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Screening of phenotypic performance, drought, and salinity tolerance in the mutagenized population of *Oryza sativa* cv. MR219 generated through ion beam irradiation

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Abstract Forward genetic approach was used to identify and characterize mutants based on phenotype traits. This approach is considered as a basic element to analyze the function and mechanism of target genes. The morphological mutants among ion-beam irradiated seeds of *Oryza sativa* cv. MR219 was identified. Seedling screening at the three-leaf stage recorded five categories of mutants with 21.97%, whereas the seedling frequency was 16.76% among 1,575 of M_2 and 150 of M_3 mutants' generation. The verification of mutation in the M_3 generation showed that the highest mutation frequency was leaf mutation (5.14%), followed by the root mutant (4.25%), and drought and salinity tolerance (3.69%). At this stage, 14 distinct mutations were involved in a significant number of mutants' tolerance to drought and salinity. By observing rice growth characteristics at the reproductive stage in the M_3 generation, 4.58% mutation covering around 10 mutant traits were marked and verified among 41 mutants. Among the verified mutants, the highest morphological frequency recorded was panicle mutation (1.45%), followed by tillering mutant (1.12%), and leaf mutant (0.89%). These findings revealed that all the examined mutants were suitable for subsequent functional gene analysis and in agronomy study.

Keywords: Drought, Forward genetic, Ion beam irradiation, MR219, Mutant collection

Introduction

Rice (*Oryza sativa* L.) is one of the significant food crops in the world. It provides more than 60% of the total daily calories needed by the human population. According to the Food and Agriculture Organization (FAO, 2020),

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Asia produces and consumes about 90% of the world's rice, with Malaysia producing a total of 2.34 million metric tons a year from 2013 to 2019 (Hirschmann, 2021). However, the global production of rice has declined due to biotic and abiotic stresses including drought stress, salinity, and various rice diseases. These events contribute immensely to the problem of food security to meet the demand to sustain the increasing population. Thus, rice breeding plays a key role in increasing food production for the growing global population. Creating superior rice with a high yield has been the major challenge among breeders to ensure food security. Substantial progress has been made toward optimal rice protocol, with mutation being a crucial method for rice breeding (Thang *et al*., 2010).

Mutations produce new or altered traits in genetic material, resulting in variation which is transmissible from parents to offsprings. Mutation is recognized as a valuable method, and its beneficial effects on crop production and cost reduction have been verified in a shorter timeframe. It is also a helpful tool for isolating and cultivating expected variants and resistances for breeding programs. The main aim of mutation breeding is to generate new varieties with high yield, higher nutritional content, and tolerance to biotic and abiotic stress. The FAO/IAEA Mutant Variety Database (MVD) recorded a total of 3,365 mutant varieties in which, 828 rice mutants were registered involving mutation induction techniques (Beyaz and Yildiz, 2017). Surprisingly, Asia produces more than 84% of rice mutant varieties, with China, Japan, and India dominating mutant production. These mutants have been established as the main crop and have been extensively cultivated to enhance food security.

Successful outcomes in mutation breeding depend on the type of mutagenic treatment. The bulk of the mutant varieties were developed and documented in the MVD using mutation induction methods that employed physical mutagens such as gamma rays and x-rays. (Li *et al*., 2019). Chemical mutagens generate high-frequency mutations and are often simple to use without advanced equipment. On the other hand, induced mutagenesis is a promising method for the rapid development of crop character compared to conventional breeding. Moreover, numerous mutant alleles are a source of genetic diversity.

Ion beam irradiation has recently attracted much attention because of its high mutation rates and survival rates, wide phenotypic variation, and targeted trait specificity (Oladosu *et al.,* 2016). Furthermore, ion beam has emerged as a modern mutation tool for inducing mutation in rice over the last two decades. The technique is commonly used in several local varieties in Japan, including Nipponbare, Hitomebore (Nakagawa and Kato, 2017), Torjan Indonesia (Sjahril *et al*., 2018), and Indira Barani Dhan 1' (Chauhan *et al.,* 2019). In contrast, Malaysian research on the use of ion beams in rice breeding is still underexplored (Mohamad *et al*., 2006; Hasan *et al*., 2020). This research aimed to classify each mutant in the M_2-M_3 population produced by ion beam-induced mutation, assess their wide-adapted ability through phenotypic evaluation, and assess their population heterogeneity through the distribution of each associated character to grain yield and abiotic stresses (drought and salinity tolerance). The current experiment was arranged with an array of several idiographic mutants that can be useful in elucidating the biological roles of new rice genes, promoting genetic, and positional cloning.

