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Comparative Response of Callus and Seedling of *Jatropha curcas* L. to Salinity Stress

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ABSTRACT : *Jatropha curcas* L. is an oil bearing species with many uses and considerable economic potential as a biofuel crop. Salt stress effect on growth, ion accumulation, contents of protein, proline and antioxidant enzymes activity was determined in callus and seedling to understand the salt tolerance of the species. Exposure of callus and seedling to salt stress reduced growth in a concentration dependent manner. Under salt stress Na content increased significantly in both callus and seedling whereas, differential accumulation in the contents of K, Ca, and Mg was observed in callus and seedling. Soluble protein content differed significantly in callus as compared to seedling, however proline accumulation remained more or less constant with treatments. The proline concentration was ~2 to 3 times more in callus than in seedling. Salt stress induced qualitative and quantitative differences in superoxide dismutase (SOD; E.C. 1.15.1.1) and peroxidase (POX; E.C. 1.11.1.7) in callus and seedling. Salt induced changes of the recorded parameters were discussed in relation to salinity tolerance.

Keywords : Antioxidant enzymes, *Jatropha curcas*, Seedling, Callus, Salt

INTRODUCTION

Soil salinity is an inevitable impediment for agriculture world wide. Excess salinity in the soil has devastating effects on plant growth, crop yield and even leading to complete crop failure in the worst affected areas (Owens, 2001). It is estimated that salinity limits the production of nearly 40% of agricultural lands world over (Serrano and Gaxiola, 1994). Growing demand for food and plant products to feed the expanding world population, with ever decreasing soil resources and dwindling fresh water supplies warrants the need for biological and technical solutions to overcome the physiological limitations, which restrict crop productivity (Reddy and Iyengar, 1999). This demands the extension of cultivation on uncultivable degraded/ saline soils, which are not suitable for conventional agriculture, identification of salt tolerant crops or increase salt tolerance in existing crops. Despite advances in increasing plant productivity and resistance to number of pests and diseases, improving salt tolerance in crop plants remain elusive, mainly because salinity simultaneously affects several aspects of plant physiology. To achieve salt tolerance three interconnected aspects of plant activity are important; (1) damage must be prevented, (2) homeostatic conditions must be re-established and (3) growth must resume (Zhu, 2001). Salt stress causes ionic imbalance (Zhu et al., 1997), with excess sodium and chloride ions having a deleterious effect on many cellular systems (Serrano et al., 1999) therefore, plant survival and growth depends on adaptations to re-establish homeostasis, osmoregulation and oxidative damage (Cherian and Reddy, 2003; Elkahoui et al., 2005; Niknam et al., 2006). To cope up with salt stress, plant responds with physiological and biochemical changes (Hernandez et al., 1993; Lutts et al., 1996; Cherian and Reddy, 2003; Elkahoui et al., 2005; Niknam et al., 2006). These changes aim at retention of water in spite of high external osmoticum and maintenance of metabolic activities (Hasegawa et al., 2000).

In vitro culture technique avoids physiological and structural complexities of the plant (Bajji et al., 1998) and

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provides an important tool for studying salt effects and its mechanisms in plants (McCoy, 1987; Chen et al., 1998). It is also reported that both cellular and whole plant mechanisms are equally important in elucidating salt tolerance mechanisms (Dracup, 1991; Bajji et al., 1998; Niknam et al., 2006). However, there were relatively few studies comparing responses of intact higher plants and their tissues (Dracup, 1991; Naik and Widholm, 1993; Gulati and Jaiwal, 1994), even fewer addressed physiological changes during salt stress (Cherian and Reddy 2003; Elkahoui et al. 2005; Niknam et al., 2006). Plant cell and tissue culture studies are also relevant to crop improvement because they offer a means of rapid selection and development of salinity resistant crops. (Cherian and Reddy, 2003; Elkahoui et al., 2005).

Jatropha curcas (physic nut) is a multipurpose species belongs to the family Euphorbiaceae and distributed in arid and semi arid areas of South America and all tropical regions. *J. curcas* recently received tremendous attention for its seed oil that can be converted into biodiesel and is considered to be a universally acceptable as an energy crop and alternative to conventional fossil fuels (Takeda, 1982; Mandpe et al., 2005). The species grows in areas with extreme climate and soil conditions that could not be inhabited by most of the agriculturally important plant species (Francis et al., 2005). However, there are no reports on existence of *J. curcas* on saline soils or studies related to salt tolerance either at whole plant or at cell level. Therefore, the present study was undertaken to evaluate the responses of *J. curcas* to salt stress both at cellular and whole plant level by studying the changes in the activity of antioxidative enzymes, accumulation of ions and proline to assess the possibility of improving salt tolerance in the species.

