

ORIGINAL ARTICLE

Role of Exogenous Proline in Ameliorating Salt Stress at Early Stage in Two Rice Cultivars

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The study evaluated the effect of proline on germination and seedling growth of two Malaysian rice cultivars (MR220 and MR232) under salt stress. The exposure of rice seeds to increasing concentration of NaCl (0, 100, 200, 300 and 400 mM) had drastically affected germination (%), root and shoot length (mm), chlorophyll content and protein content. It is evident from the result of inhibition in germination rate, reduction in root and shoot length, chlorophyll content and protein content. However, several studies have shown that exogenous application of proline has ameliorated the negative effect of salt stress by regulating cellular osmotic balance. The present study has demonstrated that rice seeds pretreated with proline (1mM, 5mM and 10mM) and grown at different NaCl concentrations counteracted the adverse effect of salt. Pretreatment of proline at a concentration of 1mM was found to be effective and stimulated cellular activities, whereas 10mM proline was ineffective in improving plant growth under high level of salt (300 and 400mM NaCl).

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Plants are exposed to several abiotic stresses during its growth and development. Among the abiotic stresses, salt stress drastically affects crop growth and poses a major threat to agricultural productivity worldwide (Epstein *et al.*, 1980; Munns, 2002; Flowers, 2004). In most crop species, stress usually inhibits seed germination, seedling growth and vigor, flowering and fruit set (Zeinali *et al.*, 2002; Sairam and Tyagi, 2004). In general, high

level of salt in soil causes imbalance in osmotic potential; ionic equilibrium and nutrient uptake (Niu *et al.*, 1995; Munns, 2002). Further, it facilitates severe ion toxicity by depositing high concentration of Na⁺ which causes membrane disorganization, inhibition of cell division and expansion. In addition, it also impairs a wide range of cellular metabolism including photosynthesis, protein synthesis and lipid metabolism (Alia-Mohanty and

Saradhi, 1992; Ashraf, 1994; Zhu, 2001; Parida and Das, 2005; Lichtenthaler *et al.*, 2005).

Plants have been classified as salt sensitive (glycophytes) and salt tolerant (halophytes) based on their ability to grow on salinity. Glycophytes employ different strategies to respond and adapt to stress. One of the prime responses to salinity stress is that it restricts plant water uptake efficiency and limits water potential (Chaum *et al.*, 2004). An imbalance in osmotic potential would result in loss of turgidity, cell degradation and consequently cell death (Cicek and Cakirlar, 2008). However, to prevent water loss from the cell and to sustain cellular functions, plants synthesis and accumulate a number of compatible solutes called "osmolytes". These osmolytes include proteins, carbohydrates, amino acids and quaternary ammonium compounds (Rontein *et al.*, 2002; Ashraf, 2004; Ashraf and Harris, 2004). It is assumed that under stress condition, these osmolytes protect the subcellular structure either by regulating cellular osmotic potential or by scavenging reactive oxygen species (ROS). ROS are highly reactive and can seriously disrupt normal metabolism of the plant through oxidation of membrane lipids, proteins and nucleic acids (Noctor and Foyer, 1998; Hernandez *et al* 2001; Arafa *et al.*, 2009).

Several strategies have been proposed to alleviate the degree of cellular damage caused by abiotic stress and to improve crop salt tolerance. Among them, exogenous application of compatible osmolytes such as proline, glycinebetaine, trehalose, etc., had gained considerable attention in mitigating the effect of salt stress (Ashraf and Foolad, 2007). Under stress condition, exogenous proline application improved tolerance of somatic embryos of celery (Saranga *et al.*, 1992) and tobacco cell culture (Okuma *et al.*, 2000). Further, foliar spray of proline counteracted the growth inhibition induced

by NaCl in rape seed (Makela, *et al.*, 2002), rice (Rahman *et al.*, 2006), wheat (Raza *et al.*, 2006) and maize (Ali *et al.*, 2007). Studies have shown that exogenous proline application effectively regulates osmotic potential and plays a vital role in sustaining plant growth under osmotic stress (Serraj and Sinclair, 2002; Ali *et al.*, 2007; Ashraf and Foolad, 2007; Hoque *et al.*, 2007).

Rice is highly sensitive to salinity and its tolerance varies with growth stages. For example, seed germination and seedling growth stages are very sensitive to abiotic stress. It is opinioned that selection of plants at early stages, *viz.*, either at germination or at seedling stage would improve the tolerance by establishing good crop stand (Munns 2002; Cuartero *et al.*, 2006). However, the information pertaining to the role of exogenous proline on germination and early seedling growth is limited. Hence the present study was initiated to examine the effect of exogenous application of proline on germination and seedling growth of rice under salinity stress.

MATERIALS AND METHODS

Seeds of two Malaysian rice cultivars MR220 and MR232 were surface sterilized in 2% sodium hypochlorite solution for 10 min. The seeds were placed in Petri dishes, each Petri dish consists of 50 seeds. The experiment was conducted in two subsets. In one set, the rice seeds were allowed to germinate in different concentration of NaCl (0, 100, 200, 30 and 400mM). In the other set, the seeds were pretreated in three levels of proline (1, 5 and 10mM) for 12 h and transferred to Petri dishes containing 0-400mM range of NaCl. All the seeds were allowed to grow for 10 days at $25 \pm 2^\circ\text{C}$ temperature. The observations were recoded on the parameters such as roots and shoot length, chlorophyll content, proline and protein content for

the following treatment to study the influence of proline pretreatment in mitigating salinity stress in rice.

- Control
- NaCl concentration (0, 100, 200, 30 and 400mM).
- NaCl concentration + 1mM proline
- NaCl concentration + 5mM proline
- NaCl concentration + 10mM proline

Plant growth measurement

Germination percentage was recorded for the two set of experimentation on 5th day after planting. Seedlings were harvested on the 10th day and their roots and shoot length were measured and recorded.

