6. Malaysia

Application of Mutation Techniques and Biotechnology for Minimal Water Requirement and Improvement of Amylose Content in Rice

[1]Rusli Ibrahim, [1]Abdul Rahim Harun, [1]Sobri Hussein, [2]Abdullah Mat Zin, [3]Sariam Othman, [4]Marziah Mahmud, [4]Mohd Rafii Yusof, [1]Siti Hajar Mohd Nahar, [4]Zarifth Shafika Kamaruddin, [5]Ana Ling P.K

[1]Agrotechnology & Biosciences Division, Malaysian Nuclear Agency, Ministry of Science, Technology & Innovation (MOSTI), Bangi, Selangor; [2]Universiti Malaysia Terengganu, 21030 Kuala Terengganu; [3]Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor; [4]Universiti Putra Malaysia (UPM), Serdang, Selangor; [5]Department of Bioscience, Faculty of Engineering and Science, University Tunku Abdul Rahman, Kuala Lumpur

6.1 Introduction

Rice is the staple food crop in Malaysia. Malaysia managed to achieve 72% self-sufficiency level in rice with the current average rice yield of 3.7t/ha/season. In this situation, about 28% of the local demand will have to depend on rice imports. In Peninsula Malaysia, rice production depends largely on the irrigated lowland production system. Currently there are 241,741ha of irrigated rice in Peninsula Malaysia which contributes more than 85% of the national rice production. However, growing irrigated rice requires large amount of water. It was estimated that about 3,000 liters of water are required to produce 1kg of rice. Unfortunately, there are signs of declining water supply that threatens the sustainability of irrigated rice production.

The largest water withdrawal (more than 75%) in Malaysia is for irrigation in the agriculture sector and is mainly confined to irrigated rice production. However, the future agriculture's share of water will decline because of increasing competition for available water from urban and industrial sectors. Water is becoming a scarce entity in the future and in some areas, it has become a limiting factor in rice production (Bhuiyan, 1992). Thus, rice will be the crop most affected by water scarcity as it depends most heavily on irrigation. Therefore, there is an urgent need to develop rice varieties adapted to water stress and still produce acceptable yield and posses all the other beneficial traits.

Water supply is affected by the loss of watersheds due to deforestration and soil erosion. There is severe depletion of valuable groundwater resources as water is taken up for agricultural and industrial purposes. Some varieties of rice can be grown without irrigation, however their growth totally depend. About 15% of the rice varieties in Sabah and Sarawak are rainfed. Rainfed rice varieties are lower in yield due to uncertainty of water supply. In view of this water shortage, there is an urgent need to develop new varieties of rice with high yield potential and stability under water stress conditions. Therefore, in an attempt to develop water stress tolerance lines, morphological and agronomic traits that are related to water stress condition, can be useful in establishing a successful breeding program for rice improvement.

Induced mutation is an important supplementary approach to plant breeding, particularly when it is desired to improve one or two easily identifiable traits in an otherwise good lines or varieties. The successful use of plant breeding for improving crops requires the existence of genetic variation of useful traits. Unfortunately, the desired variation is often lacking. However, radiation can be used to induce mutations and thereby generate genetic variation from which desired mutants may be selected. Mutation induction has become a proven way of creating variation within a crop variety. It offers the possibility of inducing desired attributes that either cannot be expressed in nature or have been lost during evolution.

Heavy ion beams, such as carbon ions, are more effective in plant for inducing mutations compared with electron beam (Hidema *et al.*, 2003). Novel mutant have been obtained by the carbon ion irradiation in several plant species (Hase *et al*., 2000). To date, a number of rice mutants with various significant improvements in morphological traits have been recommended for planting (Mohamed *et al.*, 1988) but none was targeted for adaptive traits per se. Water shortage has become the bottleneck of Malaysia's food security. The development of water-saving rice varieties to decrease water consumption in rice production is inevitably a major goal in agriculture research. Thus, to achieve long-term food security and sustainable development in Malaysia, 'Water-saving or drought-resistance' (WDR) rice varieties are urgently needed.

Drought is one of the most important limiting factors in more than 65% of paddy fields in Malaysia where super rice varieties cannot perform well under drought stress. Therefore, the development and production of drought-tolerant rice varieties, to stabilize and improve the production levels in the low-middle-yielding fields, is needed. In recent years, the field drought-resistance screening facility was established through mutation breeding program and the evaluation standard was developed. Some advanced lines of drought tolerance rice varieties were identified and will be used in both molecular mapping and breeding programmes. The objectives of this study, therefore, were to screen for mutant lines derived from gamma rays and ion beam irradiation for high yield potential and stability under water stress conditions with improved quality trait such as amylose content.

6.2 Materials and Methods

6.2.1 Mutation Induction

Seeds of popular local variety MR219 were irradiated with 300Gy of gamma radiation from the ^{60}Cobalt source at Malaysian Nuclear Agency in 2008. Irradiated seeds were sown at Mardi Research Station in TanjungKarang, Selangor. The M_1 seedlings were transplanted into the field with 25cm x 25cm planting distance. Ten thousand M_1 seedlings were planted to produce M_2 seeds and a total of 5,250 plants were selected from which 2 panicles per hill were randomly harvested from each hill.

For ion beam irradiation, four rice cultivar, MR219, Q74, MR211 and PongsuSeribu 2 were irradiated in Takasaki Japan. MR219 and Q74 were irradiated on 20th Jan 2009 for sensitivity test. A total number of 100 seeds per dose (0, 10, 20, 40, 60, 80, 100, 120, 160, 200 Gy) of MR219 and MRQ74 were irradiated with ion beam in Takasaki Japan. The seeds were evaluated at Malaysian Nuclear Agency. The shoulder dose for MRQ74 was observed at 120Gy. The optimum doses of these cultivars were identified and the second irradiation was done on 16 Jun 2009 whereby 1,500 seeds of each cultivar were irradiated with doses of 60 and 80Gy. Meanwhile Seeds of MR211 and PongsuSeribu were irradiated on 5th Feb. 2010 for sensitivity test.