Materials and methods

Ion-beam mutagenesis

Seeds of the MR219 variety that were mature, stable, and uniform were collected from the GeneBank of the Malaysian Agriculture Research and Development Institute (MARDI). The seeds were chosen for ion-beam mutagenesis and mutant library construction in the present study. A total of 2,000 dry rice seeds were placed embryo-upward on Petri dishes and coated with 7.5 meters of kapton polyimide film. The 320 MeV carbon-ion irradiation was carried out using a vertical beamline of the AVF-cyclotron (JAEA, Takasaki), which was attached to the Irradiation Apparatus for the crop. Under ambient pressure, carbon-ion irradiation at doses of 70 Gy was achieved in 3 minutes. Seeds that were not irradiated were used as sensors. Fungicides were used to pre-soak the seeds for 16 hours before being washed several times. Unirradiated seeds were marked as control. Seedlings of M_1 were transplanted and harvested in a paddy field to develop M_2 generation.

Screening of the irradiated seedling population

A total of 2,000 M_2 seeds were germinated and sown into a plastic tray in a single seed per hole in 2019. Of the total 2,000 M_2 seeds, only 1,575 seedlings were growing with a germination rate of approximately 78.75%. In a growth chamber, the seedlings were nourished using a hydroponic system with a regulated temperature regime (20/30 ℃, night/day, and 12 hours of photoperiod). After 25–30 days of sowing (i.e. about the three-leaf stage), promising mutants from the seedling population were screened. Those with different shapes of seedling or root components than the usual phenotype of MR219 cultivar were selected. At the Malaysian Nuclear Agency (MNA) experimental greenhouse, the promising mutants were independently transplanted into the field and subsequently harvested to generate M_3 seeds.

Screening for stress tolerance mutants

A kill curve study was carried out in MR219 (parent variety) against various concentrations of NaCl (0.8–1.6%) and PEG 6000 (15–35%) to determine its tolerance to salt and drought (Thang *et al.*, 2010). Seedling survival rates were used to assess crucial concentrations of NaCl (1.2%) and PEG 6000 (25%), which were then treated to ion-beam seedling populations to screen the frequency of the mutant empirically. The irradiated seedlings that survived the abiotic stresses were labeled as tolerance mutants and transplanted to a paddy field to develop M_3 generation

Screening for morphological mutation

M_2 seedlings were grown in a greenhouse at the MNA, Bangi. The seedlings were grown in a pot filled with wet soil and treated as an individual plant. The MR219 (parent), Pokalli (salinity check variety), and NMR152 (Drought check variety) were assigned in a randomized complete block design (RCBD) with three replications. The plants were sub-irrigated with 2 L per plot and the recommended NPK fertilizer was placed inside each plot. The seeds were watered with the same amount at two to three days intervals depending on the amount of water left inside the plot. No pesticide and herbicide were applied to avoid any intervention. The further response of the morphological traits was observed in M_2 plants on a single plant basis including plant height, days to flowering, the number of tillers, flag leaf area, panicle length, and days to maturity. Thereafter, the grains were manually harvested when the plants reached maturity. Grains (M_3 seeds) from each M_2 plant were packed in an envelope. The culms and leaves were cut at ground level and wrapped with a newspaper. The grains and plant parts were dried in an oven at 37 ℃ for 48 hours. Further response of the mutative traits in M_3 seedling was conducted as described previously.

Data analysis

All the morphological differences against the control plant were recorded. The frequency mutant was calculated based on the formula as follows:- Number of mutants for each character’s x 100/Total number of M2/M3 seeds.

Results

Analysis of mutation at three-leaf-stage mutant population

Diverse morphological traits on the leaf, seedling height, and root, as well as drought and salt tolerance, were analyzed in the M_2 population at the three-leaf stage. A total of 358 promising mutants were found from 1,575 seedlings, with a frequency of 21.97%. However, only 150 mutants were able to maintain their mutated traits in the subsequent M_3 generation, with a seedling mutant frequency of 16.76%. In the M_2 population, among the mutant types, long root mutants (Figure 1F) showed the uppermost frequency of mutant with 55 individuals (3.49%), followed by short root (3.17%). Others such as lateral root and none-adventitious root were also reported at a lower rate. Leaf mutation was the most common (5.36%) among the mutants confirmed in the M_3 generation, followed by root mutation (3.91%), and abiotic stress resistance (3.80%).

