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Comparison of drought-induced polypeptides and ion leakage in three tomato cultivars

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Abstract

An attempt has been made to determine if drought-induced proteins could be used as a selection marker to differentiate between tolerant and sensitive cultivars. Three Indian tomato (*Lycopersicon esculentum* Mill.) cultivars (Pusa Ruby, Arka Vikas and Pusa Early Dwarf) were subjected to drought stress *in vivo* as well as *in vitro* and the pattern of polypeptide expression was determined using one-dimensional SDS-PAGE. In all the three cultivars, a new 29 kDa polypeptide accumulated in leaves, in response to gradual drought stress and its accumulation was fastest in Pusa Ruby. Drought stress also resulted in an increase in ion leakage from leaf discs of all the three cultivars but the rate was lower in Pusa Ruby than in other two. Therefore, it was concluded that Pusa Ruby is most tolerant to drought stress among the three tomato cultivars investigated.

Additional key words: drought tolerance, *Lycopersicon esculentum,* mannitol, SDS-PAGE, water stress.

Introduction

The rate of drought-induced polypeptide accumulation and subsequent depletion during recovery may be important indicators of stress tolerance. In addition, the abundance of these polypeptides may also provide some important information about their function (Bray 1990). Some drought-induced proteins (*i.e.* dehydrins) are highly hydrophilic (Kosová *et al*. 2007) and remain soluble, a characteristic called "boiling stability" (Jacobsen and Shaw 1989).

The degree of cell membrane damage induced by any stress can be measured by electrolyte leakage from cells. The drought tolerant genotypes tend to leak electrolytes at a low rate compared to the sensitive ones (Bajji *et al.* 2002).

Genetic variability within a species offers a valuable tool for studying mechanism of stress tolerance. Although tomato plants usually require a high water potential for optimal growth (Waister and Hudson 1970, Alian *et al*. 2000), the information on response of tomato plants to drought stress is rather scarce (Perez-Alfoncea *et al*. 1993, Torreciallas *et al*. 1995). Most of the previous investigations on tomato were devoted to the study of the differential responses to salinity and drought stresses of wild versus domesticated species with the objective of selection of donors for tomato breeding programmes (Bolarin *et al*. 1991, Cuartero *et al*. 1992, Guerrier 1996). However, very few reports on the genotypic variations in cultivated tomato are available (Alarcon *et al*. 1994, Caro *et al*. 1994, Alian *et al*. 2000).

The objective of the present study was to assess if protein accumulation and electrolyte leakage could be used as selection markers to differentiate drought tolerant cultivars of tomato from the sensitive ones so that they could be cultivated in areas where sufficient water is not available for irrigating the crop.

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Abbreviations: ABA - abscisic acid; BA - N^6-benzyladenine; BSA - bovine serum albumin; DTT - dithiothreitol; EDTA - ethylenediaminetetra-acetic acid; IAA - indole-3-acetic acid; LEA - late embryogenesis abundant; MS - Murashige and Skoog; PAGE - polyacrylamide gel electrophoresis; SDS - sodium dodecyl sulphate.
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Materials and methods

Plants of *Lycopersicon esculentum* Mill. cvs. Pusa Ruby, Arka Vikas and Pusa Early Dwarf were raised in earthen pots and all the *in vivo* experiments were performed on tomato leaves excised from 40-d-old seedlings. For *in vitro* studies, leaf explants were taken from aseptically grown 6-week-old seedlings on MS basal medium.

Petioles of control (un-stressed) leaves were dipped in water for the duration of treatment. Two sets of detached leaves were drought stressed (day/night temperature of 40/35 °C) till they lost from 10 to 80 % of their original fresh mass. After respective water loss percentages, one set was frozen in liquid nitrogen and stored at -80 °C and the second set of leaves was kept in water for rehydration for the duration they took to loose 10 - 80 % mass. They were then frozen in liquid nitrogen and kept at -80 °C.

To induce drought stress *in vitro*, leaf explants were excised from *in vitro* grown seedlings and cultured on regeneration medium (MS + 3 mg dm^{-3} benzyladenine + 0.3 mg dm^{-3} indoleacetic acid) supplemented with 2 - 10 % mannitol. Twenty four explants were taken and the experiment was repeated thrice. Leaf explants with developing calli and shoots, cultured on mannitol supplemented medium for 30 d were frozen and taken for protein profile studies.