A total of 1,500 M_1 seeds of MR219 and MRQ74 irradiated with 60 and 80Gy were raised at Malaysian Nuclear Agency green house. Only M_2 seeds of MR219 with 80Gy were planted in Agriculture Department experimental Plot, TgKarang Selangor. About 10,000 of M_2 individual plants of MR219 were planted. About 5% of M_2 populations were selected for further screening in M_3 including analysis of amylose content.

6.2.2 Green-house screening

Green-house screening was done using cement troughs filled with paddy soils. A water float control was constructed to control the level of water from time to time by adjusting it in order to attain the saturated water regime as require in the study. M_2 seeds of MR219 were hand seeded at a distance of 4cm between and within rows. Flooded water level was maintained at 4cm for 7 days after seeding to allow for good establishment of seedling. Then after, water was gradually drained to provide stress to the plants. However, the water level was again raised to 4cm above soil surface at 21 and 45 days after sowing. During this time, fertilizer was applied at the rate of 60:40:30 kg/ha. The trough remained flooded about 7 days after fertilization to allow optimum distribution of fertilizer and after which the water level was again lowered to 5cm from soil level to provide additional stress to the water. Second stress was done at 30 - 40 DAS where the water level was reduced to 10cm from soil level and finally at 41 – 50 DAS until maturity, the water level was finally reduced to 15cm from soil level. At maturiy, a total of 500 panicles with good filled grains were selected for field screening.

Figure 1. Greenhouse screening of mutant lines of MR219 irradiated with gamma rays for minimal water requirement using water simulation technique.

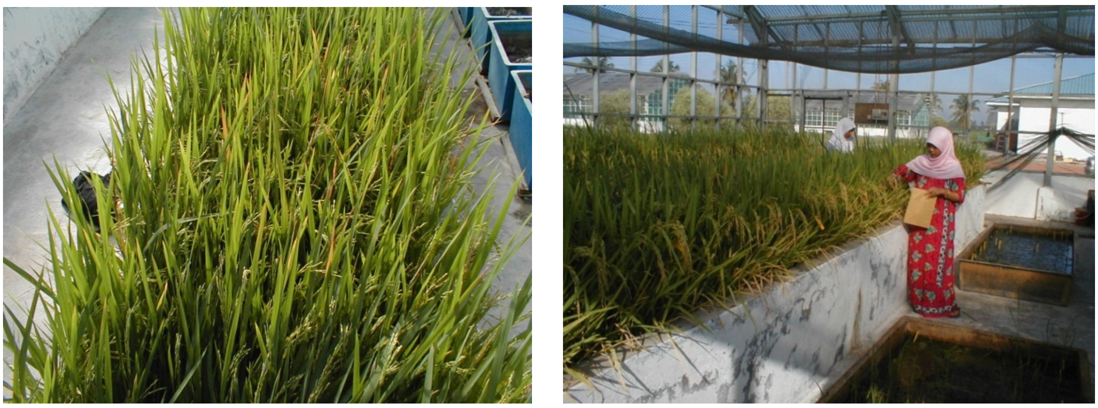

Figure 2. Selection of mutant lines of MR219 irradiated with gamma rays tolerant to minimal water requirement using water simulation in the greenhouse

