2 ºC with a 16-h photoperiod at 100 mol $m^{-2}s^{-1}$. Shoots of germinated seeds were cut and subcultured on growth regulators free MS medium, and shoots of *A. graecorum* were subcultured in MS medium supplemented with 44 µM $AgNO_3$ for shoot culture establishment.

Induction of callus formation

Leaf disks of established shoot culture *L. esculentum* were cultured in MS medium supplemented with 2 mg/l NAA and 0.2 mg/l BAP in order to get calli. On the other side, since *A. graecorum* normally has small leaves, tiny-mature-explant method (Hassanein & Mazen, 2001) was essential prerequisite to rapidly obtain masses of calli sufficient for the effect of stress on protein and isoenzyme expression study and for the indirect regeneration studies, as well.

Tiny mature explant method

Leaves from *A. graecorum* in vitro grown shoots were collected and then sliced into small pieces (0.1 - 1.0 mm) by razor and forceps into a drop of liquid V-KM medium (Binding & Nehls, 1977) modified from 8p medium of Kao and Michayluk (1975) containing 1 mg/l NAA, 0.1 mg/l 2,4-D and 0.5 mg/l BAP. The pieces were mixed with droplets of agarose solution (2% Sigma type V11). The agarose solution was

prepared by melting in V-KM (during autoclaving) and was used at 40 ºC. After cooling, the solidified medium containing tiny pieces of tissue was cultured in V-KM medium. After three weeks, thus formed small calli were separated and subcultured into solid-agar (8 g/l) MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D and 0.5 mg/l BAP.

Direct and indirect regeneration under stress condition

Internodal stem segments (0.5- 0.7 cm in length) of *A. graecorum* were placed in MS medium containing 1 mg/l BAP and 00, 43, 86, 129, 172, 200 mM NaCl or 00, 86, 172, 215, 256, 300 mM mannitol. *L. esculentum* leaf explants (0.5x0.5 cm) were cultured in MS medium containing 2 mg/l BAP and 0.2 mg/l IBA with the same concentrations of NaCl or mannitol as in the previous case. The segments were cultured at 25 ± 2 ºC with a 16-h photoperiod at 100 mol $m^{-2}s^{-1}$. In order to compare regeneration capacity under salt and drought stresses of both plant species, three NaCl concentrations (43, 86 and 172 mM NaCl) expressing similar water potential of three others of mannitol (86, 172 and 256 mM mannitol) were used (Mercado et al., 2000). After 6 weeks, the number of explants, which formed adventitious buds and the number of buds per explant were counted.

For indirect regeneration, calli (0.5 g each) of *A. graecorum* were subcultured in MS medium supplemented with 1 mg/l BAP and several different concentrations of NaCl (00, 43, 86, 129 and 172 mM). The same concentrations of NaCl were added to MS medium supplemented with 2 mg/l zeatin to regenerate shoots from tomato calli.

In order to survive under salt stress, calli (0.5 mg each) were subcultured in medium containing successive higher NaCl concentrations - beginning with 21.5 mM. After 6 weeks in each passage, about 0.5 g of healthy-green-NaCl treated tissues were transferred into MS medium containing higher NaCl concentration. Dead and brown tissues were removed during the successive passages. Transfer of the callus tissues to higher NaCl concentrations continued, each time the medium supplemented with extra 21.5 mM NaCl more than the previous concentration, until calli were subcultured in medium containing 129 mM NaCl. Then, the calli were transferred into shoot regeneration medium containing 129 mM or 172 mM NaCl. The percentage of calli that formed adventitious buds and the number of buds/callus were counted.

Preparation of calli for electrophoresis and other analyses

L. esculentum or *A. graecorum* calli (4 weeks old) were subcultured in MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D and 0.5 mg/l BAP as well as 172 mM NaCl or 256 mM mannitol for one week. The calli were cultured at 25 ± 2 ºC with a 16-h photoperiod at 100 mol $m^{-2}s^{-1}$. Then, the calli were used for the determination of peroxidase activity and electrophoresis work, or weighed and

dried to calculate the fresh and dry masses of calli and their water contents. Consequently, the dry masses were used for determination of free amino acids and proline.

