Tropical Agricultural Research Vol. 14: 87-96 (2002)

Effect of Salt Stress on Leaf Area, Rate of Photosynthesis and Proline Accumulation of Rice Varieties at Different Growth Stages

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ABSTRACT. Studies on photosynthetic parameters (leaf area and rate of photosynthesis) and proline content as affected by salinity stress in rice is essential to increase growth and yield. The present study attempts to investigate the effect of salinity on the above parameters in four rice varieties (Nona Bokra, Pokkali, At 353 and IR 28) at three growth stages (seedling, tillering and flowering). Application of salt stress significantly decreased leaf area (by 48-58%) and rate of photosynthesis (by 14-70%) and increased the proline accumulation (by 14-70%) under experimental conditions was also observed. There was a positive correlation between leaf area and photosynthesis rate. The proline accumulation was higher in leaves than in roots. Induced salinity caused an increase in proline in leaves of Nona Bokra. At tillering stage, most varieties accumulated higher amounts of proline in leaves compared to other stages. Only Nona Bokra showed a 71% increase of proline at the flowering stage at the highest salt level while Pokkali, At 353 and IR 28 showed a significant reduction at this stage. The pattern of change in the root proline was the same as that of leaf proline. The interactive effect of variety×growth stage×salt level was significant in all parameters measured throughout the growing period.

INTRODUCTION

In Sri Lanka, salinity problems are primarily associated with the coastal area and irrigated lands of the dry zone, where the total irrigated area is about 0.5 million ha. Since irrigation has been practiced in these areas from ancient times, salinity could be a problem at least in patches of irrigated land, and with the development of modern irrigation networks due to the accumulation of salt in the basins of irrigation channels. Unfortunately, water supplied is often of poor quality, and evapotranspiration leads to the concentration of salts in the soil added during irrigation. Therefore, it is possible that salinity would become a problem over the years in this region of Sri Lanka as in many other countries.

Leaf area and photosynthesis rates at different growth stages determine the growth, development and ultimately the yield of any plant. Salinity decreases both growth and net photosynthesis of higher plants (Maria *et al.*, 2000). For rice, the threshold electrical conductance (EC) is 3 dS/m, and a 1 dS/m increase in salinity over this can reduce yield by 12% while an EC value of 6-10 dS/m is associated with a 50% decrease in yield (Moorman and Van Breedman, 1978). This is due to the competition of different physiological

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processes among sink organs for the limited carbon supplies under salinity (Munns and Termaat, 1986).

Proline accumulation in plants is one of the most frequently reported responses induced by salt stress. Higher proline level in stress resistant organisms is often used as an index of resistance (Charest and Phan, 1990). Cytoplasmic accumulation of this non-toxic amino acid is thought to be involved in osmotic adjustment of stressed tissues (Kavi Kishor *et al.*, 1995). Proline is also involved in the protection of enzymes (Solomon *et al.*, 1994) and cellular structures (Van Rensburg *et al.*, 1993), and acts as a free radical scavenger (Alia Prasad and Pardha Saradhi, 1995). In higher plants, proline is synthesized from either glutamate or ornithine. Glutamate pathway is the primary route for proline synthesis under conditions of osmotic stress (Delauney and Verma, 1993). It has been revealed that the induction of the gene for P5CS (Pyrroline-5-carboxylate synthetase) precedes the accumulation of proline (Yoshiba *et al.*, 1995).

Morphological screening programs of rice have shown an inadequacy on the basis of salt tolerance and yield. Many varieties which show good salt tolerance are low yielding due to biochemical and physiological inadequacies (Erikson *et al.*, 1995). The negative influence of salinity at a particular growth stage such as the seedling stage affects significantly on subsequent growth stages. For example, if a rice variety is highly sensitive at low salt level during seedling stage, then it can not be grown even under mild salt condition. To understand the metabolic adjustments and to quantify the degree of effect of salinity during seedling, tillering and flowering stages, information on leaf area, photosynthesis rate and proline accumulation of rice under salt stress conditions is essential. However, information of such studies is scarce. The present study attempts to investigate the effects of salinity on the leaf area, photosynthesis rate and accumulation of proline in rice and to identify salt tolerant ability at different growth stages (seedling, tillering and flowering) of four rice varieties (Nona Bokra, Pokkali, At 353 and IR 28).

