Research Article

Induced Mutagenesis for Creating Variability in Thailand's Upland Rice (Cv. Dawk Pa-yawm and Dawk Kha 50) using Ethyl Methane Sulphonate (EMS)

Ali Awais, Charassri Nualsri and Watcharin Soonsuwon*

Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hatyai, Songkhla Province, Thailand

Abstract | To enhance the genetic variability and to create new and diverse characters to a natural population, researchers sometimes opt for mutation breeding. This research has been done to investigate the effective doses of EMS and to observe the phenotypic variability of the two upland rice genotypes Dawk Pa-yawm (white rice) and Dawk Kha 50 (red rice), in Thailand. Seeds of the potential genotypes has been treated with varying EMS concentrations. With the increase in concentration, there was a continuous decrease in germination, shoot and root lengths respectively. Data have been subjected to regression analysis and effective doses of EMS were recorded for both genotypes. The effective value calculated for shoot and root length in Dawk Pa-yawm was 1.34% and 1.33% and in Dawk Kha 50, 1.23% and 1.86% respectively based on the reduction of the above mentioned parameters after the EMS treatment. Furthermore, the EMS response in M_1 generation was also observed in the field condition by various quantitative measurements. The traits, especially plant height, panicle length, number of filled grains, number of grains per panicle, 1000 grain weight and yield per plant showed a clear fall off when compared to control while the panicle length in both mutants were higher than that of control. Several mutants also revealed some notable phenotypic variations in the roots, seeds, panicles and leaf morphology. Phenotypic observations determined that the Dawk Kha 50 has more potential of variability than Dawk Pa-yawm towards EMS. Later selection in the advance generations might be useful to isolate agronomically useful mutants for the future use in upland rice breeding programme.

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***Correspondence** | Watcharin Soonsuwon, Prince of Songkla University, Thailand; **Email:** watcharin.s@psu.ac.th
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Introduction

Rice (Oryza *sativa* L.) is not only a crop but also a life for an ample world's population. Its importance can be predicted from the fact that it is shaping the cultures, diet behavior and source of living for a majority of the people in the world remarkably in Asia. The United Nations in particular titled 2004 as the International Year of Rice reviewing its importance for the wellbeing of mankind. However, the estimated consumption of the rice by the year 2025 is notable (Hossain, 1995). Although numerous varieties of rice with excellent traits are available yet there is a need to enrich the existing germplasm or to carry out necessary research to contribute in its improvement.

Many plant research areas such as plant physiology, genetics and plant breeding use mutants. Mutations are fashioned by spontaneous mutation, ultraviolet light, chemical mutagens and ionizing radiations etc. Consequently, there has been an increasing interest in using chemical and irradiation mutagenesis in model

organisms for functional genomics research (Liu et al., 1999; Nadeau and Frankel, 2000).

Chemical mutagens (EMS and sodium azide) and irradiation (Gamma rays, X-rays and fast neutrons) have been used occasionally on a large scale to induced mutations. Combination of various physical and chemical mutagens may result in various synergetic and antagonistic effects (Makeen and Babu, 2010). Knott (1991) concluded that the application of the mutagens should be specific as for the elimination of the deleterious gene rather than using it when a handful of the genetic variation is present for the desired traits. Ionizing radiations induce chromosomal rearrangements and deletions while on the other hand chemical mutations are responsible for point mutations which would provide a series of change of function mutations (Bhat et al., 2007). Nilan (1981) examined Ethyl Methane Sulphonate (EMS) as a better source to produce higher proportions of choromosomal abberations. According to Kaul and Bahn (1977), EMS is more successful than gamma rays in terms of both effectiveness and efficiency. In addition to this, together with nitroso compounds, it appeared to be the most common and fruitful for in vitro mutagenesis.

EMS has been reported to be the most potent among the chemical mutagens used in rice (Kawai, 1965) and in other crops (Gaul et al., 1972; Jacob, 1965). EMS alkylates guanine bases and ultimately results in mispairing-alkylated G pairs with T instead of C, resulting in primarily G/C to A/C transitions (Bhat et al., 2007).