Determination of free amino acids and proline

Free amino acids were extracted from dry plant tissue and determined according to the method of Moore and Stein (1948) and free proline was determined according to Bates et al. (1973).

Determination of relative peroxidase activity

One g of calli was ground at 4 ºC in a mortar in 1 ml extraction buffer at 0.1 M tris-HCl, pH 7.0, and 0.002 M cysteine. The homogenate was centrifuged at 15 000 g at 10 ºC for 15 min. Supernatants were collected for immediate electrophoresis or measuring peroxidase activity. 50 µl of extracted samples in 10 ml of assay mixture were spectrophotometrically measured at 470 nm. The assay mixture for peroxidase activity contained 40 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 5 mM guaiacol, 0.3 mM hydrogen peroxide. Then, the relative peroxidase activity was determined (O.D./ gm fresh weight/ hr) as described previously (Wakamatsu & Takahama, 1993; Kar & Mishra, 1976). Peroxidase activity of grown under non-stress conditions calli was considered to be 100% and a control treatment.

Native PAGE and Isoenzyme analysis

Gels were run at 18 mA for 6 h at 10 ºC in 0.025 M Tris +0.192 M glycine buffer (pH 8.9). Four enzymes were stained: peroxidases (Siegel & Galston, 1967), esterases (Wetter & Dyck, 1983; Brewer, 1970), Glutamate oxaloacetate transaminase (Brewer, 1970; Gabriel, 1971), and malate dehyderogenase (Brewer, 1970). Native PAGEs were stained with Coomassie blue dye.

SDS PAGE analysis

SDS PAGE was performed using 10.5% acrylamide gels. As a tracking dye, protein samples (40 µg each) were mixed with an equal volume of buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and bromophenol blue. The so prepared mixture was heated in a water bath for 3 min at 96 ºC and loaded onto gel for 6 h at 10 ºC in run buffer containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Protein bands were visualized using Coomassie blue dye.

RESULTS AND DISCUSSION

Tissue culture of *L. esculentum* and *A. graecorum*

Leaf explants were used to obtain calli from both *L. esculentum* and *A. graecorum*. In *L. esculentum*, calli were obtained in MS medium supplemented with 2 mg/l NAA and 0.2 mg/l BAP or MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D and 0.5 mg/l BAP. The validity of explant tissue techniques in their salt-tolerance breeding program use in tomato was confirmed (Pérez-Alfocea et al., 1994). On the other hand, to obtain calli from *A. graecorum* leaves was difficult due to their small sizes. Therefore, 44 μM $AgNO_3$ and tiny mature explant methods were used (Hassanein & Mazen, 2001). $AgNO_3$ stimulated leaf expansion of cultured shoots, which may be due to inhibition of ethylene action (Bíriza & Machová, 1991). Slicing explant to small pieces for the tiny-mature-explant method increases the cut area that is exposed to the medium. Furthermore, aggregation of small explant pieces and even cell aggregates in agarose compensate the use of large explant. Therefore, to divide and form callus, much lesser number of cells could be used.

In *L. esculentum*, shoot regeneration from callus was obtained in a medium containing 2 mg/l zeatin, while shoot regeneration was obtained from leaf discs into a MS medium supplemented with 2 mg/l BAP and 0.2 mg/l IBA. In *A. graecorum* shoot regeneration was obtained from internodal stem segments or calli in a MS medium supplemented with 1mg/l BAP.

