International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Evaluation of Rice (Manawthukha) Germplasm under Salt Stress at Seedling, Vegetative and Reproductive Stages through SSR Markers

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Abstract: ***Salinity, one of the abiotic stresses, is a great problem for rice production worldwide incurring substantial yield loss: a great threat towards food security. In Myanmar, 3% of total rice crops are affected by salinity every year. In this study, Manawthukha was selected because Manawthukha is one of the rice cultivars commonly cultivated in Kyaukse region, which is prone to salinity. Thirteen rice germplasms including Pokkali and control were used for identification of salt tolerant rice genotypes at the seedling stage at Biotechnology Research Department, the molecular laboratory, Kyaukse. Phenotypically, on the basis of SES and % total dry matter (TDM) reduction of the genotypes viz. MK-B1, MK-D1, MK-E, MK-F2 and Pokkali were found to be salt tolerant (ranging value SES is 12); on the other hand MK-A1, MK-D and MK-D2 were identified as moderately tolerant and MK-F4 was salt susceptible. For genotyping, Three selected SSR markers already known to be polymorphic, viz., RM10694, RM3412b and RM336 were used to evaluate rice genotypes for rice salt tolerance and it was found that MK-B1, MK-D1, MK-E and MK-F2 were promising salt tolerant mutant lines. Thus, the salt tolerant lines can be used in further evaluation for salinity tolerance and the SSR markers used in this study proved to be valuable tools for identifying salt tolerant genes in marker assisted breeding.***

Keywords: Manawthukha, Salinity Tolerance, SSR Markers, Seedling stage, Vegetative Stage, Reproductive Stage, Polymorphism

1. Introduction

Rice is one of the most widely cultivated crops which provide food for one-half of the world's population [1]. The soil salinity of reclaimed paddy fields is one of the important stresses which limit rice growth and yield in Asia and Africa [2]. There are 380 million ha of saline soils on the earth's land surface which are widely distributed in arid and semi-arid areas and seasonally dry coastal areas, where they severely affect the agricultural production of many countries [3]. Myanmar produce two rice crops annually: wet season and dry season. Wet season production typically accounts for 85% of total production, while the dry season the remaining 15%. Myanmar's paddy fields can be found mostly in the delta and central dry zone areas. The drought area of rice cultivated in Myanmar is 12% about 155140 hectares. Some 14% (173560 hectares) of rice area are affected by submergence in Myanmar. The total rice crop affected by salinity is about 3% (513780 hectares) in Myanmar [4].

Manawthukha var. is growing more than 20 % rice growing areas. Most of people like very much because it has intermediate amylose content, high yield and acceptable grain quality. But it has non aromatic gene. Rice is sensitive to salinity particularly at the seedling stage. Screening/breeding of rice varieties for tolerance to salinity have been carried out for over three decades and various screening methodologies are used to screen out tolerant varieties [5]. Although salinity affects all stages of growth and development of rice, salinity at the reproductive stage depresses grain yield much more than salinity at the vegetative stages, therefore, screening for tolerance at reproductive stages has been considered to be more useful [6].

The use of physiological characters as selection criteria in salt tolerance breeding requires the identification of the contribution of each individual character to salt tolerance [7]. Plant growth, plant height or shoot biomass was reported to have dilution effects on sodium accumulation in leaves of rice. Panicle weight, tiller numbers per plant and harvest index are important agronomic characters for the prediction of final yield in rice. These yield components are severely affected by salinity [8].

Salinity affects plants at all stages of development, but sensitivity sometimes varies from growth stage to the next. Several studies indicated that rice is tolerant salinity during germination, becomes very sensitive during early seedling stage (2-3 leaf stage), gains tolerance during vegetative growth stage becomes sensitive during pollination and fertilization and then become increasingly more tolerant at maturity [9]. However, some studies reported that at fertilization, rice is not sensitive to salinity [10]. Hence, early seedling stages were used to know the response of the rice plant to salinity. To screen the salt tolerant variety, we need reliable technique. IRRI standard protocol for salinity screening is such type of technique [11]. Conventional breeding is time consuming and depends on environmental conditions. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in rice breeding. SSR or microsatellite markers are proved to be ideal for making genetic maps [12] and, assisting selection [13] and studying genetic diversity in germplasm.