Chlorophyll content

Total chlorophyll, chlorophyll *a* and chlorophyll *b* were determined for control, NaCl treated and pretreated proline seeds grown under salinity by following the method described by Harbone (1984). Fresh leaves (250mg) were homogenized in 80% acetone at 4°C. The extract was centrifuged at 10,000x g for 5 min. Absorbance of the supernatant was read at 646nm and 663nm using a spectrophotometer. The amount of chlorophyll in the leaf tissue was expressed as mg/g FW.

Estimation of free proline content

Free proline content of control, NaCl treated and pretreated proline seedlings were determined by following the method of Bates *et al.*, (1973). Leaf samples of 0.5g were used for proline extraction. Optical density of the sample extract was determined spectrophotometrically at 520nm. The concentration of proline was expressed as $\mu\text{mol g/FW}$ using a standard curve of pure proline.

Estimation of soluble protein content

Total soluble protein was determined by following the method of Bradford (1976) for the two sets of experiment along with the control. Fresh plant material of 0.5g was homogenized in 5ml of

extraction buffer (50mM Tris-HCl; pH-8, 1mM PMSF, 10% (v/v) glycerol) and centrifuged. Aliquot of the extract was used for determining protein concentration using bovine serum albumin (BSA) as a standard. SDS-PAGE was performed according to Laemmli (1970) and the gels were stained with 0.03% Coomassie Brilliant Blue.

Data analysis

The experiments were performed in completely randomized design with three replicates. Differences among the treatment as well as between the cultivars were tested using the SPSS software program (Version13.0). Data were subjected to Levene's Test to test the equality of error variances. The test has shown that the error variance of dependent variables is equal across the group. Hence analysis of variances of all the parameters was performed and differences at $p < 0.05$ were considered significant.

RESULTS

Rate of seed germination

The onset of germination was strongly affected by increasing concentration of salt (Table-1). Reduction in the rate of germination was found to be increasing with increasing salt concentrations, particularly at 300mM and 400mM NaCl respectively, compared to control (Fig.1a). Differential response of cultivar to salinity was observed at higher level (400mM) where the rate of seed germination was poor and accounted for 9.5% in MR 220 while 43.17% in MR232.

The influence of exogenous proline application on the rate of germination of two cultivars grown under varied dose of salinity is presented in Fig.1b & c. Exogenous proline significantly influenced the rate of germination (Table-1), seed pretreated with lower dose of proline

(1mM) was more effective in ameliorating salt stress compared to 5mM and 100mM. The germination response of cultivar under salt stress is presented in Fig.1d. In both cultivars, applica-

tion of minimal dose of proline (1mm) overcome the drastic effect of stress and improved the rate of germination, even at 300mM and 400mM level of salt concentrations.

Table 1 Effect of exogenous proline application on germinated rice seedlings grown under different concentrations of NaCl. The table shows the mean sum of square, F ratio and p value (< 0.05) of the determinants exposed to NaCl and exogenous proline application. The figure corresponds to mean sum of squares (MSS), F ratio and p value (≤ 0.05)

Effect	Germination %	Root length (mm)	Shoot length (mm)	Total chlorophyll content (mg/g FW)	Proline content ($\mu\text{mol g/FW}$)	Protein content
NaCl (MSS)	7813.959	4210.337	321.881	8.665	35.900	0.241
F ratio	6.991*	6.057*	0.776*	7.557*	32.131*	1.740*
Sig _(0.05)	0.004	0.007	0.561	0.003	0.000	0.206
Proline	2180.730	7185.198	3327.465	2.201	0.296	0.281
F ratio	1.951*	10.337*	8.026*	1.920*	0.265	2.029*
Sig _(0.05)	0.175	0.001	0.003	0.180	0.849	0.164
NaCl x Proline	1117.714	695.077	414.586	1.147	1.117	0.138
F ratio	31.300*	23.811*	14.813*	2.104*	6.092*	2.581*
Sig _(0.05)	0.000	0.000	0.000	0.026	0.000	0.006

Plant growth measurement

Root length

Delayed emergence of root and shoot was noticed at increase intensity of salt stress compared to control. Under controlled condition, the root elongated to a measure of 60.17mm and reduced to 2.8mm under high salt condition (400mM) in MR232 (Fig.2a.). Significant difference was noticed among the varieties for root length (Table -1), the performance of MR232 was better than MR 220 for this trait. Exogenous proline application had significant impact on root elongation in both the cultivars (Fig 2b & c). The result has proved that even at higher concentration of salt, lower dose (1mM) of proline pretreatment improved seedling root length thereafter slows down as the concentration of proline increases (Fig. 2d).

Shoot length

The response of shoot growth to salinity was similar to the trend observed in root length (Fig. 3a). Significant difference was noticed for shoot length under different salt concentrations in both the cultivars (Table -1). The shoot length was reduced from 37.1mm in control to 0.8 mm when exposed to high level of salt (400mM NaCl). Exogenous application at the range of 1 and 5mM proline improved shoot growth in both cultivars even at higher level of salinity (Fig 3b & c). It is obvious from the Fig. 3d that 1mM and 5mM of proline significantly reduced the negative effect of NaCl. However, higher concentration of proline (10 mM) was not much effective compared to other pretreatments.

