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Growth and protein pattern in cowpea seedlings subjected to salinity

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Abstract

Cowpea (*Vigna unguiculata*) seeds were put to germinate on filter paper under control (distilled water) and salt stress (100 mM NaCl) conditions. Seeds and seedlings were classified in eight developmental stages (DS), according to their morphological traits. Under control conditions, 7 d after planting, 100 % of the seedlings reached DS VIII (seedlings with radicles measuring more than 5 cm, cotyledons leaving the filter paper, hypocotyls straight and cotyledonary leaves fully open) and under NaCl stress conditions, 11 d after planting only 68 % of the seedlings were at DS VIII. The length of the main root and of shoot has decreased 23 and 44 %, respectively. The two-dimensional electrophoretic patterns of the albumins isolated from stems and leaves were determined in seedlings at DS VIII. In stems 19 proteins (14.6 to 76.3 kDa) had their relative concentration increased by salinity, 8 (31.2 to 65.0 kDa) had their relative concentration decreased by salinity and 9 (16.3 to 39.8 kDa) were apparently synthesised *de novo*. In leaves, under salt conditions 9 proteins (18.2 to 33.2 kDa) increased in concentration, one (17.1 kDa) decreased in concentration and one (21.2 kDa) was apparently synthesised *de novo*.

Additional key words: 2-D electrophoresis, NaCl-stress, *Vigna unguiculata*.

Introduction

On a world-wide basis, salinitization of soils is becoming a critical problem and it is thought to encompass up to 10 % of the irrigated areas and furthermore soil salinization is growing at a rate of 1 - 2 % a year. Salinity affects various aspects of plant metabolism and induces morphological and anatomical changes that can be seen either as adaptations or as signs of an unbalance in development (Poljakoff-Mayber 1975, Sohan *et al.* 1999). Reduction in growth and in yield are undoubtedly the most conspicuous responses of plants to the excess of salts in the soil and this can be a consequence of osmotic stress that causes water deficit and reduces water absorption. Alternatively, it can be due to specific effects of the ions that cause toxicity and nutritional unbalance (Greenway and Munns 1980, Amzallag 1997, Cainnes and Shennan 1999). Despite the efforts dedicated to clarify this question, the precise mechanism involved in the response of plants to salt stress remains unknown. Studies using seedlings in the establishment phase pose a particular problem, because salinity causes not only an inhibition in growth, but also retardation in development. The use of seedlings of the same chronological age, as it is generally done, makes it impossible to draw a distinction between these two effects (Gomes-Filho *et al.* 1983, Franco *et al.* 1999). One way to overcome this problem is to use plants in the same morphological stage, as in this way the growth reduction observed would be caused by growth inhibition and not retardation in plant development.

Several studies have established a relation between metabolic and structural changes and salt stress. As a consequence of changes in gene expression, protein synthesis is also affected by the majority of the environmental stresses, including water and salt stress (Hurkman and Tanaka 1987, Sabehat *et al.* 1998). Although we have information about the changes in the electrophoretic pattern of proteins due to water and salt stress in grasses such as rice, barley, maize and wheat (Ramagopal 1987, Ramani and Apte 1997, Ricchardi *et al.* 1998, El-Shintinawy and El-Shourbagy 2001), this

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sort of information is lacking for legumes. This present work was performed in order to determine the effect of salinity on the electrophoretic patterns of proteins from stems and leaves of cowpea (*Vigna unguiculata*) seedlings. Our studies were conducted using seedlings of the same morphological developmental stage, in order to allow us to separate the changes due to salinity itself, from those caused by growth retardation.

Materials and methods

Plants and germination: Seeds of cowpea [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba, previously surface-sterilized with sodium hypochlorite, were allowed to germinate on filter paper (Prisco and Vieira 1976), at 25 ± 2 °C and photoperiod of 12 h, irradiance of 16 μmol m^{-2} s^{-1}. For the control treatment (sowing in distilled water), seedlings were collected from 1 to 8 d and for the salt treatment (sowing in 0.1 M NaCl) they were collected from 1 to 12 d after sowing.

