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Interactive Effects of PEG-induced Water Stress and $CaCl_2$ on the Status of Calcium and Ca^{2+} Binding Proteins During Seedling Growth of Peanut (*Arachis hypogaea* L.) Cultivars

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ABSTRACT

Seedlings of two peanut cultivars (TPT-1 and TPT-4) grown in distilled water for 12 d were subjected to polyethylene glycal (PEG-6000) induced water stress. Twenty mM $CaCl_2$ was added to the stressed seedlings to examine the ameliorative effect of PEG induced water stress. The seedlings were analysed for calcium content at 2-d intervals for 7 d. Calcium-binding proteins (CaBPs) were quantified in the seedlings of different treatments in the two cultivars. Water stressed seedlings had significantly lower calcium content. The seedlings of the two cultivars showed differences in the accumulation of calcium under treatments. CaBPs fractionated through DEAE cellulose columns. The pooled fractions of the proteins separated by SDS-PAGE contained different molecular weights of polypeptides. The polypeptides were resolved on the $CaCl_2$-treated seedlings better than either control or water stressed and PEG + $CaCl_2$ treated seedlings. Calmodulin isolated from the seedlings of both the cultivars showed a single band co-migrating with 15 kDa protein of Bovine brain calmodulin. Differences between the cultivars, treatments and interaction for the parameters were significant. The two cultivars showed differences in calcium-binding proteins under PEG-induced water stress and its alleviation by $CaCl_2$ in peanut seedlings.

Key Words: Calmodulin, calcium-binding proteins, seedlings.

Water stress is one of the major abiotic constraints limiting growth and yield of many crop plants. Water deficits at the seedling stages are crucial for peanut crop production. Amendment of soil with calcium salts is known to mitigate the adverse effects of drought (Rajendrudu and Williams, 1987). In laboratory experiments, addition of $CaCl_2$ has been shown to ameliorate the PEG-induced water stress of peanut seedlings (Usha *et al.*, 1999). Enhanced accumulation of calcium-binding proteins (CaBP) in transgenic plants has been shown to confer tolerance to water stress (Fatima *et al.*, 2001).

The role of Ca^{2+} as a second messenger has been well documented (Cosgrove *et al.*, 2000). It plays a cardinal role in the signal transduction in a wide variety of eukaryotic organisms (Bush, 1995). Calcium-modulated proteins are the targets of intercellular calcium signals in higher plants (Robert and Harmon, 1992). The Ca^{2+} receptor protein calmodulin (CaM) and other calcium-binding proteins are also involved in response to environmental stresses (Braam *et al.*, 1996; Person *et al.*, 2001). More than 150 different calcium modulated proteins have been isolated and characterised (Moncriet *et al.*, 1990). This diversity of calcium receptors reflects the specialised structural and functional features required to carry on a wide variety of calcium dependent cellular responses.

CaBPs, intracellular Ca^{2+} receptor molecules, change their conformation by binding to Ca^{2+} and transmit Ca^{2+} signals to activate numerous membrane-bound enzymes to control cellular regulations (Cosgrove *et al.*, 2000). Studies on the combined effect of water stress and $CaCl_2$ on the expression of either CaM and other CaBPs are

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rare in peanut cultivars. Our hypothesis was that Ca^{2+} plays a role in modulation of CaBPs in peanut seedlings exposed to water stress.

Materials and Methods

Seeds of peanut (*Arachis hypogaea* L.) cvs. TPT-1 and TPT-4 were obtained from the N.G. Ranga Agricultural Univ., Tirupati, India. Seeds of uniform size were surface-sterilized with 0.1% (w/v) mercuric chloride solution for 2 min and rinsed thoroughly with distilled water. Seeds were germinated in bread boxes containing 20 mL of distilled water for 1 wk, and the 7-d-old seedlings were subjected to -1 MPa, 20 mM $CaCl_2$ and -1 MPa PEG + 20 mM $CaCl_2$ (9.6×10^3 erg cm^{-2} sec^{-1}) at 25 ± 2C. At a fixed time of 2-d intervals, the seedlings were removed from the bread boxes and blotted dry. The calcium, CaBPs, and CaM were measured separately both in cotyledons and in embryonic axis on the 1st, 3rd, 5th and 7th d after giving the treatments.