MATERIALS AND METHODS

Experiments were conducted in a green house at the Department of Agricultural Biology, University of Peradeniya. Seeds of the rice varieties Nona Bokra (salt tolerant), Pokkali (salt tolerant), At 353 (moderately salt tolerant) and IR 28 (salt susceptible) were obtained from the Rice Research Institute, Bathalagoda. Plants were grown under green house conditions in hydroponics using a nutrient solution (Yoshida *et al.*, 1976) circulated by a small pump. Each variety had three replicates and seedling density was 15 l^{-1} plastic pot at the beginning and later at the flowering stage it was thinned out to two plants. Pots were inserted in to the tubes of the hydroponic system. The spacing between pots in the tube was 12 inches. The temperature varied from 28-31°C during day and 24-28°C in the night.

Salt stress was applied to 14 day old plants: Chloride dominant salt solution (Peiris, 1992) was added to the nutrient medium and a range of salinity levels was induced similar to that found under natural conditions (4, 6 and 8 dS/m) by adjusting the concentration of NaCl, $CaCl_2$, KCl and $MgSO_4$. None salinized nutrient medium (Electrical conductance 1.0-1.03 dS/m) was used as the control. Conductivity and pH of growth medium (5-5.5) were checked and adjusted every two days. Solutions were renewed weekly

till the 4th week of planting and then renewed twice a week until flowering. Plant survival rate was estimated during the stress period considering all dead plants including those which showed necrosis in all leaves. The following parameters were measured at biweekly intervals synchronizing with the three growth stages (seedling, tillering and flowering). Leaf area was measured in fully expanded leaves of nine randomly selected plants by a portable leaf area metre (Li-3000, Li-Co-R, Inc.). Photosynthesis rate (μmol CO_2 m^{-2} s^{-1}) was measured by a portable photosynthesis system (Li-6400, Li-Co-R, Inc.) under full sunlight (9:30 am -11:30 pm).

Plants were collected for proline extraction at different growth stages *viz.*, seedling (19-25 days), tillering (35-42 days) and flowering (70-84 days) after salt stress was applied. Free proline content was determined according to Bates *et al.* (1973) with slight modifications. Approximately 0.5 g of plant material (leaves and root) separately was cut in to small pieces and then transferred separately into test tubes containing 3 ml of deionized water. Tubes were kept in a boiling water bath with moderate shaking for 40 min, and then were cooled to room temperature. One ml of water extract was reacted with one ml acid ninhydrin and one ml of glacial acetic acid was mixed in a test tube and kept for 30 min in a boiling water bath. The reaction was stopped by placing the tubes in an ice bath. The reaction mixture was extracted with 3 ml toluene and mixed vigorously for few seconds. The organic layer was separated and its absorbency was read at 520 nm using an UV-Visible spectrophotometer (UV 1201, Shimadzu Corp., Kyoto, Japan). The proline concentration was determined from a standard curve and calculated on a fresh weight (FW) basis as follows:

$$\mu \textit{ mol proline / g of FW material} = [(\mu g \textit{ proline / ml} \times \textit{ml tolune}) / 115.5\ \mu g / \mu mol] / [(g \textit{ sample}) / 5]$$

Data were analyzed by the general linear models (GLM) procedure using the statistical analysis system (SAS) version 6 computer package. Model was chosen for factorial analysis indicating 3 way interaction and means were compared by the least square means (LSMEANS) test at P=0.05.

RESULTS AND DISCUSSION

Leaf area, rate of photosynthesis and plant survival rate

The most important morphological character that responded to salinity was changes in leaf area (Fig. 1). Leaf area decreased by 15-19% and 25-48% over the control for all varieties, at salt levels 4 and 6 dS/m, respectively throughout the growing period (Table 1). During the experimental period leaf area reduction of Pokkali and At 353 was not significantly different at P=0.05 at 4 and 6 dS/m.