Leaf mutation

The M_2 generation yielded a total of 119 individual seedlings showing leaf mutation with eight different mutation features, including leaf color and form. In the M_3 generation, a total of 7.55% were found and they demonstrated the highest ratio in the seedling mutant library. In the case of leaf color mutation, four mutations, including albino, greenable albino, yellow and virescent were found in the M_2 generation. The yellow-green and greenable albino mutants were able to survive and turned green, while most albino mutants died as a result of their inability to carry out photosynthesis. Among the 53 albino mutants, only 22 plants (41.5% of albino) recovered by returning to green color and continue to grow (Figure 1A). Of the recovered plants, only 10 (1.12% of albino mutants) manage to develop seed and display stable state heritability, while the others died after the three-leaf stage. Similarly, only 15 of the 25 yellow-green seedling mutants (Figure 1J) were able to grow further. In addition, the leaf shape, rolled leaf, and the virescent leaf mutation were discovered in the M_2 generation. A total of 31 rolled leaf mutants were recorded but only 18 mutants demonstrated a stable heritability in the M_3 generation. For virescent leaf, 10 mutants were found in the M_2 generation but only five maintained the trait in the M_3 generation. Contrary to the MR219 parent variety, the virescent mutant had a thicker leaf and a lighter color.

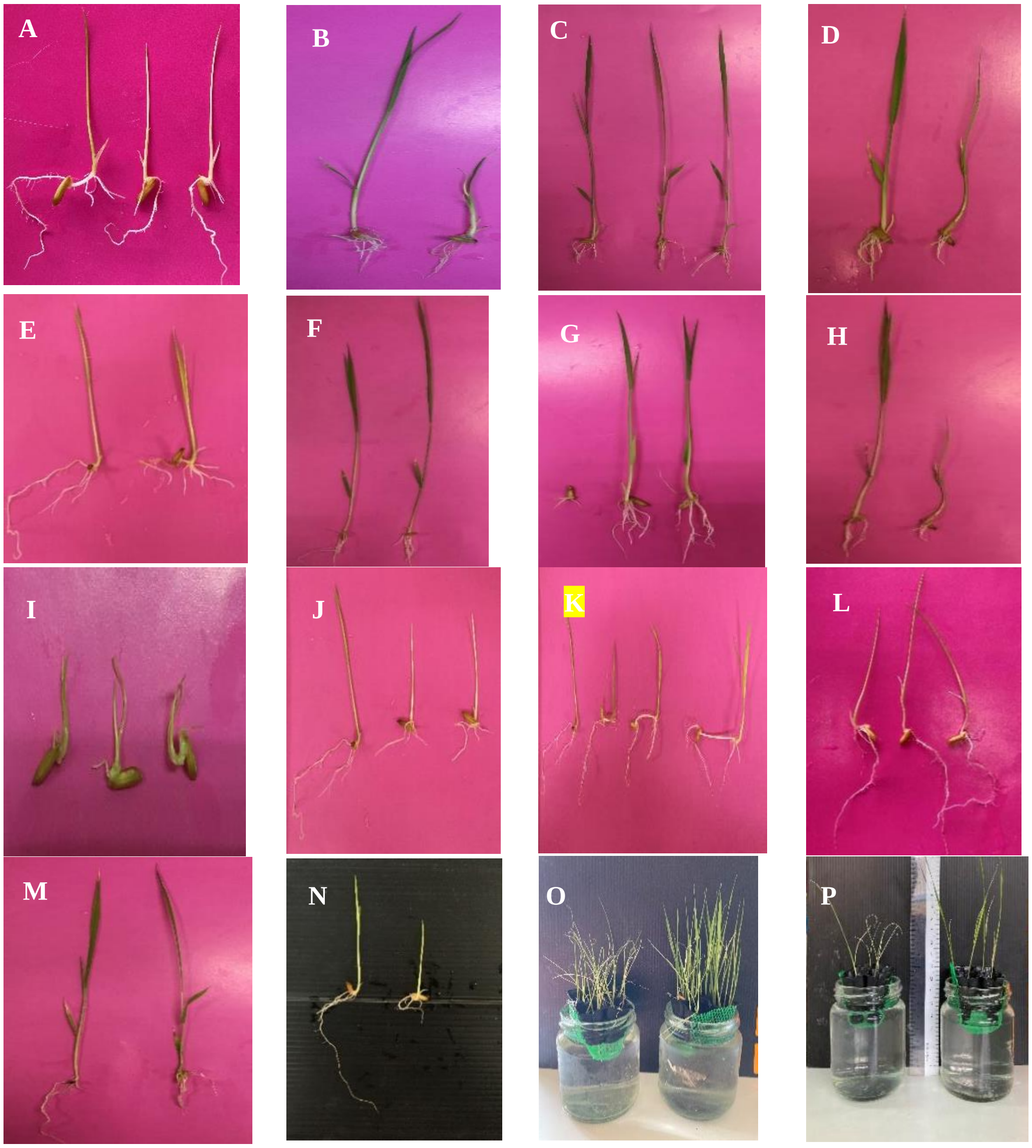

Figure 1. Mutation observed in seedlings at three-leaf stage (Leaf: Mutant; Right: wild type) (A: Albino, B: Dwarf, C: Rolled leaf, D: Less lateral root, E: none adventitious root, F: Long root, G: Severe dwarf, H: Short root, I: Incomplete twin seedling, J: Greenable albino, K: Mesocotyl mutant, L: Virescent, M: Long radical, N: Yellow green mutant, O: Salt tolerance mutant, P: Drought tolerance mutant

Seedling height mutation

In the M_2 population, dwarfism (Figure 1B) was the most common phenotype observed, accounting for 30 plants with an occurrence of 1.90%. Dwarf seedlings had an average height of 6.2 cm (ranging from 3.0 to 8.4 cm), while the parent variety had an average height of 11.7 cm. In the M_2 generation, three severe dwarf mutants (Figure 1G) with height ranging from 1.4 to 2.2 