Microsatellite marker analysis is promising to identify major gene locus for salt tolerance that can be helpful for plant breeders to develop new cultivars. From these points of view the present study were undertaken for determining the phenotypic performance of rice germplasm under salinized

conditions at the seedling stage and to identify salt tolerant rice lines from thirteen rice germplasm using SSR markers.

2. Materials and Methods

a) Plant Material

Rice cultivar viz., Manawthukha was used as the experimental material in this study. The life period of Manawthukha is about 135 days. Its original name is Mashuri -M and supposedly originated in Malaysia. It is a semi-dwarf type with a plant height of 105cm, 10 to 12 number of effective tillers, 19 g of 1000 grains weight and it has a droopy panicle axis.

b) Induction Treatment

Breeder seeds of Manawthukha (500 seeds) were treated with 100 Gy, 150 Gy, 200 Gy, 250 Gy, 300 Gy, 350 Gy and 400 Gy of gamma rays from the ^{60}Co at the Department of Atomic Energy (DAE), Kabaraye Pagoda Road, Yankin Township, Yangon. This experiment was carried out at the Department of Biotechnology, Technological University (Kyauk Se), Mandalay Division, after it has been irradiated.

c) Preparation of Peter Solution and Salinization

Water soluble fertilizer as hydroponic solution was used by Peter's (20-20-20) at a rate of 1 g per L purified drinking water. 100 mg per L of $FeSO_4$ were also added to the solution. The pH of the solution was adjusted to pH 5.0-5.1 every two days. Different concentrations of NaCl were added to the nutrient solution (7.013 g NaCl per L of nutrient solution gives an EC 12 dS m^{-1}) measuring by Electrical conductivity (EC) meter. Test entries were rated at 10 and 16 days after initial salinization.

d) Evaluation of rice genotypes at seedling stage

At seedling stage, germinated seeds were sown in hydroponic system with distilled water in a plastic box (6 litres). Fifty seeds per line, on a Styrofoam sheet with 8 lines attached a nylon net bottom. The sheet was floated on the distilled water with Peter nutrient solution. Eight treatments containing (7 rice cultivars) 1 Pokkali (tolerant check) were tested in salt stress (0, 6, 8, 10 and 12 dS m^{-1}). Salt stress evaluation was done by using different concentrations of salt such as EC 0, 6, 8, 10 and 12.

e) Modified standard evaluation score (SES)

Scoring was done according to the modified standard evaluation system (SES) used in rating of the visual symptoms of salt toxicity injury [14] (Table I). Hydroponic system with IRRI standard protocol was used at the glasshouse to evaluate salt tolerance of rice germplasm. This scoring discriminates the tolerant, moderately tolerant and susceptible rice lines.

Table I: Standard Evaluation System For Scoring of Visual Salt Injury at Seedling And Reproductive Stage in rice

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are Elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying.	Susceptible
9	Almost all plants dead or dying	Highly susceptible

f) DNA extraction from plant young leaves

Healthy leaf samples were collected from 25-day old seedlings for isolation of genomic DNA. Leaf sample was washed in distilled water stored in –80˚C freezer before use. About 200 mg of rice leaves were thoroughly ground in 1ml of KCL solution containing 7.45mg of KCl, 0.1ml of 1M Tris-HCl, 0.02ml of 0.5 M EDTA) After grinding, the samples were centrifuged at 12000rpm for 10 mins. Then the upper solution was transferred into new centrifuged tube containing equal volume of phenol: chloroform (1:1) and centrifuged at 12000 rpm for 10mins. The nucleic acid solution was transferred into 500µl ice-cold absolute EtOH and kept at -20˚C for 20 mins. Then, the tubes were centrifuged at 12000 rpm for 10 mins and the supernatant was drained. 70 % EtOH was added to the tubes and centrifuged at 12000 rpm for 5 mins twice. When the ethanol was completely evaporated, the pellet was suspended with 30µl of Nuclease free water together adding 2µl of 10mg/ml RNase and incubated at 37^{0}C for 30 mins. Finally, the extracted genomic DNA was stored at -20˚C. gDNA were checked with 1% agarose gel electrophoresis and stained with 0.5ug of 10mg/ml ethidium bromide.