Estimation of Calcium. Total Ca^{2+} content of seedlings was determined adopting the method of Piper (1957). The concentration of Ca^{2+} was measured at 422.7 nm using an atomic absorption spectrophotometer.

Calmodulin Isolation. CaM was extracted and purified as per the method of Le Roux and Dubery (1989). The protein was subjected to heat treatment for 3 min at 85 C, the resulting precipitate was removed and the protein solution was adjusted to 5 mM Ca^{2+} with $CaCl_2$. The protein passed through a phenyl sepharose CL-4B column. CaM was desorbed from the column by eluting with 5 mM EGTA in 10 mM tris-HCl buffer (pH 7.0). CaM containing fractions were pooled, dialysed, lyophilized, and UV spectra were recorded. The protein concentration was determined using the Bradford reagent (Bradford, 1976). Amino acid analysis of hydrolysed CaM at 110 C was performed following the method of Thompson *et al.* (1989) with an automatic amino acid analyser. Isolated and purified CaM activity was tested by studying its ability to stimulate cAMP phosphodiesterase. The assay was performed using the method of Schiefer (1986). Electrophoresis was performed using SDS-PAGE on 12.5% gels as per the method outlined earlier by Van Eldik *et al.* (1980).

Extraction of Calcium-Binding Proteins. The extraction of calcium-binding proteins was carried out as per the method outlined by Anderson and Cormier (1978) with slight modifications. Acetone powders of cotyledons and embryonic axis were made to remove the phenolic compounds and lipids.

Isolation. One hundred g of acetone powder was homogenized at 4 C with 300 mL of 25 mM tris-HCl buffer (pH 8.0) containing 2% polyvinyl pyrrolidine (w/v). The homogenate was filtered and centrifuged at 12,000 Xg for 30 min. The supernatant was brought to 50% saturation with solid ammonium sulphate and equilibrated for 30 min. The extract was adjusted to 1 mM Ca^{2+} with $CaCl_2$ and centrifuged. The pellet was suspended in 2 mL of homogenizing buffer. The precipitate was dialysed against 0.1 M NaCl and against distilled water separately. The protein was subjected to pre-activated DEAE cellulose column (2.5×, 18 cm) which had been pre-equilibrated with 2 mM tris-HCl buffer (pH 8.0) containing 0.1 mM EGTA. The column was washed thoroughly with the homogenizing buffer to remove unwanted proteins. The Ca^{2+} binding proteins were collected using linear gradients from 0.1 M to 0.6 M NaCl in 25 mM tris-HCl buffer (pH 8.0) containing 0.1 mM EGTA. Fractions, each with a volume of 3 mL, were run in a sephadex G-75 superfine column (2.8×, 43 cm) at 4 C using 1 mM tris-HCl buffer (pH 7.8) containing 1 mM EDTA. The resulting fractions were pooled on the basis of the calcium and the protein contents. The pooled samples were subjected to 12% SDS-PAGE as per the method outlined by Van Eldik *et al.* (1980).

Results and Discussion

Calcium. In the present study calcium content decreased in cotyledons and increased in embryonic axis from 1st to 7th d after treatment during seedling growth of both the cultivars. The PEG treatment caused a greater decrease in calcium content in the seedlings than the other treatments including control seedlings in cv. TPT-1 than cv. TPT-4 (Table 1). In other species, some of the adverse effects of PEG could be attributed to the nutritional imbalances, for example in citrus leaves and roots (Zekri, 1995). In water stressed plants the dry weight, Ca, Mg, and P contents decreased in wheat (*Triticum aestivum* L.) cultivars (Ashraf *et al.*, 1998). Higher levels of calcium were observed in $CaCl_2$-treated seedlings than other treatments in cv. TPT-4 than that of cv. TPT-1. Cytosolic Ca^{2+} levels have also shown to regulate gene expression in plant cells (Poovaiah *et al.*, 1987). Epstein (1961) noted that Ca^{2+} in the solution around the root tissue represents the normal physiological condition and the ion is essential for the integrity of the selective ion transport mechanisms. In the present study, the $CaCl_2$ treated seedlings showed higher levels of CaM and a greater number of CaBPs than the other two treatments.