cm were discovered. In the M_3 population, about 12 dwarf mutants were confirmed, with a mutation frequency of 1.34. However, tall mutants were rare as only 17 were identified in the M_2 population, while only 10 were maintained the trait in the M_3 population. Relative to control seedlings, tall mutant seedlings were slightly taller, and their leaves were also significantly longer.

Root mutation

The root system was a vital category observation. In the M2 generation, five categories of root mutation were observed: short root (Figure 1D), long root (Figure 1F), less lateral root (Figure 1H), none-adventitious root (Figure 1I) and long radicle (Figure 1M). In the M_2 generation, a total of 55 long root and 50 short root mutants were observed. However, only 23 long root mutants (frequency of 2.57) and 10 short root mutants (1.12) were confirmed in the M_3 generation. When compared to the average root length of the control, short root mutants ranged from 5.8 to 6.0 cm, while long root mutants ranged from 12.5 to 17.9 cm (9.5 cm). Short root mutations were associated with dwarf seedlings (Figure 1B) and they had more adventitious roots than the controls. A very low frequency of less lateral root mutants was recorded with only five mutants in M_2 and 2 in M_3 generation.

Salt and drought tolerance mutation

To observe the morphological mutants, the remaining seedlings were either exposed to 1.2% NaCl solution or treated in 25% Polyethylene glycol (PEG) 6,000 solution to analyze the salt and drought tolerance. The majority of the wild type, MR219, and M_2 seedlings died two weeks after the treatment. However, the surviving seedlings demonstrated a significant result. The plants that survived after treatments with NaCl solution and PEG 6,000 solution had initially shown rolled leaves due to the direct effect of the root system stress. Nevertheless, these mutants recovered rapidly after transplantation. Salt or drought-resistant mutants tended to have a relatively stable root system for husky roots. In the M_2 generation, a total of 22 salt-tolerant (Figure 1O) and 35 drought-tolerant (Figure 1P) plants were found. The findings were confirmed using resistant mutants in the M_3 population. Accordingly, a total of 13 salt-tolerant mutants and 20 drought-tolerant mutants were found homozygous.

