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Expression of late embryogenesis abundant proteins (LEA) in NaCl and ABA stress induced seedlings of *Morus indica* L.

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Abstract
Late embryogenesis abundant (LEA) proteins are involved in tolerance to drought, cold and high salinity in many different organisms. LEA proteins are well associated with the desiccation tolerance in organisms. Present study focused on analysis of protein profiles in 5 day-old germinating seedlings of *Morus indica* L. subjected to NaCl stress revealed that the protein of 21 kDa decreased in content with increase in the concentration of NaCl in contrast to seedlings grown on WPM containing NaCl where the 21 kDa protein was highly expressed at 100 and 150 mm. SDS-PAGE protein analysis in 5 day-old germinating seedlings subjected to ABA revealed that 21 kDa protein was expressed at low levels at all concentrations tested. On the other hand, the intensity of 21 kDa protein band increased when ABA was incorporated in WPM. Most of the LEA proteins have the characteristics of high hydrophilicity and thermo-stability. Hence present study provides a reference platform to understand their protective mechanisms during the adaptive response to desiccation in organisms.

Keywords: *Morus indica* L, LEA proteins, NaCl, ABA, SDS, Salinity, Stress

Introduction
As the name suggests, Late Embryogenesis Abundant proteins were originally discovered in the late stages of embryo development in cotton seeds (Dure, *et al*., 1981,Galaul *et al*., 1986) [11, 14]. In a broad sense the term 'stress' is used for any environmental factor potentially unfavorable to living organisms. The biological stress may be defined as any environmental factors capable of inducing a potentially injurious strain in living organisms. Stress is of two types. One is biotic stress and another one is abiotic stress. Abiotic stress, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment (Wang *et al*., 2003)[33].

Salinity is one of the most important factors limiting plant growth and yield. Salt stress is complicated because it induces both water deficit and specific salt damage (Munns *at al*., 2003)[23]. In addition, it also induces oxidative stress in plant tissues exposed to high salinity (Hernandez *et al*., 1995)[16]. The most prominent event of salt stress responses include transient adjustment of intracellular fluctuations in ionic balance (Wated*et al*., 1996;Binzel *et al*.,1988)[34, 3] and pronounced changes in metabolism of small molecular weight compounds like proline, betaine, sugars and ABA (Binzel*et al*., 1988) [3]. Another important consequence is the alteration in polypeptide profiles (Bruggeman and Janiesch, 1988; Singh *et al*., 1985; Ramagopal and Carr, 1991) [4, 29, 25]. These events reflect transient changes in polypeptide synthesis.

Sunder *et al*. (2003) [31] have analyzed the stress-associated protein in a high-rubber yielding guayule (*Parthenium argentatum* Gray cv. 11591) leaves. Dell'Aquila and Spada (2000)[9] howed that the expression of most polypeptides decreased following increasing stress in lentil seeds. Germination and subsequent hydrophonic growth under salt stress (100 mm NaCl) triggered an accumulation of six major stress proteins and resulted in a growth arrest of young seedlings of rice (Karuna*et al*. 2003)[18]. They identified the salt induced peptides as LEA proteins. In both cases many of the observed changes in gene expression were also induced by treatment with the plant hormone absisic acid (Bartels *et al*., 2005; Close *et al*., 1989; Piatkowski*et al*., 1990)[2, 6, 22]. One of the striking resemblances

between some of the proteins induced during stress conditions and during the onset of desiccation tolerance in seeds is their heat stability (Ried and Walker-Simmons, 1993)[26]. LEA proteins are a broad family of universal plant proteins, which accumulate at high levels during late stages of embryo development (Galau *et al*., 1986)[14]. Expression studies show that LEA proteins are generally associated with cellular dehydration in seeds and in response to water deficit in vegetative tissues. The expression of LEA genes was also induced by treating plant tissues with the plant hormone abscisic acid (Bartels *et al*. 2005) [2]. Typical for most LEA proteins is high hydrophilicity associated with solubility after boiling. LEA proteins in different plant species have been divided into groups based on predicted biochemical properties and motifs with significant sequence similarities (Cuming, 1999; Ingram and Bartels, 1996)[8, 17].