MATERIALS AND METHODS

Callus culture and growth determination

Callus of *J. curcas* were produced from in vitro grown leaf on MS (Murashige and Skooge, 1962) medium (pH 5.8) containing 0.70% agar, 5 mg/L 6-benzyl amino purine (BAP) and 1 mg/L naphthalene acetic acid (NAA) and sterilized by autoclaving at 1.05 kg/cm^2 pressure and 121℃ for 20 min. Friable callus were sub cultured for every 4 weeks after initiation and grown for further 4 weeks before being used for salinity tolerance studies. NaCl was used as salt in all experiments. At the beginning of experiment, callus (100 mg) was transferred to fresh medium amended with different concentrations of salt (0 (control), 20, 40, 60, 80, 100 mM). The callus was grown at 25± 2℃ in dark and was harvested after four weeks of growth. Callus was washed thoroughly with distilled water to remove the adhering ions and fresh mass (fm) was taken after removing the moisture by blotting. Dry mass (dm) was determined after drying the callus at 60℃ for 48 h to a constant mass.

Germination percentage and seedling growth determination

100 uniform seeds of *J. curcas* collected from a single plant, were surface sterilized with 0.1% mercuric chloride solution and then thoroughly washed with distilled water and germinated in Petri dishes lined with double layer of Whatman No. 1 filter paper. The petri dishes were moistened with 20 ml of different concentrations (0, 20 40, 60, 80, and 100 mM) of salt and maintained in dark at 28±2℃. Seeds were considered germinated when the radicle was at least 5 mm long. The experiment was continued for 10 days. Germination percentage was calculated 10 days after the beginning of experiment using the equation: final germination percentage = number of germinated seeds/ total number of seeds planted × 100. Full grown seedling of 10 cm long, were transferred to 30 plastic containers, each with five seedling, after a week the containers were randomly arranged and treated with 300 ml of Hoagland nutrient solution supplemented with above said concentrations of salt (Haogland and Arnon, 1950) and allowed to grow in controlled condition (25±2℃ temperature, 16 h photoperiod at a photon flux intensity of 46 μmol m^{-2} s^{-1} and 85% relative humidity) and the plants were continuously aerated. The nutrient solution was changed after

every week. From each treatment fresh mass and increased shoot length were measured after 6 weeks. For dry mass determination, the seedling were dried at 60℃ for 48 h to a constant mass and weighted.

Determination of ion content

Oven dried callus and seedling were digested in 10 ml of HNO_3:$HClO_4$ (10:4) on hot plate in a fume hood. After digestion the solution was filtered and final volume was adjusted to 50 ml and analyzed for Na, K, Ca and Mg using inductively coupled plasma emission mass spectroscopy (ICP, Perkin-Elmer).

Proline extraction and estimation

Proline was extracted and determined following the method described by Bates et al. (1973) using L-proline as standard. 200 mg each of fresh callus and seedling were homogenized in 4 ml of 3% aqueous sulphosalicylic acid and centrifuged at 10,000 rpm for 10 min to remove debris. 2 ml of the supernatant was mixed with 2 ml of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 ml of 6 M orthophosphoric acid) and 2 ml glacial acetic acid in a test tube and boiled at 100℃ for 1 h. The reaction was stopped by cooling the tubes in ice bath. The chromophore formed was extracted with 6 ml of toluene and the absorbance of resulting organic layer was measured at 520 nm (Shimadzu UV-160A, Japan). The concentration of proline was estimated by referring to a standard curve for L-proline.

Enzyme extractions and assays

Callus and seedling (100 mg) were homogenized at 4℃ with 2 ml of extraction buffer [(200 mM Tris-HCl, 10 mM ethylenediaminetetraaceticacid (EDTA), 0.5 M sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0% insoluble polyvinylpyrrolidone (PVP) (pH 8.5)]. The homogenate was sonicated for 5 min and centrifuged at 15,000 rpm for 15 min. The supernatant obtained was used for estimation of protein content (Bradford, 1976) and enzymes activity.