Other mutation types

The mutagenized population produced seven twin-seedling mutants. In this study, two forms of twin seedling mutants were observed: partial and complete twin seedlings. These seedlings varied in the existence of one or two radicle primordia. In incomplete twin seedlings, two plumules emerged from the embryo's ventral and dorsal (endosperm) sides and displayed a similar radicle (Figure 1I). In the latter, two radicle primordia emerged from the embryo together with the two plumules, culminating in the development of the juxtaposed twin in the same embryo. Only three mutants out of seven were able to be transmitted unto the M_3 generation with a 0.34% recurrence rate (Table 1). In the M_2 population, 12 dominant mutants with mesocotyl mutants (Figure 1K) were recorded with a wide range of shape lengths. However, there was no further verification of mesocotyl mutant in the M_3 generation.

Table 1. Morphological characteristics and the frequency of ion beam irradiated M_2 and M_3 seedling

Categories	**Mutant characteristics**	**M_2 Mutants**	**Mutants frequency in M_2 (%)**	**M_3 Mutants**	**Mutants frequency in M_3 (%)**
Leaf	Albino mutant	31	1.97	0	0
	Greenable albino mutant	22	1.40	10	1.12
	Yellow green mutant	25	1.59	15	1.68
	Virescent	10	0.63	5	0.56
	Rolled leaf	31	1.97	18	2.01
Seedling height	Dwarfism	30	1.90	20	2.23
	High height	17	1.08	10	1.12
Root	Short root	50	3.17	10	1.12
	Less lateral root	5	0.32	2	0.22
	long root	55	3.49	23	2.57
	None adventitious root	6	0.38	0	0.00
Tolerance	Drought tolerance	35	2.22	20	2.23
	Salt tolerance mutant	22	1.40	14	1.56
Others	Twin seedling	7	0.44	3	0.34
	Long mesocotyl	12	0.76	0	0.00
Total		**358**	**21.97**	**150**	**16.76**

Mutants with morphological traits

In the M_2 population, seedlings were developed by individually transplanting 1575 promising mutants to the experimental field, and morphological features were observed during the growing phase. Overall, 83 mutants were identified with a frequency of 5.27% in six different mutation types. However, only 41 mutants were confirmed in the M_3 generation, with a mutant occurrence of 4.58% covering ten distinct mutant traits. As compared to the identified mutants, panicle mutant showed the highest mutant frequency with 1.45%, followed by the tillering mutant (1.12‰), and heading date (0.89‰) (Table 2).

Table 2. Seedling morphological characteristics and ion beam mutant frequency in M_2 and M_3

Categories	Mutant characteristics	M_2 Mutants	Mutants frequency in M2 (%)	M_3 Mutants	Mutants frequency in M3 (%)
Leaf	Spotted leaf	6	0.381	6	0.00
	Cluster leaf	6	0.381	2	0.22
Tiller	Increased tillering	10	0.635	8	0.89
	Reduced tillering	3	0.190	2	0.22
Heading date	Early heading	11	0.698	4	0.45
	Late heading	5	0.317	2	0.22
panicle	Dense panicle	8	0.508	4	0.45
	Lax panicle	10	0.635	4	0.45
	Small Panicle	15	0.952	5	0.56
Grain	Short awn	9	0.571	4	0.45
Total		83	5.270	41	4.58

Leaf

A leaf is a significant plant organ because it is photosynthetic and provides nutrients for plant growth and reproduction. A few leaf mutations were recorded and categorized based on their visual appearance. Other leaf mutations, such as spotted leaf and clustered leaf, were also observed at the three-leaf level. Spotted leaf/lesion mutation (Figure 2A) was seen in two M_3 lines, which developed lesions that were identical to those triggered by pathogen invasion, wounds, and stress. Two types of lesion-like mutations were described based on their leaf position: those with patched leaves and those with spotted first internode. In the mutant population, six clustered leaf mutants were recorded (Figure 2B). These mutants formed a new leaf and began to reproduce following the removal of disturbance to the original plant height. They were classified as "non-heading" mutants because they did not go through the reproductive process to develop panicles even after harvesting (i.e. six months after sowing).