Over a couple of years, numerous research institutes have been functional in producing and improving the EMS-induced mutant rice populations. Jain (2010) stated that the determination of LD50 at the beginning is a prime step to initiate the EMS induction. Duration and varying the concentration, pH of the solution and the solvents used are some of the means to evaluate the right dose of the chemical concentration. In the absence of which results in high or low mutational frequency.

Materials and Methods

Plant materials

In this research, the seeds of the two upland rice varieties, Dawk Pa-yawm and Dawk Kha 50 which belongs to southern parts of Thailand, were selected and exposed to the EMS mutations.

EMS treatments

Dry and mature seeds of the two rice varieties (100 seeds per each dose) were placed in a 500 ml flask along with the distilled water, keeping the level of the water a bit higher than the seeds, for 24 hours under room temperature. Later on decant the water and add EMS at the concentration of 0.5%, 0.75%, 1% and 1.25% in water. Incubate this material for 12 hours under the temperature not exceeding than 22°C followed by the decantation of the EMS and washing it with distilled water twice, (5 times, 4 minutes each) and (4 times, 15 minutes each) to clean the residuals of the EMS. Seeds were then clean further under the running tap water for about 3 hours respectively before moving the seeds to the germination paper as mentioned in Table 1. Same protocol was followed, when the seeds were treated with the EMS for the field experiment. Extra care should be taken in selecting the seeds to minimize the damage caused by the EMS treatment as the broken seeds are extremely vulnerable to direct contact with the EMS and hence deteriorating the seed chemistry. Furthermore, EMS as all alkylating agents, is a highly reactive chemical. So, solutions should be prepared just prior to use.

Table 1: *EMS protocol for upland rice.*

100 seeds	**Soaking in the 500 ml ultrapure water**		**Over night**
Seeds in batches of 100 seeds/ treatment/ variety in the flasks			
0.5%	Concentrations of EMS (v/v)	Incubator (20 – 24°C)	12 hours 120rpm
0.75%			
1%			
1.25%			
	Washing with distilled water twice	100 ml	5 times/ 4 min
		200 ml	4 times/ 15 min
	Washing under running tap water		3 hours
	Germination paper sowing		Data collection after 15 days
	Measure the shoot and root lengths of the plants		

An appropriate dose of EMS

EMS treated seeds of the Dawk Pa-yawm and Dawk Kha 50 were germinated in the germination paper and examined after 15 days to measure the root and shoot lengths. The EMS concentration was used to induce mutagenesis to seeds after the preliminary experiment's results for Dawk Pa-yawm was 1.34%

and for Dawk Kha 50 1.23% and 1.86% respectively. According to the previous research studies, in order to obtain an optimal mutation yield, germination up to 70 % is considered appropriate in case of EMS mutation (Mohapatra et al., 2014; Savin et al., 1968). The concentrations 1.34% in Dawk Pa-yawm and 1.23% in Dawk Kha 50 were selected accordingly as aforementioned, but 1.86% concentration in Dawk Kha 50 was producing 50-60% reduction in germination. EMS concentration approaching to 2% is considered to be deadly for germination and successful survival of the plant.

Phenotypic variability among mutants

The M_1 seeds after EMS treatment were grown in the field at 20×25 spacing to obtain the M_2 seeds from the mutant population. The seeds of each morphologically distinct plant were kept separate. To examine and to record the phenotypic data, visual assessments were done at three different stages, viz. seedling, vegetative and reproductive stages following DUS guidelines (Mohapatra et al., 2014). Several other characters like awns, grain and panicle morphology, stem and leaf characters were visually recorded. Plant height was measured from the tip of the panicle to the base of the plant. Furthermore, the M_1 seeds of the two varieties Dawk Pa-yawm and Dawk Kha 50 mutants along with the control seeds were grown in the germination paper. Seeds were examined after 15 days of germination and the respective changing in the root structures of mutants in comparison with the control were recorded.