g) Amplification of microsatellite markers and evaluation of genotypes

In this experiment, ten SSR markers that were previously used for screening of salt tolerance_RM490, RM493, RM3412, RM10694, RM562, RM8094, AP3206, RM10784, RM336 and RM21were used for evaluation of polymorphism. Out of ten primers, three polymorphic SSR markers viz., RM10694, RM3412b and RM336 were selected to evaluate 13 rice germplasm for salt tolerance of mutant Manawthukha varieties (Table II). Each PCR reaction contained 10 µl reaction mixtures containing 0.5 µl (30ng) template DNA sample, each 1µl of 10 µM forward and reverse primer, 0.5 µl of 5U *Taq* DNA polymerase, 1µl 10 x *Taq* buffer, 0.8 µl of 2.5mM dNTPs mixture and 5.2 µl of nuclease free water. PCR reaction was carried out as initial denaturation at 94^{o}C for 5 min, followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and extension at 72^{o}C for 1 min and final extension by 5 min at 72^{o}C. Banding pattern of the genotypes was scored comparing the banding pattern of Pokkali. Similar banding pattern like Pokkali, were considered as tolerant and that with different banding patterns were considered as susceptible. The amplified PCR products were checked with 8%Polyacrylamide gel electrophoresis and the banding patterns were analyzed after staining with silver nitrate solution.

Table II: The Sequence and Size of the Microsatelite Markers Used in this Study

Primers	PCR product Size (bp)	Sequence		Annealing Temperature (C°)
		Forward Primer	Reverse Primer	
RM10694	194	TTTCCCTGGTTTCAAGCTTAGC	AGTACGGTACCTTGATGAAAGG	55
RM3412	110	TCATGATGGATCTCTGAGGTG	GGGAGGATGCACTAATCTTTC	55
RM336	175	CTTACAGAGAAACGGCATCG	GCTGGTTTGTTTCAGGTTCG	55

3. Results and Discussion

a) M1 Studies

The M1 seeds of this variety were sown in the field of Technological University (Kyaukse) with the control (non-irradiated seeds), 100 Gy, 150 Gy, 200 Gy, 250 Gy, 300 Gy, 350 Gy and 400 Gy seeds that can be seen in figure 1 and some agronomic characters of M_1 generation can be seen in Table III. According to Table III, the filled grain of 400 Gy was higher than that of other treatments but the panicle length of this variety was the lowest. At maturity, the main panicles from this variety were harvested for M_2 generation.

Figure 1: View in rice var. Manawthukha plantation in M1 generation

b) M2 Studies

For M_2 generation, the main panicles of M_1 plants were collected and 19 plants from each treatment were screened by hydroponic screening method. The mean performance of yield components was listed in Table IV. It can be observed that the plant height and 1000 grains weight of control was higher than that of all treatments. However, the effective tillers and panicle length of all treatments were decreased with the increasing dose of radiation.

Figure 2: View in rice var. Manawthukha plantation in M2 generation

Table III: Mean Values of Some Agronomic Characters from M_1 Generation in Summer Season, 2015

Treatment	Plant Height (cm)	Panicle Length (cm)	Effective Tillers	Filled Grain	1000 Grains Weight (g)
Control	89.69	22.45	12.62	90.689	21.8
100 Gy	90.5	22.37	13.07	95.1	19.96
150 Gy	87.1	21.73	10.73	88.9	20.53
200 Gy	87.72	21.79	11.59	90.517	20.25
250 Gy	84.17	22.32	13	78.467	20.31
300 Gy	85.76	21.41	10.34	72.586	19.86
350 Gy	90.47	23.6	9.2	77.133	21.5
400 Gy	89.5	21.3	11.67	111	21.72

Table IV: Mean Values of Some Agronomic Characters of Potential Lines Selected from M_2 Generation in Rainy Season, 2015

Treatment	Plant Height (cm)	Affective Tiller	Panicle Length (cm)	Filled Grain	1000 Grains Weight (g)
Control	105.86	14.68	23.59	103.27	21.8
100 Gy	106	14.15	23.25	115.8	19.96
150 Gy	103.9	11.05	21.65	107.15	20.53
200 Gy	95	10.05	21.87	100.47	20.25
250 Gy	91.45	12.05	21.95	106.6	20.31
300 Gy	95.7	9.87	22.9	87.95	19.86
350 Gy	95.8	10.5	22.9	85.4	21.5
400 Gy	94.7	7.8	22.3	62.3	21.72

c) M3 Studies

Among 62 plants from M_2 generation, the plants which were not better than the control plants were not screened for M3 generation. Nineteen plants including Pokkali, IR29 and control were selected and screened again for M3 generation and also they were regarded as the mutant lines and were cultivated in rainy season. The mean performance of yield components was listed in Table V. MK-C1 had the lowest number of effective tillers and yield per hill. Although the panicle length in MK-D was significantly higher than that of the mutant plants and control, the number of tillers in control was significantly higher than that of the mutant lines. However, MK-F3 had the highest number of 1000 grains weight .The promising lines in MK-D2 and MK-E of yield per hill was significantly higher than that of the mutant plants.