Proteins were extracted from the leaves following the protocol of Vu *et al.* (1982). Samples were homogenized with pestle and mortar in extraction buffer (50 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiotreitol and 5 mM isoascorbate). Crude protein extracts were centrifuged at 12 000 *g* for 20 min at 4 °C. Proteins in the supernatant were estimated spectrophotometrically at 750 nm according to Peterson (1977) using bovine serum albumin (BSA) as standard. For SDS-PAGE, 100 μg total proteins were precipitated by adding 4 volumes of cold acetone and then centrifuged at 10 000 *g* for 10 min at 4 °C. Pellet obtained was dried and dissolved in 0.02 cm^3 sample buffer (62.5 mM Tris HCl, pH 6.8, 2 % SDS, 2.5% glycerol and 0.01 % bromophenol blue). Boiling stable proteins were obtained by boiling total protein samples (750 - 800 μg) for 10 min and then transferred immediately to ice and kept for 5 min. Supernatant was obtained after centrifugation at 10 000 *g* for 10 min. Boiling stable proteins were precipitated by adding 4 volumes of cold acetone to the supernatant. Pellet obtained after centrifugation at 10 000 *g* for 10 min was dissolved in 0.02 cm^3 sample buffer. Before loading, the samples were heated at 100 °C for 2 min.

Each lane was loaded with 100 μg of total or 750 μg of boiling stable proteins and run at 25 mA for 2 h in *Mini protean II* gel apparatus of *Bio-Rad* (Hercules, USA). Gels were stained with 0.2 % m/v Coomassie Blue in 40 % methanol and 10 % acetic acid. They were destained in 40 % methanol containing 10 % acetic acid, photographed and analyzed in *Gel Documentation 2000* system (*Bio-Rad*).

For determination of electrolyte leakage, leaf discs of all the three cultivars (diameter 7 mm), were initially desiccated for 24 h, washed for 5 min to remove the adhering ions leached during past 24 h and then immersed in distilled water. The conductance of the immersion solution (E_L) was measured after 15, 30, 45, 60, 120, 240 and 300 min by a digital conductivity meter (*Electronic Instrumentation*, Delhi, India).

Results

Detached leaves of *L. esculentum* Arka Vikas and Pusa Early Dwarf lost more water at each point of time than leaves of Pusa Ruby (Table 1).

SDS-PAGE analysis revealed 16 polypeptides in control leaf samples of all the three cultivars ranging from 4 to 182 kDa, *i.e* 4, 11, 18, 21, 24, 26, 33, 36, 38, 41, 45, 52, 58, 72, 149 and 182 kDa. The synthesis of a majority of polypeptides was not affected up to 50 % water loss but a new 29 kDa polypeptide accumulated in leaves in response to gradual drought stress. In dehydrated leaves of Pusa Ruby a 29 kDa polypeptide appeared after fresh mass loss of 40 % and it was highly accumulated after fresh mass loss of 60 % (Fig. 1*A*). However, in cultivars Arka Vikas (Fig. 1*B*) and Pusa Early Dwarf (Fig. 1*C*) it accumulated after fresh mass loss of 50 % and the expression level was lower compared to Pusa Ruby. Dehydrated leaves also expressed another novel polypeptide of 31 kDa in all the three cultivars but its content was much less compared to the 29 kDa polypeptide. The contents of 18, 45, 52, 58 and 72 kDa polypeptide increased in dehydrated leaves of all cultivars (Fig. 1).

On rehydration, the content of the major stress induced 29 kDa polypeptide decreased in all the three cultivars (Fig. 2). In Arka Vikas, the content of 52 kDa polypeptide during rehydration was higher when compared not only to its control but also dehydrated sample as well as the other two cultivars (Figs. 2*B,* 3*A*). In fact, the rehydrated samples retained all the polypeptides of dehydrated samples but their contents were lower in all the three cultivars.

The major stress induced 29 kDa polypeptide in three cultivars was not found to be boiling stable (Figs. 2, 3*B*). Only the low molecular mass polypeptides (11, 18, 21 and 26 kDa) were boiling stable.