Statistical analysis

The statistical design for this experiment to estimate an appropriate EMS dose, was organized using 5×5 factorial in completely randomized design with four replications. Regression analysis was performed to sort out the effective dose for the field experiment to raise M_1 generation. The experimental data was analyzed using R 2.14.0 programme.

Results and Discussions

The present research was an effort to induce mutation in two upland rice varieties of Thailand, Dawk Pa-yawm and Dawk Kha 50. Analysis of variance of the studied traits like root and shoot length showed highly significant differences as in Table 2. There were highly significant differences of root and shoot length for treatment, EMS and EMS into genotype interaction. Moreover, there was a highly significant difference of root length for the genotype but a significant difference of shoot length for the genotype in ANOVA.

Table 2: *Analysis of variance of studied traits.*

Source	df	Mean squares of Traits	
		Root Length	Shoot Length
Treatment	9	48.20 **	30.79**
EMS	4	95.25**	62.13**
Genotype	1	39.48**	5.90*
EMS X Genotype	4	3.33**	5.66**
Error	30	0.64	0.98
CV %		6.04	7.38

*Significant *; Highly Significant ***

Effect of various concentrations of EMS on root and shoot lengths and decision of LD50

EMS mutagenesis induced a significant impact on the root lengths of both Dawk Pa-yawm and Dawk Kha 50 respectively. As per results obtained, there is a gradual decrease in the root lengths with the increase in the EMS concentrations. The root lengths were maximum in control for both of the genotypes Dawk Pa-yawm and Dawk Kha 50 as 16.59 cm and 20.17 cm which gradually showed a fall off to below 5 cm at 2% EMS (Figure 1). Kiong et al. (2008) revealed that the extent and nature of chromosomal damage is a highlighting factor for a survival of a plant till maturity.

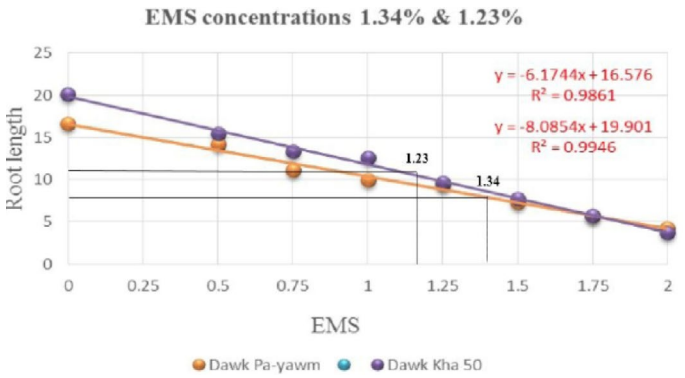

Figure 1: *Effect of various concentration of EMS on root length and decision of LD50.*

Moreover, similar results were observed when the shoot lengths of the germinated seeds were measured after the EMS treatment. There was a significant decreased in the shoot lengths of both the Dawk Pa-yawm and Dawk Kha 50 respectively. The decreasing trend was according to the findings of the researches performed in past using EMS as a mutagenic source

to induce mutations (Talebi et al., 2012). The highest shoot lengths were observed for the control population in both of the parent varieties as 18.69 cm and 16.06 cm which gradually decreases when the EMS concentration increases. At higher concentrations of EMS, the shoot lengths were almost approaching to the zero value on the graph as shown in the Figure 2. Most of the mutants showed an almost 2 times decrease in the shoot length when treated with EMS as compared to control, alongwith some mutants having improved shoot lengths (Mohapatra et al., 2014). Shoot and root lengths can be used as steady characters to investigate the optimum doses for gamma rays and EMS for a treatment on a wide scale in a breeding programme (Shah et al., 2008). Similar decreasing trend of shoot length was observed when Basmati rice was treated with mutagen of various doses and with the increase in the mutagen dose there was a continuous decrease in the shoot length (Cheema and Atta, 2003).