Figure 3: View in rice var. Manawthukha plantation in M3 generation

d) M4 Studies

For M4 generation, thirteen plants including Pokkali and control were selected and screened again from M3 generation and also they were regarded as the mutant lines and were cultivated in summer season. The mean performance of yield components was listed in Table VI for phenotypic characteristics. In M4 generation, plant height, panicle length, 1000 grain weight and yield per hill of MK-B1 was higher than the control. Although 1000 grain weight of MK-D1 and MK-E were higher than that of control, yield per hill was lower than the control and some of the resistant plants. The plant height of MK-C and MK-F2 were the lowest than the control and some of the mutant lines. And also the plants were tested with the salt tolerance primers to get salt tolerance mutant plants from eleven plants by the genetic molecular laboratory. For genotypic characteristics, among thirteen lines including Pokkali and control, four lines (MK-B1, MK-D1, MK-E and MK-F2) were salt tolerant mutant lines that were checking salt tolerant primers (RM10694, RM3412b and RM336).

Figure 4: View in rice var. Manawthukha plantation in M4 generation

e) Screening thirteen rice germplasm for salt tolerance using SSR markers

Identification of molecular markers tightly linked to salt tolerant genes can serve as landmarks for the physical localization of such genes facilitating marker assisted selection (MAS). The SSR markers were also reported as highly polymorphic in IR29 and Pokkali for tagging salt tolerant genes [15]. El-Refaee et al. (2006) [16] also reported that 80% of the tested SSR primers showed polymorphic pattern in rice while they studied 272 SSR primers on nine rice genotypes. In this experiment RM490, RM493, RM3412b, RM10694, RM562, RM8094, AP3206, RM10784, RM336 and RM253 primers were used for polymorphism survey of M4 and M5 mutant plants. Out of these primers, three polymorphic SSR markers viz., RM10694, RM3412b and RM336 were shown highly polymorphic for salt tolerance selection of tested plants. In respect of primer RM10694, 7 lines were found tolerant at salt stress whereas 4 lines were susceptible in comparison with the tolerant variety Pokkali check line (Fig 6). Similarly, the same results were found by using primer RM3412b (Fig 7). With RM336, only 4 lines that were similar to tolerant Pokkali were identified. In the same reaction, 7 saline susceptible lines were also found as same as susceptible variety control (Fig 8). These three primers i.e. RM10694, RM3412b and RM336 showed polymorphisms in the studied thirteen rice germplasms because they showed different banding patterns and discriminated tolerant lines from moderately tolerant and susceptible with reaction to the tolerant variety Pokkali. For genotypic characteristics, among thirteen lines including pokkali and control, four lines (MK-B1, MK-D1, MK-E and MK-F2) were salt tolerant mutant lines that were checking salt tolerant primers (RM10694, RM3412b and RM336).

f) Evaluation of salt stress symptoms after 10 and 16 days

Salt injury symptoms were shown after 7 to 8 days from the first day of salinization. The main symptoms were leaf rolling and formation of the new leaves, leaf rolling and whitening of the tips which lead to complete cessation of the growth and finally drying the leaves. Jubay (2012) and Islam (2004) [17] observed similar salt injury at the seedling stage of rice plant in Pokkali and IR29.