The activity of superoxide dismutase (SOD; E.C. 1.15. 1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 ml of enzyme extract. Samples were illuminated by two 15 W fluorescent lamps for 10 min. The absorbance of reaction mixture was read at 560 nm. A non-irradiated reaction mixture served as control. Log A_{560} was plotted as function of the volume of enzyme extract used in the reaction mixture (Giannopolitis and Ries, 1977). From resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to Shannon et al. (1966) by following the change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of H_2O_2 and enzyme. The assay mixture consisted of 2.8 ml of O-dianisidine-buffer (16 ml of 0.5% (w/v) o-dianisidine, 48 ml of 0.6 M sodium acetate, pH 5.5, and 416 ml of H_2O), 0.1 ml H_2O_2 (1% w/v) and 0.1 ml enzyme extract. The amount of enzyme required to change the absorption (Δ OD) by 0.01 min^{-1} mg^{-1} protein was taken as unit enzyme activity.

Separation of isoenzymes of SOD and POX was performed by Native PAGE (7.5% polyacrylamide) by loading of 50 μg of protein for POX and 100 μg for SOD. The gels were run in electrode buffer composed of 0.025 M Tris and 0.192 M glycine (pH 8.8) for 3 h at 4℃ at a constant current of 30 mA. After electrophoresis gels were stained for SOD (Misra and Fridovich, 1977) by incubating in a solution containing 2.5 mM NBT for 25 min, followed by incubation in 50 mM potassium phosphate buffer (28 μM riboflavin and 28 mM EDTA, pH 7.8) for 20 min in dark and then exposed to light for 15 min.The gels were stained for POX (Gulati 1989) by incubating in 0.02 M acetate buffer (pH 5.5) for 10 min,

then transferred to fresh 0.02 M acetate buffer (pH 5.5) containing 0.66% guaiacol. After 5 min of incubation 3% H_2O_2 is added drop wise till desired contrast was obtained and reaction was stopped by replacing the solution with distilled water.

The data are based on a mean of minimum five independent experiments. All the data were subjected to two way analysis of variance and significance was determined at 95% confidence level.

RESULTS

Effect of salt on germination percentage and growth of callus and seedling

Salt inhibited seed germination and seedling growth at all concentration and the inhibition was proportional to salt concentration used. At lower concentration (20 mM) seed germination was not effected, however, at 40, 60, 80, and 100 mM concentrations seed germination inhibited by 15, 25, 41, and 46% respectively as compared to control (Table 1). Fresh mass and dry mass of both callus and seedling also decreased gradually at all salinity levels and inhibition was proportional to salt concentration in the medium. The decrease in fresh mass was 18, 56, 70, 72, and 74%, and for dry mass it was 43, 77, 85, 83, and 84% respectively as compared to control in callus. Whereas, in seedling the decrease was 32, 49, 52, 76, and 85% for fresh mass and 44, 61, 50, 72, and 75% for dry mass respectively at 0, 20, 40, 60, and 100 mM salt concentration as compared to control (Fig. 1 a&b). Shoot growth of seedling also showed a similar trend as shown for fresh and dry mass (Table 1).

Table 1. Effect of salt stress on seed germination and shoot growth in seedling of *J. curcas*.

NaCl (mM)	Germination percentage	Increased shoot length (cm)
0	88.75±6.70	3.81±0.81
20	88.73±5.71	3.75±0.55
40	75.82±4.71	3.10±0.76
60	70.89±4.11	1.82±0.51
80	52.25±4.70	1.16±0.28
100	47.50±4.81	0.62±0.13

Each represent mean±S.E. of n=25

Effect of salt on ion accumulation

Salt provoked a dose dependent increase in Na ion accumulation in both callus and seedling and accumulation was ~1 to 1.5 times higher in seedling as compared to callus (Fig. 2a). K content decreased in callus, where as, in seedling it increased however, concentration was 2 to 3 times higher in callus than seedling at low salt concentration (Fig. 2b). The Na/K ratio increased gradually with increasing concentration of salt both in callus and seedling and ratio was ~2 times higher in seedling (Fig. 2e). Ca accumulation decreased gradually in callus whereas, in seedling it increased up to 60 mM then decreased