The reduction in leaf area could be due to the accumulation of high concentration of Na^+ and Cl^- ions which induces chlorosis and leaf senescence. This will ultimately lead to a decrease in active photosynthetic leaf area. Generally, in sensitive species, an increase in shoot Na^+ results when control fails and this failure is correlated with a reduction in growth (Flowers *et al.*, 1977). As a result, and in agreement with the finding of Munns and

Termaat (1986), the production of carbohydrate declines and productivity falls below a level capable of sustaining further growth. At EC = 8 dS/m, 58% reduction of leaf area in At 353 was observed compared to the control. However, reduction of leaf area significantly affected photosynthesis rate in all varieties.

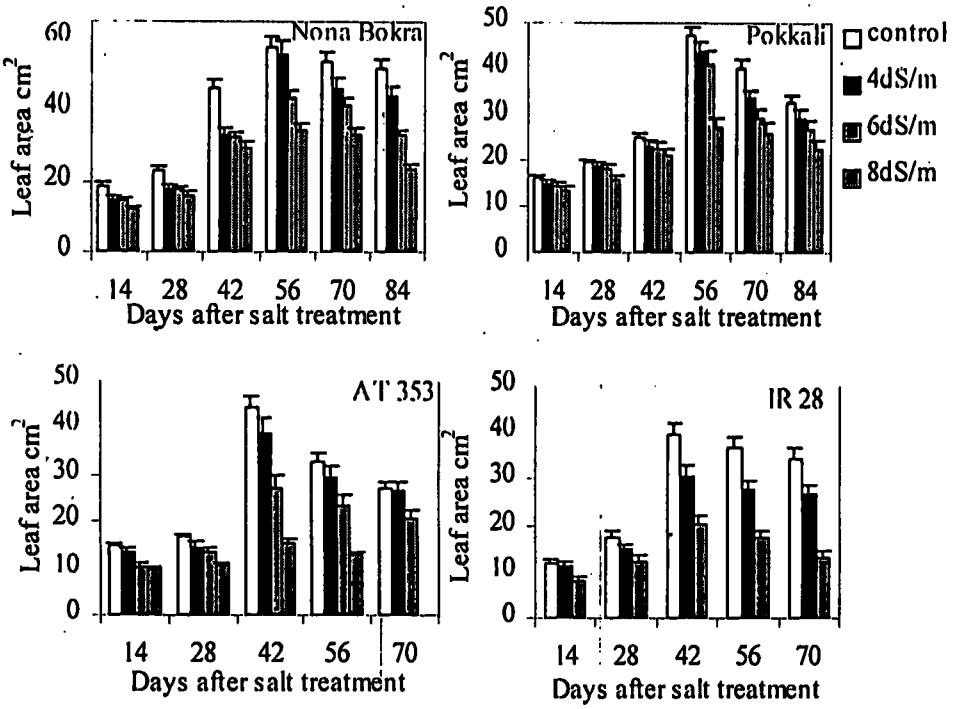

Fig. 1. Leaf area of rice varieties in response to different salt treatment.

The exposure of plants to salinity decreased the photosynthesis rate (PR) significantly in all varieties at all sampling dates (Fig. 2). At the end of the salinization period, salt level 4 dS/m significantly ($P>0.05$) reduced the PR of At 353 by 14%, Nona Bokra and Pokkali by 18% and IR 28 by 24% compared to the control (Table 1). However, the reduction was high at 6 dS/m. For IR 28 this was 70% and for all other varieties it was between 31 and 32%. Brugnoli and Lauteri (1991) have reported that photosynthetic CO_2 assimilation is known to be sensitive to environmental stresses and considerable decline of net photosynthesis under salt stress is principally due to stomata closure, which is brought by secondary osmotic stress caused by salinity.