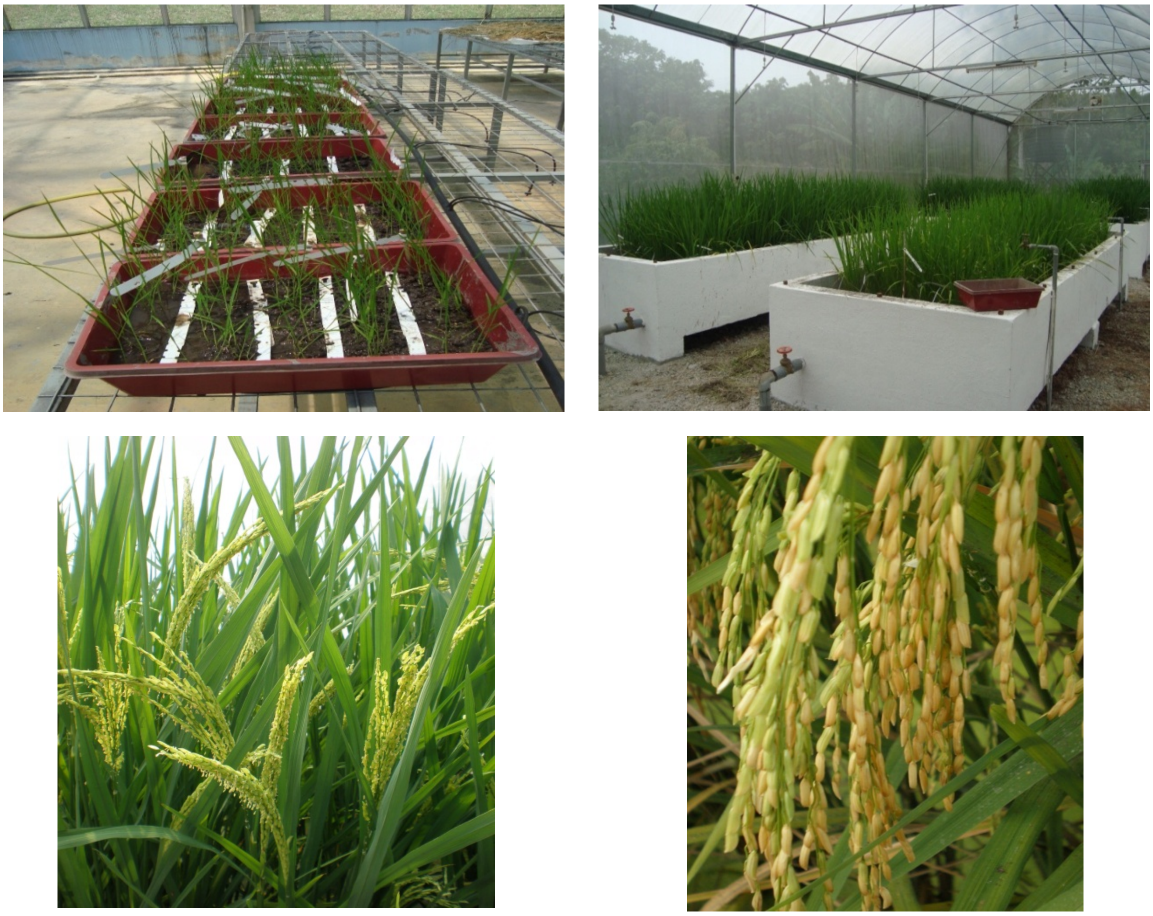

Figure 3. M_1 plants of MR219 and Q74 irradiated with 60 and 80 Gy of ion beam planted at Malaysian Nuclear Agency green house.

6.2.3 Field Screening

For field screening, the plot lay-out was constructed to simulate precise water stress regime. The screening procedure (stress treatment, fertilization methods and etc) was similar to the green house screening but 14 days old seedlings were transplanted instead. The seedlings were transplanted to the field with planting distance of 10cm x 10cm to make up for 16 plants per entry. A total of 38 lines were planted. Flooded field condition was maintained until 7 days after planting to allow for seedling establishment prior to water stress treatment at different stages of plant growth ie. at 41 - 50, 60 - 70 and 80 - 99 days after transplanting. During stress treatment the water level was maintained at 15 cm below soil level. The 1st fertilizer application (100:40:30) applied was done at 21 days after transplanting and the 2nd fertilizer application was done at 53 days after transplanting. Sampling and harvesting was done at 85% ripening. Important agronomic traits were recorded at maturity from 5 plants, sampled at random from each plot.

After several series of selection and fixation, 12 potential lines with the required adaptive traits were recovered at M_4 generation. However, only two potential lines designated as MR219-4 and MR219-9 were selected for further testing under several stress environments. Selection was mainly based on the percent filled grain because under water stress environment, it is the most sensitive indicator of tolerant lines.

6.2.4 Yield Trials

MR219-4 and MR219-9 were evaluated for yield and adaptation in MADA under saturated and flooded conditions. The aim was to study the interaction effects of rice variety and water management on yield and yield components such as panicles/sq m, spikelets/panicle, percent filled grains and the 1000-grain weight. The lines were also evaluated under aerobic soil condition in MARDI Seberang. The same mutants were evaluated for adaptation and yield under flooded conditions in KETARA, Besut, Terengganu.

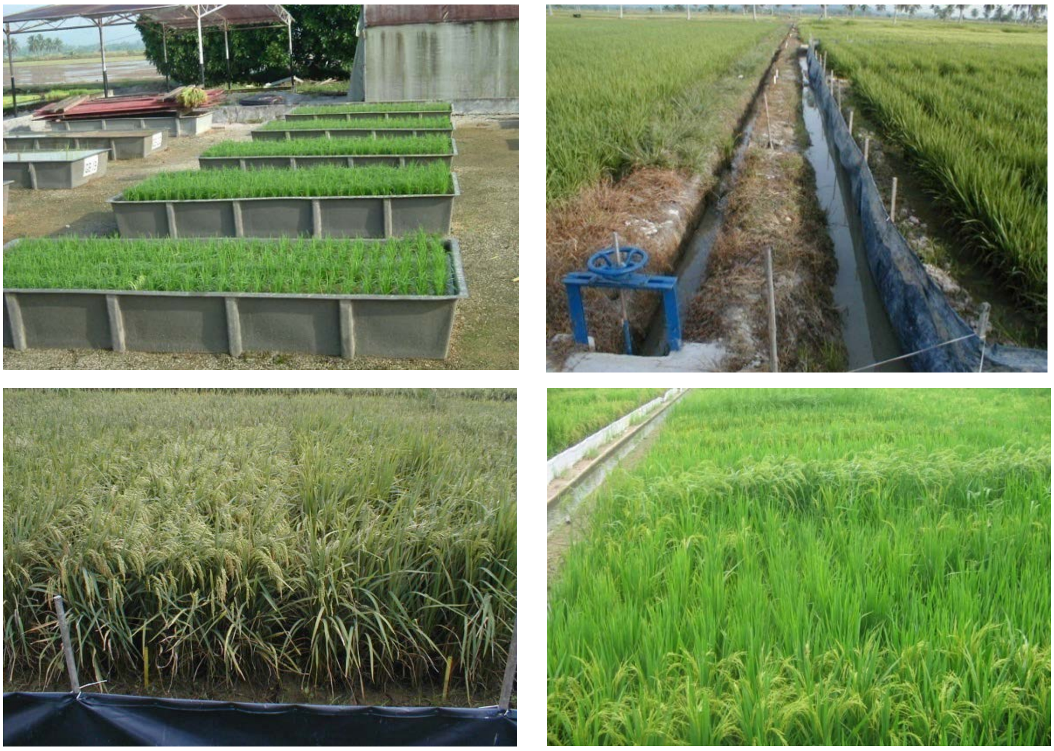

Figure 4. Field screening and selection of advanced mutant lines of MR219 irradiated with gamma rays for minimal water requirement using water simulation technique.