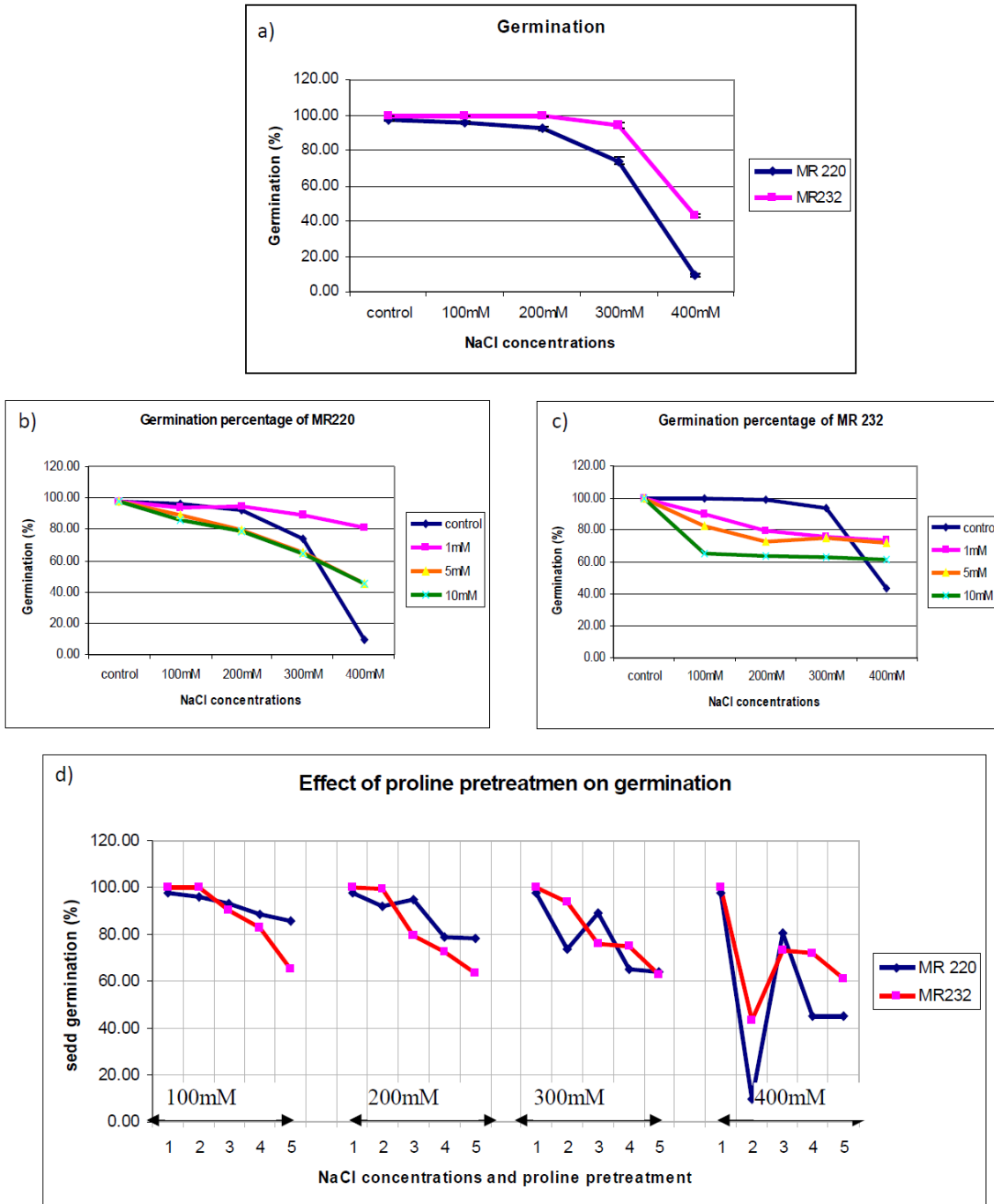


Figure 1 a) Effect of salinity on germination of two rice cultivars. b) Influence of proline pretreatment on germination in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on rate of germination of two rice cultivars grown under different salt concentration. (n=5)

Chlorophyll content

The level of chlorophyll Content was investigated for both MR220 and MR232 under

control, salt and proline pretreated seedlings and the result are presented in Fig. 4 (a, b & c). The result revealed that increased level of salinity stress caused

significant reduction in photosynthetic rate in both cultivars. Both the cultivars have shown a reduction of 93%, 84%, 26% and 7% of total chlorophyll content in 400mM, 300mM, 200mM and 100mM sodium chloride treated seedlings respectively. Chlorophyll content of both the cultivars reduced

significantly by increased concentration of salt (Table-1). Though there was a slight difference among the cultivars response towards exogenous proline application, the pigment content in both the varieties increased (Fig.4d), when the seeds are pretreated with 1mM of proline.