Plant tissue culture has been proposed as a useful, quick and economical tool for evaluating the salt tolerance via shoot organogenesis from leaf explants, and the shoot apex growth (Mercado et al., 2000). In the present work, direct regeneration of stress tolerant as well as stress sensitive plant species under stress conditions was studied

Table 1. Regeneration frequency (Reg. Freq.) and number of buds (N. buds) obtained from internodal explant of *A. graecorum* and leaf explant of *L. esculentum* cultured for 6 weeks in a shoot formation medium supplemented with different concentrations of NaCl or mannitol.

Conc.	NaCl				Mannitol				
Conc.	*A. graecorum*		*L. esculentum*		Conc.	*A. graecorum*		*L. esculentum*	
	Reg. Freq.	N. buds	Reg. Freq.	N. buds		Reg. Freq.	N. buds	Reg. Freq.	N. buds
0 (Control)	98	12	100	6	0	98	12	100	6
43	96	12	96	4*	86	98	13	97	4*
86	83	6*	45*	3*	172	89*	8*	58*	4*
129	55*	5*	9*	2*	215	63*	6*	28	2*
172	24*	3*	00	00	256	33*	3*	00	00
200	00	00	00	00	300	00	00	00	00

* Significant differences in relation to the corresponding reference control.

(Table 1). Shoot regeneration capacity of tested plants was generally inhibited by stress. Highly pronounced was the inhibition when salinity concentration rose in comparison to mannitol. This inhibition was not attributed to the water potential state because the same water potential caused by NaCl and mannitol expresses various regeneration capacities. Regeneration capacity of tomato explants was completely inhibited at NaCl concentration higher than 129 mM NaCl in total accordance with the results, reported previously (El-Enany, 1997; Mercado et al., 2000). On the other hand, regeneration from *A. graecorum* explants was possible up to 172 mM NaCl. It indicated that regeneration capacity via direct pathway of stress tolerant plant species was higher than that of stress sensitive plant species.

Regeneration capacity of *A. graecorum* via indirect regeneration pathways (Table 2) could be obtained in the presence of NaCl up to 129 mM. Shoot regeneration of *L. esculentum* was completely inhibited when the calli were subcultured in a shoot regeneration medium containing NaCl concentration higher than 43 mM. Comparison between data in Tables 1 and 2 indicated the fact that, under stress conditions, indirect regeneration was more sensitive than the direct one in both plant species, yet this sensitivity was higher in *L. esculentum* than in *A. graecorum*.

Table 2. Regeneration frequency (Reg. Freq.) and number of buds (N. buds) obtained from calli of *A. graecorum* and *L. esculentum* cultured for 6 weeks in a shoot formation medium supplemented with different concentrations of NaCl or mannitol. a) calli adapted to grow under salt stress by rising the concentration of NaCl gradually up to 129 mM, then they subcultured in the shoot initiation medium containing relatively high NaCl concentrations.

Conc.	NaCl					Mannitol			
Conc.	*A. graecorum*		*L. esculentum*		Conc.	*A. graecorum*		*L. esculentum*	
	Reg. Freq.	N. buds	Reg. Freq.	N. buds		Reg. Freq.	N. buds	Reg. Freq.	N. buds
0 (Control)	100	25	90	4	0	100	23	92	4
43	100	25	43*	1*	86	100	24	86	3
86	66*	20	00	00	172	71*	15*	6*	1*
129	10*	4*	00	00	215	22*	6*	00	00
172	00	00	00	00	256	20*	6*	00	00
129a	25*	11*	00	00	300	9*	2*	00	00
172a	16*	2*	00	00					

* Significant differences in relation to the corresponding reference control.