The three cultivars were subjected to osmotic stress during *in vitro* culture by adding mannitol to the regeneration medium. Pusa Ruby was the most

Table 1. Comparison of water loss [% of fresh mass] from three tomato cultivars, *i.e*. Pusa Ruby, Arka Vikas and Pusa Early Dwarf. Each value ± SD represents average of three experiments.

Time [h]	Pusa Ruby	Arka Vikas	Pusa Early Dwarf
1	13.30 ± 0.097	17.50 ± 0.075	19.16 ± 0.105
2	17.15 ± 0.100	22.20 ± 0.068	22.80 ± 0.087
3	20.90 ± 0.100	29.10 ± 0.065	27.50 ± 0.087
4	25.81 ± 0.100	34.10 ± 0.070	32.50 ± 0.087
5	29.41 ± 0.091	38.30 ± 0.065	35.50 ± 0.081
6	30.80 ± 0.085	41.60 ± 0.070	39.70 ± 0.081
7	33.70 ± 0.080	44.10 ± 0.075	42.80 ± 0.089
8	35.70 ± 0.090	48.30 ± 0.080	45.30 ± 0.092
24	57.30 ± 0.116	72.50 ± 0.096	67.50 ± 0.095
26	58.80 ± 0.115	74.00 ± 0.100	70.50 ± 0.089
28	61.70 ± 0.111	75.80 ± 0.100	73.30 ± 0.095
30	63.70 ± 0.105	78.30 ± 0.090	75.80 ± 0.089
32	66.10 ± 0.105	79.10 ± 0.090	75.80 ± 0.037
48	71.60 ± 0.158	82.80 ± 0.060	81.60 ± 0.030
50	72.50 ± 0.152	83.90 ± 0.060	82.00 ± 0.030

Table 2. Electrolyte leakage, EL [µS cm^{-1}], in drought stressed leaf discs in three cultivars of tomato. Means ± SD of three experiments. EL in control discs after 300 min were 12.9, 14.0 and 13.4 µS cm^{-1} in Pusa Ruby, Arka Vikas and Pusa Early Dwarf, respectively.

Time [min]	Pusa Ruby	Arka Vikas	Pusa Early Dwarf
0	19.0 ± 0.57	15.1 ± 0.20	8.5 ± 0.20
15	65.2 ± 0.32	55.4 ± 0.20	63.9 ± 0.26
30	78.9 ± 0.37	80.0 ± 0.41	81.8 ± 0.25
45	90.6 ± 0.40	93.3 ± 0.15	95.3 ± 0.40
60	96.3 ± 0.40	98.2 ± 0.26	101.1 ± 0.35
120	105.8 ± 0.10	115.6 ± 0.20	112.3 ± 0.25
180	110.8 ± 0.25	137.2 ± 0.10	120.1 ± 0.26
240	112.1 ± 0.20	138.7 ± 0.10	122.2 ± 0.30
300	112.5 ± 0.20	141.6 ± 0.40	125.0 ± 0.41

responsive cultivar showing optimum callus formation on 8 % mannitol while Pusa Early Dwarf developed least amount of callus and in very few cultures (data not shown). The control sample of Pusa Ruby showed 12 polypeptides of molecular masses 21, 26, 27, 32, 33, 35, 38, 40, 46, 50, 55 and 72 kDa (Fig. 4*A*). The expression of higher molecular mass polypeptides from 38 to 72 kDa was very low. The protein profile of leaves cultivated on 2 % mannitol was quite comparable to that of control. On 4 % mannitol, the proteins of control were expressed in higher levels along with the induction of additional bands of 22, 28 and 29 kDa. In cultures on 6 % mannitol, contents of all the polypeptides increased, compared to those on 2 and 4 % mannitol. On 8 % mannitol, protein expression was highest. On 10 % mannitol, samples retained all the polypeptides developed on 8 % mannitol but the expression of high molecular mass polypeptides