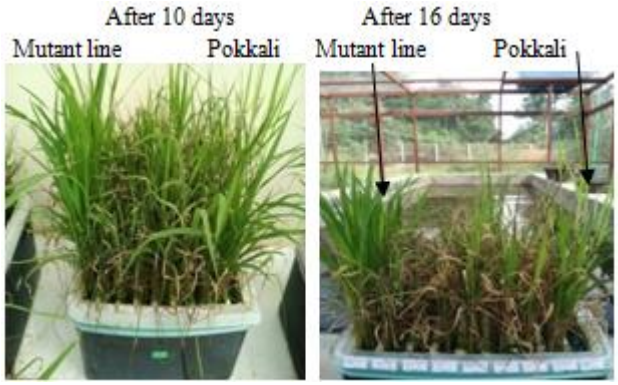

Figure 5: Potential lines of MK at EC 10 and 16 days

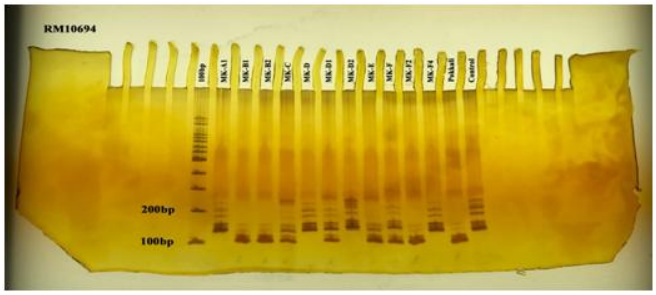

Figure 6: Banding profile of 13 rice germplasms using primer RM10694 Polymorphic bandings compare with Pokkali were MK-B1, MK-B2, MK-C, MK-D1, MK-E, MK-F and MK-F2

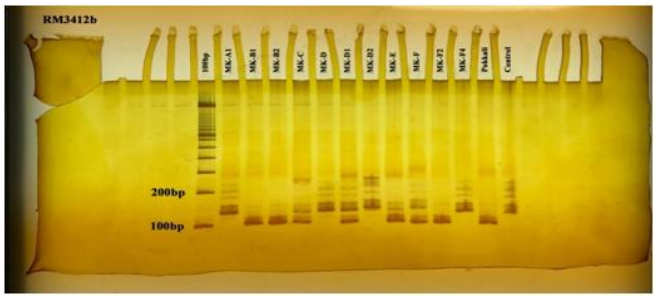

Figure 7: Banding profile of 13 rice germplasms using primer RM3412b. Polymorphic bandings compare with Pokkali were MK-B1, MK-B2, MK-C, MK-D1, MK-E, MK-F and MK-F2

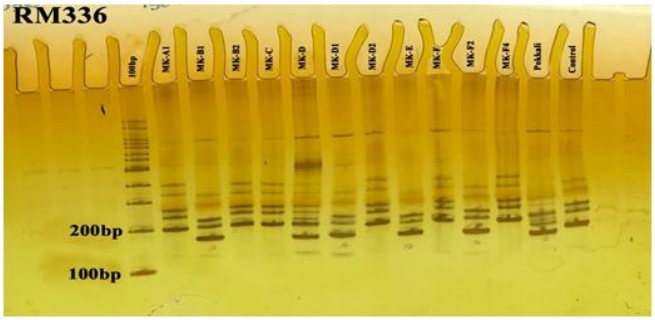

Figure 8: Banding profile of 13 rice germplasms using primer RM336. Polymorphic bandings compare with Pokkali were MK-B1, MK-D1, MK-E and MK-F2

Table V: Mean Values of Some Agronomic Characters of Potential Lines Selected from M_3 Generation In Rainy Season, 2016

Potential Lines	Plant Height (cm)	Panicle Length (cm)	No. of Tillers	No. of Effective Tillers	1000 Grains Weight (g)	Yield per Hill (g)
MTK Control	75.2	20.2	20.6	10.8	19.98	2.26
MK-A	67.67	18.33 ns	11.67*	6 ns	16.67*	3.997 ns
MK-A1	64.67*	17.54*	9.22*	4.44*	15.68*	2.69 ns
MK-A2	70.75 ns	18.63 ns	12.75*	5.75*	15.75*	3.85 ns
MK-B	69.33 ns	21.67 ns	10.67*	5.67 ns	15.6*	3.72 ns
MK-B1	71.45 ns	20.8 ns	9.25*	2.95*	19.9 ns	3.09 ns
MK-C	60.5*	17.25*	9.5*	6.25 ns	15.18*	2.63 ns
MK-C1	68.67 ns	20.23 ns	9.33*	2.67*	15.47*	2.13 ns
MK-D	68 ns	22 ns	14.5 ns	4 ns	17.38	2.84 ns
MK-D1	65.33*	19.33 ns	11.33*	5.67 ns	16.43*	4.93 ns
MK-D2	76.85 ns	19.26 ns	14*	12.42 ns	18.37 ns	5.31*
MK-E	72.5 ns	20.5 ns	10.5*	6 ns	18.37 ns	5.44 ns
MK-F	59.4*	17.21*	9.2*	6.87*	15.06*	2.96 ns
MK-F1	59*	18.5 ns	9.25*	5.75*	18.12 ns	2.73 ns
MK-F2	67 ns	19.033 ns	7.67*	3.67*	16.5*	4.13 ns
MK-F3	70.2 ns	17.64 ns	12.6*	10.8 ns	20.05 ns	2.61 ns
MK-F4	60.6*	17.4*	11.4*	7.2 ns	18.9 ns	2.8 ns