At the highest salt level, varieties Pokkali and At 353 showed decreased PR between 62 and 67% respectively compared to control, whereas Nona Bokra was less affected and its PR reduced by 47% of control during the experimental period. Tomato plants grown under 100 mM NaCl conditions have shown up to 70% reduction in the total photosynthesis (Maria *et al.*, 2000). The allocation of photosynthetic products during the early plant development could have an important implication on the overall plant growth

under salinity. Since there was reduction in PR with increasing salt levels, it is possible that salinity would cause significant reduction in yield.

Table 1. The effect of salinity on the leaf area, photosynthesis rate and plant survival rate during the total growing season.

Parameter	Electrical conductance dS/m	Varieties			
		Nona Bokra	Pokkali	At 353	IR 28
Leaf area cm^2	Control	41.76a	33.61a	29.05a	28.89a
	4	34.78a	27.20a	24.75a	23.53a
	6	30.26b	25.53a	19.60a	14.98b
	8	24.83c	21.01b	12.11b	*
	CV%	8.21	5.63	4.89	7.10
Photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	Control	8.30a	8.15a	6.72a	6.41a
	4	6.77b	6.70b	5.75b	4.87b
	6	5.69c	5.53c	4.54c	1.91c
	8	4.25d	3.06d	2.23d	*
	CV%	7.32	6.40	8.10	5.21
Survival rate %	Control	100	100	100	100
	4	100	100	100	95
	6	100	95	80	50
	8	90	80	28	*

These values are means of nine observations, which were taken biweekly during the growing season. Means with the same letter along the column are not significantly different at $P<0.05$. * - missing data.

Survival rate was approximately 100% for all varieties up to 4 dS/m and for Nona Bokra and Pokkali up to 6 dS/m also. At these two levels plants were healthy and actively growing. However, a drastic decline was observed for At 353 by 28% and IR 28 by 50% at 8 and 6 dS/m. At 353 was highly affected at the highest salt treatment (EC=8 dS/m) during tillering stage and all plants were dead in that condition before the next sampling. During seedling stage IR 28 plants were highly affected at EC=6 dS/m compared to other varieties and only 50% of plants survived throughout the growing period at this salt level (Table 1). This is because of the highest reduction of photosynthetic leaf area and PR at high salinity, which prevents plants maintaining their sustainable life.

Leaf proline content

The proline content of all varieties increased significantly when exposed to salt stress (Table 2). Free proline content generally increased with decreasing leaf area throughout the growing period, and all varieties however maintained their survival rate to a certain extent at all salt levels. This implies that proline is accumulated into the remaining

living cells to maintain the osmotic potential of the cells, and thereby plants photosynthesize with the limited photosynthetic leaf area.

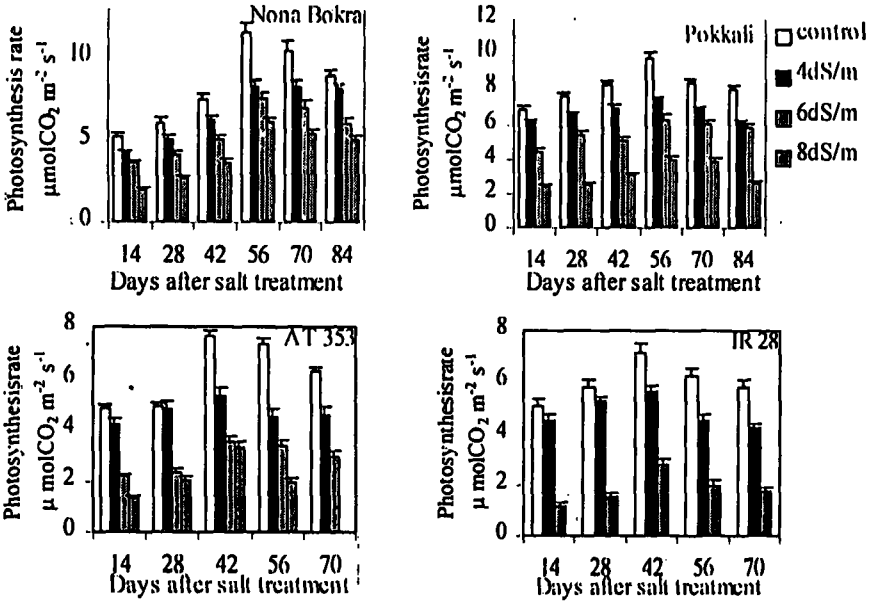

Fig. 2. Photosynthesis rate of rice varieties in response to different salt treatment.