Morphological characterization and growth measurements: Seedlings were classified according to their morphological developmental stage (DS) as defined by J.T. Prisco (unpublished results) as follows: DS 0 - quiescent seed; DS I - completely imbibed seed; DS II - radicle emerged, with length up to 2 cm; DS III - radicle bigger than 2 cm and smaller than 5 cm; DS IV - radicle bigger than 5 cm; DS V - radicle bigger than 5 cm, hypocotyls leaving the filter paper; DS VI - radicle bigger than 5 cm, cotyledons out of the filter paper, curved hypocotyl and cotyledonary leaves not open; DS VII - radicle bigger than 5 cm, cotyledons out of the filter paper, straight hypocotyl and cotyledonary leaves not open; DS VIII - radicle bigger than 5 cm, cotyledons out of the paper, straight hypocotyl and cotyledonary leaves open. Measurements of total root and shoot length, as well as biochemical analysis were performed with seedlings at DS VIII. In each treatment 5 replicates of 50 seedlings were used for growth measurements.

Protein extraction and determination: Tissues from seedlings at DS VIII from both treatments, were freeze-dried turned into a fine powder, and kept at -20 °C. Protein extraction was performed (1/20 m/v) in cold 25 mM Tris/HCl, 0.5 mM ethylenediaminetetraacetic acid, 0.15 M NaCl, 1 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.002 % sodium azide, 5 % polyvinylpyrrolidone, pH 7.4 for 3 h. After filtration and centrifugation (9 500 *g*, 15 min, 4 °C), the crude extract was brought to 90 % saturation with ammonium sulphate. The proteins were collected by centrifugation (12 000 *g*, 30 min, 4 °C) and dialyzed (Mr cut off 3 000 *g*) against distilled water for 48 h. After centrifugation (16 500 *g*, 30 min, 4 °C) the albumins were collected, freeze-dried and kept at -20 °C until used. Protein measurements were made according to the method of Bradford (1976), using bovine serum albumin as standard.

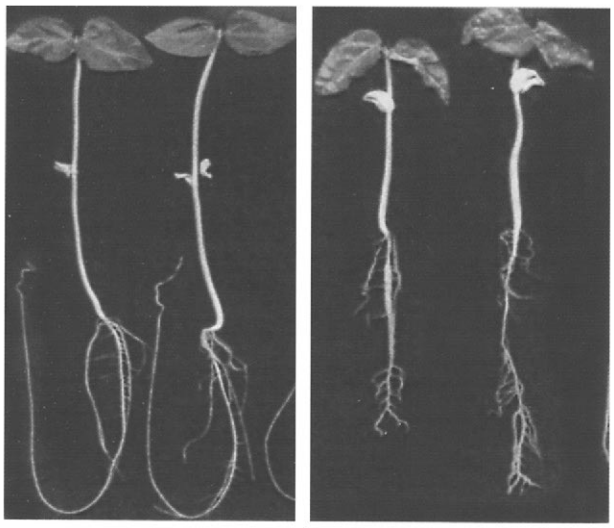
Two-dimensional (2-D) electrophoresis: Isoelectric focusing (IEF) was performed in gels having diameter of 2.5 mm and length of 110 mm, prepared according to O'Farrell (1975). For the formation of the pH gradient, prior to sample application, the gels were pre-run for 15 min at 200 V, followed by 30 min at 300 V and 30 min at 400 V. Protein samples were dissolved in lysis buffer (9.5 M urea, 0.4 % Triton X-100, 0.04 % ampholytes, pH 3 - 10, and 5.1 % 2-mercaptoethanol). After sample (40 μg) application, 0.01 cm^3 of a solution made of 9 M urea and 1 % ampholytes pH 3 - 10, was carefully layered on the top of the sample. IEF was performed at 400 V for 17 h, at 24 °C. The second dimension separation was performed in SDS-PAGE slab gels (165 × 185 × 0.5 mm), prepared according to Laemmli (1970). The separation gel consisted of a linear gradient of 10 to 18 % acrylamide. Before the electrophoretic run, the IEF gel rod was equilibrated in 62.5 mM Tris/HCl, pH 6.8, 2 % SDS, 5 % 2-mercapto-ethanol, 0.001 % bromophenol blue and applied on top of the application gel. Electrophoresis was developed at 30 mA, at 24 °C. After the run the gels were put in fixation solution (45 % methanol, 10 % acetic acid) for 16 h, under gentle stirring. The gels were then developed with silver nitrate, according to Schevchenko *et al.* (1996).