Figure 5. Field planting of M_3 mutant lines of MR219 irradiated with 80Gy ion beam at Tanjong Karang, Selangor Experimental Station

6.2.5 Analysis of Amylose Content

To determine the apparent amylose percentage a standard curve using amylose from potato was applied. The method described by Juliano (1971) was used to make a standard curve and amylose-iodine solution with rice flour was prepared manually by grinding rice grains in mortar and pestle. Then the samples were sieved with 45μm aperture. Using this method, 0.04g of standard amylose was weighted and put into a volumetric flask. 1ml of 95% ethyl alcohol was added and gently mixed to spread the flour. 9ml of 2 N Naoh were added to the mixture. The standard solution was then transfer to magnetic stirrer and stir for 10 minute after adding distilled water to 100ml. The absorbance of the solution was measured at 620nm using a spectrophotometer, setting blank at zero absorbance. Data obtain were recorded and standard solutions against amylose content were plotted.

For the analysis of amylose content in a test sample, a portion (10g) from each of the sample was reserved. Seeds from individual panicle were harvested and a portion was subjected to analysis of amylose content. Apparent amylose content determination was performed using a near-infrared reflectance spectrophotometer According to this procedure, 0.1g of rice flour was transferred into a 100ml dry volumetric flask and mixed with 1ml of 95% ethyl alcohol. 9ml of 2 N Naoh were added to the mixture. The test solution was then transfer to magnetic stirrer and stir for 10 minute after adding distilled water up to 100ml. In another 100ml volumetric flask was added in 70ml distilled water, 2ml of 1N glacial acetic acid, 2ml iodine solution and 5ml aliquot of the test solution. The solutions were added distilled water to the volume of 100ml and let stand for 10 minute.

In the presence of amylose, a blue-black color will be observed. The intensity of the color can be tested using a spectrophotometer which reflects the concentration of starch present in the solution. The test solutions were measured using a spectrophotometer at 620nm by setting the absorbance of blank solution at zero. The blank solution was prepared by adding 2ml glacial acetic acid, 2ml iodine solution and distilled water were adjusted to 100ml. The amylose percentage was evaluated with the standard curve.

Methodology for determination of amylose content
(By Juliano, 1971)

Method for determination of amylose content

1. Sample preparation (de-husking & grinding)
2. Weight 0.1g rice flour into 100ml dry Erlenmeyer flas
3. Dissolve in 1 ml 95% ethanol and 9 ml 2M Sodium Hydroxide (NaOH)
4. Boil the solution mixture for 10 min at 95ºC
5. Homogenize the solution and add distilled water till 100ml
6. Transfer 5ml of the sample aliquot into a new 100ml dry Erlenmeyer flask
7. Add 2ml 1M Acetic acid and 2ml Iodine solutions
8. Add distilled water till 100ml
9. Measure the absorbance of amylose-iodine complex at wavelength 620nm