Induced salinity caused an increase in proline in leaves of Nona Bokra by 139% at tillering stage and Pokkali by 431% at seedling stage over the control at EC=8 dS/m. IR 28 had the lowest (86%) at flowering stage. The rate of accumulation of proline from control to the other salt levels was significantly different (Table 2). Since significant variety×growth stages×salt level interactions were observed, leaf proline contents varied depending on the growth stages, salt levels and varieties. At seedling stage, Nona Bokra and Pokkali showed increase free proline values of 14-331% and 46-131% compared to control respectively, whereas At 353 and IR 28 had 4-87% and 0-4% with the increase of salinity levels.

It was noticed that growth of Nona Bokra was less affected than Pokkali and IR 28 was more severely affected than At 353. It appeared that Nona Bokra and Pokkali are the most tolerant. Roy *et al.* (1992) reported that in the presence of NaCl, germinating seed of the salt tolerant rice cultivar CSR-4 accumulated higher amount of proline than those of the salt susceptible cultivar Ratna. At tillering stage, most of the varieties accumulated significantly high amounts of proline compared to the seedling stage. However, Nona Bokra accumulated less, 4-127% compared to control during tillering at 4, 6 and 8 dS/m. The increase of proline in At 353 was 72-60% and IR 28 was 71-78% during tillering compared to seedling stage at EC 4 and 6 dS/m. During tillering, Pokkali showed a slight increase of 4-48% compared to seedling stage at EC=4, 6 and 8 dS/m (Table 2). Therefore, the salt tolerant varieties do not necessarily accumulate large amount of free proline relative to salt sensitive variety during tillering stage.

Table 2. The effect of different salinity treatments on proline concentration (μmol/g FW) in rice leaves at different growth stages.

Growth Stage	Electrical conductance dS/m	Varieties			
		Nona Bokra	Pokkali	At 353	IR 28
Seedling	Control	99.17a	117.40a	186.22a	179.43a
	4	113.36b	258.74a	194.54a	205.50b
	6	200.62c	330.56b	213.99b	178.76c
	8	427.99d	410.78c	348.09c	*
	CV%	6.20	8.30	10.73	6.25
Tillering	Control	104.85a	308.38a	319.03a	92.54a
	4	109.83b	385.16b	335.52a	351.86b
	6	202.72c	416.47c	343.92b	319.60c
	8	238.41d	430.14d	203.66c	*
	CV%	10.61	9.79	8.61	9.51
Flowering	Control	259.62a	169.00a	322.79a	86.13a
	4	319.85b	288.61b	415.77b	194.22b
	6	340.09c	390.32c	409.65c	240.73c
	8	409.86d	198.57d	*	*
	CV%	6.68	10.12	10.82	7.50

These values are means of nine observations. Means with the same letter along the column are not significantly different at P<0.05. * - missing data.

It was interesting to note that in At 353, during tillering stage proline content decreased significantly (P>0.05) to 42% of seedling stage at EC= 8 dS/m. Plants gradually started to die from the sixth week of planting and ultimately at this salt level all plants died before the flowering stage. Therefore, it seems that free proline accumulation plays an important role in maintaining plants alive. The contribution of proline from tillering to flowering stage is of prime importance when plants yield components are determined under high saline condition. During flowering, Nona Bokra and At 353 showed higher accumulation of proline than Pokkali and IR 28 compared to tillering stage (Table 2). However, accumulation was less in IR 28. The reductions were 25-45% in IR 28 and 6-25% in Pokkali compared to tillering stage at EC=4 and 6 dS/m. At the same EC levels, Nona Bokra and At 353 showed an increment of proline of 91-67% and 24-19% (of tillering) respectively. Only Nona Bokra showed an increase of 71% proline during flowering stage compared to tillering at the highest EC level. This may be due to the plant develops mechanisms of increased resistance to higher salinity ('adaptation').