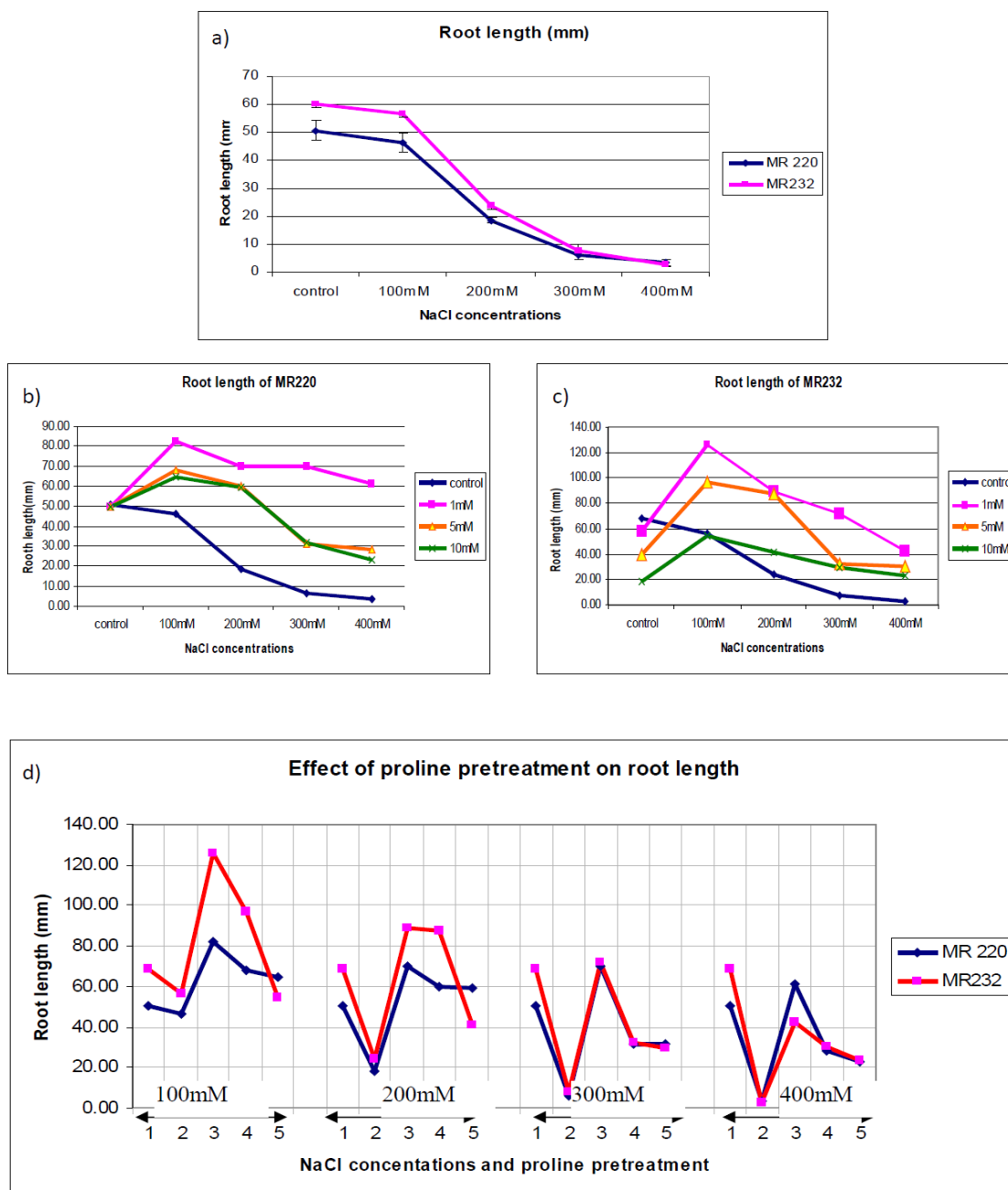


Figure 2 a) Effect of salinity on root length of two rice cultivars. b) Influence of proline pretreatment on root length in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on root length of two rice cultivars grown under different salt concentration. (n=5)