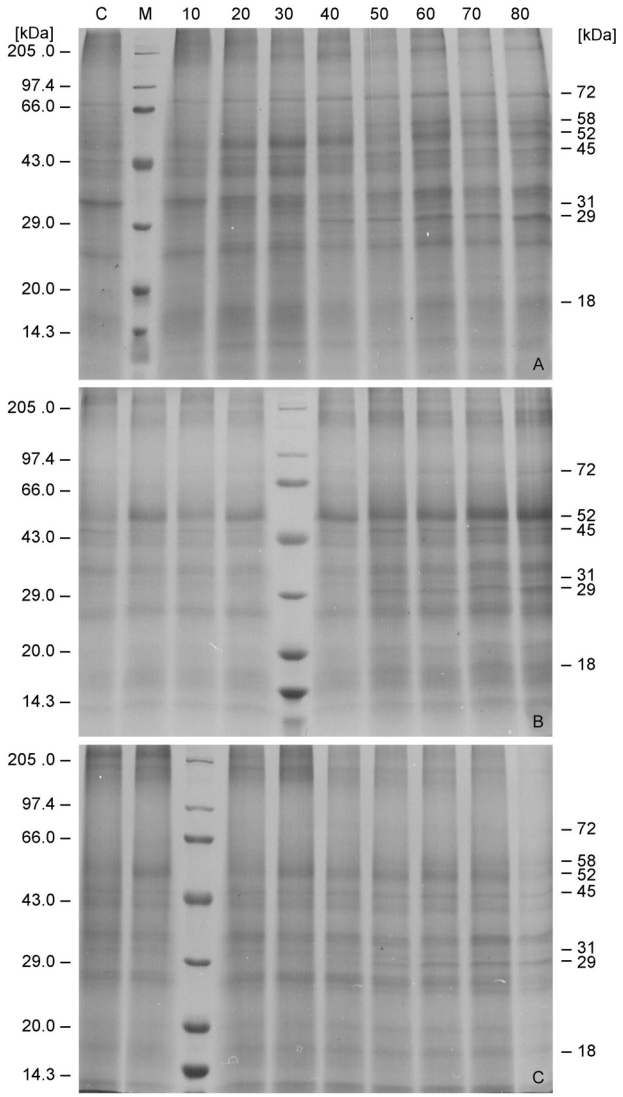

Fig. 1. SDS-PAGE analyses of total soluble proteins of *Lycopersicon esculentum* leaves after loosing 10 - 80 % of fresh mass. *A* - Protein profile of Pusa Ruby leaves: *lane* C - control, *lane* M - molecular mass marker, *lanes* 3 - 10 - protein profiles of leaves losing 10, 20, 30, 40, 50, 60, 70 and 80 % of fresh mass. *B* - Protein profile of Arka Vikas leaves: *lane* 1 - control (C), *lanes* 2 - 4 and 6 - 10 - leaves representing 10, 20, 30, 40, 50, 60, 70 and 80 % mass loss, lane 5 - molecular mass markers. *C* - Protein profile of Pusa Early Dwarf leaves: *lane* 1 - control (C), *lanes* 2, 4 - 10 - leaves representing 10, 20, 30, 40, 50, 60, 70 and 80 % mass loss, respectively, *lane* 3 - molecular mass markers (M). Molecular masses of the markers are marked on the left. In *B* and *C* the M line is shifted to the fourth and third positions, respectively.

(38, 40, 46, 50 and 55 kDa) was poorer. The protein profiles of Arka Vikas (Fig. 4*B*) and Pusa Early Dwarf (Fig. 4*C*) showed similar patterns of polypeptides but with lower expression than in Pusa Ruby.

Drought stress resulted in an increase in ion leakage from leaf discs of all the three cultivars, compared to their