Root proline content

All varieties showed increase of the proline levels in roots correlated with increase in EC levels at seedling stage (19-25 days). Like in the leaves, the highest proline accumulation of roots also was observed in Nona Bokra and Pokkali at the flowering stage.

These were 51% in Pokkali and 140% in Nona Bokra, compared to the control at EC=8 and 6 dS/m, respectively (Table 3). Proline concentrations in the root showed a significant variety×growth stage×salt stress interactions. This implies that the effect of salt stress on proline accumulation varies among different growth stages and varieties. Roots, however, could be a site of proline biosynthesis but not a site of accumulation since proline could be transported to other parts of the plant *via* the transpiration stream (Hua *et al.*, 1997).

Table 3. The effect of different salinity treatments on proline concentration (µmol/g FW) in rice roots at different growth stages.

Growth Stage	Electrical conductance dS/m	Varieties			
		Nona Bokra	Pokkali	At 353	IR 28
Seedling	Control	28.72a	94.70a	100.78a	65.00a
	4	69.80b	125.68a	119.60a	82.53b
	6	74.20c	184.04b	131.53a	176.36c
	8	94.69d	189.48c	162.23b	*
	CV%	10.15	7.50	7.02	8.61
Tillering	Control	51.79a	144.57a	174.11a	92.62a
	4	79.09b	181.24b	213.82b	162.03b
	6	150.66c	211.82c	243.74c	153.46c
	8	155.38c	257.78d	124.32d	*
	CV%	8.34	8.13	10.72	9.07
Flowering	Control	198.86a	120.40a	137.77a	49.78a
	4	269.07b	285.72b	219.02b	189.01b
	6	292.53b	288.85c	199.90c	148.10b
	8	299.53b	159.55d	*	*
	CV%	11.28	8.19	9.57	11.59

These values are means of nine observations. Means with the same letter along the column are not significantly different at P<0.05. * - missing data.

Although Nona Bokra maintained the highest proline levels at flowering stage it was low at seedling and tillering stages (Table 3). At the flowering stage increase was not significantly different at P=0.05 between the three salt levels compared to control. The pattern of proline production in roots at seedling and tillering stages was almost similar to that of the leaf but variation was observed at flowering stage. The proline level in roots of At 353 and IR 28 significantly increased by 59% and 279% of control respectively at EC= 4 dS/m during flowering stage. However, at 6 dS/m it decreased by 18% and 4% compared to tillering for At 353 and IR 28 respectively (Table 3). During flowering, in Pokkali proline content rose from 20-137% and 140% respectively at control, 4 and 6 dS/m (Table 3). Shah *et al.* (1990) have found that the salt tolerant cells will accumulate higher amounts of proline and will thus benefit from the enhanced osmoprotection afforded by proline.

CONCLUSIONS

The study showed that high salinity levels significantly reduced leaf area and photosynthesis rate, but increased leaf and root proline contents. Reduction of photosynthetic leaf area directly affected the leaf photosynthesis. This limited PR would reduce allocation of carbon resources to overall plant development and yield. Therefore, with the increase of salinity, survival of plants decreased. Growth of At 353 and IR 28 was highly affected at salt levels 8 and 6 dS/m during tillering and seedling stages respectively. This implies that these varieties can not be grown at these salt levels. In general, free proline content increased in salt tolerant varieties compared with that in salt sensitive varieties and thus may be a suitable marker in examining salt tolerance in rice compared to other parameters such as leaf area and PR. However, higher variation was observed in proline content during tillering and flowering stages than seedling stage. Therefore, for a convenient and rapid screening of rice varieties for salt tolerance, accumulation of proline at seedling stage may be used as an index. The study did indicate, that if many rice accessions were screened for salt tolerance, even though tremendous variability could be anticipated, this can be used for salt tolerance rice varieties. The validity of these results must be verified under field conditions for wider application of growing rice in saline areas of Sri Lanka.