Characteristic for group 1 LEA proteins is a 20 amino acid motif. Group 2 LEA proteins, also refereed as dehydrins. Group 4 and Group 5 LEA proteins are less frequently represented.

Abscisic acid accumulates in seeds of many species during the development (Xu and Bewley, 1991)[37]. This growth regulator is important in preventing precocious germination, stimulating storage protein synthesis (Zeevaart and Creelman, 1988)[39] and inducing the synthesis of proteins which may protect seed embryos from injury during maturation drying (Skriver and Mundy, 1990) [30]. Osmotically active substances also increase in developing seeds, causing a decline in osmotic potential (Ryczkowski, 1998; Yeung and Brown, 1982)[27, 38]. Developing seeds or embryos often will not germinate when placed in osmotic solutions at high concentrations, but will germinate precociously on solutions at high (less negative) osmotic potential, or on water (Cook *et al*., 1988; Xu and Bewley, 1991)[7, 37]. In seeds of some species, osmoticum can replace ABA in promoting storage protein synthesis (Finkelstein and Crouch, 1986; Goffner *et al*., 1990)[13, 15] and some ABA responsive proteins can also be induced by high concentrations of osmoticum (Skriver and Mundy, 1990)[30].

Xu and Bewley (1995)[36] suggested that the co-existence in developing seeds of high concentrations of ABA and low osmotic potential makes it difficult to differentiate between their roles in seed development. In *Brassica*, the effects of osmoticum on accumulation of some storage proteins are mediated through an increase in ABA (Wilen*et al*., 1990)[35], but this is not universally accepted as its mode of action. The role that ABA plays in embryogenesis is ambiguous. Alone, it can not fully prevent developing pea embryos from completing germination events at a concentration of 10^{-4} M (Barratt *et al*., 1988)[1], and it is argued that ABA affects the embryo by controlling water uptake, which can also be achieved by osmotica (Finkelstein and Crouch, 1986)[13].

In the present investigation, stress induced polypeptides in germinating seedlings under NaCl and ABA stresses were studied. In addition, protein profiles during different stages of seed development were analyzed.

Materials and Methods

Seeds were collected from ripened fruits of mulberry cultivar S-36 established in plant culture facility of Department of Plant Sciences, University of Hyderabad. The seeds were surface sterilized in 70% ethanol for 4 min and rinsed 4-5 times with sterile distilled water. This was followed by treatment with 0.1% bavistin for 15 min and 0.1% mercuric chloride for 5 min under sterile conditions. The seeds were then rinsed 4-5 times in sterile distilled water with duration of 5 min each. The sterilized seeds were placed on Whatman No. filter paper in bottles containing 15 ml of NaCl and abscisic acid (ABA) individually in different concentrations, such as 50 mm NaCl, 100 mm NaCl, 150 mm NaCl, and 50 µM ABA, 100 µM ABA, 150 µM ABA, respectively. While the bottles containing NaCl are autoclaved, ABA was added to sterile bottles after filter sterilization. In another set of experiment, the sterilized seeds were placed on Woody plant medium (1980) with same concentrations as mentioned above. Protein profiles were analyzed from the extracts of mature seeds (0 day), and from the 5 day-old germinating seedlings grown in the presence of different concentrations of NaCl and ABA.As a control, 5 day-old germinating seedlings grown in the presence of GA3 alone or WPM supplemented with 3 mg/l GA3 was used. The observations on germination percentage shoot and root length were recorded after 5 days of culture in different concentrations of NaCl and ABA.

For analyzing the protein profiles during different stages of seed development, seeds were collected from mulberry cultivar-S36 (*Morus indica* L.) after 10, 15, 20 and 25 days after fertilization.