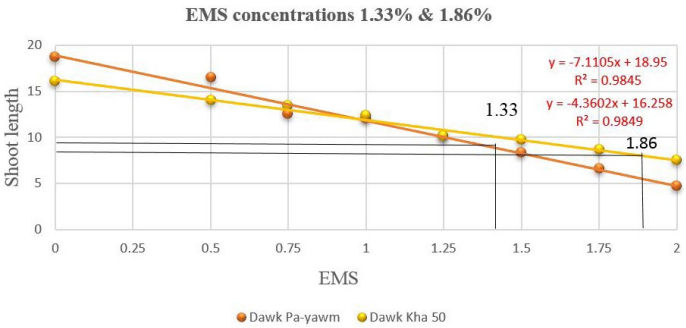

Figure 2: *Effect of various concentration of EMS on shoot length and decision of LD50.*

Mean values comparison of different traits in M_1 generation

It can be noticed in the field conditions, EMS induced a significant impact on various characters of the rice population. Figure 3 showed the mean values comparison of different traits of mutants with their control genotypes. Figure 3(a) shows the plant height deviation of the mutants of both the genotypes from the normal control population. Both the mutants of Dawk Kha 50 and the mutant of Dawk Pa-yawm showed the decreased plant height as compared to the control. In case of Dawk Kha 50 mutants, the mutants of 1.23% showed the relatively more height than the mutants of 1.86% EMS. Figure 3(b) surprisingly showed the increase number of tillers in Dawk Pa-yawm mutant as compared to control population while in Dawk Kha 50 the control has the edge over both of the mutants. Again the number of panicles in M_1 generation manifested a varying trend in Dawk Pa-yawm in which the control population has reduced number of panicles than the mutant but for Dawk Kha 50, the mutants exhibited less number of tillers as compared to the control population. In Figure 3(d), the panicle length of mutants of both Dawk Pa-yawm and Dawk Kha 50 1.23% amazingly showed an increase trend than the control population while the mutant 1.86% of Dawk Kha 50 showed the decrease of panicle length than the normal control population. Figure 3(e) showed the clear decrease in the no. of filled grains in Dawk Pa-yawm mutant while in Dawk Kha 50 the mutant 1.23% showed low level of filled grains than the Dawk Kha 50 control. Figure 3(f) represented the less value of no. of grains/ panicle in both Dawk Pa-yawm and Dawk Kha 50 1.23% mutants.

Mutants exhibiting phenotypic variability in M_1

Besides showing the similarity with the normal control population, there were some mutants which showed a striking new variability regarding the phenotypes as shown in Figure 4. Swaminathan et al. (1970) noticed EMS as the most efficient mutagen in inducing the mutagenesis followed by gamma rays and Nitroso-Guanidine. In Dawk Kha 50, some mutants displayed the presence of awns in the panicles while the control population lack this character. The awns were characterized as white and purple. The panicles bearing the white awns were fertile producing the healthy grains while on the other hand, there was no seed production in the panicles bearing the purple color awns. Moreover, some mutants have produced the panicles with curved seeds while some mutants offered variation in the panicle color, exhibiting the white and yellow grains respectively. Some of the panicles as a whole were purple but interestingly there was no evidences of fertilization and were completely sterile. Furthermore, in Dawk Pa-yawm the visible phenotypic variation was the mutant showing the stripped nature of leaves with half leaves green and half white, a clear evidences of chlorophyll mutation. Earlier researchers also reported this kind of chlorophyll mutation (Gustafsson, 1940; Rajarajan et al., 2014). According to Gautam et al. (1992) and Ratnam and Rao et al. (1993) the increase in the dose of the mutagen is directly proportional to the mutagenic efficiency and hence increases the frequency of chlorophyll mutation. EMS is 2-2.5 times more efficient than gamma rays (Gautam et al., 1992).