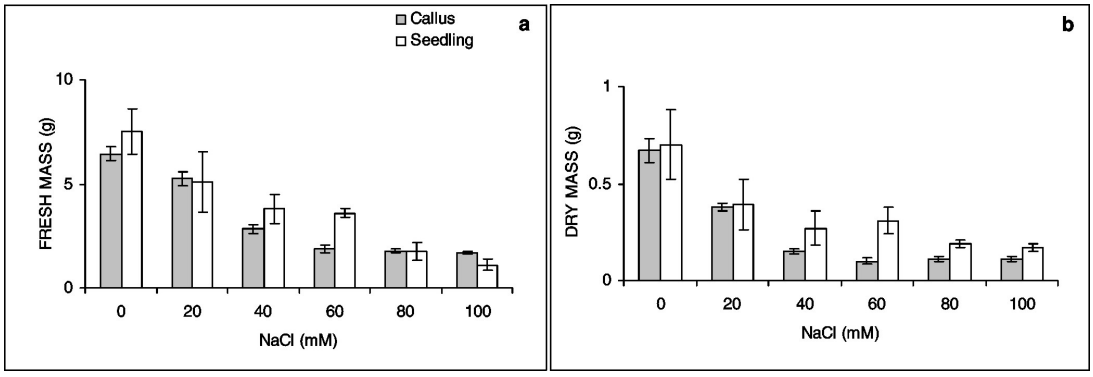

Fig. 1. Effect of salt stress on fresh mass (a) and dry mass (b) of *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