Protein Extraction

All the samples mentioned above were weighed 100 mg each and ground in a pre-chilled motor and pestle in 1ml of 50 mm Tris HCl buffer (pH 5.7) containing 5 mm $MgCl_2$, 2 mm K_2HPO_4, 1 mm EDTA, 5 mm DTT, 2 mm KH_2PO_4, 5 mm DTT, 2% PVP, 20% glycerol, 10 mm NaF, 10mm β-mercaptoethanol and 2 mm PMSF. After homogenization, the samples were centrifuged at 4°C for 20 min at 12,000 rpm. The supernatant was taken and soluble protein content was estimated by Lowry's (1951)[21] method with minor modifications as given below.

Protein estimation by Lowry's method

Solution A consisted of 4% Sodium carbonate in 0.2 N Sodium hydroxide. Solution B consisted of 1% Cupric sulphate, and Solution C was 2% Sodium potassium tartrate and Solution D was 1N Folin's reagent (commercial).The working solution was obtained by mixing solutions A, B, C in a ratio of 23:1:1 and this solution were used within 24 hr of preparation. One ml of the working solution was added to one ml of protein sample, mixed well and allowed to stand for 10 minute. Then 0.2 ml of solution D was added rapidly while vortexing the sample. After 30 minutes, absorbance of the sample was recorded at 650 nm. Bovine serum albumin (BSA Fraction V) was used as a standard protein (20-200 µg/ml).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed following the method of Laemmli (1970)[20] with minor modifications. The separation of proteins was performed in 5% stacking gel and 10% resolving gel. Both the resolving and stacking gel contained 2.4% bisacrylamide as a cross linker and 0.1% SDS. The final buffer concentrations were 0.45 M Tris HCl pH (8.9) in resolving gel and 0.2 M Tris HCl (pH 6.7) in stacking gel. Ammonium persulphate and N,N,N,N-tetra methylethylene diamine (TEMED) were used as polymerizing reagents in final concentration of 0.05% and 0.1%, respectively. The electrode buffer comprised of 0.0247 M Tris HCl and 0.19 M

Glycine and 0.1% SDS in one liter of distilled water (pH 8.3).The samples were mixed with sample buffer consisting of 0.5 M Tris HCl (pH 6.8) and boiled at 90°C for 3 min. The samples (10-20 µl) having 50 µg protein were loaded in slab gel wells of the gel of 8 x 8 x 0.1cm dimension which was polymerized in plain glass plates and was fixed to Broviga (India) vertical slab gel apparatus. Gels were run at room temperature at a voltage of 75 and 100 DC (direct current) for stacking and resolving gel, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached about 3-4 mm from the bottom of the gel. Then the gels were removed, and stained overnight with 0.25% w/v Commassie Brilliant Blue R 250 in methanol: glacial acetic acid: water (50:7:43) v/v. After staining the gels were destained with methanol: acetic acid: distilled water (50:12:38) until color is removed. The gels were then placed in water for storage. Medium range molecular weight marker (Bangalore Genei Pvt. Ltd.) was used for calibration.

Results and Discussion

In the present study, the germination percentage of the seed was affected by the concentration of NaCl when placed in bottles as well as WPM. Seeds placed on NaCl at 50-150 mm exhibited germination with a frequency varying from 63 to 26% in comparison to control seed that exhibited germination with a frequency of 70%.Germination percentage decreased from 76% to 55% with the increasing concentrations of NaCl on WPM. Seed grown in the presence of 50 µM ABA exhibited germination with a frequency of 6.6% where as the germination was totally suppressed at 100 µM and 150 µM ABA. Differential results were obtained when ABA was incorporated in the WPM. Germination was observed with a frequency of 51 to 32% on WPM containing 50-150 µMABA while the seeds cultured on WPM containing 3 mg/l GA3 germinated with a frequency of 91%. Growth inhibition under saline stress is one of the first responses described by many authors in intact plants and in tissue cultures (Ramagopal, 1990)[24]