Calmodulin. The CaM content decreased in cotyledons and increased in embryonic axis from the 1st to 7th d in all the treatments in both the cultivars. The PEG-treated seedlings showed lower levels of CaM content than the other two treatments in cv. TPT-1 and cv. TPT-4 (Table 2). PEG-induced stress caused greater damage to the membranes of seedlings of rice (*Oriza sativa* L.) (Reddy *et al.*, 1998). $CaCl_2$ treated seedlings maintained higher levels of CaM than the other two treatments, including control seedlings, in cv. TPT-4 and cv. TPT-1. This may be due to inadequate availability of Ca^{2+} for the formation of a Ca-CaM complex for

Table 1. Effect of PEG (-1 MPa), $CaCl_2$ (20 mM), and their combination on the changes in calcium content of cotyledons and embryonic axis of two peanut cultivars (TPT-1 and TPT-4) during seedling growth (μg g^{-1} fresh wt.).[a]

DAT[b]	Seedling part	TPT-1 treatments					TPT-4 treatments					Correlation coefficient
		T_1[c]	T_2[c]	T_3[c]	T_4[c]	C.D.[d]	T_1	T_2	T_3	T_4	C.D.	
1	COTY	3.062 ±0.01	1.018 ±0.009	2.056 ±0.01	3.647 ±0.01	0.0459	3.647 ±0.01	1.744 ±0.01	2.556 ±0.01	4.342 ±0.01	0.0413	r_1 0.9546
	E.A.	0.741 ±0.01	0.452 ±0.02	0.643 ±0.01	0.877 ±0.01	0.0517	0.951 ±0.01	0.662 ±0.01	0.848 ±0.01	1.247 ±0.01	0.0401	r_2 0.9517
3	COTY	2.536 ±0.01	0.877 ±0.01	2.045 ±0.001	3.042 ±0.01	0.0481	2.964 ±0.01	0.962 ±0.01	2.142 ±0.01	3.631 ±0.01	0.0471	r_1 0.9624
	E.A.	0.849 ±0.01	0.653 ±0.01	0.759 ±0.01	1.070 ±0.01	0.0475	1.557 ±0.01	0.854 ±0.01	1.248 ±0.01	2.560 ±0.01	0.0420	r_2 0.9220
5	COTY	2.040 ±0.08	0.651 ±0.01	1.544 ±0.01	2.743 ±0.01	0.514	2.346 ±0.01	0.751 ±0.01	1.846 ±0.01	2.958 ±0.01	0.0401	r_1 0.9806
	E.A.	1.855 ±0.01	0.845 ±0.01	1.051 ±0.01	2.256 ±0.01	0.0429	2.069 ±0.01	1.158 ±0.01	1.650 ±0.01	3.251 ±0.01	0.0448	r_2 0.9322
7	COTY	1.742 ±0.01	0.476 ±0.09	1.238 ±0.01	2.047 ±0.01	0.0356	2.070 ±0.01	0.560 ±0.01	1.464 ±0.01	2.547 ±0.01	0.0517	r_1 0.9146
	E.A.	2.246 ±0.01	1.038 ±0.01	1.362 ±0.01	2.954 ±0.007	0.0506	2.751 ±0.01	1.559 ±0.01	1.856 ±0.01	3.964 ±0.01	0.0463	r_2 0.9610

[a]Values in the table indicate Mean ± Standard Error. r_1 r_2 = Corr. coeff. between COTY and E.A. for cvs. TPT-1 and TPT-4, respectively.
[b]DAT = Days after treatment.
[c]T_1 = Control, T_2 = -1 MPa(PEG), T_3 = -1 MPa (PEG) + 20 mM $CaCl_2$, T_4 = 20 mM $CaCl_2$.
[d]C.D. = Critical difference at $P \leq 0.05$. All mean differences of treatments exceed C.D. and hence differ significantly at $P \leq 0.05$.