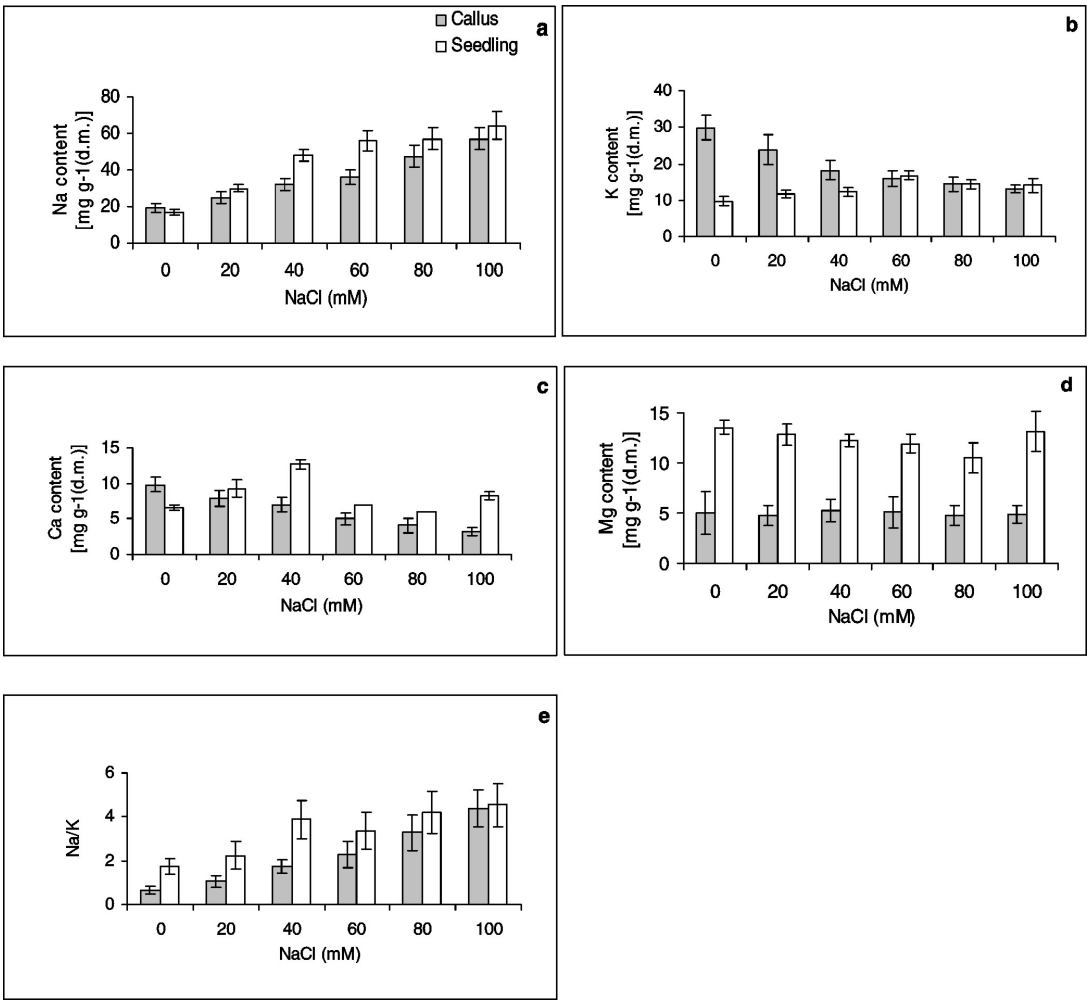

Fig. 2. Effect of salt stress on Na (a), K (b), Ca (c), Mg (d) and Na/K (e) accumulation in *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

however, accumulation was more in seedling than callus (Fig. 2c). Mg content also decreased with increasing salt concentration in both callus and seedling; however, Mg concentration was ~2 to 3 times higher in seedling than callus (Fig. 2d).

Effect of salt on protein and proline accumulation

Salt stress resulted in accumulation of soluble proteins in callus up to 80 mM and decreased at 100 mM, where as in seedling protein content remained more or less constant at all the salinity levels. The increase in protein content in callus was 21, 45, 63, and 24% at 20, 40, 60, and 80 mM salt concentrations respectively (Fig. 3a). No significant change was observed in proline content at all salinity level in both callus and seedling. Proline and protein accumulation was ~2 to 3 times more in callus as compared to seedling (Fig. 3b).

Effect of salt on the activity of antioxidant enzymes

The activity of both SOD and POX was determined by both spectrophotometric assay and gel staining following native PAGE. SOD and POX activity increased as function of external salinity in callus whereas, in seedling the activity gradually decreased at all salt concentration. SOD activity increased ~1.5 to 2 fold at 40, 60, and 80 mM salinity level and decreased slightly at 100 mM as com-

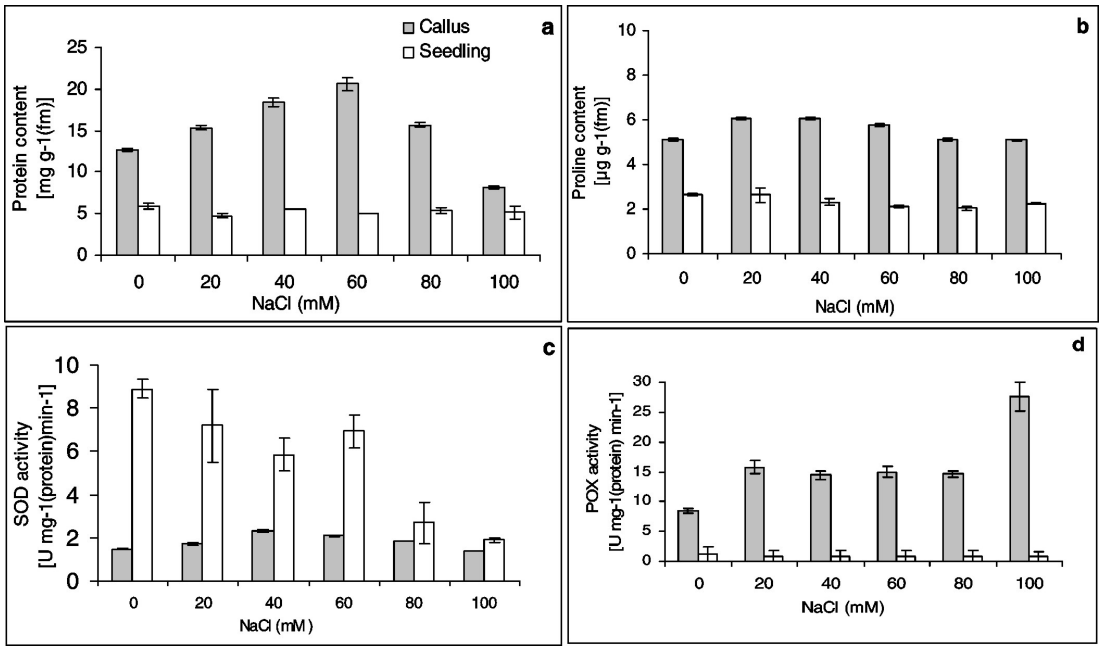

Fig. 3. Effect of salt stress on protein (a) and proline content (b), SOD (c) and POX (d) activity in *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

