Effects of Ethyl Methane Sulfonate (EMS) on Mutation of Upland Rice (*Oryza sativa* L.)

Awais Ali

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Plant Science
Prince of Songkla University
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Major Program	Plant Science

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Abstract

Mutation is a tool that has been used occasionally in genetics to enhance the genetic variability and to add something new and diverse to a natural population. This research aims to induce variability in two high yielding upland rice genotypes of Thailand, Dawk Pa-yawm (white rice) and Dawk Kha 50 (brown rice). Seeds of these genotypes were treated with various concentrations of EMS 0.5%, 0.75%, 1% and 1.25% respectively. With the increase in the applied concentrations, there was a continuous decrease in shoot and root lengths. Regarding to regression analysis LD_{50} of EMS for Dawk Pa-yawm was 1.34% and 1.23% for Dawk Kha 50. Several traits showed a clear fall off when compared to the control population in M_1 like plant height, panicle length, number of filled grains and number of grains per panicle, 1000 grain weight and yield per plant while the panicle length was higher in both of the mutants when compared to their control counterparts. Likewise in M_2, Dawk Pa-yawm mutant performed better only in plant height as compared to the control population while in rest of the traits mutants of Dawk Pa-yawm were lagging behind the control population. The overall performance of the Dawk Kha 50 mutants were better than the control population in majority of the studied traits like plant height, number of tillers, panicle length, number of panicles, number of filled grains, number of grains per panicle and yield per plant while in 1000 grain weight the control population of Dawk Kha 50 was leading the mutants. SSR analysis using 11 primers was also done to investigate the genetic variation caused by EMS. Two primers, RM 316 and RM 8225 showed the positive variability among mutant and the parent genotypes. Furthermore, there were several mutants in this research which showed some striking new phenotype as well. Hence later selection in the advance generation might be useful to isolate agronomically useful mutants for future use.

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CHAPTER 1

INTRODUCTION

1. Research Background / Rationale:

Majority of the human population use rice as a staple food, especially in Asia and the Africa. Rice seems as life to nearly 8 billion people and a secondary staple source of food for a further 900 million of the world (Shehzad *et al*., 2011). Almost 17 countries in Asia and the Pacific, 9 in North and South America and 8 African countries use rice on daily basis. It is an estimate that by the year 2025, rice consumption will be breathtaking (Hossain, 1995), as in major Asian countries, demand will more than the population growth. Various countries massively depend on upland rice and have no resources to change the rainfed to irrigate systems (Pingali *et al*., 1997). Rice importance can be predicted from the fact that it has the third-highest worldwide production, after sugarcane and maize (FAO, 2009). The 95% of the rainfed lowland rice area is in Asia. Boosting the economies, enhancing wealth of the consumers are key factors which flare up the call for high quality rice imports. China's imports are increasing steadily (Anon, 1998).

Among the top rice producing countries, Thailand ranks the sixth in productions. Thai rice is described by its long slender and clear grain which, when cooked, becomes fluffy and remains soft for a relatively long time. Hence, Thai rice has become distinguished throughout the world for its admirable quality. Although a number of varieties with excellent grain quality exist, there is a need to advance the plant by developing types with shorter straw, fitter resistance to disease and better response to fertilizers. Attempts to effect variety improvement have been made through irradiation in addition to the conventional breeding program. More than 434 rice varieties were released by the end of the last century using mutagenesis to cope the increase demand of rice (Maluszynski *et al*., 2000).

1.1 Nutritional profile / composition:

Rice is an essential grain when it comes to human nutrition and intake of calories. Rice furnishes 20 percent of the world's dietary energy supply. On the other hand, wheat supplies 19 percent and maize only 5 percent. Rice is also favorable source of thiamine, niacin and riboflavin (FAO, 2009). Its nutritional value per 100g contains 1527kJ energy of which carbohydrates are 80g, fat, 0.66g protein, 7.13g vitamins (B1, B2, B3, B5 and B6), minerals like calcium, iron, magnesium, manganese, phosphorus, potassium, zinc and water up to 11.61g.

Rice cultivation can be done in contrasting environments, depending upon water availability. Lowland rainfed, Lowland irrigated deep water, coastal wetland and upland rice. It is the main food for the people of Thailand essentially associated with their culture, rites and rituals.

In the past three decades, Asia's rice production has been more than tripled, mainly resulting from the "Green Revolution" which increased the rice productivity in irrigated systems (Khush, 1999 and 2001). Some total of more than 250 million farm families are involved in rice cultivation in Asia (Pingali *et al*., 1997). The publication of the draft rice genome presented astonishing opportunities to give function to each of the estimated 50,000 genes, which ultimately is successful and useful for rice improvement as well (Feng *et al*., 2006; Goff *et al*., 2002; Yu *et al*., 2014). To accomplish this objective, rice growing countries along with plant breeding institutes developed near isogenic lines, mutant populations, mapping and also enriching germplasm. Also they helped in the trait improvements by identifying the genetic variation.

The major constraints which the rice crop is facing now a days in Asia region are germplasm availability and varietal development, declining production resources, deteriorating soil health, low efficiency of nitrogen fertilizers, ever changing balance of rice and pests, aging of rice farmers, increasing cost of production, rice trade and price incentive, post-harvest losses, weeds and biotic and abiotic stresses. Varietal improvement of rice is no doubt very pivotal to increase yield by creating variability in the available germplasm and appropriate selection procedures. The usefulness of plant breeding program depends specifically on the amount of genetic variability that may

exist in the nature or how much a plant breeder can generate variability in the treated population so as to perform meaningful selection. The green revolution technologies are almost exhausted for any further productivity gains (Cassman, 1994).

Mutation is the prime source of producing variability in the plants. The breeding, using mutation is useful method for crop improvement. Mutation facilitates in two ways. It provides the new starting material that help to produce new cultivars and also it is helpful in identifying new genes for studying gene's nature and their role in regulating biochemical pathways (Konzak, 1984; Konzak *et al*., 1984; Rick, 1986; Old and Primose, 1989). Several improvements reported for mutant cultivars after Micke *et al*. (1990) like increased yield, early maturity, resistance against pests and pathogens, seed traits like morphology and quality, plant architecture in terms of reduced plant height. Other improvements are like adaptability, threshability, easier harvesting, cold tolerance and winter hardiness.

1.2 Mutagens:

Mutants are widely used in plant research areas, such as plant physiology, genetics, and plant breeding. Mutations can be induced by various means like, ultraviolet light, chemical mutagens, such as ethyl methane sulfonate (EMS), and ionizing radiation, i.e., X-rays, gamma-rays and so forth. Another way to induce is the use of biological mutagen, such as T-DNA insertions and transposon tagging. Sometimes it helps in creating a character, which is not found even in the natural population. Moreover, its contribution in fracturing tight linkages, producing translocations for gene transfer cannot be neglected (Sears, 1956). Furthermore, induced mutation has been applied for inducing genetic variation in rice (Oka *et al.*, 1958). Consequently, using chemical and irradiation mutagenesis in model organisms for functional genomics research is gaining more interest now a day (Liu *et al*., 1999; Nadeau and Frankel, 2000). Knott (1991) concluded that mutagens should be applied in particular conditions, like the elimination of deleterious genes and should not be applied when sufficient genetic variation is present for the desired traits.

The number of chemical mutagens is very great and increasing interestingly. Ethyl methane sulphonate has been reported to be the most potent among the chemical

mutagens used in rice (Kawai and Sato, 1965) and in other crops (Gaul *et al*., 1972; Jacob, 1965). EMS is a colorless, liquid compound, with 124 molecular weight, and 8% solubility in water. At pH 7 and 20 degree Celsius, its half-life is 93h. Like all alkylating agents, EMS is very reactive and, as a consequence, solutions should be prepared just before use. EMS causes the alkylation of the guanine bases and results in mispairing alkylated G pairs with T instead of C, which ultimately causes G/C- to-A/T transitions. Chemical mutagens such as EMS or N-methyl-N-nitrosourea (MNU) mainly responsible for base substitution by DNA base alkylation (Greene *et al*., 2003; Slade *et al*., 2005; Till *et al*., 2007). Gaul *et al*. (1972) described for Barley that EMS not only responsible for the high mutation rates, but it is four to five times more practical than X- rays.

Rice productivity has been showing a falloff in monocrop rice areas as well as under rice-wheat rotation (Cassman *et al*., 1997). Enormous lands in countries like Bangladesh, China, India, Myanmar, Nepal, Pakistan and some in Vietnam and Thailand are under rice-wheat rotation. The process like induced mutation is an effective technique for generating substantial genetic variability in plant species. This technique has been successfully utilized by several research institutes on different crops (Das *et al*., 1999; Azad *et al.,* 2012). In Bangladesh, nearly 3000 crop varieties have been released till to date using this technique of which 634 are rice varieties (Mohamed *et al*., 2006).

1.3 Molecular markers:

Molecular markers, an essential tool for analyzing genome diversity. Moreover, it can be used to reveal some characteristics about the respective source. Microsatellites (SSRs) are one of the most obstructive technique which helps to study polymorphism between DNA sequences (Tautz, 1989). SSR, short tandem repeats (STR) and simple sequence length polymorphisms (SSLP) are present in both prokaryotes and eukaryotes. They are available widely throughout the genome, especially in the coding and non-coding nuclear and organellar DNA and euchromatin of eukaryotes (Pérez-Jiménez *et al.*, 2013; Phumichai *et al.*, 2015). These molecular markers with the help of PCR reaction, reveals *loci* variations of repetitive sequences. Microsatellites have numerous advantages like appealing low amount of DNA, automation for high screening, may be trade between laboratories, and are immensely transferable between populations (Gupta *et al*. 2003). They also found there use in forensics both for human and wildlife cases (Evett and Weir, 1998).

2. Review of Literature

2.1 The origin of rice:

Rice or *Oryza sativa* L. is basically not a tropical plant but is still linked with a wet, humid climate. Rice as wild grass, was originated around 130 million years ago. Probably in India about 10,000 years ago, rice was originally cultivated. Till date the original form is not into account, but thousands of rice varieties are now known, both cultivated and escaped (Yoshida, 1981).

From India, the plant moved to China and then further to Korea, the Philippines (about 2000 B.C.), Japan and Indonesia (about 1000 B.C.). Persians were the first importers of the grains. From there its fame captured the Mesopotamia and Turkestan. It was the Arab travelers who took it to Egypt, Morocco and Spain and from there it had a journey all across Europe. The Portuguese and Hollanders took rice to their colonies in West Africa. From Africa it moved to America through the 'Columbian Exchange' of natural resources, rice being a gift from the Old World to the New. Now it has been cultivated in the USA for the last three hundred years. The process of puddling soil and transplanting seedlings was likely refined in China. With these advancements, rice became truly domesticated. In Southeast Asia, rice was originally grown under dry land conditions in the uplands, and after that it came to occupy the vast river deltas. In Asia, rice is classified into three sub species known as Japonica, Javanica and Indica (Gupta and Toole, 1986). The traditions of wetland rice cultivation were carried to the Philippines by the migrants from South East Asia during the second millennium B.C., and Deutero-Malays may have carried this practice to Indonesia about 1500 B.C. The crop was introduced to Japan around 100 B.C. from China or Korea.

In the Indian subcontinent major portion of the cultivated land is given to rice. It is a very vital element of the daily food in numerous parts of the country. The rice grain is handled with honor in the subcontinent and in Asia because here the failure of the rice crop is an economic setback as well as an element for creating a famine-like situation.