Table VI: Mean Values of Some Agronomic Characters of Potential Lines Selected from M_4 Generation in Summer Season, 2017

Potential Lines	Plant Height (cm)	Panicle Length (cm)	No. of Tillers	No. of Effective Tillers	1000 Grains Weight (g)	Yield per Hill (g)
MK-A1	116.5	22.5	16.5	10.5	14.972	1.43
MK-B1	125.06	25.71	17.45	15.67	20.76	2.69
MK-B2	100*	20	14*	8.77*	13.124	0.78*
MK-C	100.33*	20.67	14.33*	8.67*	14.55	0.88*
MK-D	113.48	24.46	9.79*	9.17*	19.13	2.46*
MK-D1	108.73	21.92	17.43*	13.77*	16.39	1.72*
MK-D2	116.04	24.17	17.93	16.04	19.65	2.15
MK-E	120	22.315	9.45*	7.9*	18.62	1.94*
MK-F	125.9	23.36	12.5*	10.25*	18.52	1.97*
MK-F2	106.67*	20.53	11.67*	9.33*	15.32	1.63*
MK-F4	120	21.3	8.49*	20	13.875	0.87*
Control	123.8	22.85	25	20.5	15.96	2.295

4. Conclusion

Salinity tolerance seedling stage showed wide variation in phenotypes with salinity scores ranging from 3 to 9. Among the genotypes, (MK400-2) mutant line was found tolerant, (MK100-2, 150-2, 200-2, 250-2 and 350-2) resistance lines were found moderated tolerant and (MK300-2) was sensitive to salt stress. M1 seeds of this variety were sown in the field of Technological University (Kyaukse) with the control (non-irradiated seeds), 100 Gy, 150 Gy, 200 Gy, 250 Gy, 300 Gy, 350 Gy and 400 Gy seeds. For M_2 generation, the main panicles of M_1 plants were collected and 19 plants from each treatment were screened by hydroponic screening method. So for seven treatments, a total of 136 plants, including control, IR29 and Pokkali were screened. Among them, only 62 plants survived. They were screened again for M_3 generation. Among 62 plants from M_2 generation, the plants which were not better than the control plants were not screened for M3 generation. Nineteen plants including Pokkali, IR29 and control were selected and screened again for M3 generation and also they were regarded as the mutant lines and were cultivated in rainy season. In M4 generation, among 19 promising lines, 13 plants including control were selected and cultivated in summer season. However, when genotypic analysis were performed with salt tolerant SSR markers, only 4 mutant lines (MK-B1, MK-D, MK-E and MK-F2) were observed as same as Pokkali. Some plants were collected by phenotypic screening in the field. The promising salt stress tolerant mutant lines will be cultivated in next generation to confirm if they can be tolerant of salt stress and then Yield Trials of these mutant lines will be tested. Comparison with the irradiated mutant lines, the non-irradiated control ones were completely under salt affected although they can reach to heading stage.

5. Acknowledgment

I would like to express heartfelt gratitude to Dr. Aye Aye Khai, my supervisor and main source of inspiration, for her tireless, interest, encouragement and patience on this research work. I also thank to Dr. Myat Minn, Dr. Nay Chi Win and other technical staff members in the laboratory at Biotechnology Research Department (Kyaukse) for the help in laboratory and farm. My sincere gratitude to Dr. Myo Myint, other teachers and friends from Department of Biotechnology, Mandalay Technological University for their helpful facilities and suggestions. I would like to express special thanks to Daw Thin Thin Soe, principal scientist, and other technical staff members in the Department of Atomic Energy, Yangon for the permission and help for gamma chamber application. I also thank to Dr. May Sandar Kyaing, Dr. Honey Thet Paing Htwe, Dr. Moe Moe Myint, Ma Khin Pyone Yi and other technical staffs from the molecular laboratory at Biotechnology Research Department (Kyaukse) for the help in the laboratory.

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