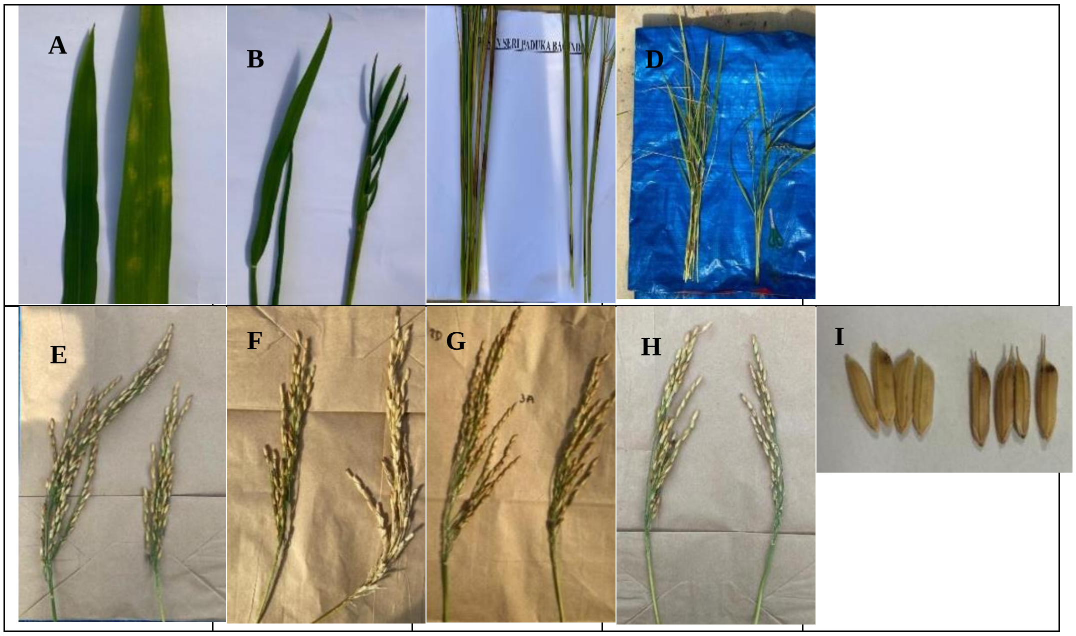

Figure 2. Morphological trait found in M_2 and M_3 mutant population (Mutant: left, Wild: right) (A: spotted, B: cluster leaf, C: Reduced tillering, D: Dwarf, E: small panicle, F: palealess spikelets, G:dense panicle, H: Lax panicle, I: Short awn)

Tillering

Two types of tillering were noticed in this study, specifically “reduced tillering” (Figure 2c) and “increased tillering” mutants. M_2 generation generated a total of 0.83% of tillering mutant with 10 and three mutants showing increased and reduced tillering mutants, respectively. As presented in Table 2, only 10 of the mutants showed a stable inheritance in the M_3. The increased and reduced tillers ranged between 12.4 to 18.8 and 3.4 to 4.2, respectively as compared to the average number of effective tillers (8.4 tillers/hill) in the parent variety, MR219. This variation could be due to the effect of genotype and environmental interaction. Still, some reduced tiller mutants had a stable inheritance in the M_3 generation with a lesser number of tillers per plant.

Culm

In rice, dwarfism leads to a reduction in the length of each intern and panicle. In field conditions, all types of dwarf mutants resulting from M_2 were established for M_3 segregation. Plant heights at maturity extending from 44.8 to 78 cm were established as a mutative phenotype for dwarfism inequalities, while the parent variety reached 90.2 cm. In the current experiment, mutants recorded an averaged height of 102.2 cm, which was higher than the parent variety, and unusual in M_3. Owing to their very low plant stature up to maturity, two mutative plants were considered as "severe dwarfs" (plant height ranged from 30 to 44 cm). Other peculiar features that distinguished dwarf phenotypes included small panicles, early heads, deformed leaf shapes, and increased tillering. In the paddy field, several mutants with fewer tillers and smaller plants were found, with one of them confirmed as homozygous in the M_3 generation.

Heading time

Plants with mutant heading dates that varied from an average of over 7 days were labeled as mutants. Early heading and late heading mutants were included in this group, with a prevalence of 0.31% and 0.70% of total mutants screened in the M2 generation, respectively. The mutant frequency was reduced by 0.22% and 0.45% in the M3 generation. Presently, only four early heading and two late heading mutant lines were identified (Table 2).

Panicle

The utmost prominent irregular phenotype in the field was the panicle form (2.69% in M_2 generation). Big panicles ranged from 27.8.1 to 20.4 cm in length with an average of 25.2 cm, whereas the average (range) of spikelets per panicle was 130.8 (120.4–131.2) (Figure 2E). Small panicle mutants (Figure 2E) averaged 14.2 cm (12.2–16.4 cm) in length, with 65.0 spikelets per panicle (60.2–78.4 spikelets). The average length of a wild form panicle was 23.4 cm, with 120 spikelets per panicle. There were several "low fertile panicle" mutations. In the area, ten lax panicle mutants (Figure 2H) with low panicle density were also recognized and homozygous in M_3.