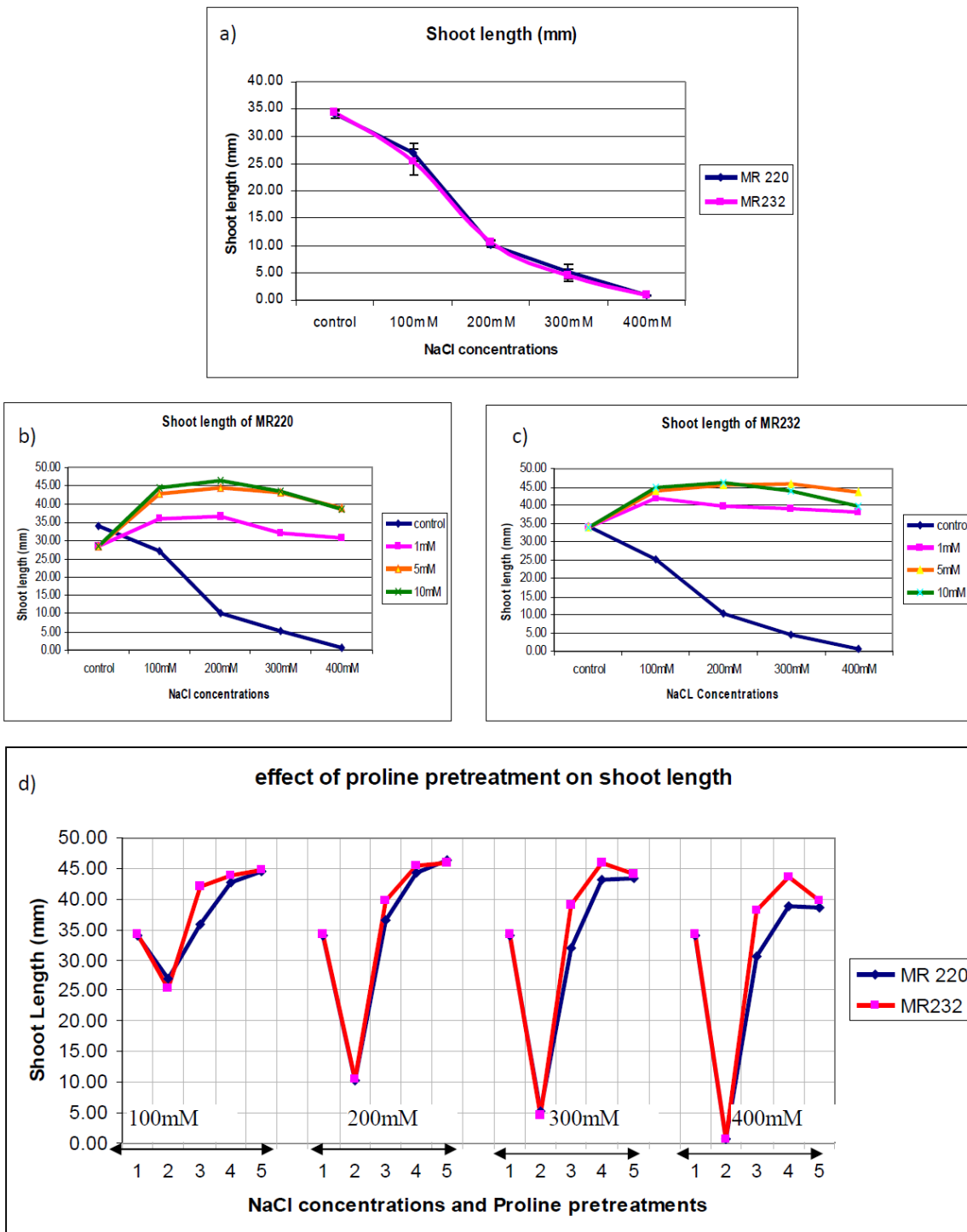


Figure 3 a) Effect of salinity on shoot length of two rice cultivars. b) Influence of proline pretreatment on shoot length in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on shoot length of two rice cultivars grown under different salt concentration. (n=5)

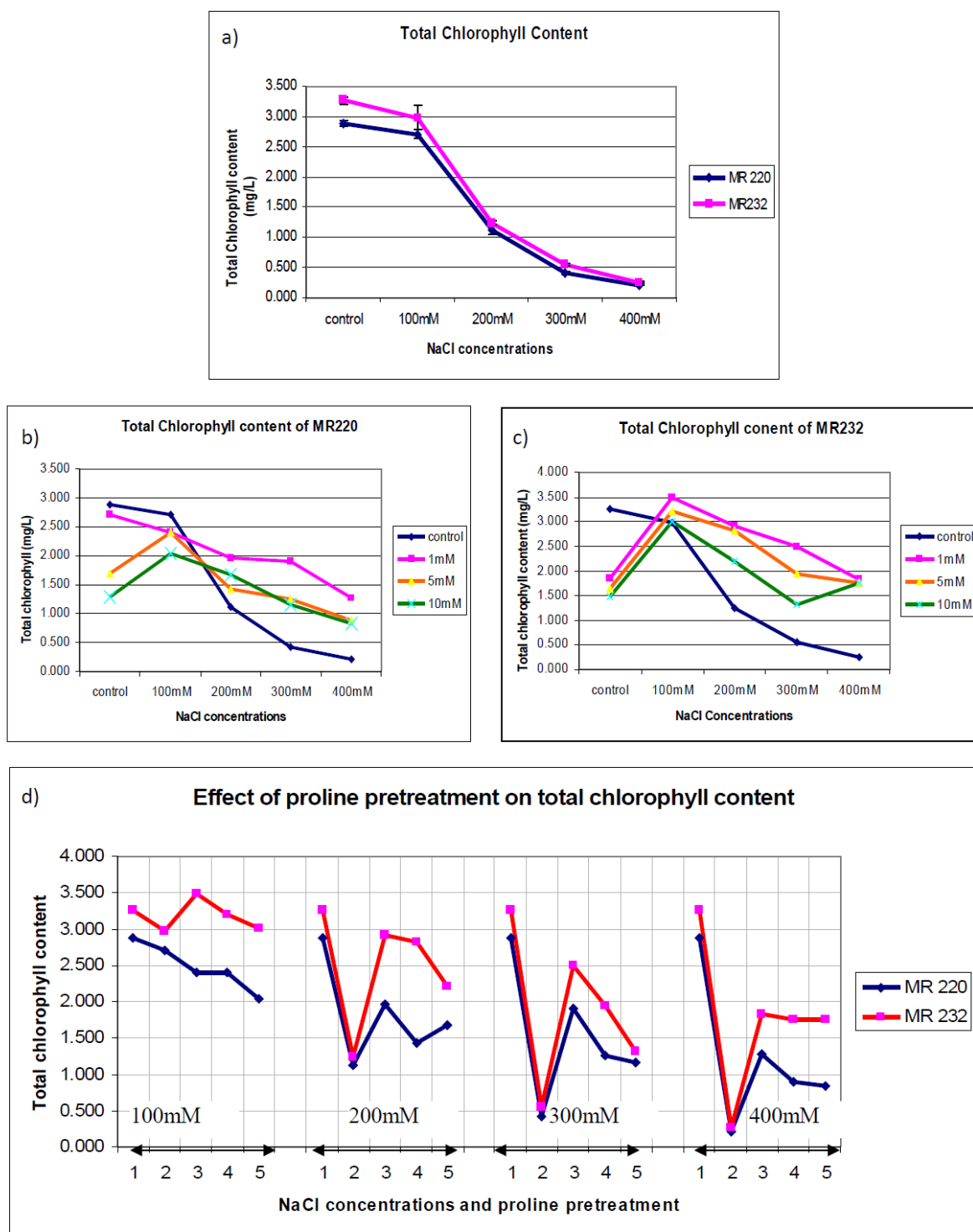


Figure 4 a) Effect of salinity on total chlorophyll content of two rice cultivars. b) Influence of proline pretreatment on total chlorophyll content in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on total chlorophyll content of two rice cultivars grown under different salt concentration. (n=5)