Gradual subculturing at successively higher NaCl concentrations (21.5 mM/ 6 weeks) probably enabled some cells to adjust the osmotic pressure to survive and form callus, while tomato calli exposed to 86 or more mM NaCl, died in three weeks. Rahman and Kaul (1989) obtained the same results. Consequently, tomato calli can adapt to survive and grow in a medium containing 172 mM NaCl. Adaptation of

tomato calli did not change the capacity of tomato to regenerate at relatively high NaCl concentrations. On the other hand, adaptation of *A. graecorum* calli improved their regeneration capacity in a MS medium supplemented with 129 mM NaCl, where regeneration frequency and buds number increased 2.5 fold in comparison to those of calli subjected via sudden exposure to 129 mM NaCl (Table 2). In addition, while sudden exposure to172 mM NaCl completely inhibited the formation of adventitious buds, it did not inhibit adventitious bud formation of calli subjected previously to gradual increase of NaCl concentrations. Similar results were obtained by Basu et al. (1997). Such an improved response to high NaCl concentration at cellular level could be related to the use of Na or Cl ions as osmotic agents (Pérez-Alfocea et al., 1994; Mercado et al., 2000).

Callus growth and water content

In a long-term experiment, 172 mM NaCl and 256 mM mannitol inhibit growth and morphogenesis of resistant and sensitive plant species calli. In both cases, the callus turned brown or black, and eventually died. The calli of *L. esculentum* died in three weeks, and those of *A. graecorum* died in eight weeks. In the presence of light, if the plant tissue contained a relatively high phenol content, oxidized by phenol oxidases, the tissue turned brown or black (Hassanein et al., 1999) and died. Lindfors et al. (1990) found that the onset of tissue browning, before the concentration of phenolic compounds became high, did not cause a loss of vitality.

In one week, callus fresh mass of both species became smaller, probably due to the loss of water content (Table 3). The loss in water content in *A. graecorum* was lesser than that of *L. esculentum.* The low loss of water content of the stress resistant

Table 3. Fresh weight (FW), dry weight (DW), water content (WC), relative peroxidase activity (RPA), free amino acids (AA) and proline (Pro) of *A. graecorum* and *L. esculentum* cultured for one week in a medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D and 0.5 mg/l BAP and 172 mM NaCl or 256 mM mannitol.

Plant ssp	Treat.	FW (mg/ callus)	DW (mg/ callus)	WC (%)	RPA (%)	AA (mg/g DW)	Pro (mg/g DW)
L. esculentum	Control	1125	66	94	100	8	3
	NaCl	553*	64	87*	134	19*	11*
	Mannitol	604*	65	89*	125	17*	7*
A. graecorum	Control	1253	65	93	100	12	4
	NaCl	679*	66	90*	176	20*	12*
	Mannitol	677*	67	90*	146	25*	14*

* Significant differences in relation to the corresponding reference control.

plant *A. graecorum* may be due to the rapid response of plant to avoid severe water loss.

Proline and amino acids

Proline and amino acids are generally thought to have an important role under abiotic stress, therefore, stress-sensitive *L. esculentum* and stress-tolerant *A. graecorum* were compared with respect to their capacity to accumulate proline and amino acids. In a MS medium with or without mannitol or NaCl, *A. graecorum* calli contained higher proline and amino acids contents than those of *L. esculentum*. Sumaryati et al. (1992) reported that when grown in a non-selective medium, all resistant lines of tobacco showed higher proline values than the wild type. In their studies of *in vitro* selection for salt tolerance in *Lycopersicon* ssp, Hassan and Wilkins (1988) reported that proline content of the selected salt tolerant line was much higher than that of the nonselected one. Opposite results were obtained by Tal (1884) and Liu & Zhu (1997). Adapted to grow under relatively high concentration of NaCl calli contained more proline not adapted callus (Basu et al., 1997). Proline may play a role as an enzyme-stabilizing agent in a salt stress medium (Demir & Kocacaliskan, 2001). Furthermore, proline has the ability to mediate osmotic adjustment, stabilize sub-cellular structure and scavenge free radicals (Hare et al., 1996). In the present work, the sharp increase in proline content attributed to the sharp increase in amino acids content which may be due to increased protein hydrolysis (Irigoyen et al., 1992). In addition, the high content of proline may come from NO_3 and organic acids (Venekamp & Koot, 1988; Pérez-Alfocea et al., 1994). Accumulation of proline and amino acids in *L. esculentum* calli was higher under salt stress than under drought stress. On the other hand, in *A. graecorum* calli, accumulation of proline was higher under drought stress than under salt stress.