Grain

One grain mutation type namely short awn was observed in the M_2 generation and four mutant plants were validated in the M_3 generation. Other agronomical traits in this mutant type, such as seed setting, plant height, and seed weight, were similar to the parent variety (Table 2).

Discussion

Forward genetics is the study of the genetic cause of an altered or pathological phenotype (i.e. phenotype-genotype) caused by chemical or irradiation treatments. Functional experiments and the discovery of novel alleles suitable for breeding will benefit from a mutant library with a high mutation density. The most widely used mutagen is gamma rays. However, gamma rays, characterized by low LET radiation cause distant events of ionization and may result in plant DNA spread-out damage (Oono *et al.,* 2020). High LET radiation emanating from protons and other ion beams at the ionization site cause extreme clustered DNA damage. Also, such ionization has a greater range of DNA damage relative to gamma rays. Heavy-ion beam irradiation has recently appeared as a novel mutagen in seed plant mutation breeding. It is an effective means of initiating mutations in several plant organisms due to its high frequency and wide range. As a result, ion beam mutagenesis has been used in combination with mapping and cloning methods to separate and depict genes (Yang *et al*., 2019).

By screening the M_3 mutant in the inherited population, a few mutative phenotypes that were not noticed in the M_2 generation, such as spotted leaf and thick panicle were discovered in the M_3 segregated population. Similarly, Thang *et al*. (2010) recorded spotted leaf, dense panicle, and red-brown rice in M_3 mutants of *Oryza sativa* cv. Nipponbare. Interestingly, morphological mutations such as small panicle heading time, dwarfism, and spotted leaf mutations appeared more frequently than others. This was also observed in the study by Potupureddi *et al*. (2020) who demonstrated a variation in different morphological characters including height (tall, dwarf, bonsai) and pink apiculus from M_2 to M_5 generations and mutants from Samba Mahsuri. These findings were also consistent with the plant height variations identified by Mohapatra *et al.* (2014). Observations from the present study suggested that some mutants might have mutations with large phenotypic effects leading to a significant exclusion from the phenotype of the wild type.

In Jia *et al.* (2009), this study, the mutation frequency of many mutant types decreased following the progression from M_2 to M_3. The frequency of sub-lethal *et al.* phenotypes such as virescent and albino reduced significantly in $M_{3,}$ compared to those in the M_2 generation, indicating that the former was lethal. Similar studies have reported a high frequency of chlorophyll deficiency mutants by using ion beam irradiation treatment in rice (Maekawa *et al.,* 2003;

Hayashi *et al.,* 2007). It is also noticeable that numerous morphological trait mutants were segregated in the M_3 generation, while mutants with unremarkable appearances were discarded including none-adventitious root and mesocotyl mutants. One of the possible explanations is the assortment for stable mutants in a single seed descent. Brooks (2008) observed a reversion phenomenon evidenced by a sickle shape seed phenotype in the M2 generation, which reverted to wild type seed shape by the M_5 generation. However, in this study, we did not notice any reversion phenomenon in the M_3 generation.

Furthermore, the results herein demonstrated that characteristics such as plant height, heading time, and tillering ability were continuously segregated in the subsequent generation depending on the nature of the trait involved. However, previous studies (Soomro *et al.,* 2002: Roy *et al*., 2018) reported several high-yielding mutant lines with more tillers number and high grain number. For instance, Roy *et al.* (2018) identified eight high yielding aromatic mutants in the background of Tulpanji using gamma irradiation. Soomro *et al.,* (2002) identified two high-yielding mutant lines derived from IR6 through gamma rays. Several high-yielding mutants are also popular in many countries such as Vietnam (55 mutant varieties), Bangladesh (44 mutant varieties), and Thailand (two aromatic mutant varieties (RD6, RD15) where they have been cultivated over millions of hectares (Oladosu *et al.*, 2016). In India, the PNR-381 and PNR-102 aromatic rice varieties are early maturing rice mutants derived from the Basmati 370 PNR-17-3 cultivar and have become popular in Haryana and Uttar Pradesh States. In contrast, one study suggested that the environmental effects were linked to quantitative traits, thus the expression of such traits might differ depending on the environmental fluctuation. Likewise, linkage phenomena were observed in this study including dwarfism with increased tillering. This might be due to the capability of ion beam irradiation to elicit a pleiotropic phenomenon (Hirano *et al.*, 2015).