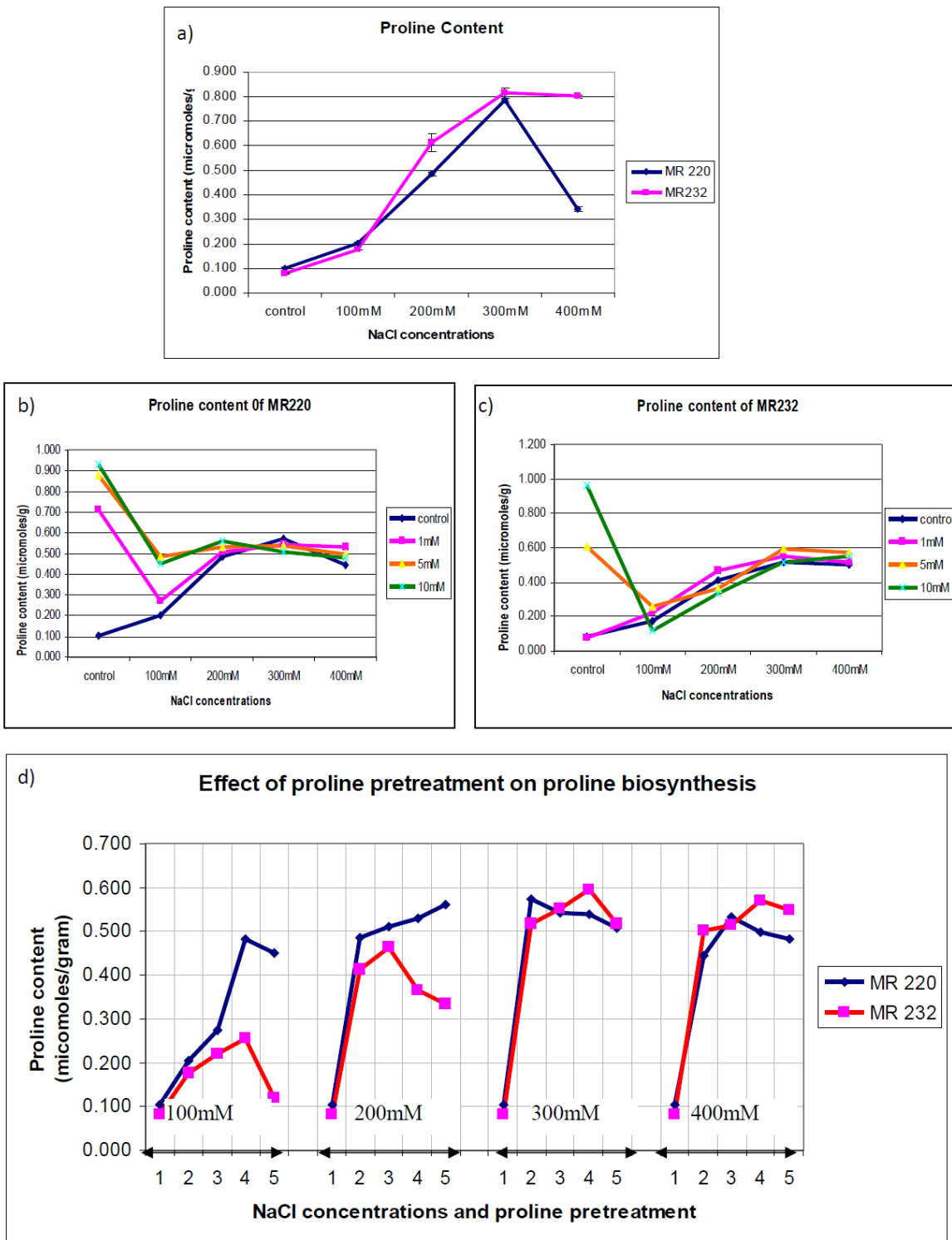


Figure 5 a) Effect of salinity on proline biosynthesis of two rice cultivars. b) Influence of proline pretreatment on proline biosynthesis in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on proline biosynthesis of two rice cultivars grown under different salt concentration. (n=5)