Results

One day after sowing, both in the control and in the salt treatment, 100 % of the seedlings were at DS I (Table 1). However, in the second day, while in the salt treatment all the seedlings remained in DS I, those from the control treatment were at DS II (80 %) and III (20 %). From the second to the sixth day, in the control treatment and from the third to the twelfth day in the salt treatment, on a given day we could find seedlings in various DS. This was particularly conspicuous in the salt treatment. Under control conditions, from the seventh day all the seedlings were in DS VIII, while in salt conditions, only at the eleventh day 68 % of the seedlings were at DS VIII (Table 1).

Table 1. Number of cowpea seeds or seedlings in different developmental stages (0 - VIII) at 0 - 12 d after sowing (DAS) under control and NaCl stress conditions. The numbers within parentheses represent percentage.

	DAS	0	I	II	III	IV	V	VI	VII	VIII
Control	0	50 (100)								
	1		50 (100)							
	2			40 (80)	10 (20)					
	3				19 (38)	31 (62)				
	4		1 (2)		2 (4)	38 (76)	8 (16)	1 (2)		
	5					2 (4)	2 (4)	8 (16)	8 (16)	30 (60)
	6						1 (2)			49 (98)
	7									50 (100)
	8									50 (100)
NaCl	0	50 (100)								
	1		50 (100)							
	2		50 (100)							
	3		19 (38)	31 (62)						
	4			4 (8)	37 (74)	9 (18)				
	5				7 (14)	43 (86)				
	6		1 (2)		3 (6)	46 (92)				
	7				9 (18)	30 (60)	3 (6)	4 (8)	3 (6)	1 (2)
	8				7 (14)	16 (32)	10 (20)	8 (16)	3 (6)	6 (12)
	9		1 (2)		4 (8)	6 (12)	11 (22)	8 (16)	6 (12)	13 (26)
	10				2 (4)	7 (14)	20 (40)	6 (12)	4 (8)	11 (22)
	11							7 (14)	9 (18)	34 (68)
	12				2 (4)	2 (4)	4 (8)	4 (8)	10 (20)	28 (56)



CONTROL SALT TREATMENT

Fig. 1. Cowpea seedlings in DS VIII. The seedling on the left is 7-d-old and was raised in distilled water (control). The seedling on the right is 11-d-old and was raised in 0.1 M NaCl (salt treatment).

DS VIII seedlings from the salt treatment are smaller than those from the control treatment (Fig. 1). Salinity decreased the length of the main root, stem and whole seedling (main root + shoot) by 23, 44 and 32 %, respectively (Fig. 2).

In the 2-D electrophoresis patterns of the albumins from stems of seedlings at DS VIII, from both salt and control treatments, it can be seen that at least 36 proteins had their deposition pattern altered due to the salt treatment (Fig. 3). From these, 19 (14.6 to 76.3 kDa) had their relative concentrations increased, 8 (31.2 to 65.0 kDa) had their relative concentrations decreased and 9 (16.3 to 39.8 kDa) were apparently synthesized *de novo*.

In the 2-D electrophoresis patterns of the albumins from leaves of seedlings in DS VIII, eleven proteins had their deposition pattern altered due to the salt treatment (Fig. 4). From these, 9 (18.2 to 33.2 kDa) had their relative concentration increased, one (17.1 kDa) had its relative concentration decreased and one (21.2 kDa) was apparently synthesized *de novo* (Fig. 4).

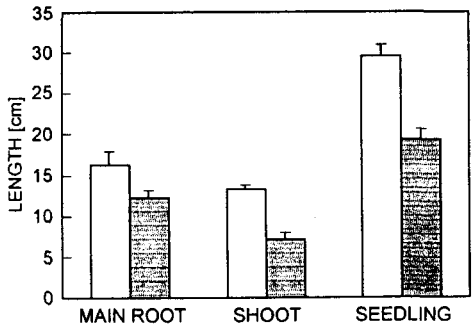


Fig. 2. Growth measurements of the main root, shoot and whole cowpea seedlings in DS VIII. Seedlings raised in distilled water (*empty columns*) and 0.1 M NaCl (*stripped columns*) are 7- and 11-d-old, respectively. Means ± SE, $n = 5$.