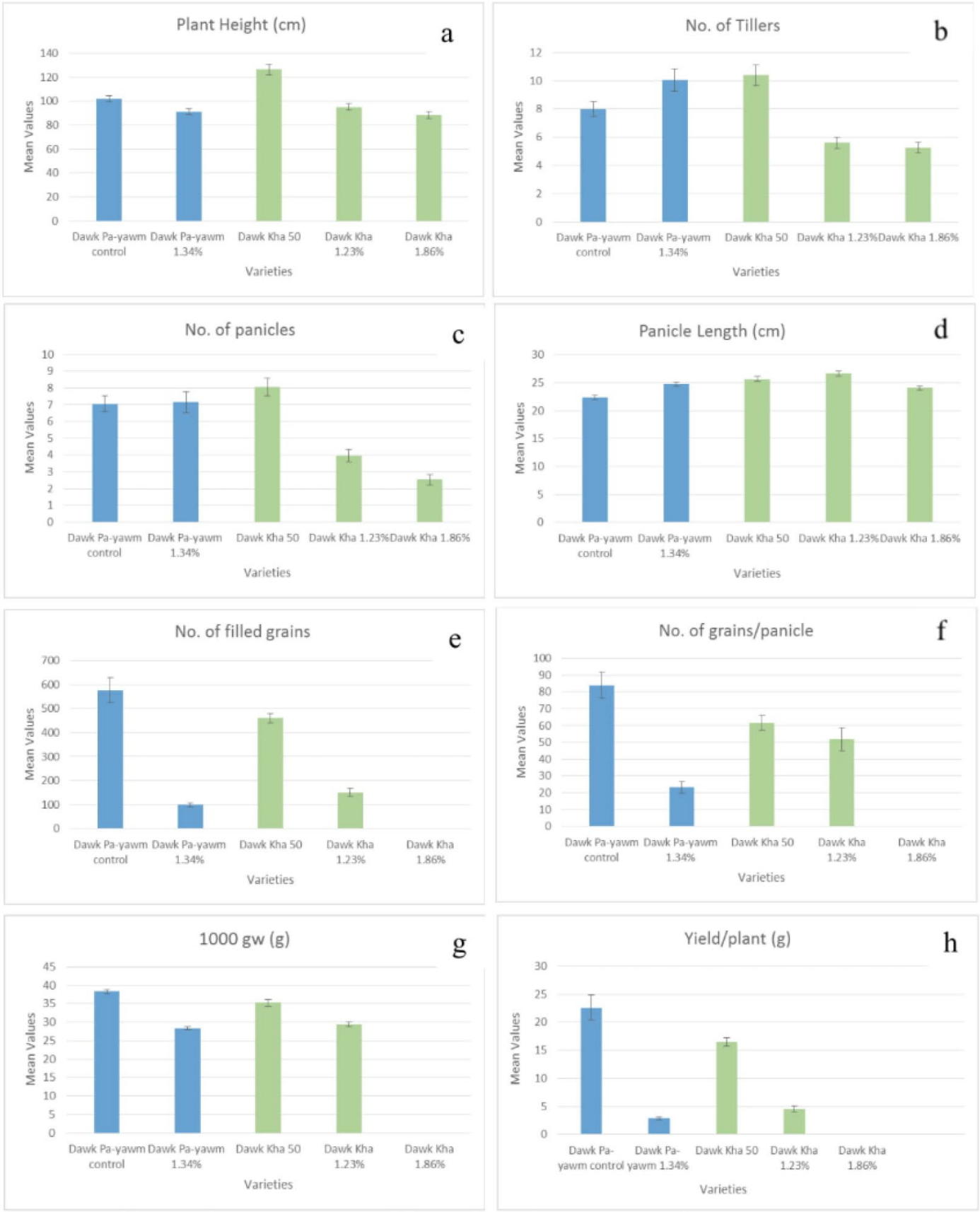

Figure 3: *Mean values comparison of the various studied traits in Dawk Pa- yawm and Dawk kha 50.*

Also there is an increase in the efficiency of chlorophyll mutation when subjected to a definite optimum dose of mutagen and decreases with the increase in the further dose (Cheema and Atta, 2003). Mutants observed for this behavior had white panicle with 0- 20% fertility. Also there were some mutants in which the seed size was relatively small as compared to the normal parental population.