The present study showed a higher mutant frequency in the root. Roots are crucial for plant growth and production because they anchor the plant to its growth substrate, promote water and nutrient uptake from the soil, and respond to environmental signals including biotic and abiotic stresses (Meng *et al,* 2019). Similarly, Thang *et al.* (2010) reported a high number of root mutant frequencies in the M_2-M_3 mutant population of Nipponbare. However, the root morphology was not further explored. The hydroponic approach in this study demonstrated the feasible approach to investigate the morphology of roots without obstacles. Thus, the present finding could be applied for functional genome analysis, especially the long root mutant which is useful for molecular-level research.

Recently, abiotic stress has gained significant interest in rice breeding programs to increase crop productivity. The creation of a new vareity with high levels of stress tolerance requires an understanding of the related genetic regulatory factors (Parmar *et al.,* 2017). Hence, the identification of mass stress-resistant gene mutation is a prerequisite and this feature is crucial to accommodate the increasing demand. Abiotic stress mutation was also among the higher mutation frequency found in this study. Our findings aligned with the mutation study conducted by Wu *et al.* (2005), in which rice mutants that are resistant to Blast, BB, and tungro disease were identified upon irradiating IR64 with gamma rays. Similarly, Mohapatra *et al.* (2014) identified BB resistance mutants in an EMS-induced Nagina 22 population. Our study is consistent with the above-mentioned studies which indicate that mutagenized populations harbor a great amount of variability that can be recovered if the mutant population is subjected to appropriate screening techniques.

The phenomenon of the unique shape of mesocotyl was recorded in the M_2 generation in this study. To our knowledge, this is the first study to report the unique shape of mesocotyl mutant in MR219 variety using ion beam irradiation. In monocots, the mesocotyl is responsible for moving buds out of deep water and soil after germination. Besides, mesocotyl elongation is an important feature in deep sowing resistance and its complex relationship with phytohormones. An earlier study was reported that the long mesocotyl mutates using EMS mutagenesis (Patil *et al.,* 2019). However, the mechanism of mesocotyl elongation and domestication is still poorly understood and might spark a greater research interest.

Seven twin-seedling mutants were noticed in M_2 with three of them appearing to be stably inherited in the M_3 generation. A similar trait mutation was also identified by Thang *et al.* (2010) with two types of twin seedlings (complete and incomplete). Two-seedling in rice was modified by several genes, and dominated by two recessive genes, the fractions which convey the quantitative inherent character (Luo *et al.,* 2021). Furthermore, the separate twin rice strains were inherited differently. Twin inheritance had a cytoplasmic influence, while rice twin heredity is related to apomixes. These two distinct mutants represented a significant phase in the process of defining mutants based on morphological features. This could assist researchers in confirming enigmatic theories that are regarded to gene control by heredity.

Grain type is important in varietal improvement since the visual characteristics of rice grains is equally a significant attribute that affects consumer’s preference. A previous study reported the development of different grain type mutants by irradiating IR8 with gamma rays (Azad *et al.* 2012). Different grain types (short, long, bold) were also reported from the Nagina22 mutant population (Mohapatra *et al*., 2014). A mutant line derived from Kitake rice variety using fast-neutron (FN) mutagenesis carried a new grain shape 9–1 (gs9–1) allele that also affects grain size (Jiang *et al*., 2019). However, only one type of grain mutants was isolated in the present study.

In conclusion, findings in this study showed the importance of a forward genetic approach in developing a collection of mutants based on the phenotypes data (agronomy). This data might help plant breeders to identify some key regulatory genes applicable in developing new molecular markers. Also, single nucleotide polymorphism (SNP) analysis and marker-assisted selection (MAS), selection of germplasm or breeding genotypes will benefit from the sequence shift in mutants (Shen *et al.*, 2001). The importance of mutant collection in the forward genetic approach remains a concern for further functional gene analysis. M_2 plants are often used to identify mutants because it allows the widest selection of traits. However, the M_2 plants were used in a single trial because it is challenging to maintain some of the traits in subsequent generations. Therefore, it is recommended that the M_4 and M_5 be used in screening for mutants involved in disease resistance, yield, and quality.

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