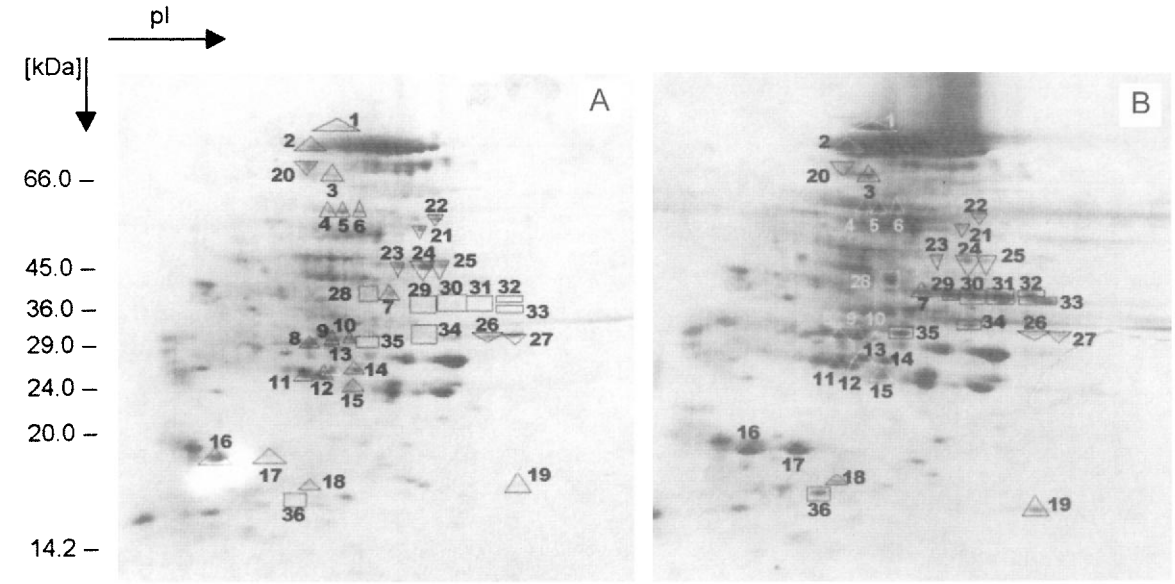


Fig. 3. 2-D electrophoresis of the albumin fraction from stems of cowpea seedlings in DS VIII. Seeds were germinated in distilled water for 7 d (*A*) or in 0.1 M NaCl for 11 d (*B*). The symbols Δ, ∇ and □ indicate proteins that increased or decreased in concentration or that were apparently synthesized *de novo*, respectively, due to salinity. The numbers on the left indicate the molecular masses of the proteins. A total of 40 mg of protein was loaded on the gel.