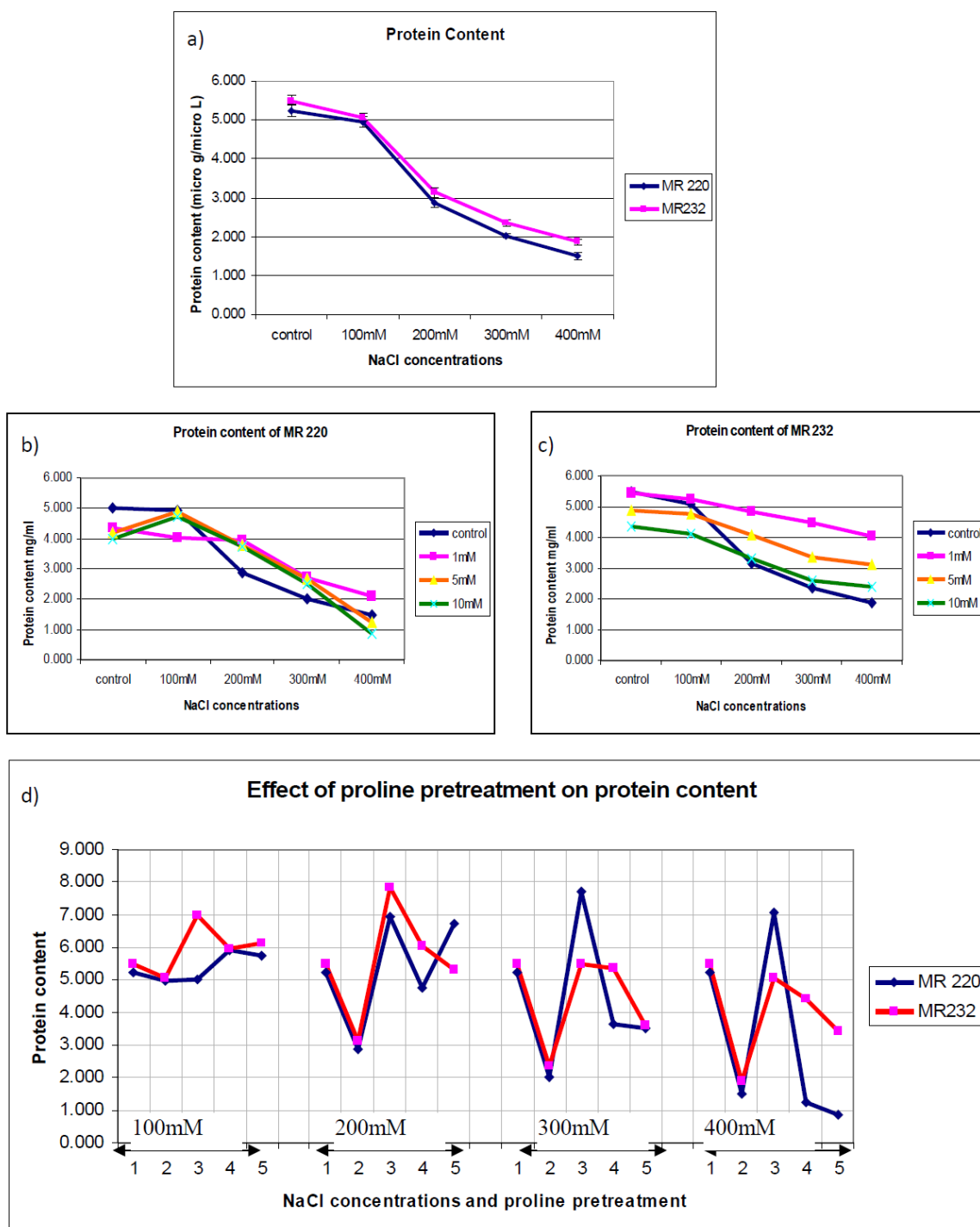


Figure 6 a) Effect of salinity on protein content of two rice cultivars. b) Influence of proline pretreatment on protein content in MR 220 and c) MR 232. d) Mitigating effect of exogenous proline on protein content of two rice cultivars grown under different salt concentration. (n=5)

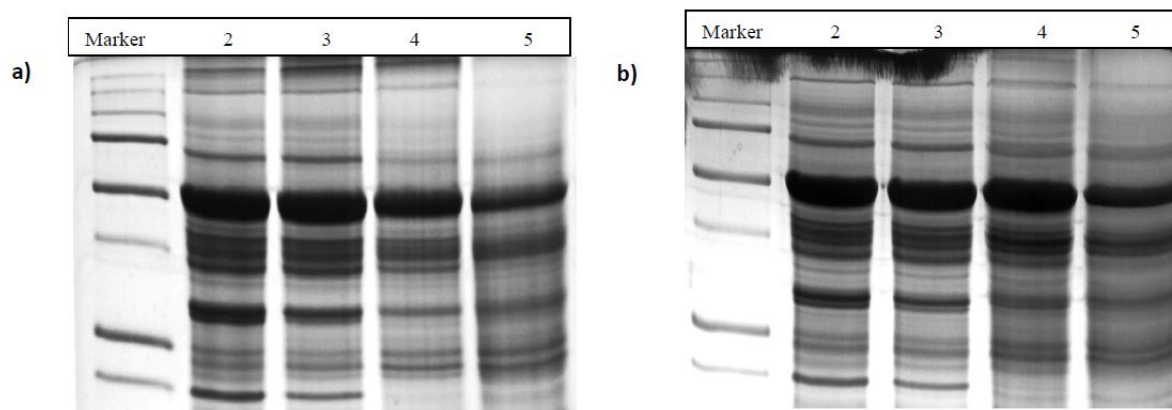


Figure 7 (a & b): SDS PAGE profile indicating the changes in protein expression of two rice cultivars a) MR220 and b) MR 232 under different level of NaCl concentrations. Lane 1: molecular marker (Precision plus protein # 161-0373), Lane 2: 100mM NaCl, Lane 3: 200mM NaCl, Lane 4: 300mM NaCl and Lane 5: 400mM NaCl.

Proline biosynthesis

Salinity stress markedly increased proline accumulation in leaf tissues (Fig 5a). This increase was significantly elevated at progressive level of salt in both the cultivars and becomes static at higher concentration (400mM) in MR232. In MR220, accumulation of proline dropped at 400mM NaCl (Fig. 5b & c). Although proline accumulation was minimal in control, exposure to external proline increased its content drastically in leaf tissues of both the cultivars (Fig 5d).

Protein content

The effect of salt stress on protein content depends on the concentration of NaCl. Increase in concentration of salt in the growing medium generally caused a decrease in protein content of the leaf in both the cultivars (Fig. 5a). The SDS –PAGE analysis revealed a considerable difference in the protein pattern under different level of salt concentration (Fig.7).

Exogenous application of different levels of proline caused significant reduction in the protein content of the leaf tissues (Table-1) and the effect was more obvious in MR232 (Fig 5b & c). The

exogenous proline application improved the protein content in leaf tissues; especially lower dose level (1mM) is found to be effective for maintaining plant function at higher level of salinity stress (Fig. 5d).

DISSUSSION

Rice is very sensitive to salinity during seed germination and it impairs seedling emergence and establishment. The onset of seed germination was delayed and germination percentage was decreased due to increasing levels of salt concentration. Further the study has shown that inhibition of germination rate varied greatly with increased salinity. Germination of seeds was better in lower concentration of salinity than at higher levels (300mM and 400mM NaCl). Increasing salinity has either delayed or reduced the rate of germination (Greenway and Munns, 1980). Inhibition of germination is due to imbalance in water uptake which limits the hydrolysis of food reserves and immobilizes the translocation of food reserve from storage tissue to developing embryo axis (De larcercda *et al.*, 2003). Moreover, increasing salinity decreases osmotic potential and often accumulates with toxic ions which may retards seed germination

(Dodd and Donovan, 1999; Okcu *et al.*, 2005). It has been shown that exogenous application of proline mitigates the adverse effect of salinity by detoxifying the ions and protects the plant cells by maintaining osmotic balance (Ashraf and Foolad, 2007; Hoque *et al.*, 2007; Okuma *et al.*, 2000; 2004). In the present study the seeds pretreated with proline provided significant evidence for assessing salt tolerance at germination stage.