2.2 Botany of rice:

Rice crop is a member of Poaceae family which is primarily grassland. It consists of aerenchymatic tissues and a moderately aquatic plant. It is well adapted to growing in flooded soils, but it can perform well in non-flooded soils. The parts of the rice plant are divided into vegetative and reproductive organs. Roots of the rice plant are fibrous and have the root hairs. The culm or the stem has a series of nodes and internodes but in alternate order. It bears a leaf and a bud which may grow into a tiller or shoot. The primary tillers grow from the lowermost nodes and give rise to secondary tillers. The leaves originated at an angle on the culm in two ranks, one at each node. Rice plants possess both auricles and ligule while the opponent grassy weeds in rice fields don't have both. It has a panicle which is a group of spikelets borne on the uppermost node of the culm. Varieties may vary in the weight and density of the panicle. However, the individual spikelet consists of two very small outer glumes with all other floral parts lying between or above them. At the base of the flower there are two transparent structures called as lodicules. The rice grain consists of ripened ovary, the lemma and palea, rachilla, sterile lemmas and awn. Plant height of rice may vary from 1 to 1.8 m, often more or less of this depend on the cultured genotype and soil fertility level. 50 to 100 cm long slender leaves present and breadth is about 2 to 2.5 cm. Rice grain may be 30 to 50 cm long along inside with the kernel length ranging from 5 to 12 mm (FAO, 2009b).

2.3 Mutation breeding:

Mutation breeding is to create a broad variability and selection of mutants is based on desired characters. The success of plant breeding depends on the genetic variability of plants. To achieve the goal of breeding it is necessary to know the value of prevailing genetic diversity. According to Konzak and Mikaelson (1980), mutation breeding is a method used for the genetic variability that is very narrow. But the conventional methods are advantageous than mutation breeding.

Mutations can occur naturally or artificially induced. Artificial induction of mutation can increase the genetic diversity of plants through changes in the composition of genes derived from the plant itself. Spontaneous or natural mutation not able to provide genetic diversity speedily and precisely, therefore, methods to induce mutations is an important issue to be known in order to increase and enhance the yield of crop plants (Ahloowalia and Maluszynsky, 2001).

2.3.1. **Induced mutations in plant breeding**:

In plant breeding research induction of mutation has become a real way of improving cultivars. Treatment of plant organs (seed, pollen, etc.) with mutagens alters or breaks chromosomes of that particular organ.

Mutation has been used effectively in breeding of several food crop varieties, ornamentals and export crops (Mohamad *et al*., 2005). Mutation breeding is one of the effective ways of inducing genetic variability and new mutant lines with improved traits (Mei *et al*., 2007; Mohamed *et al*., 2006).

Most of the induced mutants used in plant breeding show the Mendelian inheritance that plant breeders could regularly exploit (Konzak, 1976). Some of the induced mutation may be directly used as cultivars while others may require modification for further recombination and selection before use as improved cultivars.

The induced mutant cultivars have also demonstrated to be outstanding parents for further cultivar development in various crops (Konzak *et al.,* 1984; Micke *et al.,* 1987, 1990). The main benefits of using this technique are that the elementary genotype of a variety is only slightly altered while the improved characters are added (Sigurbjornsson *et al.,* 1992) and the time required to breed an improved variety is shorter than that taken in hybridization technique to achieve similar result.

Limitation of a crop with narrow genetic base can be stunned by using the induced mutation technique. It gives an opportunity of inducing desired characters that are new in nature or have been disappeared during evolution. For example, when a gene for resistance to a particular disease or stress is not found in nature plant breeders have only option to utilize induced mutation techniques (Novak and Brunne, 1992).

2.3.2 Mutagenic agents:

Physical mutagens, such as ionizing radiation, or chemical mutagens, including alkylating substances, for example, EMS are the sources to induce mutations (Predieri, 2001; Wu *et al*., 2007; Talebi *et al*., 2012). Among chemical mutagens, EMS is the widely use because of the fact that it is easy to use and have potential for creating high level of substitution and irreversible mutations (Henikoff and Comai, 2003; Talebi *et al*., 2012). Chemical mutagens are ideal for inducing dominant mutant alleles, while physical mutagens are ideal for recessive mutations. Recurrent mutagenic treatment are widely use as it is helpful in creating mutation frequency and consequently may introduce new advantageous mutations (Kondo, 2009).

The use of mutation induction is to enhance mutation rate in developing new plant varieties in a short time. The occurrence of spontaneous mutation frequency rate is very low and difficult to use in plant breeding. Typically mutation are induced by physical (gamma radiation) and chemical (EMS) mutagen treatment of both seed and vegetatively propagated crops. Mutations are helpful to create new helpful characters in plants and animals or to improve the already existing characters. Sometimes for mutation induction, high energy beams are used which largely produce deletion mutants. According to International Atomic Energy Agency (IAEA) mutant database, in cereals, fruits and vegetables, around 3000 mutant's varieties have been commercially released. By invitro selection, within a short span of time some tolerant mutants for buoyic and abiotic stresses have been isolated. Also in the regenerated plants, developing new improved plant varieties are an important tool to balance up the food requirement of the growing population (Mohan, 2010). Ionizing radiations and chemical mutagens were used to induce mutation in different crops such as wheat, rice, barley, cotton, peanuts and beans. The irradiations are also used for those crops that are in-vitro cultured. These are also effectively used in micro propagated plants. Molecular markers such as RAPDs, AFLP and SSRs are used for the analysis of mutants (Ahloowalia and Maluszynski, 2001).

2.3.3 Application of EMS:

EMS is one of the most commonly used chemical agents for creating mutations. This alkylating agent can efficiently cause the chemical alteration of nucleotides, which results in various point mutations, including nonsense and missense mutations. Subjective alkylation of guanine (G) residues results in the formation of O6-ethylguanine, which can pair with thymine (T) instead of with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be changed with adenine (A)/T (Greene *et al.*, 2003). The majority (99%) of times, EMS induces C-to-T changes resulting in C/G to T/A substitutions (Krieg, 1963; Greene *et al.*, 2003). At a much lower frequency, EMS also generates G/C to C/G or G/C to T/A transversions by 7-methylguanine hydrolysis or A/T to G/C transitions by 3-methyladenine pairing errors (Krieg, 1963). EMS can be used to create loss- or gain-of-function mutants and to understand the functional role of single amino acid residues. EMS mutagenesis has been demonstrated to create useful breeding lines (Lee *et al.*, 2003).

Various degrees of sensitivity for different characters such as survival, pollen sterility, spikelet sterility to gamma radiation has been showed by all the varieties but to EMS treatment it was less sensitive to all the above mentioned characters. The wild specie *O. spontinae* in response to gamma treatments resulted great resistance in first mutant generation injury. However the lowest was recorded for japonica (Norin 27). Some varieties also showed the medium values of injuries in mutant generation in this case. Frequency and spectrum in response to gamma treatment for chlorophyll mutation was same for both low and high fertility groups. However there was a clear difference in both frequency and spectrum of chlorophyll mutation when treated with the EMS mutagen for these fertility groups,

Mohapatra *et al*. (2014) conducted an experiment using EMS in the upland rice variety Nagina 22. This experiment was carried out under the supervision of six research institutes. The specificity of this collaborative effort is phenotyping of traits that has led to identification of mutants for plant growth and architecture, flowering, maturity, grain number, shape and size, yield, phosphorus use efficiency, resistance to blast and bacterial leaf blight diseases and tolerance to drought, salinity and herbicide. A bundle of 22,292 mutagenized lines generated under this strategy are

phenotype for the above traits. Isolation was done for the few favorable mutants which are being characterized. A series of fruitful mutants are now avaliable for various traits such as phosphorus use efficiency, tolerance to drought, salinity, herbicide spray and resistance to bacterial leaf blight. This resource is giving a hope to sort and search out useful genes and alleles.

Talebi *et al*. (2012) conducted an experiment on a variety MR219, seeds of this genotype were treated with EMS at concentrations of 0.25%, 0.50%, 0.75%, 1%, 1.25%, 1.5% and 2%. Several measurements were taken to find out EMS sensitivity in M_1 generation. With the increase in the applied EMS concentration in M_1 generation, there was a clear fall off in germination, seedling height, root length and emergence as compared to the non EMS treatment. Furthermore the increased EMS treatment was also responsible for the decreased in characters such as plant height and root length in linear fashion. Certain traits such as seed germination, seedling height and root length was measured to find out the lethal dose in field conditions. In this experiment, as a regular procedure quantitative determinations were done. Data was recorded about seedling height, root length and percent of germination. Observed mean's variability was recorded. As a result, varying EMS concentrations significantly affected the above mentioned traits ($p < 0.01$). The LD_{25} and LD_{50} values were observed based on growth reduction of seedlings after EMS treatment with 0.25% and 0.50% on the rice variety.

Jian-Li *et al.* (2005) conducted an experiment on a rice variety IR64. They used chemical mutagens (EMS) and irradiation (gamma rays) to induce mutation and identified mutational changes in traits of agronomical importance. They did mutation against quantitative traits and disease resistance. They worked on forward and reverse genetics to close the gap between genotypes and phenotypes. They were concluded that the value of IR64 mutant will be effective with increased usage and extensive testing under a wide range of conditions.

Peng *et al.* (2014) reported that a large number of rice mutants has been generated by mutagenic agents. Mutant genes are involved in plant improvement for many characters. Many rice cultivars with high yields and superior grain quality has been developed and effectively used for rice production. EMS and Irradiations were

used to create rice mutants and many other methods have been developed for the analysis of rice mutants.

2.4 Molecular markers:

An important tool to study the genetic control of any trait is the use of Molecular Markers which ultimately leads to highlight certain genes and the metabolic chains involved. These technologies are helpful in controlling the chromosomal transmissions in the progeny. Genetic markers can assist in the development of new traits that can be put into mass production and would be very pivotal. These novel traits can be identified using molecular markers and maps. They have been used for mapping of genomic regions comprising genes of agricultural interest (Charcosset and Moreau, 2004). Recently, microsatellites have been used in genetic divergence studies of several crops for analysis of its diversity..

Almost any sequence variation between individuals can be used to design a marker that will allow the documentation of the parent that contributed a precise segment of the chromosome in a recombinant line. Until recently, the most popular markers were the simple sequence repeats, also known as microsatellites.

PCR-based markers are short tandems repeats, SSR's or STMS. Short nucleotide notifs (2-6 bp/nucleotides) are randomly repeated as per their characteristic. Also in both animals and plants occassionaly di-, tri-, and tetra- nucleotide repeats are present throughout the entire genomes with some unique characteristics. There is a huge difference when it comes to the copy number of these repeats in different individuals which ultimately provide the polymorphism base of the plants. These DNA sequence regions are highly conserved, specific to some primers which make them helpful in amplification through PCR reactions. Elevated level of allelic variations are considered to be an ambient feature od microsatellite loci, which labelled them as the valuable gentic markers. SSR loci are independently amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences bordering the SSR sequence. SSR markers are categorized by their hyper-variability, reproducibility, co-

dominant nature, locus-specificity, and random genome-wide distribution in most cases. (Song *et al.*, 2010)

2.5 Mutagenesis in various crops:

Borzouei *et al*. (2010) disclosed that gamma ray induce a significant impact on the shoot length and root length in wheat. Decreased in Shoot length both Roshan and T-65-58-8 genotypes upto 46 cm, at a radiation dose of 200, 300 and 400 Gy. They also described that radiation and interaction of radiation and genotypes imposed a significant effect on root length. Decreased in Root length was seen after all doses of irradiation as compared to control and a minimum length of the root were found in both genotypes subjected to 400 Gy. In another study about the effect of gamma radiation on chickpea seeds by Toker *et al*. (2005) seedlings irradiated with 200 Gy had some significant increase in their shoot length, but at 400 Gy a glaring depression in shoot length was observed.