Protein Pattern

Native PAGE profile (Fig. 1) indicated that stress tolerant plant species *A. graecorum* expressed the same protein pattern in response to relatively high NaCl or mannitol. Tomato (Fig. 2) expressed one extra band with high RF value (0.94) under drought and salt stresses (lanes 2 and 3) and another band with low RF value (0.20) only under salt stress (lane 3). These data suppose that the expression of macromolecules in stress tolerant plant species was more stable than in stress sensitive plant species.

Fig. 3 shows denatured polypeptides of both *A. graecorum* and *L. esculentum* tissues separated by SDS-PAGE. In *A. graecorum*, both NaCl and mannitol gave the same effect where two polypeptides with apparent molecular weight of 98.3 and 19.6 KD were detected (Fig. 3; lanes 5 and 6). On the other hand, the response of tomato to NaCl and mannitol was different, and extra polypeptide with high molecular weight

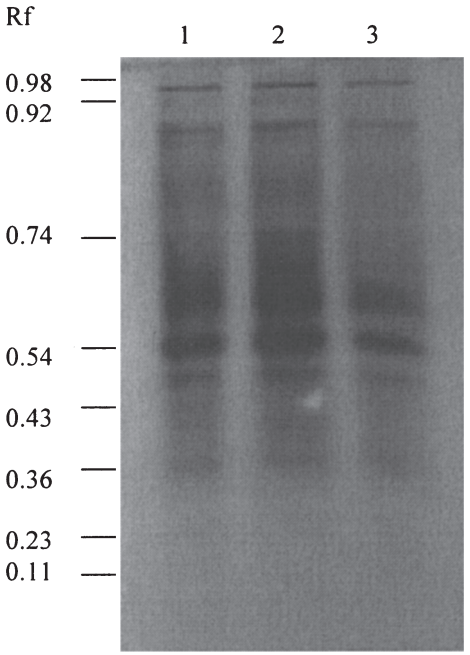

Figure 1. Protein profile on native PAGE of *A. graecorum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

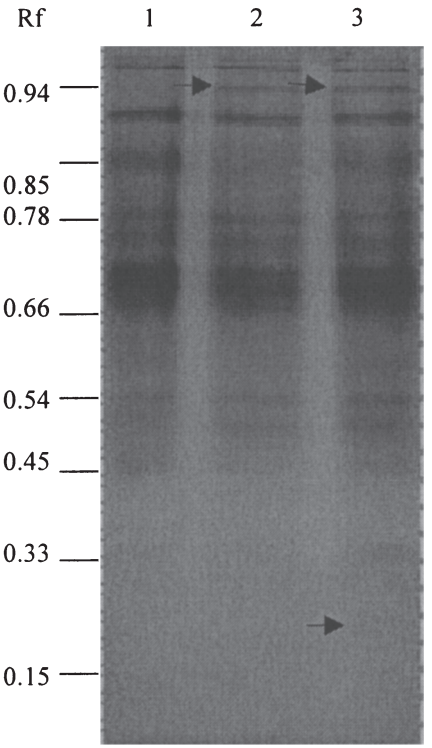

Figure 2. Protein profile on native PAGE of *L. esculentum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

(94.4 KD) was detected in calli grown in a medium containing 172 mM NaCl (Fig. 3; lane 3) but one extra polypeptide with relatively low molecular weight (18.6 KD) was detected in calli subjected to 256 mM mannitol (Fig. 3; lane 2). In addition, polypeptide with molecular weight of 16.7 was detected under the influence of mannitol or NaCl (Fig. 3; lanes 2 and 3).