Table 2. Effect of PEG (-1 MPa), $CaCl_2$ (20 mM), and their combination on the changes in calmodulin content of cotyledons and embryonic axis of two peanut cultivars (TPT-1 and TPT-4) during seedling growth (μg g^{-1} fresh wt.).[a]

DAT[b]	Seedling part	TPT-1 treatments					TPT-4 treatments					Correlation coefficient
		T_1[c]	T_2[c]	T_3[c]	T_4[c]	C.D.[d]	T_1	T_2	T_3	T_4	C.D.	
1	COTY	20.89 ±0.01	16.07 ±0.01	27.66 ±0.03	29.07 ±0.01	0.0737	25.58 ±0.02	20.11 ±0.02	30.59 ±0.03	35.68 ±0.02	0.0871	r_1 0.9634
	E.A.	7.05 ±0.01	6.61 ±0.01	9.16 ±0.02	10.80 ±0.03	0.0725	11.63 ±0.06	9.731 ±0.03	4.54 ±0.03	16.77 ±0.02	0.1345	r_2 0.9953
3	COTY	17.42 ±0.01	14.91 ±0.01	25.39 ±0.008	27.24 ±0.03	0.0639	23.37 ±0.02	17.47 ±0.06	27.64 ±0.03	31.74 ±0.03	0.1326	r_1 0.9679
	E.A.	8.78 ±0.09	7.17 ±0.01	10.81 ±0.02	12.92 v0.02	0.1655	14.74 ±0.05	11.47 ±0.04	16.39 ±0.02	19.34 ±0.02	0.1304	r_2 0.9946
5	COTY	15.27 ±0.03	12.75 ±0.02	23.28 ±0.03	25.12 ±0.01	0.966	20.59 ±0.02	15.61 ±0.01	25.44 ±0.04	28.56 ±0.03	0.1082	r_1 0.9683
	E.A.	10.12 ±0.01	9.05 ±0.02	12.18 ±0.02	14.24 ±0.02	0.0611	17.48 ±0.06	15.43 ±0.03	20.42 ±0.02	23.64 ±0.04	0.1540	r_2 0.9834
7	COTY	12.20 ±0.02	10.90 ±0.04	19.14 ±0.02	22.88 ±0.04	0.1114	18.74 ±0.05	13.54 ±0.07	20.46 ±0.02	23.32 ±0.03	0.1690	r_1 0.9973
	E.A.	11.77 ±0.02	10.78 ±0.03	14.57 ±0.01	16.12 ±0.01	0.727	20.24 ±0.02	18.33 ±0.05	25.65 ±0.05	27.70 ±0.03	0.1493	r_2 0.5959

[a]Values in the table indicate Mean ± Standard Error. r_1 r_2 = Corr. coeff. between COTY and E.A. for cvs. TPT-1 and TPT-4, respectively.
[b]DAT = Days after treatment.
[c]T_1 = Control, T_2 = -1 MPa(PEG), T_3 = -1 MPa (PEG) + 20 mM $CaCl_2$, T_4 = 20 mM $CaCl_2$.
[d]C.D. = Critical difference at $P \leq 0.05$. All mean differences of treatments exceed C.D. and hence differ significantly at $P \leq 0.05$.