Chaomei and Yanlin (1993) reported that seeds treatment with high rates of gamma radiation decline germination with a corresponding decrease in growth of plants on wheat (*Triticum aestivum* L.). The potency of mutation breeding programs largely depends on the amount of genetic variability available in the crop species and the efficiency of the selection techniques used. The outstanding works of Muller (1927) and Stadler (1928) revealed new vistas in plant breeding by introducing artificial induction of mutation.

A huge number of varieties developed by mutation breeding so far have arisen from material treated with ionizing radiations. Substantial work with chemical mutagens has begun only since 1960 following the introduction of ethyl methane sulphonate. The alkylating agents are more suited than ionizing radiations for breaking down the buffering characteristics of a germplasm and for creating a maximum of genic diversity and allelic interaction between homologous loci (Mac Key, 1967).

Chemical mutagens, especially alkylating agents, in contrast to radiations, produce wide range of mutations. Swaminathan *et al*. (l962) reported that EMS gives higher frequencies of gene mutations as compared to ionizing radiations. Swaminathan *et al*. (1962) examined chromosome and chromatid aberrations when different wheat

cultivars of various ploidy levels were treated with multiple concentration of ethyl methane sulphonate.

Yadav (1987) treated the seeds with different doses of EMS, DES and gamma rays separately and in combination in mung bean. In general the mitotic index and seedling vigor declined with increasing mutagenic doses. However, the factors like frequency and spectrum of chlorophyll mutation increased in the M_2 generation. Singh and Raghuvanshi (1987) treated the seeds with gamma rays alone or followed by treatment with EMS in *Vigna mungo*. Two mutants with pentafoliate leaves, increased yield and higher number and dry weight of nodules were isolated. Narasimha and Bhalla (1988) got a male sterile mutant in the M_2 generation in EMS treated population of pigeon pea. (Venkateswarulu *et al.*,1988) isolated different types of chlorophyll mutants after irradiated with gamma rays and treated with EMS singly and in combination in *Carthamus roseus* L. Pande and Raghuvanshi (1988) treated the seeds of *Vigna radiata* variety K581 with gamma rays and EMS. A dwarf mutant obtained in M_2 and found true breeding in M_3 generation showed elevated number of pods per plant and seeds per pod.

Jana (1963) working with gram, obtained different chlorophyll mutants, after the X-ray irradiation of seed of variety T-9 of black gram. The chlorophyll deficient mutants included albina, xanthoalba, xantha, chlorina, variegata and virescent type. Goud (1967) reported that the EMS is more operative in producing biological damage than gamma rays in bread wheat. Sato and Gaul (1967) reported that sterility induced by EMS and chemical mutagens might be due to cryptic deficiencies and specific gene mutations. Prasad (1968) pinpointed a yellow rust resistant mutant in the M_2 generation of EMS treated *Triticum durum* variety NP 404**.** Izvorska (1969) irradiated the seeds of the eggplant variety Delicacy with numerous doses of X-rays and reported that the germination was earlier in several treatments and M_1 plants flowered earlier and produced excessive fruits than controls.

Sharma (1970) observed actions of gamma rays and EMS on pollen sterility. Siddiqui (1972) worked out considerably on chemical mutagenesis and obtained high yielding mutants of *Solanum melongena*. Gustafsson (1963) obtained erectoid mutants in barley, which are of immense useful in barley breeding. Kaul and Bhan (1977) studied the effect of gamma rays, the effect of EMS and of DES singly and in

combination upon seedlings of three rice varieties. Patel and Shah (1974) irradiated brinjal seeds and noticed morphological and structural variation in shoot apex. Reddy and Reddy (1972) after working hydrazine induced various grain shape mutants in rice.

Haq and Shakoor (1980) irradiated the seeds of Cicer with gamma rays and end up with a mutant, which was resistant to blight disease. Singh and Chaturvedi (1980) treated two inbreds of mung bean with different kinds of mutagens i.e. EMS, NMU and gamma-rays and reported that irrespective of the varieties involved, EMS was found to be most efficient for germination, NMU for pollen fertility and seedling height in M_1 generation and NMU for chlorophyll mutations in M_2 generations. Singh and Chaturvedi (1980) treated two inbreds of mung bean [*Vigna radiata (L.)* Wilczek] viz. Pusa Baisaki and S-8 with EMS, NMU and gamma rays to study their relative mutagenic susceptibility and specificity. Sharma and Sharma (1981) induced polygenic variability in Lentil for yield and its components including chlorophyll mutation by different mutagenic treatments.

Chowdhury (1978) reported that chemical mutagens are more effective in inducing high frequency mutation in bread wheat. Singh *et al.* (1978) in pearl millet observed the biological damage and morphological mutations induced by gamma rays and EMS. Khan and Hashim (1979) reported the mutagenic effectiveness and efficiency of gamma rays, Ethyl methane sulphonate and hydrazine in *Phaseolus aureus.* Sharma and Sharma (1979) carried out a comparison between the effectiveness and efficiency of NMU in lentil and found that NMU was about three times more effective than gamma rays. Farook (1979) reported the rise in the mean values of quantitative characters and variation of protein characters in mutated material as compared to control in chickpea.

Khan *et al.* (2000) studied that how much of biological damages and polygenic variability induced by MMS in green gram. A dose dependent damage in M_1 and a higher coefficient of heritability and variability values for quantitative traits was seen in M_2 generation of treated population. Rizwana *et al.* (2005) noticed that when compared to EMS, gamma ray treatments are more responsible for variation in the quantitative characters in cowpea.

Kumar *et al.* (2003) observed that the EMS and gamma irradiated plants of *Lens culinaris* depict varying amount of meiotic irregularities almost at all doses. The frequency of meiotic irregularities was found to be higher in the sets of combined

treatments as compared to the single ones. The mutagenic effect of sodium azide has been known in bacteria. However, the mutagenic property of azide in plants has now been an established fact. The effect of sodium azide as well as its metabolite in barley and bacteria seems to be unique among the present day known mutagens (Kleinhofs *et al.*, 1974).

Sideris *et al.* (1973) observed that the mutation frequency acquired with sodium azide treatments was increased to about 20% in Barley by using solutions at pH values below the pKa of azide. Nilan *et al.* (1973) observed mutation frequencies on M_1 spike of up to 46 percent with time period of 4h germination with azide treatment in barley. Conger (1973) reported the effect of ascorbic acid on azide – radiation synergism on seedling growth in barley.

Bandyopadhyaya and Bose (1983) isolated tall vigorous mutants with some variation in leaf shape, early flowering and high pod production in M_2 generation of black gram treated with EMS and X-rays. Khan (1982) noticed a considerable increase in genetic variance and heritability in mung bean after treatment with gamma rays. Rao (1984) subjected varieties of pigeon pea to various doses of gamma rays and isolated wide range of mutants with varying forms of habit and profuse branching with high yield. Verma and Singh (1984) reported the induced variability in green gram using gamma rays. The M_3 population showed elevated variance than the control for pods per plant, seeds per plant and yield per plant.

Bahl and Gupta (1984) irradiated two inbreds of mung bean ML 5 and K 851, with gamma rays and also treated with EMS separately and in combination and found that albina and chlorina chlorophyll mutations occurred in the M_2 segregation population. Khan (1984) while working with mung bean treated the seeds with gamma rays, EMS and hydrazine. The mean values and variances of number of days of flowering, number of fertile branches per plant and number of pods per plant was greater than that in the control. Sharma and Kaul (1984) noticed that the EMS induced mutant of IR8 rice shows higher salt tolerance than the parent variety.

Grover and Virk (1986) reported several viable mutants after treatment with MNNG (N-methyl-Nnitro- N-nitrosoguanidine), EMS and HA (Hydroxyl amine) in mung bean. Khamankar (1984) studied the spectrum of induced mutations and found significant differences for rates at specific loci by hydrazine and hydroxylamine while,

gamma-rays gave highest frequency of lethal, EMS showed chlorophyll mutation at high rate.

Nadarajan *et al.* (1985) worked with the two varieties of *Cajanus cajan* and treated them with gamma-rays and diethyl sulphate and concluded that low doses of the mutagen slightly increased M_1 plant height but higher doses reduced the height. Khan (1986) observed the various agronomic characters like days to flowering, chlorophyll concentration etc obtained after treatment with Gamma rays.

Jayabalan and Rao (1988) isolated different chlorophyll mutants after the treatment with gamma-rays, EMS and n-nitroso-n-methylurea in two cultivars of tomato. The incidence of various types of chlorophyll mutants did not follow dose related trend.

Bahl (1988) has reviewed the genetic advances, heritability and correlation chickpea and has noticed that most of the part of studies were based on broad sense heritability and breaking of negative correlation by the mutagenic treatment. Chary and Bhalla (1988) while working with EMS reported a male sterile mutant from the M_2 generation of pigeon pea. Reddy (1989) identified several different types of chlorophyll mutants in *Triticale* after treatment with gamma rays and EMS. Reddy and Gupta (1989) observed that EMS produced high frequency of mutation as compared to gamma rays in *Triticale*. lgnacimuthu and Babu (1989) isolated many high protein mutants after the treatment of seeds with gamma rays and EMS in *Vigna mungo* and *Vigna radiate* and these possessed high level of lysine.

Natarajan and Palaniswamy (1990) treated two varieties of mung bean with EMS and in combination with gamma rays. In M_2 generation, it was observed that the 30 KR gamma ray treatments produced the highest frequency of micro mutations. Combination treatment of low doses of EMS and gamma rays were recommended for bringing mutation in yield components. Singh and Chaturvedi (1980) have calculated the mutagenic efficiency of EMS, NMU and gamma rays and their combined treatment in *Vigna radiata*. Srivastava *et al.* (1973) obtained a number of agronomical important mutants and studied dose response on gram by EMS and MMS.

Sharma and Singh (1992) gave post irradiated heat treatment after 30 KR gamma-ray dose and observed that treatment administered with higher temperature (60'~) caused maximum reduction in M, parameters and induced higher frequency of

chlorophyll and viable mutations in M_2 generation of mung bean. Sharma and Singh (1992) isolated two male sterile mutants from 30 KR gamma ray treated M_2 population of mung bean. Sharma and Singh (1992) reported induction of a number of important mutants *i.e.* bushy type, tall type, long pod, high yielding clustered pod, synchronous maturing and early maturing plants in mung bean from EMS and gamma ray treated population. Srivastava and Singh (1993) got higher estimates of variability and heritability in M_2 population after mutagenesis in Pigeon pea. Srivastava and Singh **(**1993) described synchronous maturing mutants in pigeon pea by subjecting the seeds to gamma rays, EMS and NMU. Micke (1995) analyzed the role of induced mutations in crop improvement and concluded that radiation and other mutagens are efficient in producing genetic variation useful for plant breeders.

Khan and Siddiqui (1995) isolated different morphological mutants by using chemical mutagens viz., EMS, MMS and sodium azide. These mutants show deviations from control and also among themselves in height, growth and flowering habit. Gaikwad and Kothekar (2003) reported various morphological mutants in EMS and sodium azide treated M_2 and M_3 generation of *Lens culinaris.* Out of 9 morphological mutants obtained, the early maturing, high yielding and bold seed type mutants have the ability to be used in breeding programs. Khan and Wani (2004) reported increase genotypic coefficient of variation, heritability and genetic advance for 3 quantitative characters in EMS, methyl methanesulphonate and sodium azide treated M_2 and M_3 populations in mung bean.