6.3 Results and Discussion

6.3.1 Radiosensitivity Test of MR219 irradiated with gamma irradiation

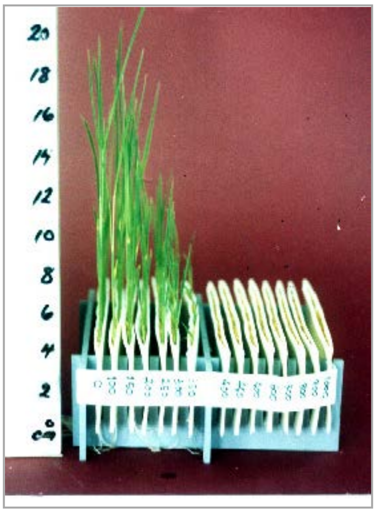

Figure 6. Radiosensitivity test of MR219 using sandwiched blotter technique

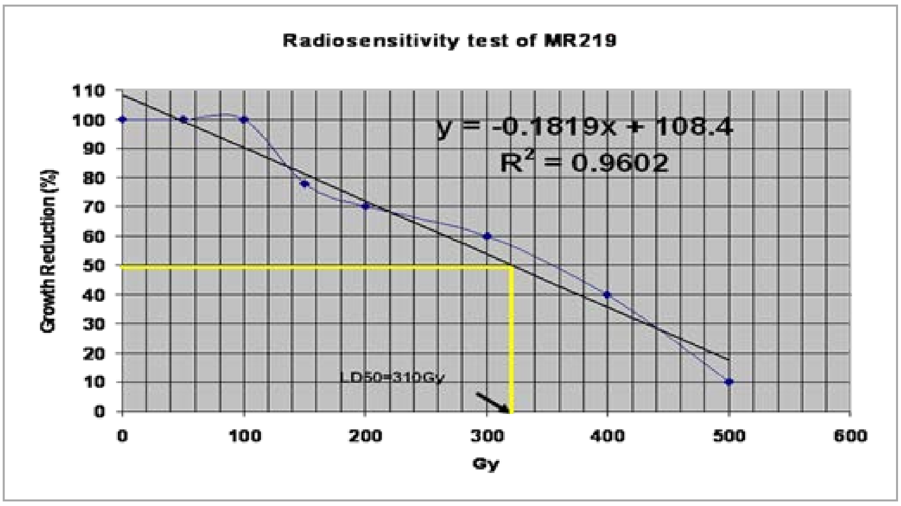

Figure 7. LD50 of MR219 irradiated with gamma rays was calculated to be 310Gy

6.3.2 Dose-response curves of MR219 irradiated with different doses of with ion beam

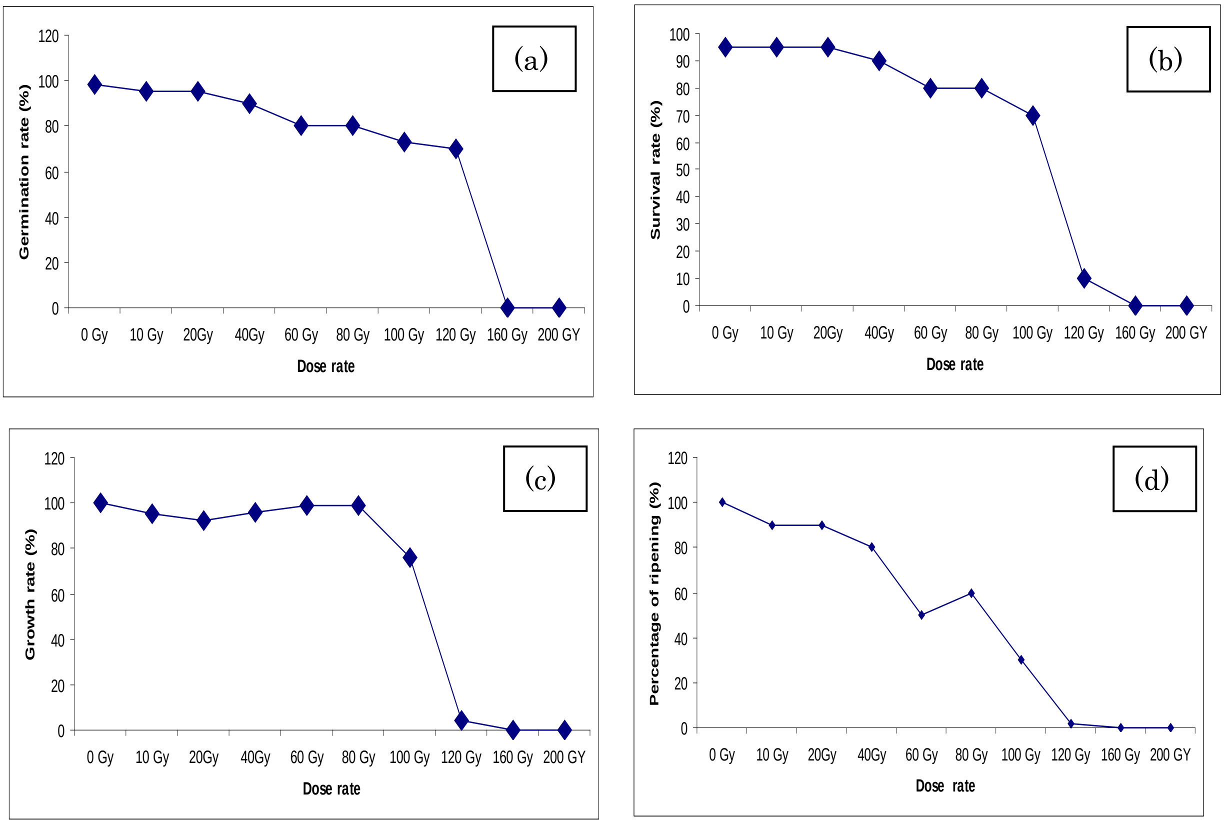

Figure 8. Dose-response curves of MR219 irradiated with different doses of with ion beam: (a) germination rate, (b) survival rate with different dose, (c) growth rate and d) percentage of ripening