Isoenzyme expression

In the present work, four enzymes belonging to three classes were studied. A class of hydrolysis was represented by esterase. A class of transferases was represented by glutamate oxaloacetate transaminase. A class of oxidoreductase was represented by malate dehyderogenase and peroxidase.

Reactive oxygen species under stress conditions result in a significant damage of the cellular constituents and even cell death if the protective mechanisms fail to detoxify them (Polle, 1995). Peroxidases are important components in the detoxification process. Under non-stress conditions, tissues of both stress-tolerant (*A. graecoru*) and sensitive (*L. esculentum*) species expressed equal values of peroxidase activity. Subjecting the cultured tissues to high NaCl or mannitol concentrations resulted in an increase of the peroxidase activity, being higher in stress tolerant than in stress sensitive plant species (Table 3).

A comparison between peroxidase activity data (Table 3) and peroxidase pattern (Figs 4 and 5) indicated that the increase in peroxidase activity may be due to the

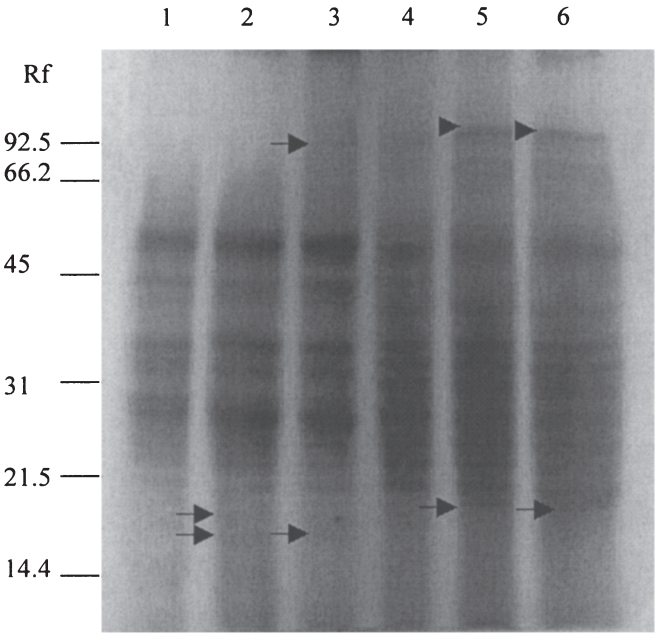

Figure 3. Protein profile on SDS PAGE of *L. esculentum* (Lanes 1, 2and 3) and *A. graecorum* (Lanes 4, 5 and 6) calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1 and 4) and 256 mM mannitol (Lane 2 and 5) or 172 mM NaCl (Lane 3 and 6).

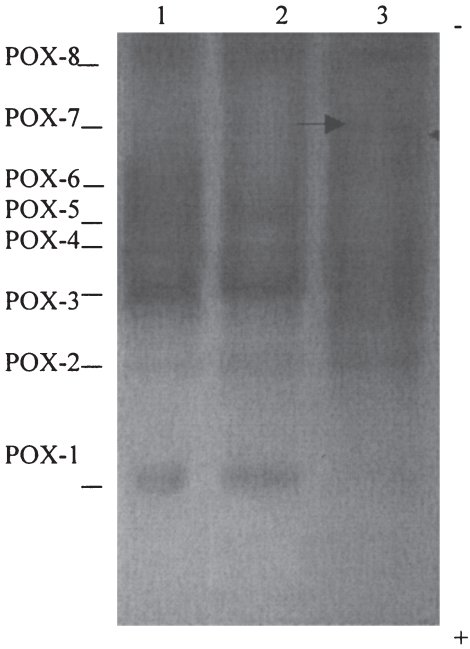

Figure 4. Native gel electrophoresis of peroxidase isoenzyme (POX) of *A. graecorum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

increase in the number of peroxidase isoenzyme forms, or the rise in expression of some of them which was represented by the increase of staining intensity of these bands (Khavkin & Zabrodina, 1994; Hassanein, 1997). In *A. graecorum*, one extra peroxidase band (PER-7) was detected in calli cultured under salt stress (Fig. 4; lane 3). In *L. esculentum*, one extra peroxidase band (PER-5) was detected under the in-