3. Objectives:

The present work has been designed for the following objectives:

1. To record the degree of response between Dawk Pa-yawm and Dawk Kha 50 rice varieties towards EMS mutagen.
2. To investigate the optimum working and lethal dose of EMS in M_1 generation of the Dawk Pa-yawm and Dawk Kha 50 varieties.
3. To study of the certain morphological characters in response to the EMS and selection of desirable mutants from segregating population in M_2 generation using SSR marker.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Plant Materials:

Two rice varieties Dawk Pa-yawm and Dawk Kha 50 (Figure 1) were used as the experimental materials. The main features of the two varieties are mentioned below:

1.1.1 Dawk Pa-yawm:

Non-glutinous rice, photoperiod sensitive local variety in southern Thailand, plant height 150 cm, days to maturity 145-150, straw colored husk, seed dormancy 2 weeks, seed size length x width x thick 10.3 x 2.4 x 2.0 mm, brown rice seed 7.3 x 2.2 x 1.8 mm, 24% amylose, yield 250 kg/rai (6.25 rai = 1 hectare), Resistance to rice blast disease, brown spot disease and narrow brown spot disease, susceptible to bacterial leaf blight disease or bacterial blight disease and leaf scald disease (Rice department, 2019)

1.1.2 Dawk Kha 50:

Dawk Kha 50 is photoperiod sensitive rice variety. It has 396 kg per rai, straight clum 145 cm plant height and 145 - 150 days maturity, green leaves, 9 seeds per panicle, straw colored husk, 21.23 % amylose content and susceptible to rice blast disease. (Suangul et al., 2018)

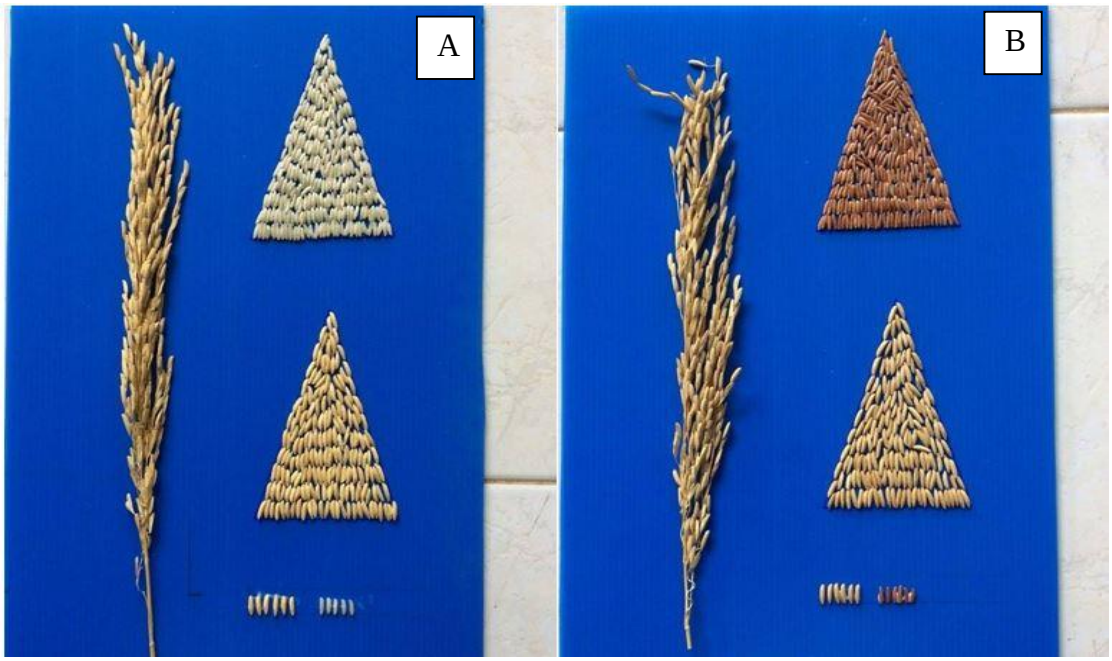

Figure 1. Pictorial representation of seeds of upland rice (A) Dawk Pa-yawm (B) Dawk Kha 50

1.2 Laboratory Materials:

EMS, Seed germination paper, CTAB extraction buffer, 2-mercaptoethanol, chloroform, Ethanol, Absolute ethanol, TAE buffer, Ethidium bromide, Gel loading dye, DNA marker solution.

1.3 Equipments:

Incubator, Pestle and mortal, Gloves, Forceps, Centrifuge, Micropipette, Tips, Water bath, Spinner, Paper bag, Scissors, Plastic bags.

2. Methodology:

The experimental place and data collection were done in the Plant Molecular Laboratory and field of Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hatyai Campus, Songkla, Thailand.

2.1 EMS protocol and LD_{50} Experiment:

The experiment was designed as the factorial experiment using complete randomized design for LD_{50} experiment. 100 seeds per treatment were used for this preliminary experiment. Seeds of the above two mentioned varieties were treated with EMS at different doses of 0.50%, 0.75%, 1.0% and 1.25% using four replications. The non-treated (control) seeds of both varieties were used along with the treated seeds. For EMS mutagenesis, rice seeds were placed in a 500 ml flask and ultrapure water up to about 5 cm levels above the seeds were added. Seeds were soaked for overnight at room temperature for 20 hours. Subsequently, water was decanted and 50 ml of 0.50%, 0.75%, 1%, 1.25%, concentrations of EMS (v/v) were added in water. Mixture of EMS and seeds were incubated for 12 hours at room temperature, followed by decantation of the EMS and rinsing with 100 ml of ultrapure water (5 times, 4 minutes each) and 200 ml of ultrapure water (4 times, 15 minutes each). Seeds were then rinsed under running tap water for 4 hours before placing in a group of three papers with the paper containing the seeds, sandwich between the upper and lower paper. Take final reading not more than 15 days.

EMS treated seeds were initially germinated in the germination paper. The paper was first dipped in the water tub and excessive water was squeezed out. Seeds were then placed in lines on the surface of the paper. After finishing, the paper was rolled and tagged properly.

2.2 Field experiment:

2500 seeds of both varieties, Dawk Pa-yawm and Dawk Kha 50 were used in field. The concentrations used were according to the results obtained after the LD_{50} experiment.

2.2.1 Land preparation:

Ploughing was done twice before sowing to make soil less clustorous and porous.

2.2.2 Application of fertilizer:

Fertilizer N-P-K (15-15-15) was applied on periodic basis after 15 days when the plants were about 1 month older, by continuous examining the fertility level of the soil and by examining the condition of the growing plants.

2.2.3 Field layout:

Seeds were sown in simple rows by maintaining row to row distance as 5 cm and plant to plant distance 10 cm, approximately one seed per hill was grown along with peat to ensure better germination. Seeds were treated with the EMS which gave rise the M_1 seeds as shown in the Figure 2. Only those plants from M_1 plants were selected which had produced the seeds while the others were rogue out. Seeds were kept separate and grown as M_2 (8 seeds per plant) which resulted in M_3 seeds. At the end SSR analysis was performed on M_2 plants.

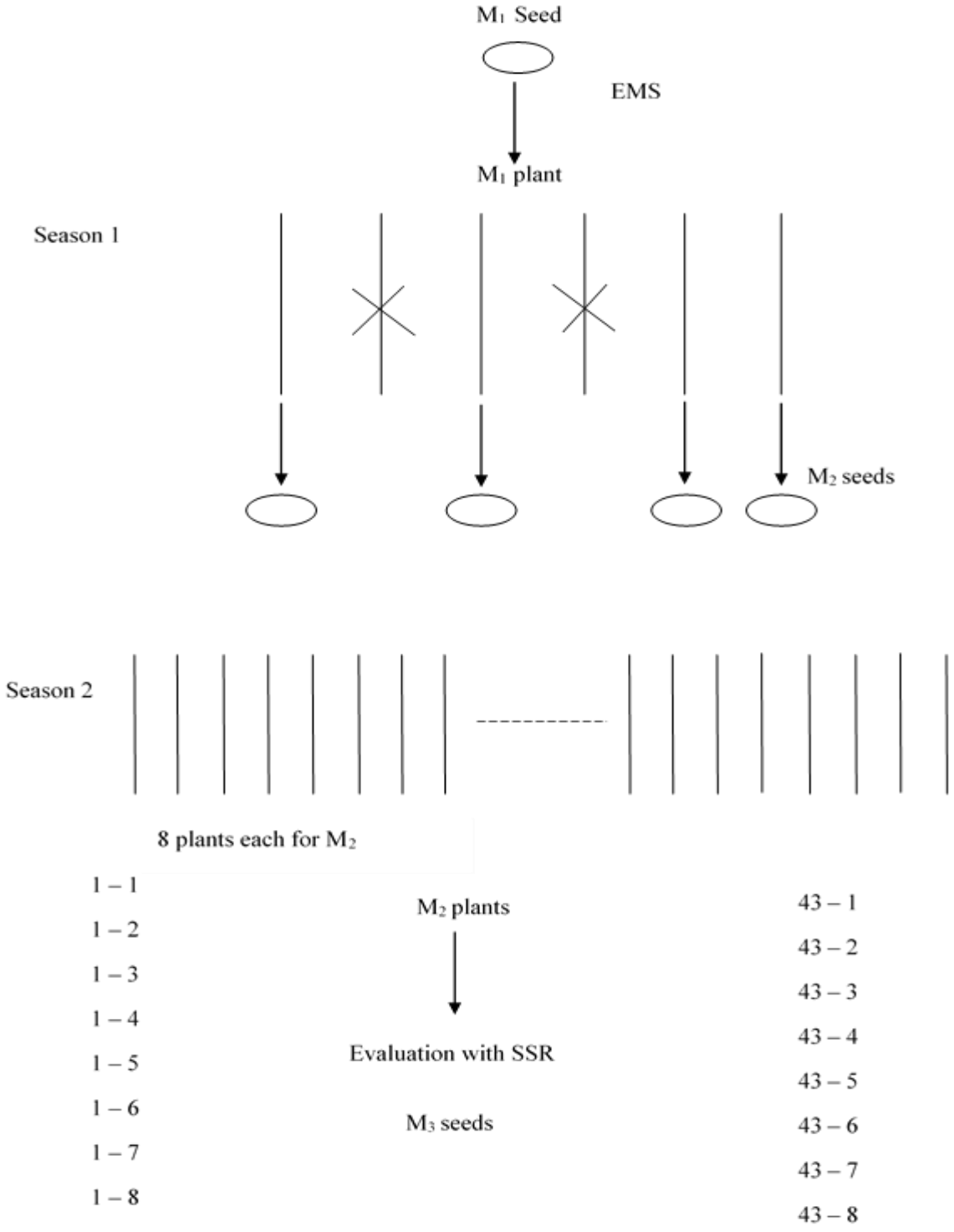

Figure 2 Diagrammatic scheme of the research work from M_1 seed till SSR analysis

2.2.4 Harvesting:

In M_1 generation, the plants which survived up to maturity and produced viable seeds under different doses of treatments were harvested separately. From the control population 10 plants from each variety were also selected and harvested. In M_2 generation, priority of harvesting was given to those plants which had shown defined mutational changes in the phenotype. Those plants were harvested and tagged separately.

2.3 Data collection:

2.3.1 Data Colletion for LD_{50}

For the LD_{50} experiment, two essential parameters were recorded and data was analyzed by Factorial design using complete randomized design. Those parameters are as under.

2.3.1.1 Root length*:*

After unrolling the paper, roots were extended lengthwise with the ruler. Reading were taken in cm, after 15 days of seed treatment.

2.3.1.2 Shoot length*:*

Shoots were extended lengthwise with the ruler and the length of shoot was measured in cm.

2.3.2. Data collection in M_1 generation:

2.3.2.1 Germination of seeds

Germination percentage of seeds were recorded in M_1 generation by the total number of seeds germinated by the total number of seeds sown and multiplied by one hundred.