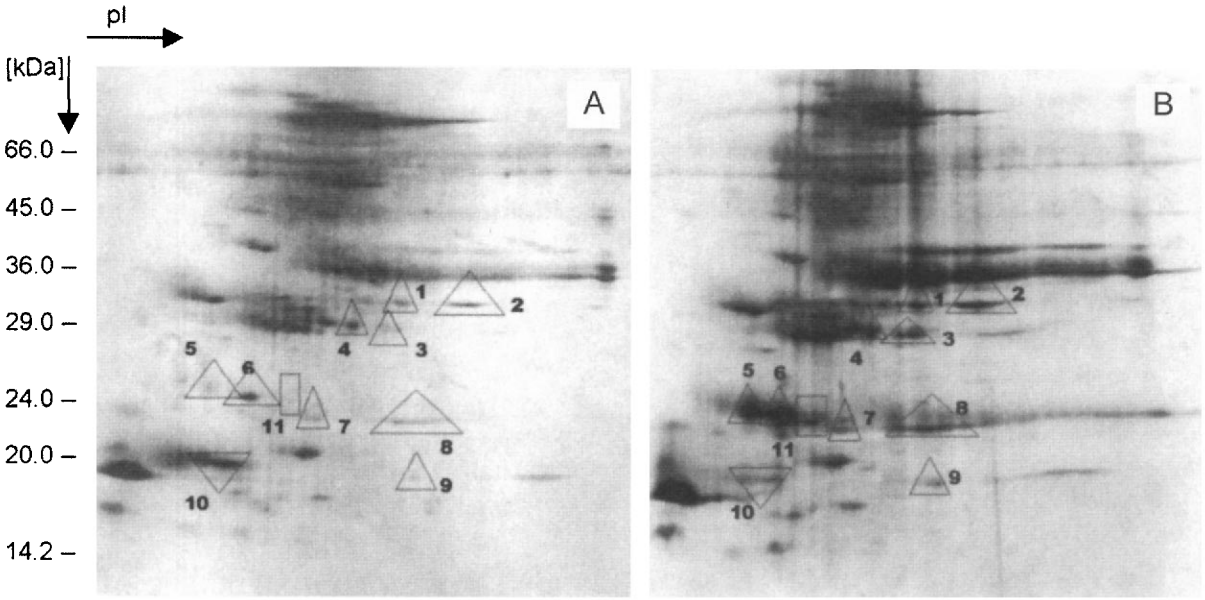


Fig. 4. 2-D electrophoresis of the albumin fraction from leaves of cowpea seedlings in DS VIII. Seeds were germinated in distilled water for 7 d (*A*) or in 0.1 M NaCl for 11 d (*B*). The symbols Δ, ∇ and □ indicate proteins that increased or decreased in concentration or that were apparently synthesized *de novo*, respectively, due to salinity. The numbers on the left indicate the molecular masses of the proteins. A total of 40 mg of protein was loaded on the gel.

Discussion

Although the salinity level used practically did not affect seed germination it strongly retarded seedling development. While under control conditions, 100 % of these seedlings were in DS VIII at the seventh day after sowing, under NaCl stress only 2 % of these were at DS VIII and the majority of the seedlings (60 %) were at DS IV (Table 1). Under salinity the majority of seedlings (68 %) reached DS VIII only by the end of the

experimental period (11 d after sowing). From the seventh until the twelfth day after sowing, with exception of the eleventh day, seedlings under NaCl stress showed a higher morphological variation, having DS from III to VIII (Table 1). These results are in agreement with those obtained by other authors, showing that in glycophytes germination is less affected by salinity than seedling establishment (Maas and Hoffmann 1977).

Besides retarding seedling development, salinity also inhibited seedling growth, as observed by other authors (Greenway and Munns 1980, Franco *et al.* 1999, Dash and Panda 2001). Length measurements of seedlings at the same DS (Figs. 1, 2), have shown that those seedlings from the NaCl treatment were on average 32 % smaller than those from the control treatment. Furthermore, as was previously observed (Gomes-Filho *et al.* 1996), salinity caused a more conspicuous reduction in the length of the shoot than in the length of the main root.

Salt stress induced changes in the electrophoretic pattern of the albumins from stems (Fig. 3). While some proteins had their relative concentrations increased or diminished under NaCl stress, others were synthesized *de novo*. Those that increased in concentration or that were apparently synthesized *de novo* and that presumably are critical for plant adaptation to unfavorable conditions (Amzallag and Lerner 1994), have molecular masses ranging from 14.6 to 76.3 and from 16.3 to 39.8 kDa, respectively. The albumins from leaves also had their deposition pattern affected by salinity (Fig. 4). In this case, the proteins that increased in concentration due to salinity have molecular masses ranging from 18.2 to 33.2 kDa, while the one apparently synthesized *de novo* had a molecular mass of 21.2 kDa. Iuchi *et al.* (1996) analysed the expression of two genes in cowpea leaves, which were induced by osmotic stress. One of these genes codes for a protein belonging to the LEA family. Proteins from this family are thought to protect cells against dehydration. The other gene codes for a protein that had similarities to two related enzymes involved with the biosynthesis of anthocyanin, medicarpin and one phytoalexin.

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