6.3.3. Germination rate of MRQ74 irradiated with ion beam

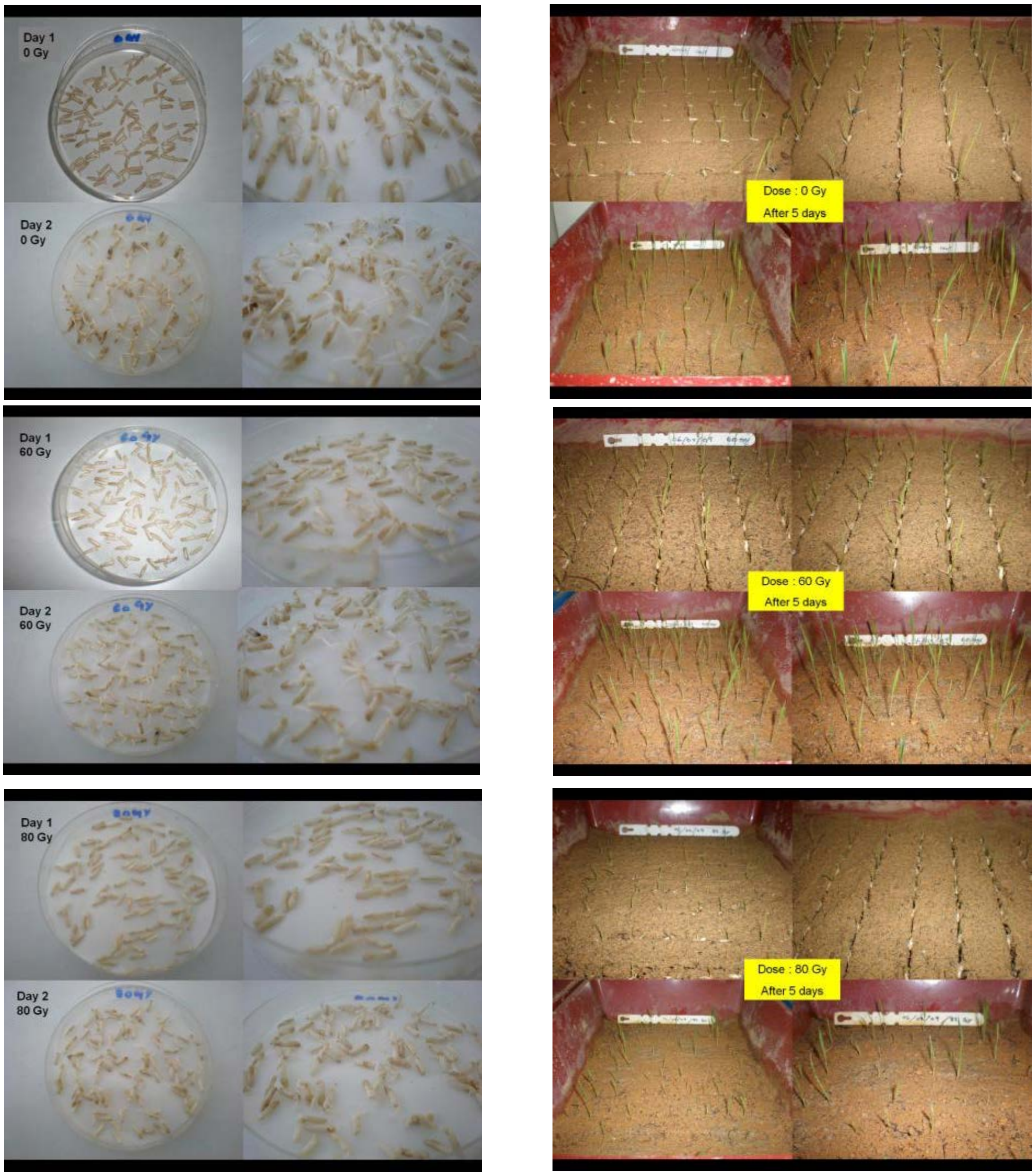

Figure 9. Germination rate of control and irradiated seeds of MRQ74 at day 1, 2 and 5

6.3.4 Plant type

The general morpho-agronomic and genetic evaluation traits of mutants and parent are as tabulated in Table 1. Panicle length, flag leaf width, leaf width are longer or broader than the parent. Similarly, the culm number for both mutants were comparatively or more than the parent.

Table 1. Morpho-agronomic characteristics of advanced mutant lines compared to its parents.

Plant Characters	MR219-4	MR219-9	MR219	Difference Over parent
Flag leaf length(mm)	34.2	36.4	40.1	lower
Flag leaf width (mm)	13.7	16	14.3	Higher(MR219-9)
Leaf length(cm)	41.3	40.6	41.4	Lower
Leaf width (mm)	15.3	12.3	13.7	Higher(MR219-4)
Culm length (cm)	74.3	75	72	Higher
Culm number (No)	15	13	14	Higher(MR219-4)
Culm diameter (mm)	3.61	3.62	3.62	same
Blast Reaction	Resistant	Resistant	Resistant	same
BPH Reaction	Resistant	Resistant	Resistant	same
Culm Strength	Strong	Strong	Strong	same
Panicle length	28.2	28.4	26.4	Higher

6.3.5 Yield and yield components

Yield trial in MADA, has indicated that the yields of MR 219-4 and MR219 -9 were comparable to the yield of MR 220. However, lines MR 219-4 and MR219-9 had significantly higher percentage of filled grains and 1000-grain weight than MR 220 (Table 2). There was no significant interaction effect of rice variety and water management on yield and yield components. Rice grown under saturated condition yielded as high as rice grown under normal flooded condition. Similarly, the yield components (panicles/sq m, spikelets /panicle, percent filled grains and the 1000-grain weight) did not differ significantly between flooded and saturated conditions. However, rice plants are significantly taller (how many percent) when they are grown under saturated than flooded condition.The growth and yield performance of MR 219-4 are similar to that of MR 219-9.The lines were also evaluated under aerobic soil condition (sprinkler-irrigation) under dry land regime in MARDI SeberangPerai and performed satisfactorily. Grain yield as high as 6.3t/ha for MR219-4 and 3.4t/ha for MR219-9 were recorded under aerobic condition.

Table 2. Yield and yield components of MR219-4 and MR219-9 in Muda (direct seeding method)

Treatment	Yield (kg/ha	Yield component				Plant height (cm)
		Panicle /sq.m	Spikelets/panicle	Percent filled grain	1000-grain wt (g)	
Saturated	6480 a	503 a	107 a	68.2 a	28.4 a	97.5 a
Flooded	6799 a	500 a	104 a	70.2 a	28.3 a	95.1 b
MR 219 – 4	6542 a	447 a	107 a	73.3 a	29.0 a	97.1 a
MR 219 – 9	6460 a	512 a	99 a	77.7 a	28.9 a	98.6 a
MR 220	6815 a	517 a	109 a	61.7 b	28.1 b	95.4 a
Water Management x variety	ns	ns	ns	ns	ns	ns

Performance of yield components and yield for KETARA are as tabulated in Table 3. Both mutants yielded lower than MR219 but they were better than MR219 for some traits such as grain width, percent filled grain, early maturation.

Table 3. Growth and yield performance of MR219-4 and MR219-9 in KETARA (direct seeding method).

Parameters	MR219-4	MR219-9	MR219	Difference Over parent
Panicles /plant	6.5	7.4	9	lower
Plant ht	100.1	100.4	103.4	lower
No. of tiller/hill	9	8	9	lower
Percent Filled Grain	78.1	87.7	73.4	higher
1000 grain wt(gm)	30.3	30.3	28	higher
Grain Length(mm)	9.69	9.69	9.86	lower
Grain width (mm)	2.38	2.41	2.35	higher
Panicle length	n.a	n.a	n.a	n.a
Yield (kg)	5259	6000	6444	lower
Maturity	110	112	116	lower

Table 4. Yield performance of MR219-4 and MR219-9 at different field condition.