2.3.2.2 Survival of plants

Percentage survival of plants was estimated in M_1 generation on the basis of counting the total number of survived seedlings divided by total number of germinated seeds and multiplied by one hundred. The data was estimated at 21 and 30 days after sowing.

2.3.2.3 Number of effective tiller and non-effective tiller

The panicle bearing plant with mature seeds at harvesting was considered as effective tiller while the rest were considered as non-effective tiller.

2.3.2.4 Panicle length *(*cm*)*

Panicle length was measured by ruler in centimeter from the base of the panicle to the tip of panicle.

2.3.2.5 Number of filled grains and unfilled grains

The well developed and fully matured ripen grains were considered as filled grains whereas, white peppery, shrunken and damaged grains were consider as unfilled grains. The counting was done per panicle.

2.3.2.6 Plant height *(*cm*)*

Plant height was measured in the field from the ground level to the tip of a plant in cm at maturity.

2.3.2.7 Yield/Plant *(*g*)*

All the matured seeds produced by a plant were weighed by the help of an electrical balance in gram and that was considered as yield per plant.

2.3.2.8 1000 seed weight *(*g*)*

A thousand seeds were counted and weighed by the help of an electrical balance in gram.

2.3.3 Data collection in M_2 generation:

For recording the data of different- quantitative characters in M_1 generation, desirable plants were selected from the treated population grown in rows. Data from the control plants were also be taken for comparison between the treated and untreated plants. The following characters were studied in M_2 generation:

2.3.3.1 Days to maturity

It was the number of days required from the date of sowing to the date when 80% pods were matured.

2.3.3.2 Plant height *(*cm*)*

It was measured in centimeter from ground level to the tip of the plant.

2.3.3.3 Number of effective tiller and non-effective tiller

The panicle bearing plant with mature seeds at harvesting were considered as effective tiller while the rest were considered as non-effective tiller and they were counted as per hill.

2.3.3.4 Panicle length *(*cm*)*

It was measured in centimeter from the base of the panicle to the tip of panicle.

2.3.3.5 Number of filled grains and unfilled grains

The well developed and fully matured ripen grains were considered as filled grains whereas white peppery, shrunken and damaged grains were considered measured from the ground level to the tip of a plant in cm at maturity.

2.3.3.6 Yield/Plant *(*g*)*

All the matured seeds produced by a plant were weighed by the help of an electrical balance in gram and that were considered as yield per plant.

2.3.3.7 1000 seed weight *(*g*)*

A thousand seeds were counted and weighed by the help of an electrical balance in gram.

2.4 DNA extraction:

DNA was extracted from the leaf samples using the modified conventional method. Approximately 0.2g of each sample was grounded with liquid nitrogen in the mortar using a pestle. A total of 700µL extraction CTAB Buffer *(*1.0g PVP-40, 8.12g $NaCl_2$, and 4.0 ml 0.5M Na_2EDTA pH8.0, 10.0 ml 1.0 Tris-HCl, 2*%* β-mercaptoethanol*)* were added to the ground leaf. A mixture was then transferred to 1.5

ml microcentrifuge tube and kept at incubation at 65 °C for 1 hour. Subsequently, 700µl chloroform*:* isoamyl (24*:*1) was added to the mixture and centrifuged at 12,000 rpm for 15 minutes at room temperature. A total of 400 µl supernatant was then transferred into a new 1.5ml microcentrifuge tube containing800 ul isopropanol. The mixture was centrifuged again at 5000 rpm for 5 minutes at room temperature. The supernatant was then discarded, and the pellets will be rinsed 2 times with 70*%* ethanol. Subsequently, pellets were dried and re-suspended in 50 µl. The quantity of the DNA was measured using Nanodrop and quality of DNA was analyzed by agarose gel electrophoresis.

2.5 SSR analysis:

The DNA extracted from the leaves of different concentration of EMS of treated plants were genotyped using 11 SSR rice primers according to McCouch *et al*. *(*2002*)* as shown in Table 2. Each PCR of 19 µl contained 13.2 µl H_2O, 1.0 µl of 100Mm dNTPs, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 1.5 unit of Taq polymerase and 1 µl DNA template. The PCR reaction was performed using a Biometra thermo cycler with the following program*:* initial denaturation at 94 ^{0}C for 4 minutes, 31 cycles of denaturatitaon at 94 ^{0}C for 1minute, annealing at 60 ^{0}C for 1 minute, extension at 72 ^{0}C for 2 minutes and final extension at 70 ^{0}C for 5 minutes. The PCR products were separated using 3% agarosegel at 100V for 50 minutes with ethidium bromide as a stain.

Table 1. List of 11 primers used for SSR analysis of rice

Maker	Chr.	Forward Primer	Reverse Primer	Anneal temp (°C)	PCR Cycles	Min Allele	Max Allele
RM5	1	TGCAACTTCTAGCTGCTCG	GCATCCGATCTTGATGGG	67	30	94	138
RM19	12	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	55	30	192	250
RM44	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	53	30	82	132
RM215	9	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	55	30	126	161
RM259	1	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT	55	30	133	186
RM283	1	GTCTACATGTACCCTTGTTGGG	CGGCATGAGATGCTGTGATG	61	30	130	176
RM307		GTACTACCGAACCTACCGTTCAC	CTGCTATGCATGAACTGCTC				
RM316	9	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	55	30	194	216
RM413	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	53	30	71	114
RM455	7	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC	57	30	127	144
RM8225	6	ATGCGTGTTCAGAATTAGG	TTGTTGTATACCTCATCGACAG	52	30	210	280

Source: http://archive.gramene.org/markers/microsat/ssr.html

2.6 Data Analysis:

Data was analyzed statistically and by the use of SSR marker analysis for the M_2 generation.

2.6.1 LD_{50} Analysis:

The experiment was designed as the Factorial experiment using complete randomized design for LD_{50} experiment. The data was analyzed by factorial design using CRD and regression analysis (Steel and Torrie, 1980).

2.6.2 Statistical Analysis:

After harvesting M_2 the yield was subjected to various statistical parameters like Means Comparisons, Standard Deviation, and Coefficient of Variation (Steel and Torrie, 1980).

2.6.3 SSR Data Analysis:

Moreover, after the DNA extraction, PCR and gel electrophoresis the bands of control population were compared with the bands shown by the mutant population.

CHAPTER 3

RESULTS AND DISCUSSIONS

1. LD_{50} Values

1.1 Analysis of Variance and Mean comparison of root and shoot length

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	
		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

* Significantly different

**Highly significant

Furthermore, the EMS×genotype interaction for the root length, shoot length and germination indicated that there was a clear decline in the means of each traits for successive increase in the EMS concentrations. Means for shoot length, root length and germination were maximum when the seeds were untreated with the chemical mutagen. While on the other hand, on higher concentrations of EMS such as 1% and 1.25% there is a clear fall off in the means as compared to the untreated seeds as shown in table 3, 4 and 5. Regression analysis was performed to sort out the LD_{50} values for both the

genotypes. The dose selected for Dawk Pa-yawm after regression analysis was 1.34% and 1,23% for Dawk Kha 50. The doses were selected where both the genotypes were giving approximately 70% of germination keeping in mind the rocky texture and poor nutritional profile of the soil.

Table 3 Effect of EMS at various concentrations on germination of seeds of rice

Cultivars	Concentrations of EMS (%)					Means of genotypes
	0	0.5	0.75	1	1.25	
Dawk Pa-yawm	23.75	23.75	24.00	20.75	18.00	22.05
Dawk Kha 50	24.50	24.00	23.00	19.50	18.25	21.85
Means of concentrations	24.12	23.88	23.50	20.12	18.12	
C.V.		6.90 %				
$LSD_{.01}$ (Treatment)		2.95 cm				
$LSD_{.01}$ (EMS)		2.08 cm				

Table 4 Mean comparison of the EMS effect at various concentrations on the root length

Genotypes	Concentrations of EMS (%)					Means of genotypes
	0	0.5	0.75	1	1.25	
Dawk Pa-yawm	16.59	14.17	11.11	9.97	9.43	14.24
Dawk Kha 50	20.17	15.50	13.32	12.59	9.62	12.25
Means of concentrations	18.38	14.84	12.21	11.28	9.59	
C.V.		6.04 %				
$LSD_{.01}$ (Treatment)		1.56 cm				
$LSD_{.01}$ (EMS)		1.10 cm				
$LSD_{.01}$ (Genotype)		0.70 cm				

Table 5 Mean comparison of the EMS effect at various concentrations on the shoot length

Cultivars	Concentrations of EMS (%)					Means of genotypes
	0	0.5	0.75	1	1.25	
Dawk Pa-yawm	18.69	16.50	12.54	12.00	10.14	13.97
Dawk Kha 50	16.04	13.99	13.42	12.41	10.17	13.21
Means of concentrations	17.30	15.24	12.98	12.20	10.15	
C.V.	7.30 %					
$LSD_{.01}$ (Treatment)	1.92 cm					
$LSD_{.01}$ (EMS)	1.36 cm					
$LSD_{.05}$ (Genotype)	0.64 cm					

1.2 Effect of various concentrations of EMS on root and shoot lengths and decision of LD_{50}

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 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 3). (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.

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).

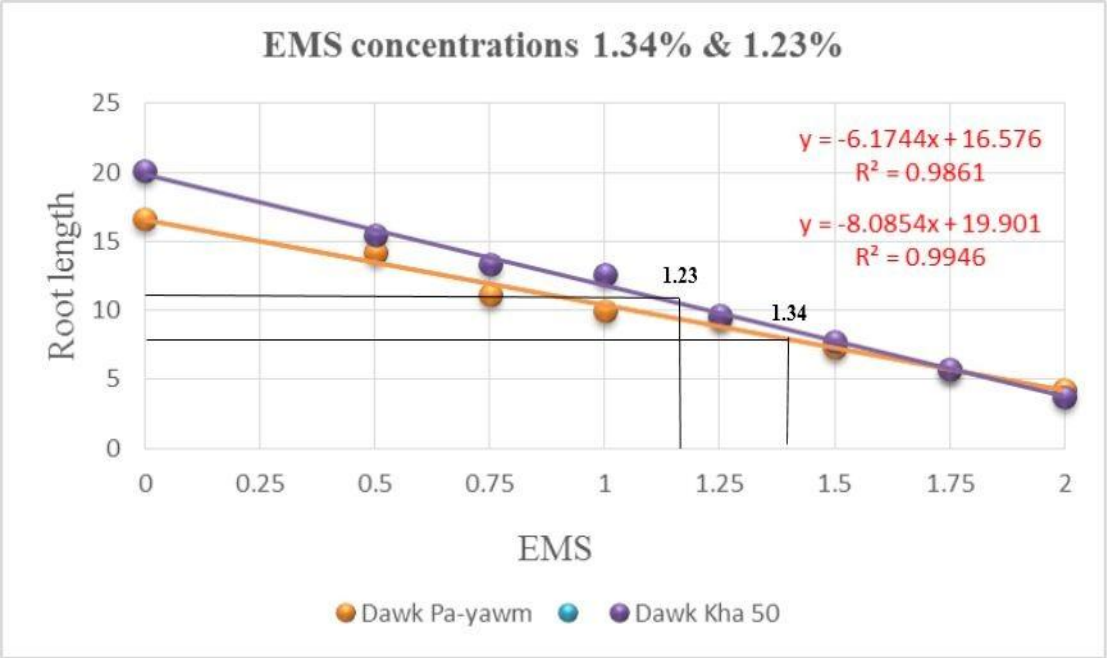

Figure 3 Effects of various concentrations of EMS on LD_{50} in root length

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 4. Most of the mutants showed an almost 2 times decrease in the shoot length when treated with EMS as compared to control, along with 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).