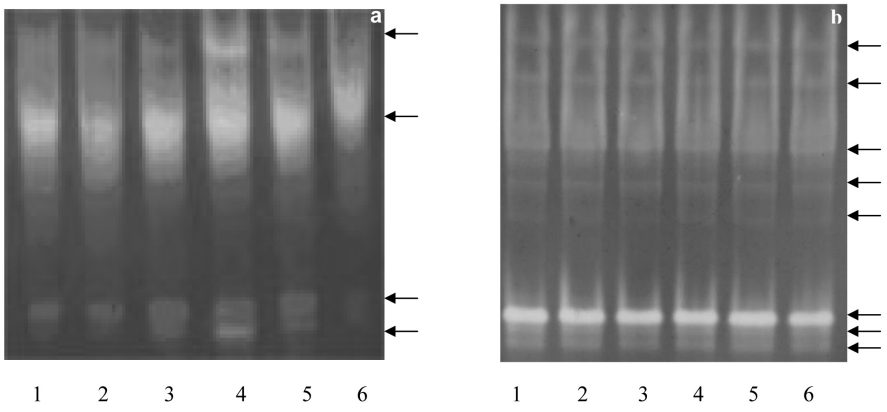

Fig. 4. Effect of salt stress on SOD isoenzymes separation in callus (a) and Seedling (b) of *J. curcas*: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and 100 mM NaCl treated callus and seedling respectively.

pared to control in callus whereas, in seedling the decrease was 1.2, 1.5, 1.2, 3.2, and 4.7 fold at 20, 40, 60, 80, and 100 mM concentration respectively and the activity was ~2 to 4 times higher in seedling as compared to callus (Fig. 3c). In native PAGE separation, four SOD isoenzymes were observed with salt treatment as compared to three isoenzymes in control callus (Fig. 4a). In case of seedling eight isoenzymes were observed in both control and salt treatments (Fig. 4b). The POX activity increased 2 to 3 fold due to salinity stress in callus in contrast to ~1.5 to 2 times decrease in seedling (Fig. 3d). In callus native PAGE two isoenzymes were observed due to salt treatment as compared to one isoenzyme in control cells (Fig. 5a), whereas, five isoenzymes were recorded in seedling irrespective of salt treatment (Fig. 5b). In callus both SOD and POX isoenzyme were in agreement with spectrophotometric assay but in seedling no relation between activity and intensity of band was observed.

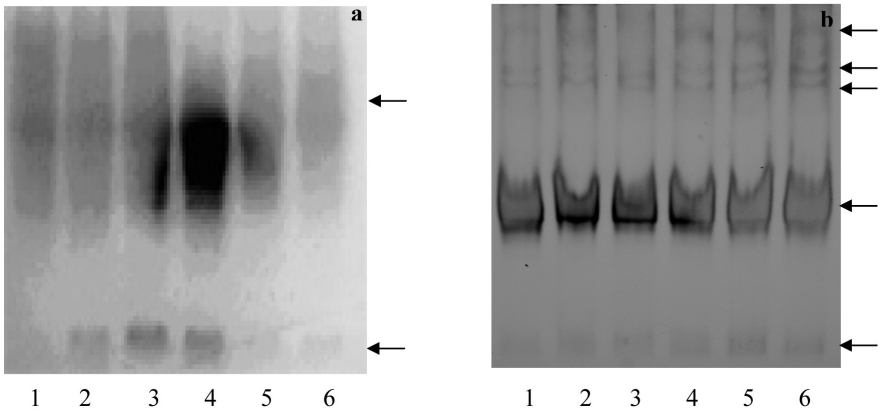

Fig. 5. Effect of salt stress on POX isoenzymes separation in callus (a) and seedling (b) of *J. curcas*: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and 100 mM NaCl treated callus and seedling respectively.