ACKNOWLEDGEMENTS

This research was funded by the Asian Development Bank (ADB). The assistance by the staff of the Department of Agricultural Biology is gratefully acknowledged.

REFERENCES

Alia Prasad, K.V.S.K. and Pardha Saradhi, P. (1995). Effect of zinc on free radicals and proline in *Brassica* and *Cajanus*. Phytochemistry. 39: 45-47.

Bates, L.S., Waldren, R.P. and Teara, I.D. (1973). Rapid determination of free proline for water stress studies. Plant Soil. 39: 205-207.

Brugnoli, E. and Lauteri, M. (1991). Effects of salinity on stomatal conductance, photosynthetic capacity, and carbon isotope discrimination of salt tolerant (*Gossypium hirsutum* L.) and salt sensitive (*Phaseolus vulgaris* L.) C_3 non-halophytes. Plant Physiol. 95: 628-635.

Charest, C. and Phan, C.T. (1990). Cold acclimation of wheat (*Triticum aestivum*): properties of enzymes involved in proline metabolism. Physiol. Plant. 80: 159-168.

Delauney, A.J. and Verma, D.P.S. (1993). Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215-305.

Erikson, J., Rogers, W.J. and Perera, A.L.T. (1995). Genetic screening of rice varieties using RAPD markers for indication of salt sensitivity/resistance. Sri Lankan J. Agric. Sci. 32: 16-29.

Flowers, T.J., Troke, P.F and Yeo, A.R. (1977). The mechanism of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28: 89-121.

Hua, X.L., Van de Cotte, B., Van Montangu, M. and Verbruggen, N. (1997). Developmental regulation of pyrroline-5-carboxylate reductase gene expression in Arabidopsis. Plant Physiol. 114: 1215-1224.

Kavi Kishor, P.B., Hong, Z., Miao, G.H. and Verma, D.P.S. (1995). Over expression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108: 1387-1394.

Maria, E.B., Jose Dell, A., Maria, C.B. and Francisco, P.A. (2000). Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. Physiol. Plant. 110: 503-511.

Moorman, F.R. and Van Breedman, N. (1978) Soils and land properties that affect the growth of rice. IRRI, Los Banos, Philippines pp 120-121.

Munns, R. and Termaat A. (1986). Whole plant responses to salinity. Aust. J. Plant Physiol. 13: 143-160.

Peiris, B.D. (1992) The effect of saline media of varying ionic composition on stomatal resistance of rice (*Oryza sativa* L.) leaves. J. Natn. Sci. Coun. Sri Lanka. 20(1): 127-131.

Roy, D., Bhunia, N., Basu, N. and Banrjee, S.K. (1992). Effect of NaCl salinity on metabolism of proline in salt-sensitive and salt-resistant cultivars of rice. Biol. Plant. 34: 159-162.

Shah, S.H., Wainwright, S.J. and Merrett, M.J. (1990). The interaction of sodium and calcium chlorides and light on growth, pottassium nutrition, and proline accumulation in callus cultures of *Medicago sativa* L.. New Phytol. 116: 37-45.

Solomon, A., Beer, S., Waisel, Y., Jones, G.P. and Paleg, L.G (1994). Effects of NaCl on the carboxyalting activity of Rubisco from *Tamarix jordanis* in the presence and absence of proline related compatible solutes. Physiol. Plant. 90: 198-204

Van Rensburg, L., Kruger, G.H.J. and Kruger, H. (1993). Subcellular location of Δ^1- pyrroline-5-carboxylate reductase in root nodule and leaf of soybean. Plant Physiol. 99: 1642-1649.

Yoshiba, Y., Kiyosue, T., Katagiri, T., Ueda, H., Mizoguchi, T., Yamaguchi, S.K., Wada, K., Hatada, Y. and Shiozaki, K. (1995). Correlation between the induction of a gene for Δ^1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J. 7: 751-760.

Yoshida, S., Forno, D.A., Cock, J.H. and Gomez, K.A. (1976) Laboratory manual for physiological studies of rice. IRRI, Los Banos, Laguna, Philippines.