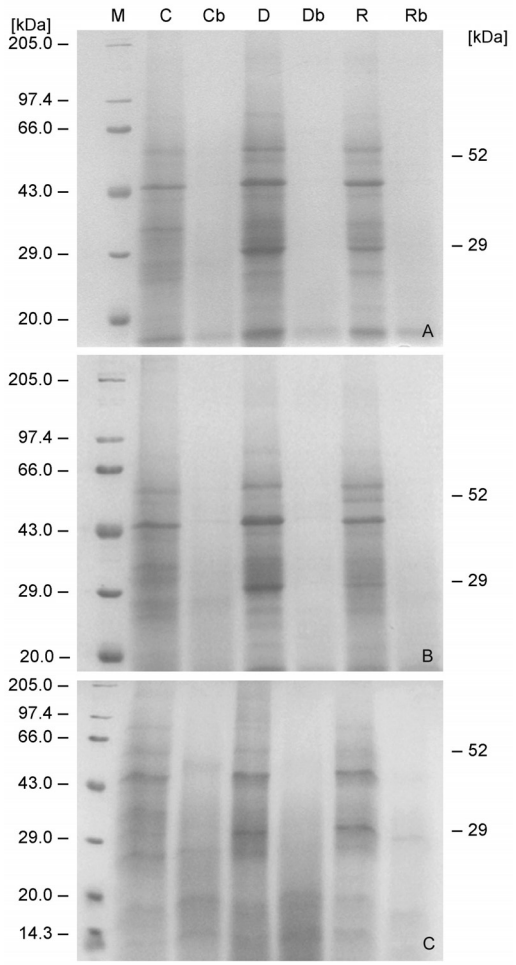

Fig. 2. Analysis of total soluble and boiling stable proteins of three tomato cultivars (*A* - Pusa Ruby, *B* - Arka Vikas and *C* - Pusa Early Dwarf) after 24 h of dehydration and rehydration. Lanes were loaded with 100 µg total proteins and 750 - 800 µg boiling stable proteins. Protein profile of leaves desiccated for 24 h and rehydrated for the same duration. The major 29 kDa polypeptide was induced after desiccation (D) whose band intensity was reduced after rehydration (R) and it was not boiling stable. Cb, Db and Rb represent boiling stable proteins of control (C), dehydrated (D) and rehydrated (R) samples, respectively. A high intensity 52 kDa polypeptide band developed during rehydration compared to its control and dehydrated samples of Arka Vikas.

respective controls, but the rate and final values of electrolyte leakage from Pusa Ruby were lower than those of Pusa Early Dwarf and Arka Vikas (Table 2).

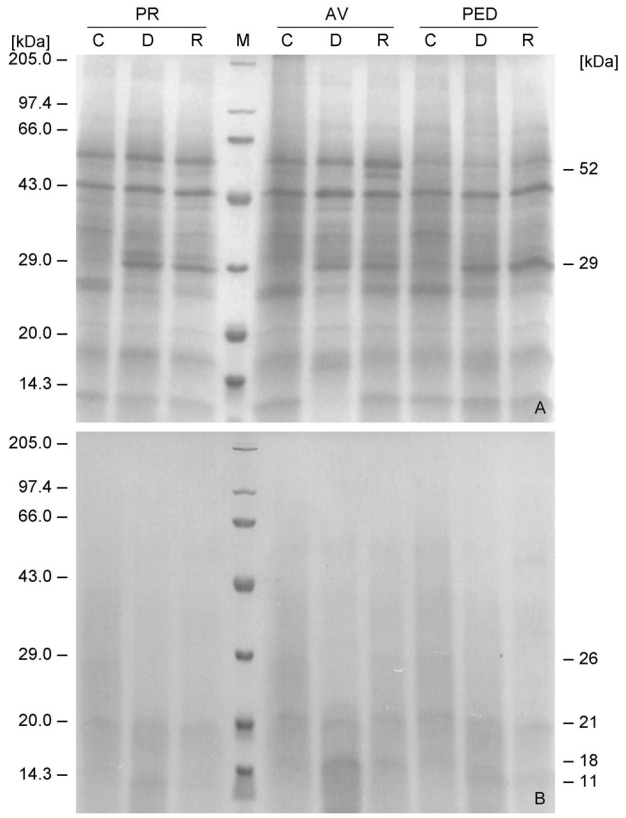

Fig. 3. Total soluble (*A*) and boiling stable (*B*) proteins of desiccated and rehydrated leaves of three tomato cultivars. Lanes were loaded with 100 µg total soluble proteins and 750 - 800 µg boiling stable proteins. *Lane* 1 - control (C), *lane* 2 - leaves dehydrated for 24 h (D) and *lane* 3 - leaves rehydrated for 24 h (R) of Pusa Ruby (PR), *lane* 4 - molecular mass markers (M), *lanes* 5 - 7 the same of Arka Vikas (AV) and *lanes* 8 - 10 of Pusa Early Dwarf (PED). The major stress induced 29 kDa polypeptide decreased after rehydration in all the cultivars. A high intensity 52 kDa polypeptide band developed during rehydration compared to its control and dehydrated samples of Arka Vikas. The 29 kDa polypeptide is not boiling stable.