Field condition	**MR219-4**	**MR219-9**
Normal flooded conditions	4.8 t/ha	5.5 t/ha
Saturated soil	5.9 t/ha	6.8 t/ha
Flooded condition	7.2 t/ha	6.1 t/ha
Aerobic soil condition	6.3 t/ha	3.4 t/ha

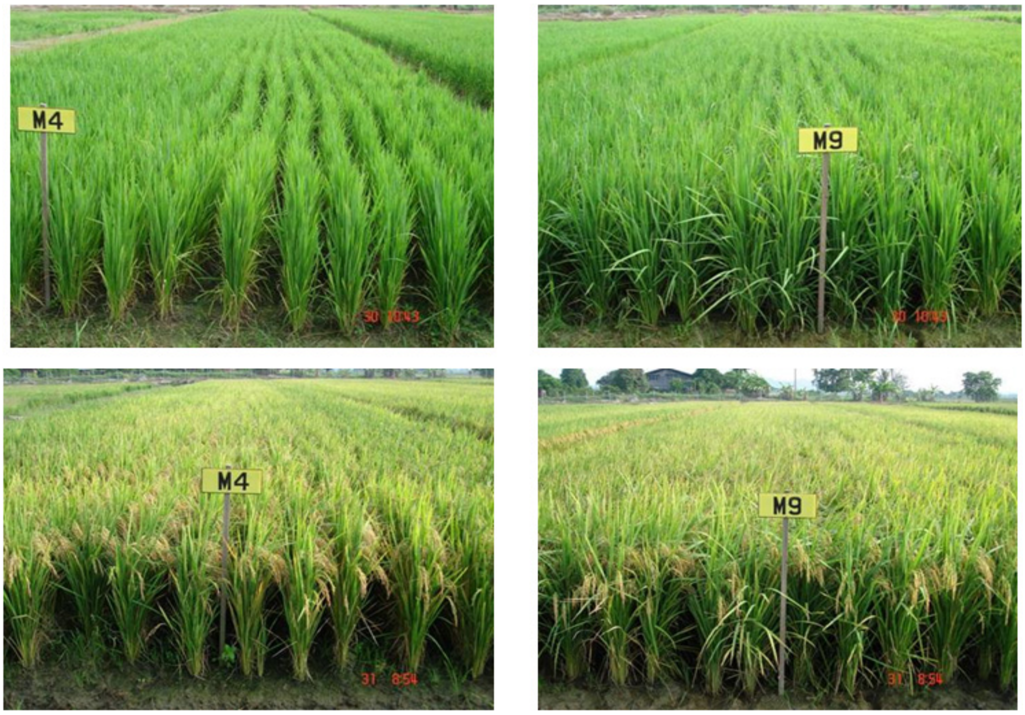

Figure 10. Evaluation of yield performance of advanced mutant lines MR219-4 and MR219-9 under low water requirement

Figure 11. Field Screening of mutants lines under simulated water stress condition at MARDI Seberang.Perai

Figure 13. Advanced mutant of MR219-4 under water stress (aerobic) condition

To produce a variety of rice mutants induced by carbon-ion beams (12C+6; 320 MeV), it is important to investigate the effects of the carbon-ion on several biological end points such as germination, survival, ripening and subsequent mutations in rice (Hidema *et al*., 2003). For MR219 which had been irradiated with ion beam, a total of 31 mutant lines were selected in M_3 generation based on their agronomic traits. However, physico-chemical characteristics were not significantly different from the parental variety except for amylose content which showed high variation among all lines. Based on overall data recorded, mutant line ML21 had the greatest performance in term of yield. Further screening should be carried out in M_4 to select for potential mutant lines which are resistance to blast disease and high yielding.

6.3.6 Analysis of amylose content

Traditional Malaysian rice varieties, such as Mahsuri and PongsuSeribu with 6 months of maturity period have high amylose content of 25.4% and 22.2% respectively in comparison to low amylose content of glutinous rice (13.3%), Carreon (13.3%), Columbia (16.6%). Original parental cultivar MR219 has intermediate amylose content (20.0%), whereas MRQ74 has high amylose content (30.0%). Mutant lines from MR219 irradiated with gamma rays have higher amylose content (22.5 to 23.7%), whereas mutant lines irradiated with ion beam have low amylose content (MR219/I2 - 18.0% and MR219/I12 - 18.6%). Parental variety MRQ74 with high amylose content (30.0%) after irradiation with ion beam was able to produce mutant lines with low amylose content (MRQ74/8 - 21.4%, and MRQ74/26 - 22.8%). This shows that ion beam irradiation is much more effective than gamma rays in producing lower amylose content.]

Table 5. Amylose content of Standard Rice samples

Standard rice samples	% Amylose
Koshihikari (Japonica rice	17 (low)
MR219 (Local indica variety)	20 (intermediate)
Mahsuri (Traditional indica variety)	25 (high)

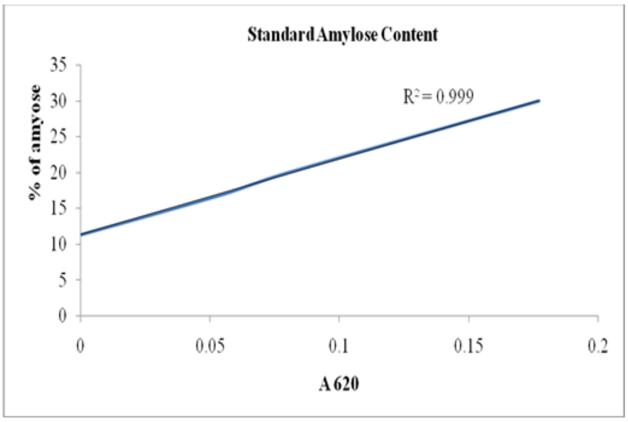

Figure 14. Standard curve of amylose using UV-VIS Spectrophotometer

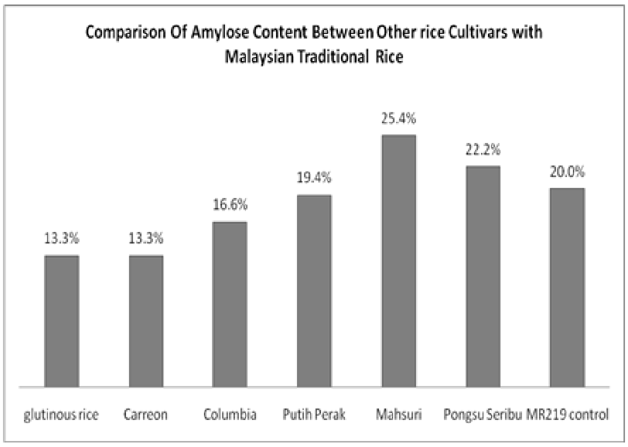

Figure 15. Comparison of amylose content between other popular rice cultivars with Malaysian traditional rice