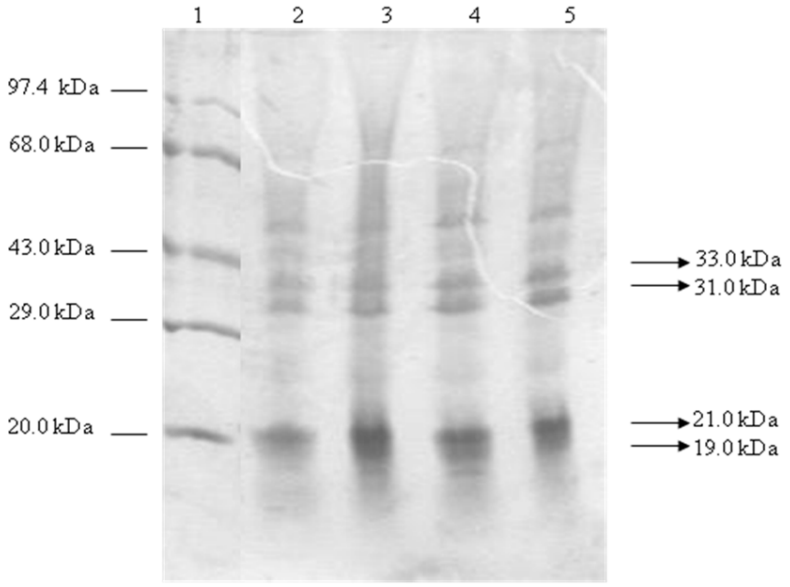

Fig1: SDS-PAGE analysis of protein extracts S-36 cultivar seed at different stages of development
Lane 1: Molecular weight marker in kilodaltons
*Lane 2-5:*Protein extract of seed collected after 10, 15, 20 and 25 days after fertilization

In order to cope with environmental stress, plants activate a large set of genes leading to the accumulation of specific stress-induced proteins that accumulate upon water, salinity, and extreme temperature stress. They have been shown to play a role in cellular protection during the stress (Close, 1997; Ingram and Bartels, 1996)[5, 17].

SDS-PAGE analysis of protein extract of S-36 cultivar seed at different stages of development revealed differences in the expression of few proteins (Fig. 1).Proteins of 31 and 33 kDa increased in content during later stages of seed development. The most striking difference was noticed with respect to 21 kDa protein, which increased in abundance at second stage of seed development and expressed at more or less at the same level during the subsequent stages of development. Kermode (1990)[19] contended that dehydration in developing seeds is a critical switch, from a developmental to germination program. The developmental switch is acquired at seed ages closer to full maturity, during which desiccation occurs and specific proteins accumulate. Filho*et al*. (2003)[12] studied the expression of SALT peptide, a 14.5 kDa mannose binding lectin in rice plants submitted to different stresses. High levels of expression of SALT protein were detected in sheath extracts treated with 170 mm NaCl, 170 mm KCl dehydration, wounding, heat (42°C) or abscisic acid. SALT protein was also detected in extracts from seeds collected at early stages of development.

Wang *et al*. (1999) [33] characterized a stress-induced protein (LEA) associated with desiccation in lily pollen. Skriver and Mundy (1990)[30], Shninozaki and Yamaguchi (1997)[28] identified various types of drought inducible proteins. Among these, dehydrins are known to accumulate immediately before desiccation during seed development (Close *et al*. 1989; Dure, 1993)[6, 10].It has been suggested that these proteins play a role in protecting plant structures during water loss. Most genes that respond to drought are also abscisic acid (ABA) responsive (Ingram and Bartels, 1996)[17].