Discussion

Analysis of water loss percentage revealed that detached leaves of *L. esculentum* cultivars Arka Vikas and Pusa Early Dwarf lost more water at various intervals of time compared with Pusa Ruby, suggesting that Pusa Ruby is more drought tolerant. This was confirmed by accumulation of 29 kDa polypeptide (drought stress related dehydrin) during water stress in all the three cultivars but earlier in Pusa Ruby than in Arka Vikas and Pusa Early Dwarf. The drought tolerant genotypes expressed the dehydrin faster and in higher quantity than the related sensitive genotypes, as has also been reported earlier (Pelah *et al.* 1997, Suprunova *et al.* 2004). The mechanism by which earlier expression of the polypeptide confers resistance in tolerant genotypes can be explained by early perception of drought stress, more efficient signalling pathways and transcription activators as well as higher expression of dehydrin related genes. The total protein profile also indicated that Pusa Ruby retained more polypeptides compared to other two cultivars.

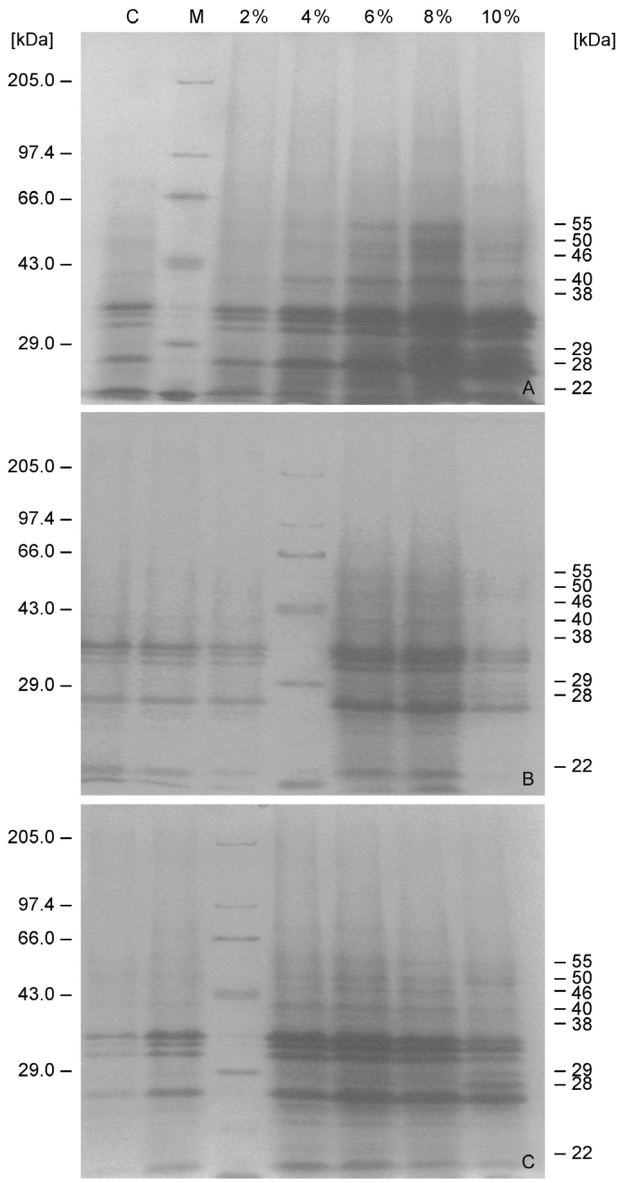

Fig. 4. Effect of *in vitro* mannitol stress (2 - 10 %) on total protein profile of leaves of the three tomato cultivars (*A* - Pusa Ruby, *B* - Arka Vikas and *C* - Pusa Early Dwarf), after a month of culture. The 29 kDa polypeptide was induced during *in vitro* mannitol treatment in all the cultivars with highest intensity in Pusa Ruby and lowest in Arka Vikas. *Lane* 1 - control (C), *lane* 2 - molecular mass markers (M), *lanes* 3 - 7 - leaves cultured on 2, 4, 6, 8 and 10 % mannitol supplemented regeneration medium. Molecular masses of the markers are marked on the left. In *B* and *C* the M line is shifted to the fourth and third positions, respectively.