Most investigations have shown that rice crop respond highly to salinity during early stages of growth as compared to germination. The imposition of salinity had an inhibitory effect on the root and shoot growth of rice seedling and its effect differed significantly between rice cultivars. The result indicated a decline in seedling growth as concentration of salinity increased. However exogenous application of proline at 1mM counteracted the adverse effect of mild salt stress (100mM NaCl) and manifested an increase in root length compared to control. The present finding is agreement with earlier reports that the exogenous application of proline alleviates the adverse effect of stress on plant growth (Kavi Kishore *et al.*, 1995; Hoque *et al.*, 2007). In addition, Mussig *et al* (2003) suggested that exogenous application of proline might function both as promotive as well as inhibitory on root growth. The present investigation revealed that at higher concentration proline did not ameliorate the adverse effects of salinity. The result obtained with increase level of proline on salinity was in confirmation with the findings of Qayyum *et al* (2007). Further, Lin (2001) indicated that the inhibitory effect of root growth may be attributed to greater accumulation of proline which may interfere with osmotic adjustment. According to Amzalleg (2002) the effectiveness of proline on the growth of salt stressed plants depends on the type of plant

species, growth stage, concentration of osmoprotectant and mode of application.

Reduced plant growth is a common phenomenon when grown under increased salinity and usually expressed as stunted shoots. It is due to the fact that Na⁺ and Cl⁻ sequestered in the vacuole could decrease the internal osmotic potential and cause partial dehydration of cytoplasm. Such dehydration impairs the cellular metabolism and ultimately reduced the growth of the seedlings (Le Rudulier, 2005). Further, earlier reports have shown that under moderate salt stress, cell division is unaffected and it is indicated by initiation of leaf rather than the cell elongation. The observed increase in shoot length could be attributed to positive effect of exogenous proline application which stimulated cell elongation and division (Fig.). The physiological effect of this amino acid on cell elongation was supported by Ozdemir *et al.*, (2004) under salt stress in rice.

Measurements of chlorophyll content provided quantitative information about photosynthesis. The reduction in growth observed in the present investigation subjected to excess salinity is often associated with a decrease in rate of photosynthetic capacity. Earlier studies have (Flexas *et al.*, 2004, 2007) opinioned that it might be due to decreased CO₂ diffusion in the leaf tissues which limits stomatal opening and alter the leaf photochemistry. Further, Munns *et al.*, (1995) suggested that accumulation of Na⁺ in the vacuoles affects net photosynthesis and limit the supply of carbohydrate in the growing cells thereby inhibits the growth. Reduction in chlorophyll pigment due to stress was also reported on crops such as maize, wheat, canola, etc., (Ali *et al.*, 2007). However pretreatment of rice seeds with proline at 1mM considerably enhanced the photosynthetic pigments under salinity stress. Increase in total chlorophyll content due to exogenous proline application primarily increased

the rate of CO₂ diffusion and favored higher photosynthetic rate (Ali *et al.*, 2007; Sharkey *et al.*, 2007)

Proline content significantly increased in rice seedlings exposed to increased salt stress. It was shown that proline accumulates in larger amount than any other amino acids and regulates osmotic potential of the cell (Ali *et al.*, 1999; Abraham *et al.*, 2003). It is also hypothesized that proline, besides being an osmolyte is also involved in scavenging free radicals and protects plant cell against adverse effect of salt by maintaining osmotic balance (Okuma *et al.*, 2004; Ashraf and Foolad, 2007). It is obvious from the study that salt stress up-regulated the enzymes involved in biosynthesis and elevated the levels of proline and it is in accordance with the findings of Hare *et al.*, 1999; Chen *et al.*, 2000 and Munns, 2005. It has also shown that salt tolerant strains generally exhibit higher proline content than the salt sensitive (Shereen *et al.*, 2007). To verify whether exogenous proline modifies the internal amino acid content, proline content of leaf tissue was determined. Different concentration of proline applied exogenously increased the measurement under saline stress, suggesting that accumulation of compatible solutes often forms a basic strategy for the protection and survival of plants under stress (Hanson and Hitz, 1982).

Increasing of NaCl substantially decreased proteins content in leaves and the effect is more pronounced at 300 and 400 mM NaCl. The decrease synthesis of proteins in leaves tissues might be due to change in amino acid metabolism due to salinity stress. Further it limits the supply of CO₂ and impairs the photosynthetic apparatus. Down regulation of protein synthesis is the common phenomenon under stress and it is clearly reflected in protein profiling analysis. Exogenous application of proline at 1mM improves the protein content in

leaf tissues at higher level of salt. It is suggested that proline pretreatment can cause alteration in cytoplasmic viscosity of the cell

CONCLUSION

The result presented above indicated that salt stress at higher concentration, especially 300-400mM NaCl is toxic to germinating seeds of both rice cultivars and resulted in reduced emergence of rice seeds. Further, it is also evident that higher concentration of salt decreased root and shoot growth and impair physiology of cells. In addition to morphological features, pigment content, proline and protein biosynthesis were also adversely affected by increased salt concentration. Exogenous proline significantly interacted with different levels of salinity, thereby mitigates detrimental effect of salt on growth and photosynthetic ability of two rice cultivars. Though proline pretreated at lower dose (1mM) improved cellular functions, it is found to be toxic and impair various functions if added at high concentration.

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