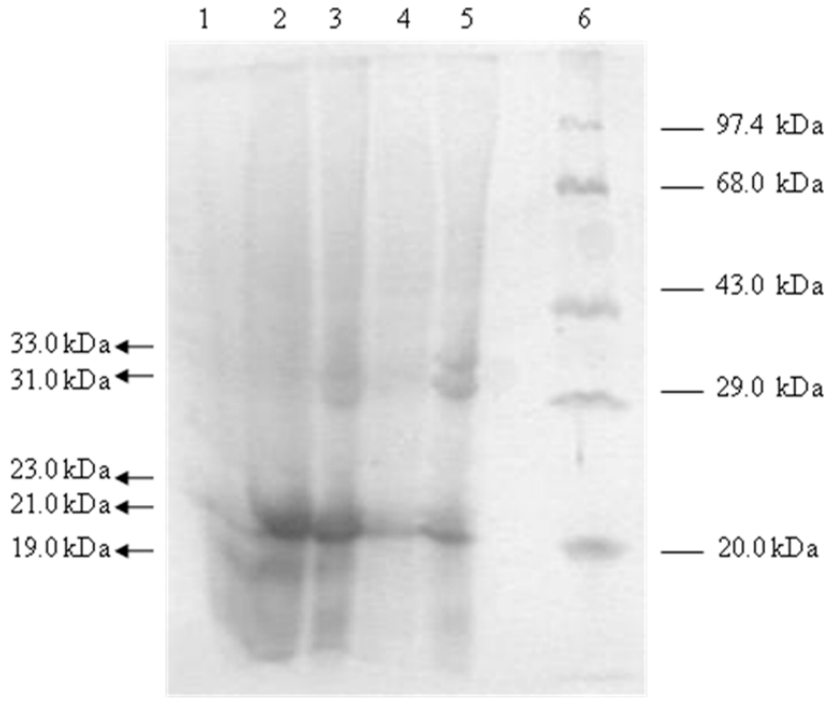

Fig2: SDS-PAGE of protein extracts of 5 day-old germinating seedlings of S-36 cultivar grown in the presence of3 mg/l GA3 (control), and different concentrations of NaCl

Lane 1: Mature seed of S-36 cultivar
Lane 2: Five day-old seedlings grown in the presence of 3 mg/l GA3
*Lane 3-5:*Five day-old seedlings grown in the presence of 50 mm,100 mm and 150 mm NaCl, respectively
Lane 6: Molecular weight marker in kilodaltons

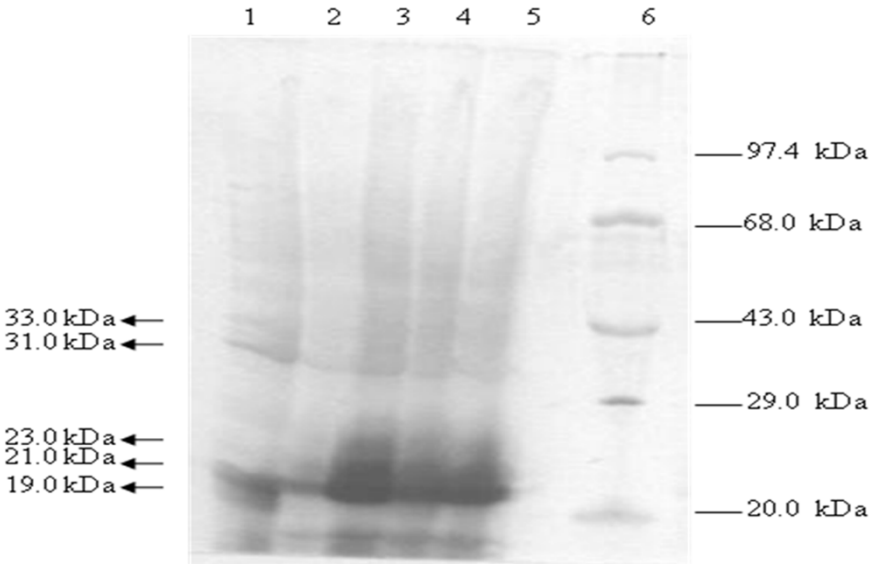

Fig3: SDS-PAGE of protein extracts of 5 day-old germinating seedlings of S-36 cultivar grown on Woody Plant Medium (WPM) containing 3 mg/l GA3 (control), and WPM containing different concentrations of NaCl