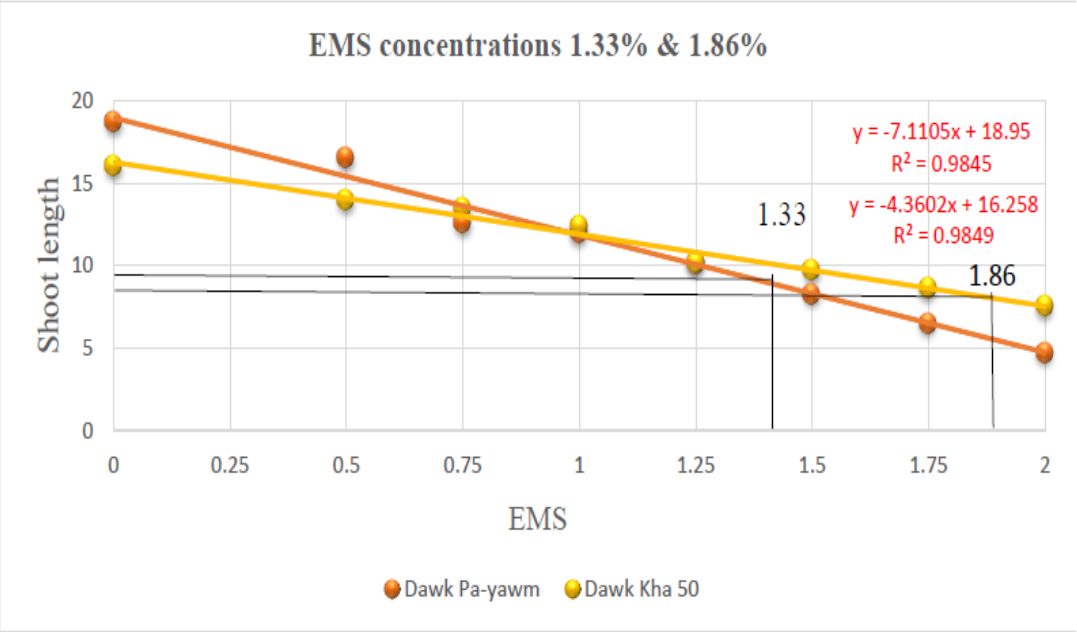

Figure 4 Effects of various concentrations of EMS on LD_{50} in shoot length

2. M_1 generation

2.1 Effect of EMS on germination percentage

The germination percentage for both Dawk Pa-yawm and Dawk Kha 50 reduced after EMS treatments. For Dawk Pa-yawm the selected dose was 1.34% and 2500 seeds were treated for M_1. Out of those, 1526 seeds were germinated. Data was recorded after 25 days of sowing and germination percentage came up to be 61.04% for Dawk Pa-yawm M_1 mutants.

For Dawk Kha 50, two concentrations were selected which were 1.23% and 1.86%. Total of 2500 seeds for both treatments were subjected to EMS and data was recorded. In case of 1.23%, out of 2500, 1771 seeds were germinated, and germination percentage appeared as 70.84%. For 1.86%, only 730 seeds germinated and had the germination percentage value of only 29.2%. These results are similar to the research of (Jadhav *et al.*, 2012; A. Singh *et al.*, 2000) where reduction in germination percentage was recorded when okra seeds were treated with EMS.

2.2 Effect of EMS on survival percentage

The data for the survival of the plants was taken after 30 days of sowing. Drastic results were obtained as the EMS affected the survival percentage of the mutants. Out of 1526 seeds germinated for Dawk Pa-yawm, only 172 survived giving the survival percentage as 11.27% for Dawk Pa-yawm.

In Dawk Kha 50 for 1.23% EMS, germination was recorded as 1771 seeds out of 2500 but only 134 plants survived giving the survival percentage as low as 7.56%. Moreover for 1.86%, mortality rate was very high as the survival percentage got a lower value of only 5.61% because out of 730 germinated seeds only 41 survived. Previous researchers also noticed the drastic reduction in the survival of the germinated seeds after the EMS treatments (Jadhav *et al*., 2012). Furthermore, the findings of (Warghat *et al*., 2011) revealed that the control population has a better survival rate than mutants

2.3 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 5 showed the mean values comparison of different traits of mutants with their control genotypes.

Figure 5a showed 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 5b 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.

In Figure 5c 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 5d 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 5e 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 5f represented the less value of no. of grains/panicle in both Dawk Pa-yawm and Dawk Kha 50 1.23% mutants while there was no production in 1.86% Dawk Kha 50 mutant at all.

Figure 5g showed the 1000 grain weight of the mutants and control in which the mutants of both genotypes have less 1000 grain weight while the mutant 1.86% did not show any value because of no seed production.

Figure 5h described the drastic decrease in the yield of M_1 mutant population of both genotypes than the control.

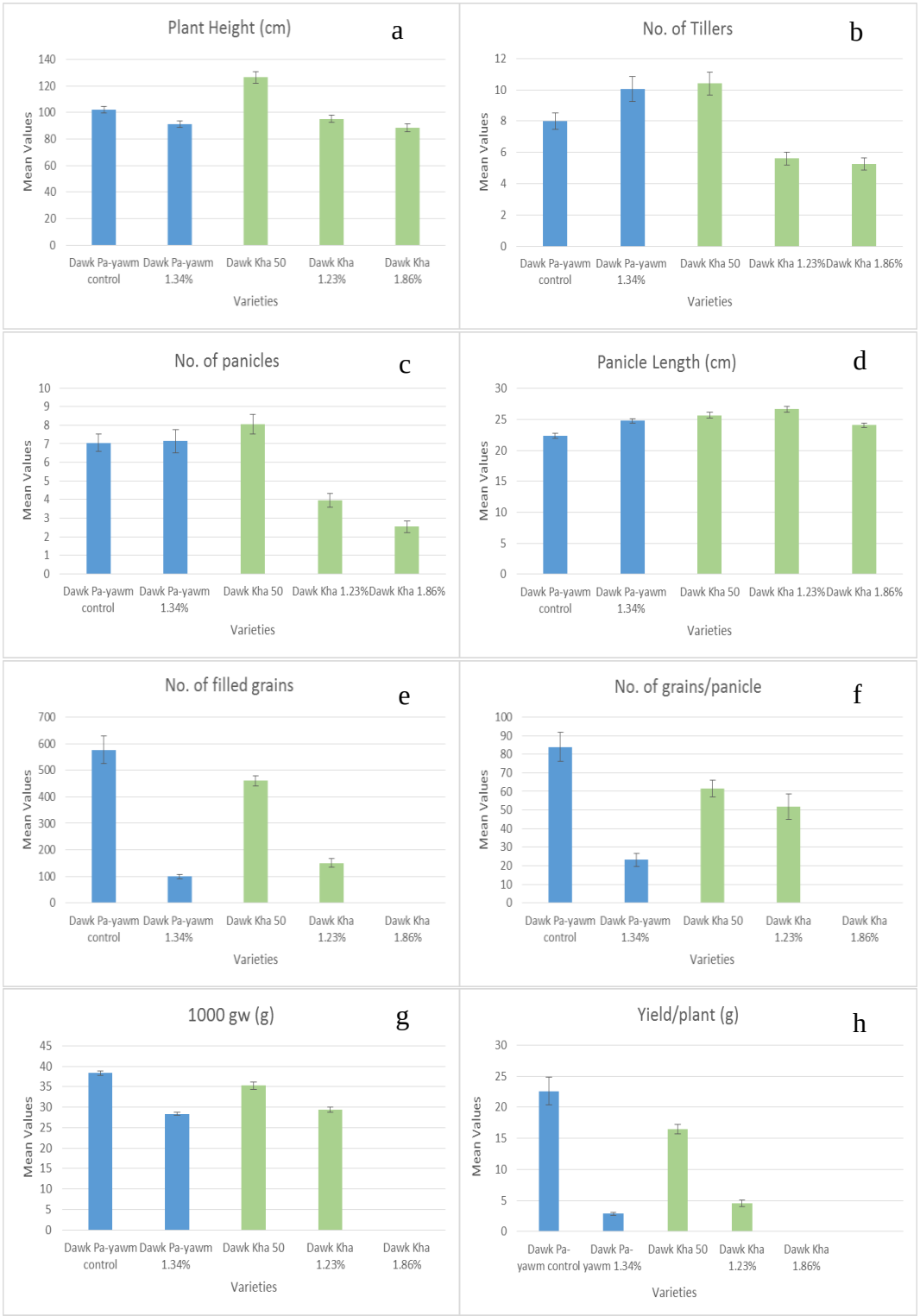

Figure 5 Mean values comparison of the various studied traits in Dawk Pa-yawm and Dawk Kha 50. (Bar = Standard error).

2.4 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 6. 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 in case of Dawk Kha 50. 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). 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 6 Representative variation for various traits in mutagenized population of Dawk Pa-yawm 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 showing curved grains. e) Dawk Kha 50 purple panicle mutant. f) representitve variation in panicle colour of mutants. g) striated chlorophyll mutant of Dawk Pa-yawm h) representitive vaariation in grains of Dawk Kha 50 mutants with control seed on extreme left and mutants at its right.

2.5 Mutants exhibiting a change in root morphology in M_1

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 enormous brace roots as compared to control after 15 days of sowing as represented in Figure 7. 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 mutants, the seedlings showing best morphological differences were selected. The mutant 12 showed up the tap roots only instead of fibrous root, while the mutants 24, 25 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.

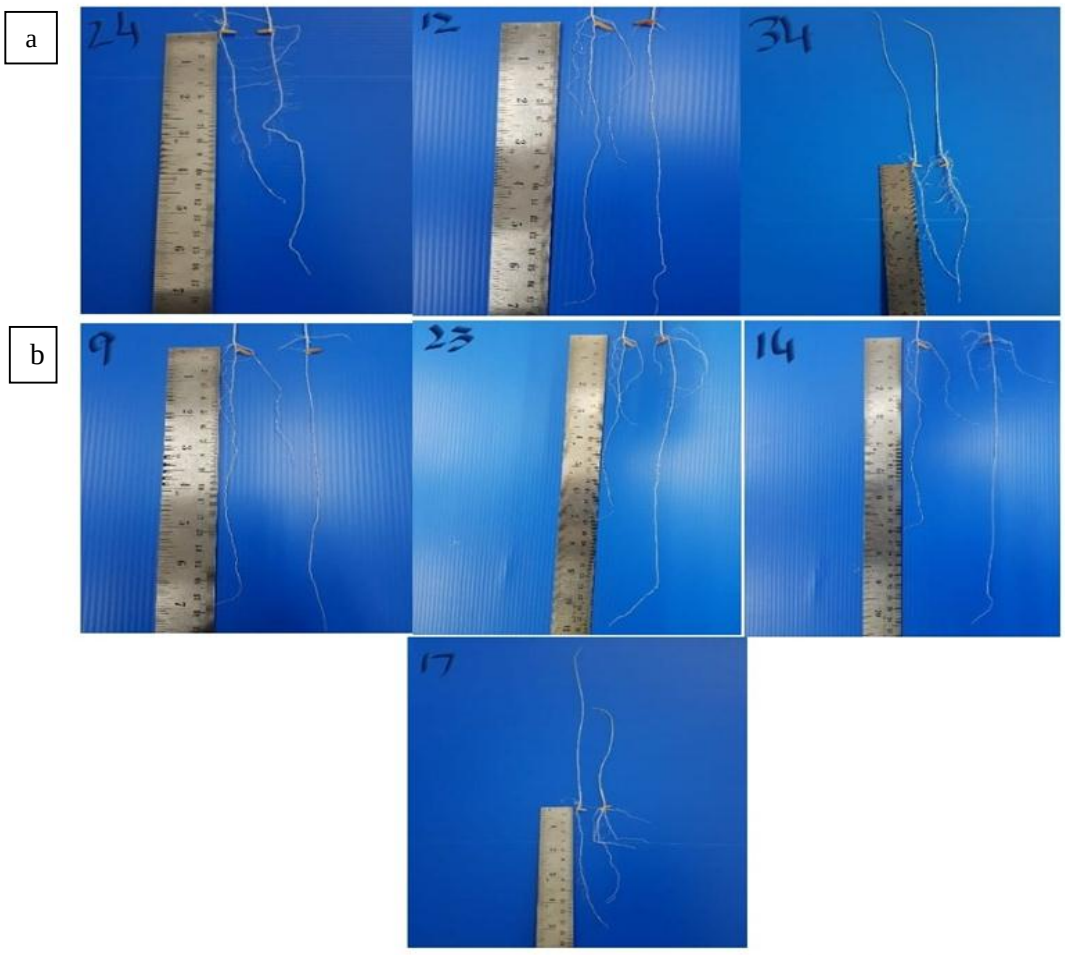

Figure 7 Representative variation for various root structure in mutagenized population of Dawk Pa-yawm and Dawk Kha 50. (Left control – right mutants)

a) 24, 12 and 34 Dawk Pa-yawm mutants; b) 9, 23,14 and 17 Dawk Kha 50 mutants.