Figure 4: *Representative variation for various traits in the mutagenized population of Dawk Pa-yawn and Dawk Kha 50. a): control panicle of Dawk Kha 50 showing no signs of awns; b): mutant of Dawk Kha 50 with purple awns; c): mutant of Dawk Kha 50 with white awns; d): Dawk Kha 50 mutant panicle; e): Dawk Kha 50 purple panicle mutant; f): representative variation in panicle colour of mutants; g): striated chlorophyll mutant of Dawk Pa-yawm; h): representative variation in grains of Dawk Kha 50 mutants with control seed on extreme left and representative variation in grains of Dawk Kha 50 mutants with control seed on extreme left and mutants at its right.*

Mutants exhibiting a change in root morphology

The seeds of the total 45 M_1 mutants of the variety Dawk Pa-yawm were germinated. As in control seeds, the germinated seedling showed the normal fibrous root system while in mutants, some of the mutants exhibit the elongated tap roots only, while some of them showed the elongated and dense fibrous and enormousbrace roots as compared to control after 15 days of sowing as represented in Figure 5. Previous studies also confirmed the alteration in post-embryonic root development of Arabidopsis when treated with EMS, which resulted in abnormal root cells expansion (Benfey et al., 1993). Furthermore, in Lotus japonicas, EMS treatment not only affected the fungal development on the root surface but also affected root exodermis and cortex (Sanoo et al., 2000). Furthermore, single crown root was observed instead of the normal fibrous root system in rice variety Nagina 22, when the rice seeds were subjected to EMS mutation (Mohapatra et al., 2014). In addition to structural changes, there were changes in root characteristics which were later appear as useful tool for drought tolerance (Mohapatra et al.,2014).

Among the controls and mutant lines, the seedlings showing best morphological differences were selected. The mutants 12 showed up the tap roots only instead of fibrous root, while the mutants 24, 25