Lane 1: Mature seed of S-36 cultivar
Lane 2: Five day-old seedlings grown in the presence of 3 mg/l GA3
*Lane 3-5:*Five day-old seedlings grown in the presence of 50 mm,100 mm and 150 mm NaCl, respectively
Lane 6: Molecular weight marker in kilodaltons

SDS-PAGE analysis of protein extract of 5 day-old germinating seedlings grown in the presence of NaCl revealed differences in the expression of proteins in comparison to seedlings obtained on GA3 alone (Fig. 2).The content of 21 kDa protein was higher in 5 day-old seedlings grown in the presence of GA3 alone. There was no change in the intensity of 21 kDa protein band at 50 mm NaCl whereas at high levels of NaCl (100 and 150 mm), the relative intensity of 21 kDa protein band decreased. It was interesting to note that 31 and 33 kDa proteins expressed in greater amounts in the presence of 150 mm NaCl whereas at low concentrations of NaCl they were expressed in low amounts. On the other hand, the content of 21 kDa protein considerably increased in the 5 day-old seedlings grown on WPM containing 3 mg/l GA3 at all the concentrations of NaCl tested (Fig. 3).

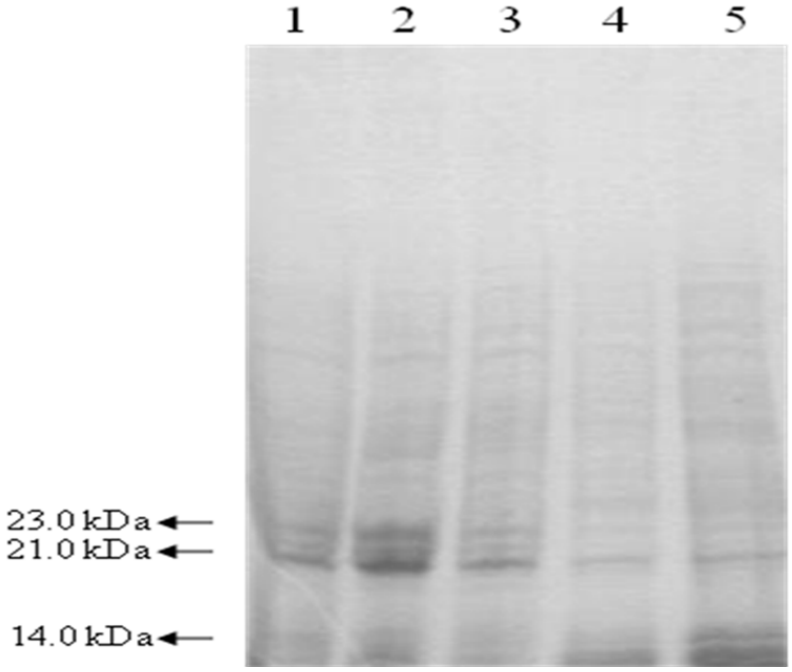

Fig4: SDS-PAGE of protein extracts of 5 day-old germinating seedlings of S-36 cultivar grown in the presence of different concentrations of abscisic acid (ABA)

Lane 1: Mature seed of S-36 cultivar
Lane 2: Five day-old seedlings grown in the presence of 3 mg/l GA3
Lane 3-5: Five day-old seedlings grown in the presence of 50 µM, 100 µM and 150 µM ABA, respectively