3. M_2 generation

3.1 Mean values comparison of different traits in M_2 generation

Figure 8 showed the mean values comparison of different quantitative traits of an average of five best control plants and the top performing five mutants for both Dawk Pa-yawm and Dawk Kha 50 respectively as shown in Tables 6 and 7. Interestingly the deviation in the performances of the mutants has been seen in the certain traits when compared to the M_1 performances, which indicated that the EMS is showing its effect in the later generation. According to the Table 6 there are four

mutants of Dawk Kha 50 which were early maturing than the control population while for Dawk Pa-yawm in Table 7 only 2 mutants which matured earlier than the control population. Other traits has been explained in Figure 8 as follow;

Figure 8a is representing the plant height in M_2 generation where both the mutants of Dawk Pa-yawm and Dawk Kha 50 has improved height than control population while this was not the case in M_1 generation where the mutants exhibited short height as compared to the control.

Figure 8b is about the number of tillers of the M_2 mutants and the control population in which Dawk Pa-yawm mutants has showed the less number of tillers than control population while on the other hand the tillers in Dawk Kha 50 mutants exhibited better performance than control. This result is totally opposite to what M_1 generation showed.

Figure 8c is a representation of the number of panicles in M_2 generation. It is very clear from the results that the panicle performance in mutants of Dawk Kha 50 is better than its control population as compared to Dawk Pa-yawm where mutants were not as good as the control plants. This result is opposite to the result, obtained in M_1 generation for this trait.

Figure 8d represents the values of the panicle length of both mutants and the control. Interestingly mutant performances for both Dawk Pa-yawm and Dawk Kha 50 are better than their corresponding control. Similar results were obtained when M_1 mutants were analyzed for this trait.

Figure 8e is a representation of the number of filled grains for the mutants and control. Surprisingly, the Dawk Kha 50 has showed a very good performance in case of mutants when compared to the control. For Dawk Pa-yawm mutant, the performance was not good than the normal control plants as it showed less number of filled grains. The mutants for both varieties in M_1 had decreased number of filled grains than control population.

Figure 8f is about numbers of grains per panicle where the Dawk Pa-yawm mutants has the value closed to their counter control population but the Dawk Kha 50

mutants were good in this trait as compared to control population and showed a complete edge in their graph as compared to the counter control. In M_1, mutants for both varieties showed less values than control population for this trait.

Figure 8g is about the 1000 grain weight for both control and the mutants. Interestingly the results are very much similar to the M_1 results. In both generations the mutants showed the decreased values than their normal controls.

Figure 8h is a clear representation for the yield per plant for the mutants and control. Surprisingly, the results for the Dawk Pa-yawm mutant is very similar to the previous results of M_1. The mutants for this variety has less value for this trait when compared to the control population. This is not the same case with the Dawk Kha 50 mutants where the performance of the mutants is far better than the normal control population and hence a better representation for the improvement in the yield of Dawk Kha 50.

Table 6 Top five best performing mutants for Dawk Kha 50

Plants	No. of tillers	No. of panicles	Plant Height (cm)	Panicle length (cm)	No. of filled grains	No. of grains/panicle	1000 grain weight (g)	Yield/plant (g)	Days to Maturity
2_1	21	17	121	29.31	1660	97.6	33	54.78	100
36	10	10	151	33.05	1557	155.7	25	38.92	90
43_4	11	6	145	28.18	1306	217.6	28.18	36.8	72
43_1	8	8	142	29.42	1147	143.75	30	34.41	72
43_2	9	7	147	26.7	1454	207.7	19	27.62	72
Dawk Kha 50	10.8	8	133.8	26.65	448.4	56.04	33.6	14.884	104

Table 7 Top five best performing mutants for Dawk Pa-yawm

Plants	No. of tillers	No. of panicles	Plant Height (cm)	Panicle Length (cm)	No. of filled grains	No. of grains/panicle	1000 grain weight (g)	Yield/plant (g)	Days to Maturity
7	7	5	112	24.78	387	77.4	28	10.386	100
11_1	3	3	101	24.6	240	80	27	6.48	85
12_3	8	3	136	24	260	86.67	24	6.24	85
17_3	8	6	145	22.9	167	27.91	33	5.51	100
26	4	2	122	23.9	193	96.5	28.5	5.5	100
Dawk Pa-yawm	8	6.4	105	22.356	509.2	83.126	37.2	19.032	104

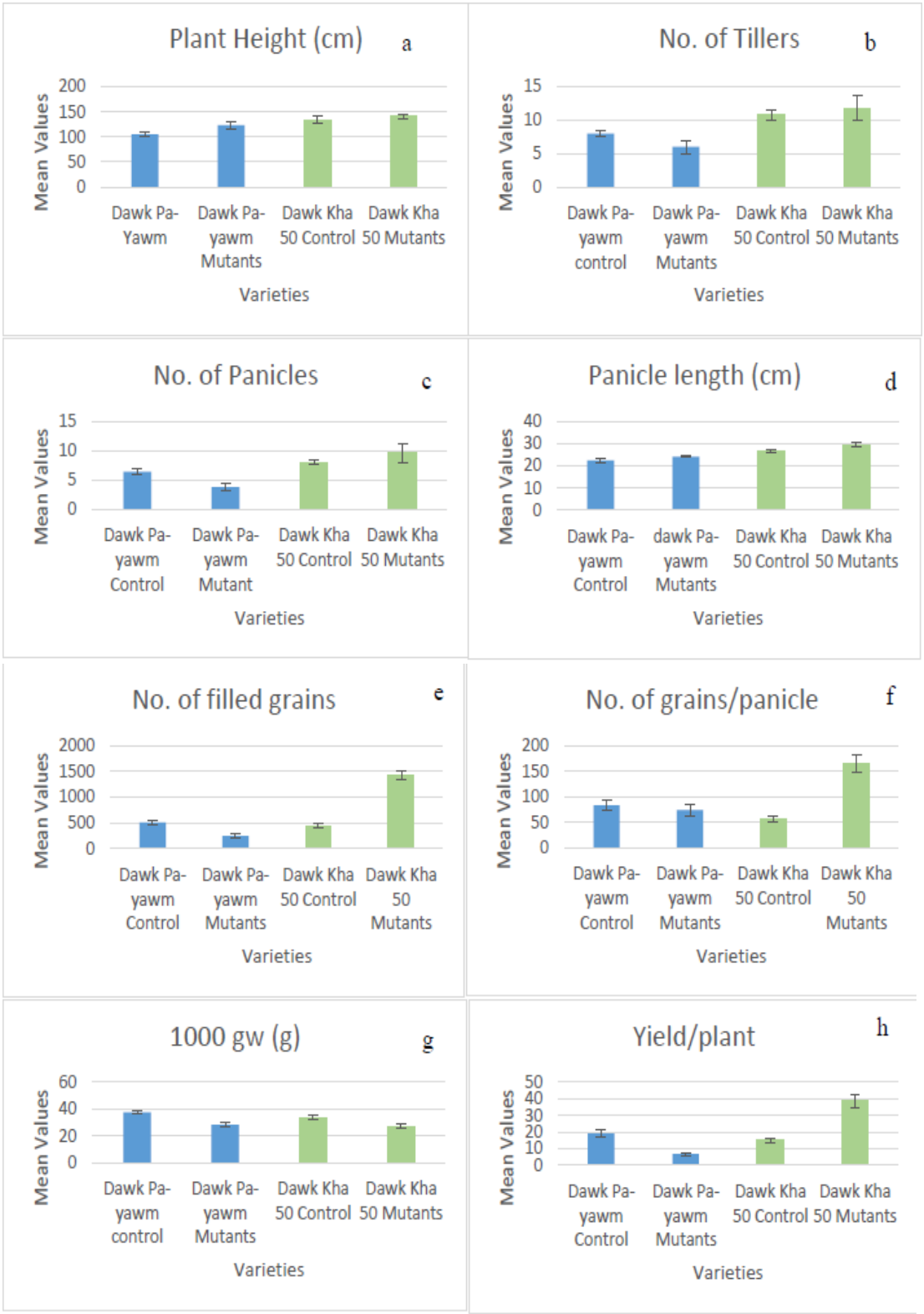

Figure 8 Mean values comparison of various studied traits in Dawk Pa-yawm and Dawk Kha 50 in M_2 (bar = Standard error)

3.2 Identification of the M_2 mutants by the use of SSR

Eleven sets of primers and 8 mutants (4 mutans/variety) were used to search out the seggregation of mutants from their parents apart from examining the morphological characters only. Out of these ten rice primers as shown in Table 2, RM 316 and RM 8225 found to be very accurate to assess the genetic variability and diversity of the respective 8 mutants. The bands shown by the mutant with RM 316 and RM 8225 are shown in the Figure 9.

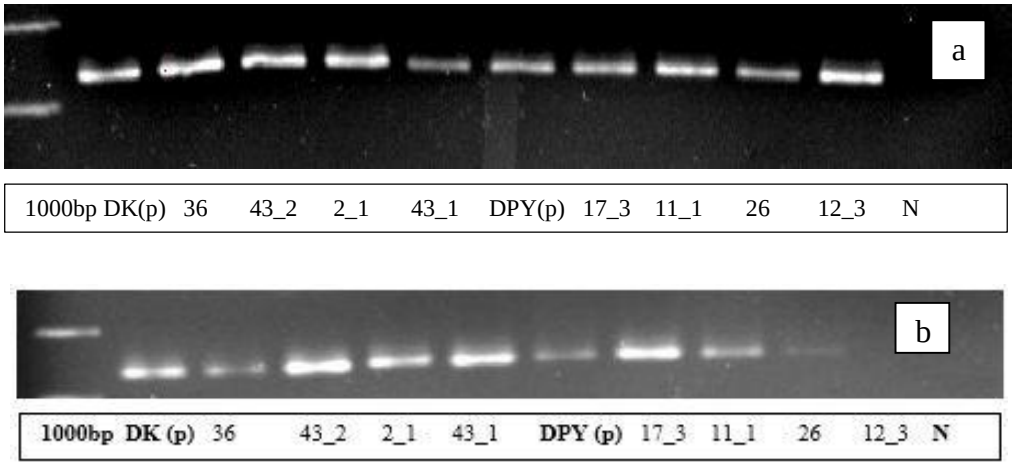

Figure 9 SSR analysis and representation of the mutants and the parents.