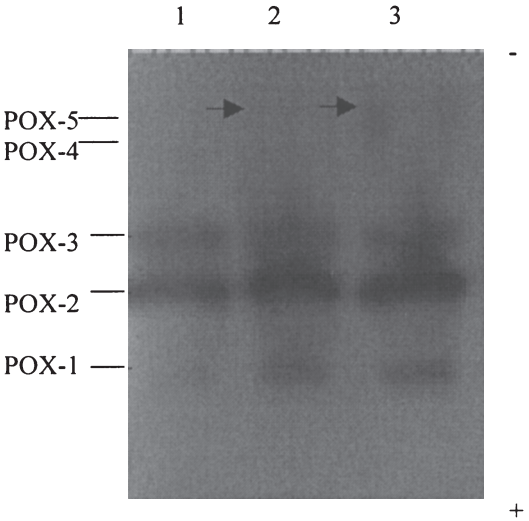

Figure 5. Native gel electrophoresis of peroxidase isoenzyme (POX) of *L. esculentum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

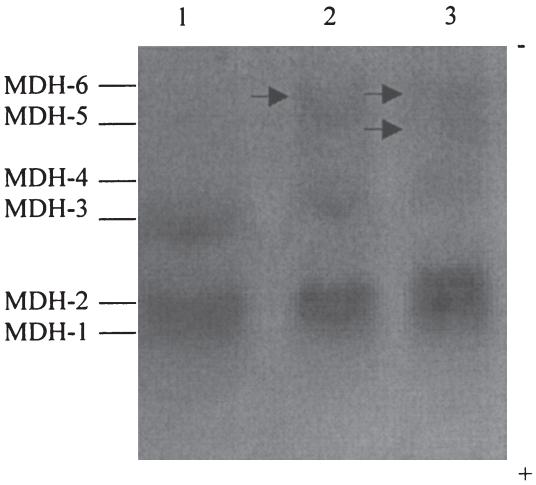

Figure 6. Native gel electrophoresis of malate dehyderogenase isoenzyme (MDH) of *L. esculentum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

fluence of mannitol or NaCl (Fig. 5; lanes 2 and 3). Drought-susceptible plants may suffer from oxidative damage more than the resistant ones because they are less capable of down regulation of electron transport via Mehler peroxidase pathways (Pastori and Trippe, 1993; Polle, 1995). Rapid death after dark browning of *L. esculentum* calli in comparison to the one of *A. graecorum* indicted that the ability of stress sensitive plant species to detoxify the accumulated oxygen free radicals was lower than that of stress tolerant plant species. Correlation between the increase in peroxidase expression and the resistance to stress condition has been established in many plant species (Quartacci et al., 1994; El-Katib et al., 1999; Hassanein et al., 1999; El-Tayeb & Hassanein, 2000).

In *A. graecorum*, malate dehyderogenase (MDH) expression pattern of calli cultured in a MS medium supplemented with relatively high concentration of NaCl or mannitol showed the same isoenzyme pattern as the unstressed calli. On the other hand, MDH pattern of *L. esculentum* under stress conditions showed a quite different pattern than that of unstressed calli (Fig. 6), where two isoenzyme forms with less negative charge (MDH-5, MDH-6; lanes 2 and 3) were detected. Malate dehyderogenase has been proposed as a component of an apoplast system that may generate H_2O_2 required for peroxidase-mediated lignification (Fry, 1986). Due to the contribution of malate dehydrogenase in H_2O_2 generation (Fry, 1986) and scavenging systems (Polle, 1995), the staining intensity of bands was darker especially when the stress resistant plant was subjected to stress. This represents the enzyme activity of peroxidase and malate dehyderogenase (data not shown).