DISCUSSION

In the present study salt stress provoked an inhibition of seed germination, growth, fresh and dry mass accumulation and both callus and seedling behaved in a similar manner to salt stress. Decrease in seed germination, fresh mass and dry mass due to salt stress was reported in different species (Niknam et al., 2004, 2006). Comparison of salt tolerance of whole plant with that of cell cultures derived from indicated that for certain species salt tolerance was similar for whole plant and cell lines indicating salt tolerance is based on intrinsically cellular process (Tal et al., 1978). Cherian and Reddy (2000, 2003) have shown that salt tolerance at whole plant is substantially greater than cell lines. According to Flowers et al. (1985) and McCoy (1987) cellular tolerance did not always correlate with whole plant tolerance. A negative correlation where, the whole plant is salt tolerant and isolated cells are salt sensitive was regarded as indicator for the operation of the mechanism depending upon organization of cells in whole plant. Hence the salt tolerance trait selected at cell level may not express in whole plant and subsequent sexual progenies, where the whole plant and cultured cells both are salt sensitive or tolerant is interpreted as indicator for the operation or lake of operation of similar mechanisms on both levels (Gulati and Jaiwal, 1994). More or less similar response observed in whole plant and cell cultures in the present study indicates the salt tolerance mechanism operating in whole plant and cell are similar. The decrease in growth was associated with an increase in Na concentration both in callus and seedling, demonstrate that response of this species to salt stress is linked to Na accumulation. Excessive accumulation of Na causes ion imbalance and metabolic disturbances (Cherian and Reddy, 2003; Elkahoui et al., 2005). K content was higher in callus as compared to seedling at all salinity level. The decrease in Ca accumulation observed in our study may be due to displacement of Ca from cell membrane by Na (Cramer et al., 1985; Shibli et al., 2007). Decreased Mg accumulation under saline condition is in agreement with earlier results (Shibli et al., 2007) and the higher concentration of Mg observed in seedling than the callus indicated the requirement of Mg for chlorophyll formation. The differences observed in various ions content in callus and seedling can be attributed to their growing conditions and nature of the plant. The imbalance in ion uptake and Na/K ratio in callus and seedling might have disturbed the growth of callus and seedling (Cherian and Reddy, 2003; Elkahoui et al., 2005). Protein content in callus and seedling showed dissimilar trend. In seedling no significant change was observed whereas, in callus it increased up to 80 mM and then decrease at 100 mM salt concentration. Cherian and Reddy (2003) and Nikman et al. (2004, 2006) also reported similar trend in callus cultures of *Suaeda nudiflora*, *Nicotiana tabacum* and *Tigonella species*. The decreased protein content at high salinity observed in

callus cultures may be due to the decrease in the synthesis of protein (Hall and Flowers, 1973), whereas, the increase in protein content at 20, 40, 60 and 80 mM may be due to stress induced synthesis of proteins (Cherian and Reddy, 2003; Niknam et al., 2006). Although accumulation of proline as a compatible solute has been reported for several plant species (Cherian and Reddy, 2000; Maggio et al., 2000; Cherian and Reddy, 2003; Niknam et al., 2006), the increase in proline pool upon salt treatment does not seem to be a general phenomenon (Stewart and Lee, 1974) and the significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Meloni et al., 2004). No significant differences observed in both callus and seedling due to salt stress in the present study is in agreement with the results of Lutts et al. (1996) and Lacerda et al. (2001). Since *J. curcas* is very succulent material with approx 90% water, the proline content estimated may not be actual, due to dilution effect of xylem sap the real concentration of cellular proline might have been under estimated (Maggio et al., 2000). Salt stress provoked a dose dependent increase in SOD activity in callus but decreased in seedling however the activity was higher in seedling than callus at all salinity level. Cherian and Reddy (2003) also reported increased SOD activity in seedling and callus cultures of *S. nudiflora* under saline conditions. Higher POX activity observed in callus than seedling indicates that cells have a higher capacity for the decomposition of H_2O_2 generated by SOD in callus than whole plant (Cherian and Reddy, 2003; Niknam et al., 2006). Salt stress induced synthesis of new isoenzymes in both callus and seedling under salt stress are in conformity with the results of Elkahoui et al. (2005) and Niknam et al. (2006). The differences in the isoenzymes pattern of SOD and POX observed between seedling and callus suggest tissue specific isoforms. The qualitative and quantitative differences observed in the present study might be a useful adaptation in the defense mechanism of *J. curcas* against active oxygen species. However salt induced changes observed in antioxidant enzymes activity may not be strong enough to eliminate all the deleterious effects provoked by salt, only alleviated the impact of stress, thus allowing cell/plant growth to occur. Many similarities observed between callus and whole plant suggests the existence of cellular based salt induced response in *J. curcas*. However, the differences in the isoenzymes pattern of SOD and POX observed between callus and seedling suggest tissue specific expression of isoenzymes which needs further studies.

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