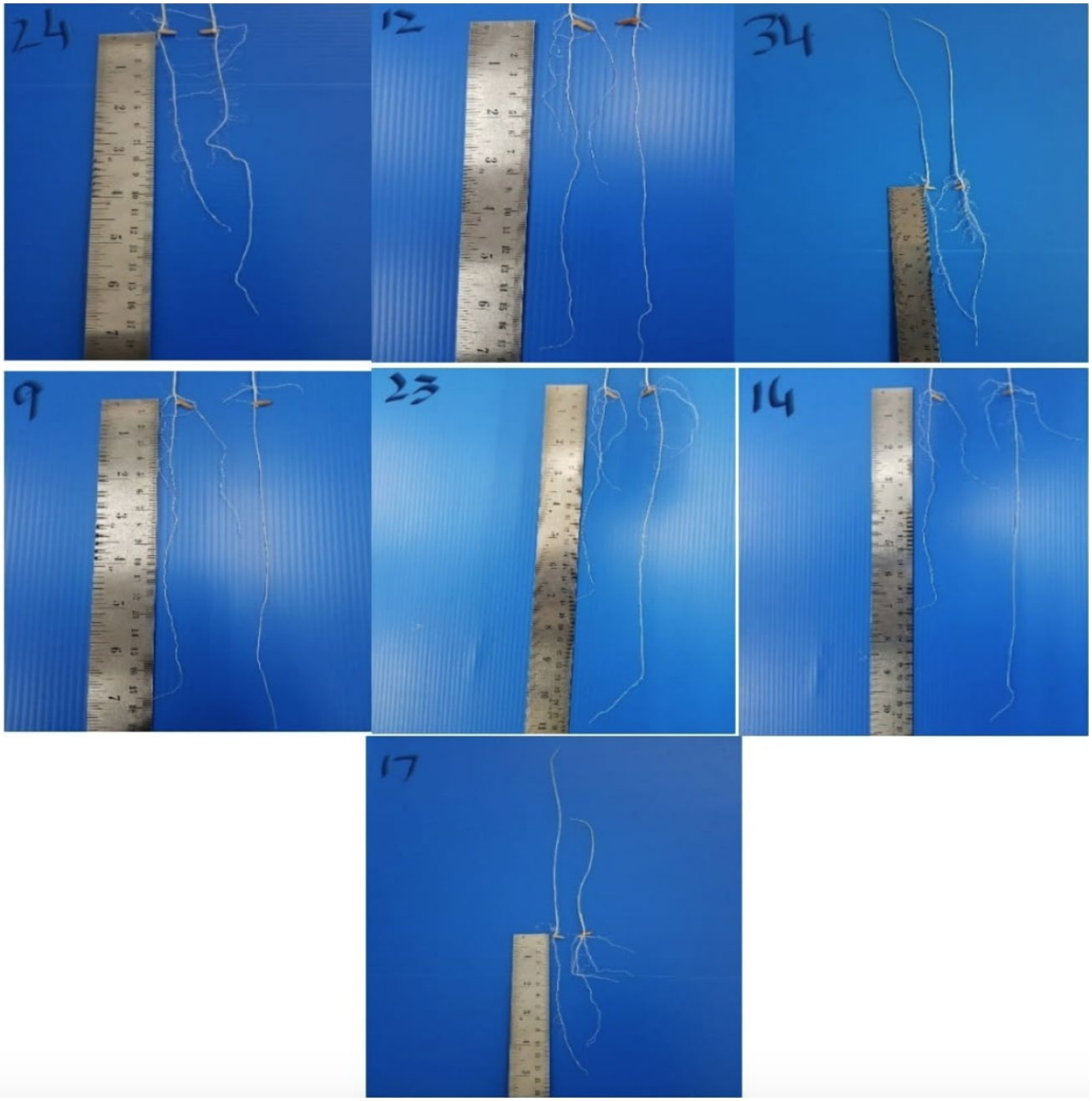

Figure 5: *Representative variation for various root structures in mutagenized population of Dawk Pa-yawn and Dawk Kha 50. (left; Control-Right; Mutants); a): 24, 12, 34 Dawk Pa-yawm mutants; b): 9,23,14,17 Dawk kha 50 mutants.*

and 34 showed the better fibrous roots after 15 days of sowing. Among all of these mutants the mutants 25 and 35 displayed the better shoot growth than the control population while in rest of the mutants the shoot growth is less than the normal control population.

On the other hand, in Dawk Kha 50, 40 M_1 mutants were examined for the changes in the root morphology after 15 days of germination. The Dawk Kha 50 mutants 9 and 14 displayed the tap root kind of nature with no root hairs at all while the mutants 17 and 23 revealed the best root generation along with the good root hairs formation and a perfect display of the fibrous root system.

Conclusions and Recommendations

It can be deduced from the research that in both the varieties Dawk Pa-yawm and Dawk Kha 50, EMS significantly affected shoot and root lengths ($p < 0.01$). Decreasing trend was observed in both parameters, when compared to the normal control population. Variability on observed means were calculated and compared with the control which indicated a clear

decline in majority of the quantitative traits such as plant height, panicle length, number of filled grains and grains per panicle, 1000 grain weight and yield per plant. Some traits like panicle length showed and increase value than control in both genotypes while the increased trend was also observed for number of tillers in Dawk Pa-yawm only.

There were some coherent appearances of different phenotypes in the mutant population. Phenotypic observations determined that the Dawk Kha 50 has more potential of variability than Dawk Pa-yawm towards EMS. Later selection in the M_2 and M_3 might be useful to isolate agronomical useful mutants for the future use.

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Author's Contribution

AA did research design, implementation, data analysis and prepared the manuscript. CN contributed in research design, research summary and recommendation and assisted in manuscript writing. WS worked in research design, data analysis, research summary, recommendation and manuscript writing.

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