Staining intensity and the number of glutamate oxaloacetate transaminase (GOT) bands of *A. graecorum* calli increased under stress conditions (Fig. 7), and GOT-2 and GOT-6 were detected. Furthermore, GOT-1 was marked stronger under stress conditions (Fig. 7; lanes 2 and 3). Under high concentration of NaCl or mannitol, *L.*

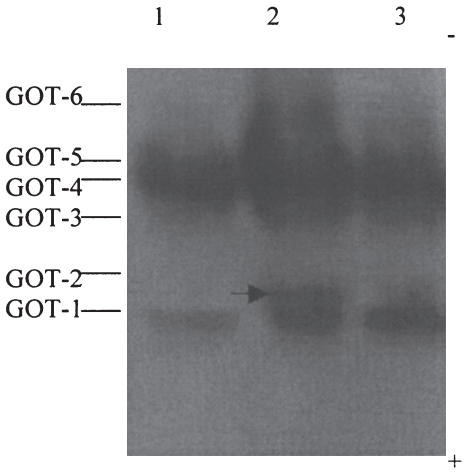

Figure 7. Native gel electrophoresis of glutamate oxaloacetate transaminase isoenzyme (GOT) of *A. graecorum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3).

esculentum calli showed GOT pattern, corresponding to that of calli under non-stressed conditions (data not shown). Comparison between GOT pattern and proline data gives an indication about incorporation of GOT enzyme in mechanisms that go on stress elevation. Studies with detached leaves indicate that the synthesis of proline from carbohydrate via α-ketoglutrate and glutamate is the main source of the free proline accumulation during drought stress (Boggerss et al., 1976; Venekamp & Koot, 1988).

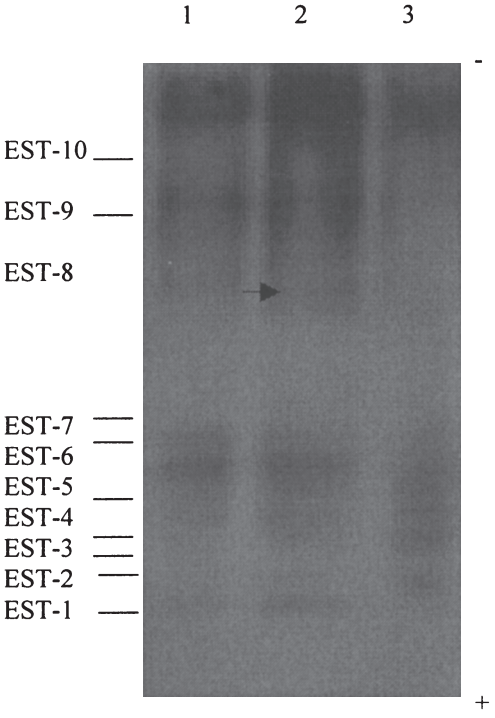

Figure 8. Native gel electrophoresis of esterase isoenzyme (EST) of *A. graecorum* calli cultured for one week in a MS medium supplemented with 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.5 mg/l BAP (Lane 1) and 256 mM mannitol (Lane 2) or 172 mM NaCl (Lane 3)

Consequently, the GOT expression in stress-tolerant plant species should be influenced strongly by stress condition.

In both plants, the number of esterase bands was not influenced by mannitol stress. In *L. esculentum*, the staining intensity of esterase bands decreased under salt stress. This decrease in staining intensity was accompanied with the disappearance of one esterase band (EST-8) in *A. graecorum* (Fig 8). Esterase isoenzyme patterns in higher plants differ between tissues and distinct physiological stages (Tansksley and Rick, 1980; Hassanein, 1999). The number and/or the staining intensity of several isoenzymes, including esterase, was affected by atrazine stress more in atrazine sensitive than atrazine resistant lines of *Solanum nigrum* (Hassanein, 1997).

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