On rehydration, the expression of the 29 kDa polypeptide decreased in all the three cultivars. In cultivar Arka Vikas, a new polypeptide of 52 kDa was induced during rehydration. Specific protein synthesis has been shown to occur during recovery period (Misra and Bewely 1986, Caruso *et al.* 2002) as recovery from drought stress can be characterized by specific patterns of gene expression. This also indicated that Arka Vikas required novel protein synthesis for recovery and hence indirectly point out that it might be most sensitive among the three cultivars.

Though the overall pattern of polypeptides was not altered during drought stress in tomato leaves but the newly expressed polypeptides were readily detected. The general protein profile is also not altered under other stresses such as salt and low temperature but alters dramatically during heat shock (Wahid and Close 2007).

The protein profiles were quite different during *in vivo* and *in vitro* studies. The total number of polypeptide bands observed in leaf samples during *in vivo* drought stress was higher than that in samples from *in vitro* treatment. This was quite expected because of the difference in these 'drought stress experiments'. The conditions of the two experiments might be expected to produce different cellular changes leading to gene expression. The air-drying of the plants during *in vivo* drought stress should stimulate 'cytorrhysis', *i.e.* whole cell collapse, while culturing of plants on mannitol or PEG supplemented medium is likely to result in 'plasmolysis' of cells (Bray 2004).

A comparison of studied cultivars indicated that the protein profiles under *in vitro* stress supported the results of *in vivo* studies. During *in vitro* stress, more major stress-induced proteins were detected in Pusa Ruby, compared to the other two cultivars. Several studies have shown upregulation of transcripts characteristic of different abiotic stresses in tolerant genotypes while their absence in the sensitive (Kawasaki *et al.* 2001). Similar results were obtained during the present study. It is believed that resistant cultivars can overcome the stress due to their ability to induce earlier protein synthesis while sensitive cultivars show a delayed response (Kawasaki *et al.* 2001).

Drought stress induced ion leakage can serve as a quantitative measure of stress-induced damage to cell membranes (Bramlage *et al.* 1978, Pelah *et al.* 1997) as cell membranes are one of the first targets of many stresses. Maintenance of integrity and stability of cell membrane under drought stress is a major component of drought tolerance in plants (Bajji *et al*. 2002). It has been reported that drought stress tolerant genotypes tend to leak electrolytes at a lower rate compared to the sensitive genotypes (Pelah *et al*. 1997).

In the present investigation, it was found that drought stress resulted in ion leakage from leaf discs of all the three cultivars, but the leakage was significantly lower from leaf discs of Pusa Ruby than Arka Vikas and Pusa Early Dwarf. These findings support the hypothesis that Pusa Ruby is more tolerant than Arka Vikas and Pusa Early Dwarf, the latter being the most sensitive.

Conductance of the immersion solution increased many folds in drought stressed leaves compared to their respective controls. It increased rapidly in first 15 min followed by a slow rate and finally attained a steady state. The major percentage of electrolyte released during first 15 min could be attributed to the surface adhering

electrolytes as well as those released by damaged cells and apoplasts (Bajji *et al.* 2002). After 15 min, the lower and almost regular percentage of leakage could be from intact cells due to simple effect of immersion and hypo-osmotic shock induction by deionised water. Electrolyte leakage enhanced with increase in stress treatment. The increase in conductivity in stressed tissues can be best explained on the basis of the complex changes in structure of the plasma membrane and the tonoplast and by assuming that the intrinsic membrane proteins controlling K^+ and Na^+ transport are damaged during drought stress.

Overall, drought stress resulted in reduced water loss, lower electrolyte leakage and higher accumulation of the major 29 kDa polypeptide in Pusa Ruby compared to Arka Vikas and Pusa Early Dwarf, therefore, it was concluded that the Pusa Ruby is most tolerant among the three genotypes. Additional studies that address the functions of this drought-induced polypeptide are being undertaken.

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