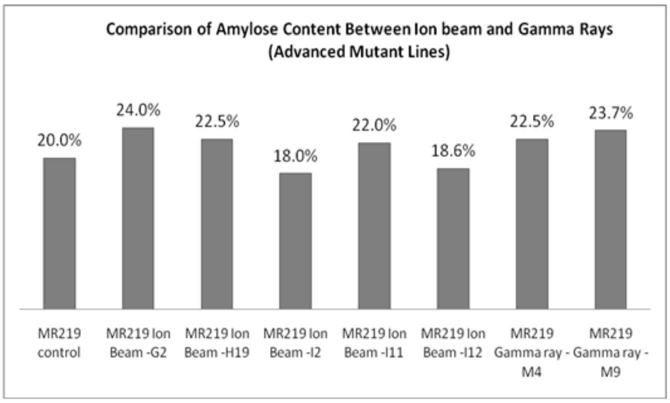

Figure 16. Comparison of amylose content between ion beam and gamma rays

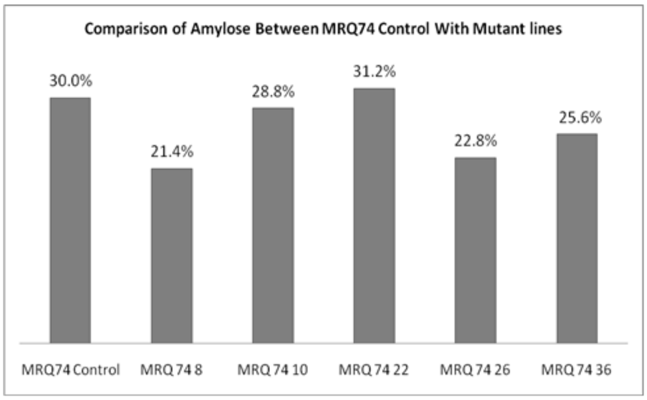

Figure 17. Comparison of amylose content between MRQ74 control with mutant lines

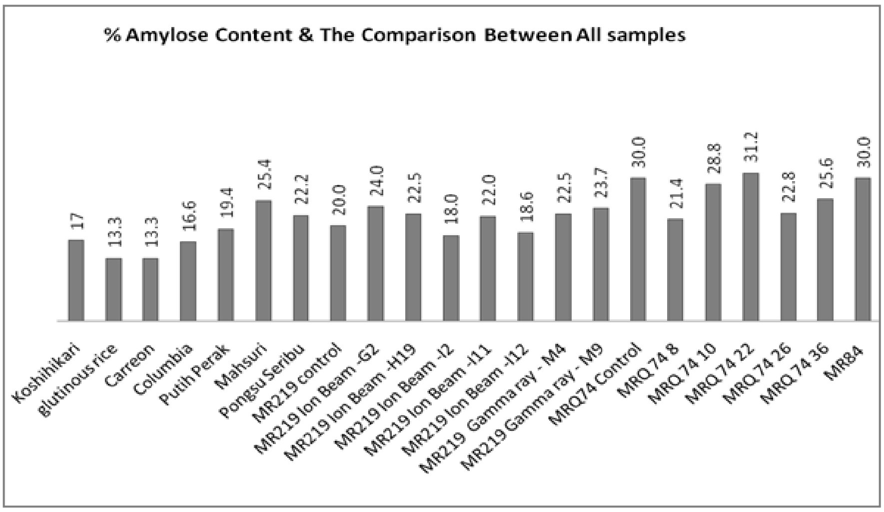

Figure 18. Percentages of amylose content and comparison between all samples

6.4 Conclusion

Main objectives of FNCA Plant Mutation Breeding Sub-Project on Composition or Quality in Rice are to select potential mutant lines of high yielding which are tolerance to minimal water requirement and improvement of amylose content. Under flooded condition, mutant line MR219-4 recorded a yield of 7.2t/ha and MR219-9 recorded 6.1t/ha compared to 5.8t/ha from the parental variety MR219. Under saturated condition, MR219-4 recorded 5.9t/ha whereas MR219-9 recorded 6.8t/ha of yield performance. The most striking observation made was when both mutant lines were tested under aerobic condition and mutant line MR219-4 performed much better than MR219-9, recorded a yield of 6.3t/ha in comparison to MR219-9 with 3.4t/ha. This shows that gamma irradiation was able to induce potential mutant lines suitable for minimal water condition with improved yield. These two advanced mutant lines will be further evaluated for stability in yield performance and other agronomic traits before they can be registered as new mutant varieties to be released to the farmers. In terms of improvement of quality of rice with respect to amylose, ion beam is more effective than gamma rays in producing lower amylose content. Mutant lines from MR219 irradiated with gamma rays have high amylose content (22.5 to 23.7%), whereas mutant lines irradiated with ion beam have low amylose content (18.0 - 18.6%). Similarly, when MRQ74 seeds with high amylose content of 30.0% were irradiated with ion beam, mutant lines recorded lower amylose content (21.4 - 22.8%). Further screening and selection in M_4 to M_6 generation will be carried out to select for potential mutant lines with high yield and resistance to blast disease.

6.5 Acknowledgement

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