maintaining membrane stability. Calmodulin present in the stroma of pea (*Pisum sativum* L.) chloroplasts require Ca^{2+} for the activation of calmodulin-dependent enzymes (Jarret *et al.*, 1982). Increasing levels of CaM in organ and organisms decreased the cell division time by about 40% both in G1 and S phases, indicating the importance of CaM in cell growth and proliferation (Rasmussen and Means, 1989). The involvement of Ca^{2+}/CaM dependent kinases were reported in the seedling growth of black gram (*Vigna radiata* L.) (Rama Kumar and Prasad, 2001). On SDS-PAGE (12.5% gel), CaM showed a single band with 15 kDa molecular weight in cotyledons and embryonic axis of both the cultivars which was comparable to that of 16 kDa of Bovine brain CaM (Fig. 1). Amino acid composition of CaM isolated from two peanut cultivars showed 148 residues and is similar to the composition of the calmodulin extracted from different plant sources (Sreenivasa Rao and Savithramma, 1999). The concentration required for half maximal activation of phosphodiesterase by CaM isolated from cv. TPT-1 and cv. TPT-4 was 1.1 to 1.5 µM.

Calcium-Binding Proteins. In the present study a greater number of calcium-binding proteins were found in the embryonic axis than the cotyledons during seedling growth of two cultivars. The $CaCl_2$ treatment resulted in increased numbers of CaBPs, and PEG-induced water stress caused a decrease in the number of CaBPs during seedling growth of both cultivars. PEG-treated seedlings showed fewer CaBPs than the other two treatments including control seedlings. Ca^{2+} amendment to soil increases the number of CaBPs in peanut pegs (Savithramma and Swamy, 1995). Calcium-binding proteins are the potential receptors of calcium and mediate a number of cellular reactions (Cheung, 1980). Ca^{2+} binds