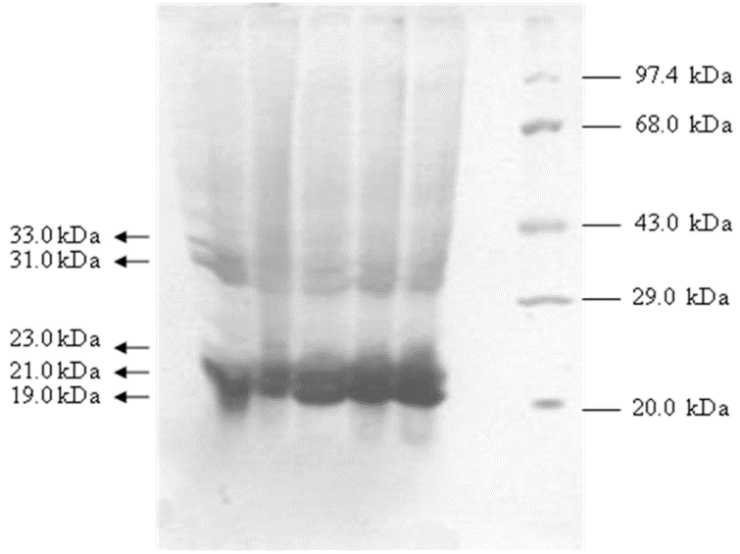

Fig5: SDS-PAGE of protein extracts of 5 day-old germinating seedlings of S-36 cultivar grownon Woody Plant Medium(WPM) with 3 mg/l GA3 (control), and WPM containing different concentrations of abscisic acid (ABA)

Lane 1: Mature seed of S-36 cultivar
Lane 2: Five day-old seedlings grown on WPM with 3 mg/l GA3
*Lane 3-5:*Five day-old seedlings grown on WPM containing 50 µM, 100 µM and 150 µM ABA, respectively

SDS-PAGE analysis of protein extract of germinating seedlings grown on different concentrations of ABA alone or on WPM containing different concentrations of ABA (50 µM, 100 µM and 150 µM) revealed differences in the expression of 21 and 23 kDa proteins. These proteins were expressed in relatively low levels in the 5 day-old seedlings grown on ABA at all the concentrations tested (Fig. 4).An increase in the intensity of 14 kDa protein bands was noticed in the seedlings grown on 150 µM ABA whereas the intensity of the protein band was low in the control seedlings as well as the seedlings grown on 50 and 100 µM ABA. On the contrary, an enormous increase in the level of 21 and 23 kDa proteins was noticed in seedlings grown on WPM containing ABA (Fig. 5).In isolated alfalfa embryos both osmoticum and ABA in the presence of an appropriate nutrient supply maintained the synthesis of developmental proteins (Xu, 1991)[37].Singh *et al*. (1985)[29] opined that the altered phenotype of cells with enhanced ability to survive and grow in the presence of high levels of NaCl is largely the result of altered gene expression. Thus, the first step in

understanding alterations of this type should be the establishment of a correlation between different gene products (mRNA or protein) and the degree of adaptation to NaCl. In tobacco cells adapted to various concentrations of NaCl, there was an increased accumulation of a 26 kDa polypeptide that has been named osmotin (Singh *et al*., 1985)[29].

Conclusion

Late Embryogenesis Abundantproteins (LEA proteins) are proteins that protect other proteins from aggregation due to desiccation or osmotic stresses. The multi-functional capacity of LEA proteins are suggested, as protein stabilization, protection of enzyme activity, membrane association and stabilization, antioxidant function, metal-ion binding or DNA protection, etc.

In the present study LEA proteins were expressed more during stress conditions than normal conditions. SDS-PAGE analysis of proteins during different stages of seed development showed that the content of 21 kDa protein increased during seed maturation. Analysis of protein profiles in 5 day-old germinating seedlings subjected to NaCl stress revealed that the protein of 21 kDa decreased in content with increase in the concentration of NaCl in contrast to seedlings grown on WPM containing NaCl where the 21 kDa protein was highly expressed at 100 and 150 mm. SDS-PAGE protein analysis in 5 day-old germinating seedlings subjected to ABA revealed that 21 kDa protein was expressed at low levels at all concentrations tested. On the other hand, the intensity of 21 kDa protein band increased when ABA was incorporated in WPM.

Here, we conclude that use of both genetic manipulation and traditional breeding approaches will be necessary to unravel the mechanisms involved in salinity tolerance and to develop salt-tolerant cultivars better able to cope with the growing soil salinity constraints.

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