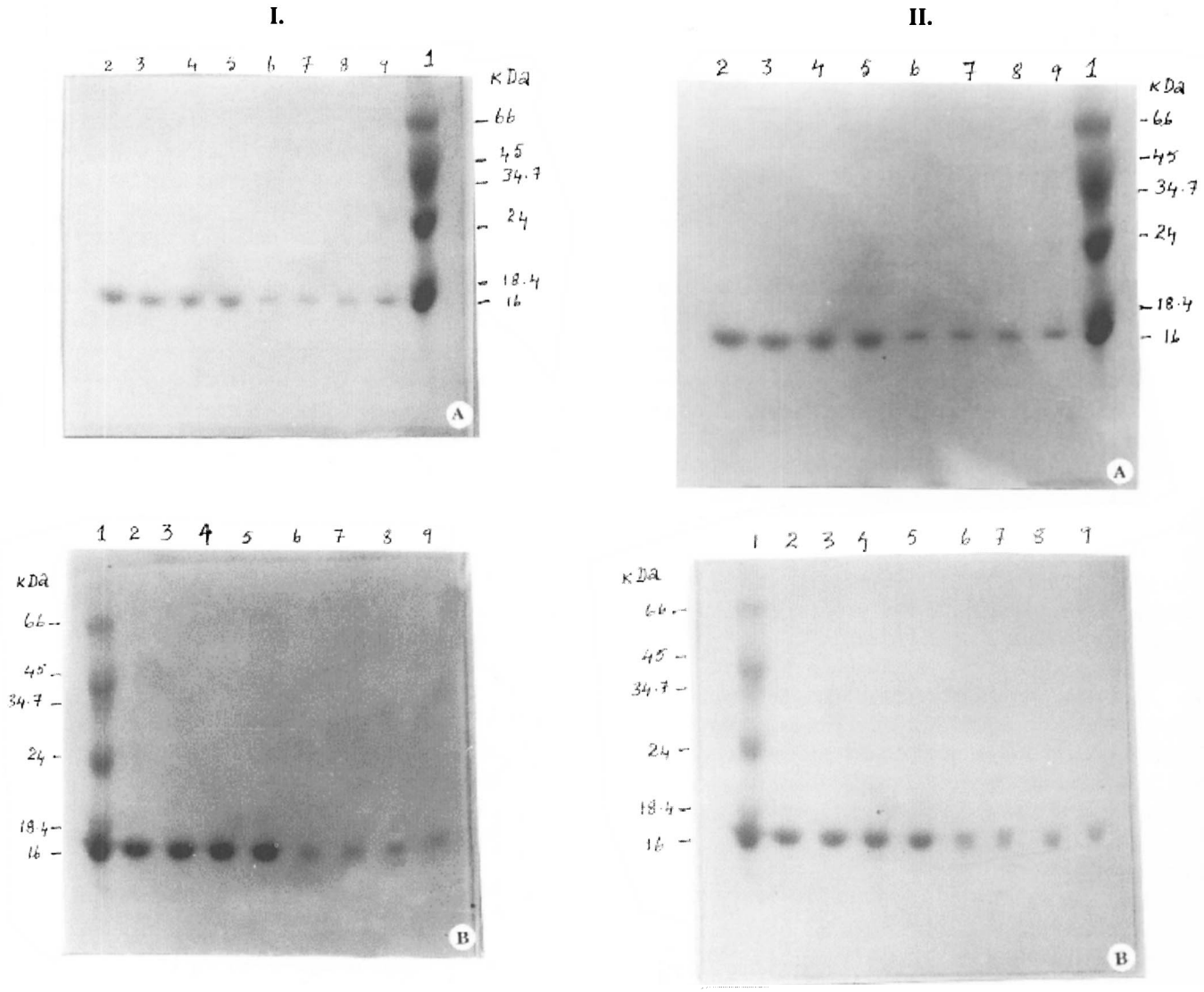

Fig. 1. I - 1st d and II - 7th d. SDS-PAGE separation of CaM purified from cotyledons and embryonic axis of two peanut cultivars during seedling growth after treatment. A = cv. TPT-1, B = cv. TPT-4. Lane 1 = molecular markers, 2 = cotyledons control, 3 = -1MPa PEG, 4 = -1MPa PEG+ 20 mM $CaCl_2$, 5 = 20 mM $CaCl_2$, 6 = Embryonic axis control, 7 = -1MPa PEG, 8 = - 1MPa PEG + 20 mM $CaCl_2$, 9 = 20 mM $CaCl_2$.

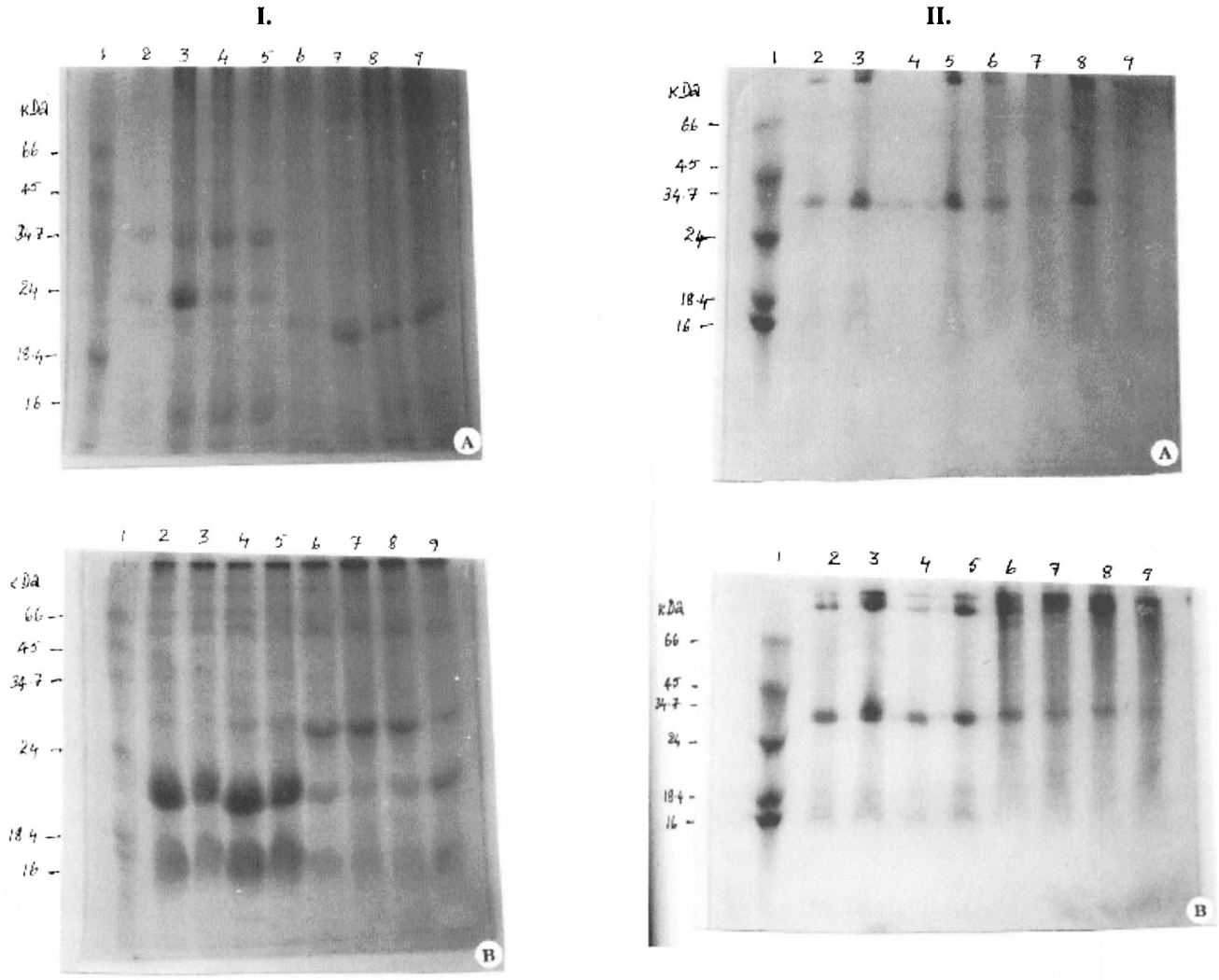

Fig. 2. I - 1st d and II - 7th d. SDS-PAGE separation of calcium-binding proteins purified from 1st d and 7th d of cotyledons and embryonic axis of two peanut cultivars during seedling growth after treatment. A = cv. TPT-1, B = cv. TPT-4. Lane 1 = molecular markers, 2 = control cotyledons, 3 = control embryonic axis, 4 = 1MPa PEG cotyledons, 5 = -1MPa PEG embryonic axis, 6 = -1MPa PEG + 20 mM $CaCl_2$ cotyledons, 7 = -1MPa PEG + 2 mM $CaCl_2$ embryonic axis, 8 = 20 mM $CaCl_2$ cotyledons, 9 = 20 mM $CaCl_2$ embryonic axis.

to the proteins whenever their concentration raises in the cytosol and forms Ca^{2+} protein complexes. These complexes would interact with proteins in the cell and cause alteration of functions.

The seedlings of the two cultivars chosen for the present study showed major differences between the treatments and cultivars with respect to CaBPs. Seedlings treated with PEG showed an extra 20 and 26 kDa CaBPs in cv. TPT-1 and 30 kDa, 68 CaBPs in cv. TPT-4. The $CaCl_2$ treated seedlings showed 33, 25, 22, and 18 kDa CaBPs in cv. TPT-1 and 28, 24, 23, and 18 kDa CaBPs in cv. TPT-4 were observed on SDS-PAGE separation (Fig. 2). The observations may be due to the differential expression of new proteins in the PEG-induced stressed and $CaCl_2$ treated seedlings, the remaining proteins could have been degraded, or the synthesizing machinery may have ceased to function. In conclusion, both CaM and CaBPs were decreased by water stress in the seedlings of peanut. Addition of Ca^{2+} to the stressed seedlings increased both types of proteins.

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