(a) RM 316, (b) RM 8225. DK (p) = Dawk Kha 50 Parent; DPY(p) = Dawk Pa-yawm parent

Seetharam *et al*., (2009) observed the genetic diversity of several rice genotypes by morphologoical as well as by SSR analysis. One of the prominent primer was RM 215 which due to its polymorphic ability is a perfect choice for estimating genetic diversity in rice. Also RM 5 was used by Nguyen *et al*. (2012) in their studies on Vitenamese upland rice to estimate the genetic differences amonf various rice genotypes.

CHAPTER 4

CONCLUSION AND SUGGESTIONS

It can be deduced from this research work that EMS significantly affected shoot and root lengths of both Dawk Pa-yawm and Dawk Kha 50. There was a clear decreasing trend in both parameters when compared to a normal control population. It showed us the degree of response, the EMS had on both the varieties. Also with the help of regression analysis we concluded and used the optimum doses of EMS for both the genotypes which resulted in various morphological characters which were absent in the parental genotypes. The effective dose after regression analysis came out to be 1.34% for Dawk Pa-yawm and 1.23% for Dawk Kha 50.

Data analysis of M_1 and M_2 generation revealed certain interesting facts related to increase and decrease values of certain quantitative traits in mutants as compared to the control population. Dawk Kha 50 showed a very positive response in M_2 in which majority of the traits were performing better than the control population and the mutants also yielded better than the control population while some of the mutants were early maturing and for Dawk Pa-yawm, results were not as good as in control population as majority of the mutants showed drastic effects in terms of yield and other quantitative traits.

So it is suggested that the concentration for Dawk Pa-yawm should be change to recheck further deviation of the mutants from the control. Another suggestion is about sowing the M_1 in pots instead of field to maximize the survival of the mutants which in this case were lost during weeding and intense field conditions. At the end it is highly recommended that the mutants of these two genotypes should be advance for the next generations to enhance the genetic variability and ultimately the performance of these genotypes.

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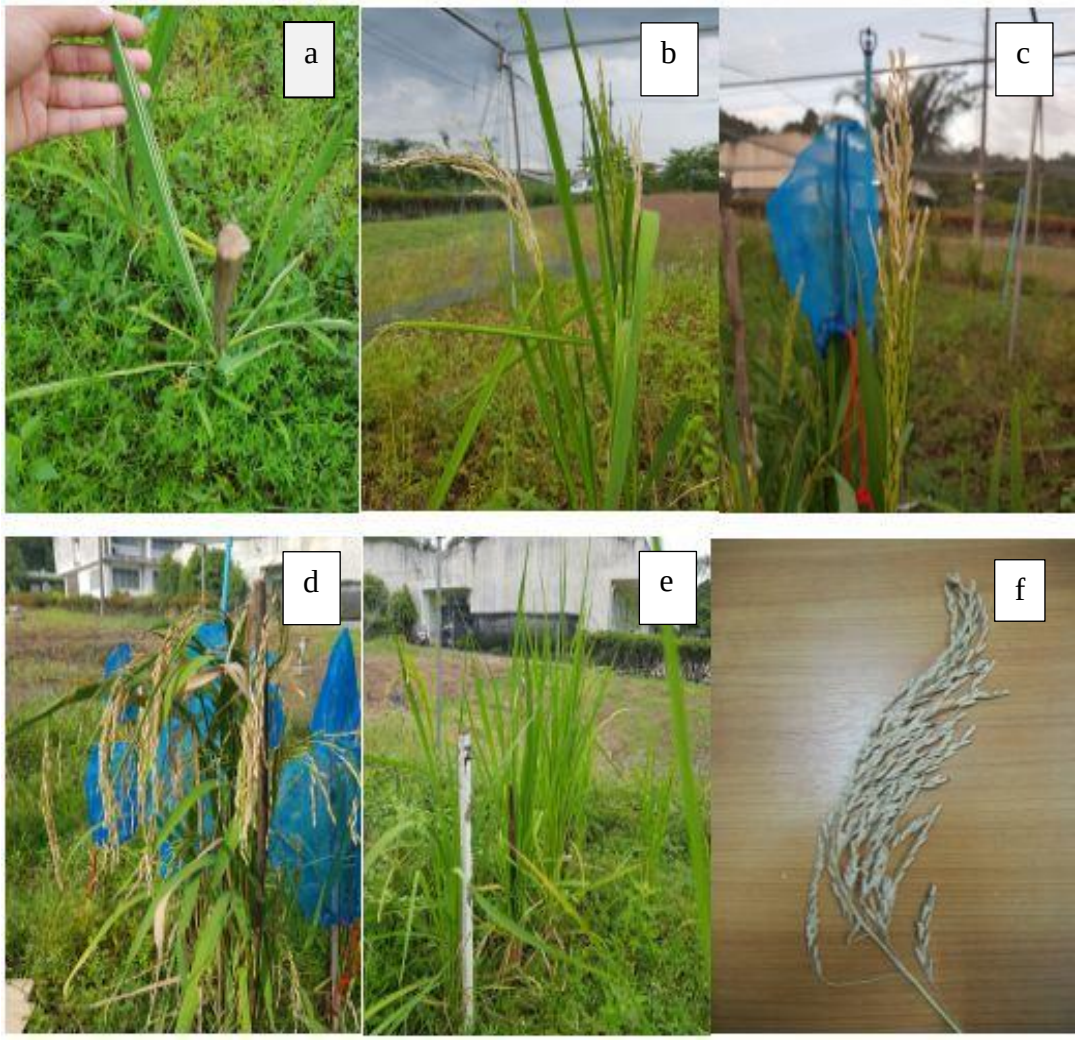
Appendix

List of the Dawk Pa-yawm M_2 mutants

Plants	No. of tillers	No. of panicles	Plant height	Panicle length	No. of filled grains	No.of grains/panicle	1000gw	Yield/plant	Days to Maturity
7	7	5	112	24.78	387	77.4	28	10.386	100
11_1	3	3	101	24.6	240	80	27	6.48	85
12_2	8	4	138	23.67	146	48.6	27.5	4.015	100
12_3	8	3	136	24	260	86.67	24	6.24	85
17_3	8	6	145	22.9	167	27.91	33	5.51	100
18_2	8	3	131	22.93	170	56.66	24	4.08	105
18_3	6	4	142	25.32	140	36.67	33	4.63	105
20_1	7	3	116	25.93	63	21	22.5	1.41	105
20_2	7	5	121	26.34	230	46	23.5	5.4	100
21	5	2	113	26.55	108	54	21.5	2.322	100
26	4	2	122	23.9	193	96.5	28.5	5.5	100
28	9	7	111	24.8	148	21.14	19.5	2.88	100
31	9	3	151	27.76	130	43.3	27	3.51	105
32	6	3	102	23.56	80	26.66	16.5	1.32	105
36	6	3	99	29.6	115	38.33	25.5	2.93	100
37_2	14	9	127	23.87	142	15.7	19	2.67	105
39_1	10	5	132	24.3	100	20	22	2.2	100
39_2	9	6	97	19.8	95	15.83	14.5	1.37	100
40	5	2	129	22.7	57	28.5	28.5	1.62	100
45	12	7	133	23.74	157	22.42	24	3.76	105

List of the Dawk Kha 50 M_2 mutants

Plants	No. of tillers	No. of panicles	Plant height	Panicle length	No. of filled grains	No. of grains/panicle	1000 gw	Yield/plant	Days to Maturity
1_1	3	3	128	30.06	328	109.33	25.5	8.364	100
1_2	15	11	118	25	688	62.54	23.5	9.11	100
2_1	21	17	121	29.31	1660	97.6	33	54.78	100
2_2	14	10	145	29.26	190	19	28.5	5.41	100
3_1	8	4	141	27.27	295	73.75	26.5	7.81	100
4_2	8	4	138	26.06	259	64.75	28.5	7.38	100
8_1	10	4	121	22.1	85	21.25	25	2.12	100
12_2	8	3	131	17.03	98	32.66	29.5	2.89	100
12_2	16	5	128	27.42	86	17.2	25.5	2.19	100
17_1	10	3	133	21.3	103	34.5	31	3.34	100
21_2	7	4	116	22.25	104	26	24	2.49	100
25_1	4	3	132	22.73	110	36.66	30	3.3	100
25_2	10	5	153	25.42	205	41	29	5.94	100
26_2	8	8	148	23.8	86	10.75	28.5	2.45	100
27_1	6	2	131	27.93	72	36	26	1.87	100
29	3	3	65	24	164	54.66	20	3.28	100
32	6	5	68	21.94	317	63.4	21	6.65	100
36	10	10	151	33.05	1557	155.7	25	38.92	90
40_1	4	2	136	25.25	89	44.5	28.5	2.53	100
41_1	8	5	132	30.12	602	120.4	30	18.06	100
41_2	7	4	128	25.07	231	57.75	26	6	100
42_3	5	5	125	24.6	225	45	24	5.4	100
43_1	8	8	142	29.42	1147	143.75	30	34.41	72
43_2	9	7	147	26.7	1454	207.7	19	27.62	72
43_3	8	5	140	27.72	751	150.2	27	20.27	100
43_4	11	6	145	28.18	1306	217.6	28.18	36.8	72
43_5	12	4	147	26.2	360	90	27.5	9.9	100



M_2 variations in mutants of Dawk Kha 50 and Dawk Pa-yawm. (a) Striated Dawk pa-yawm mutant (b) Plant bearing sterile panicles of Dawk Kha 50 (c) Immature sterile panicle of Dawk Pa-yawm (d) High yielding 12_1 Dawk Kha 50 mutant (e) Early maturing Dawk Kha 50 Mutant (f) 17_3 Dawk Pa-yawm mutant's panicle

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)

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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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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.

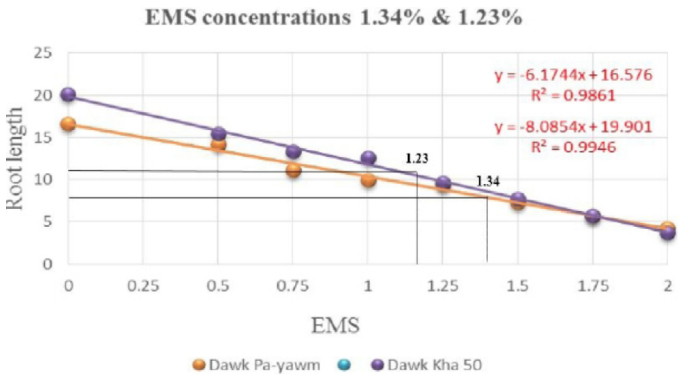

Figure 1: *Effect of various concentration of EMS on root length and decision of LD50.*

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).

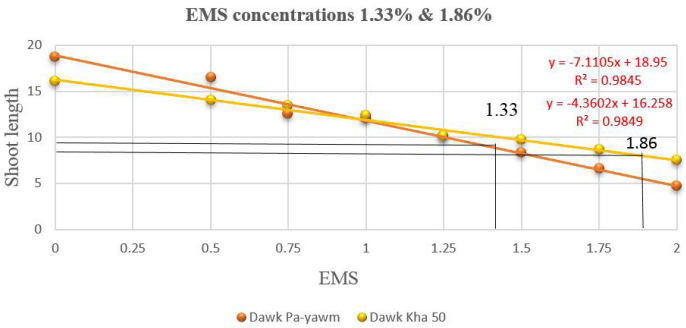

Figure 2: *Effect of various concentration of EMS on shoot length and decision of LD50.*

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).

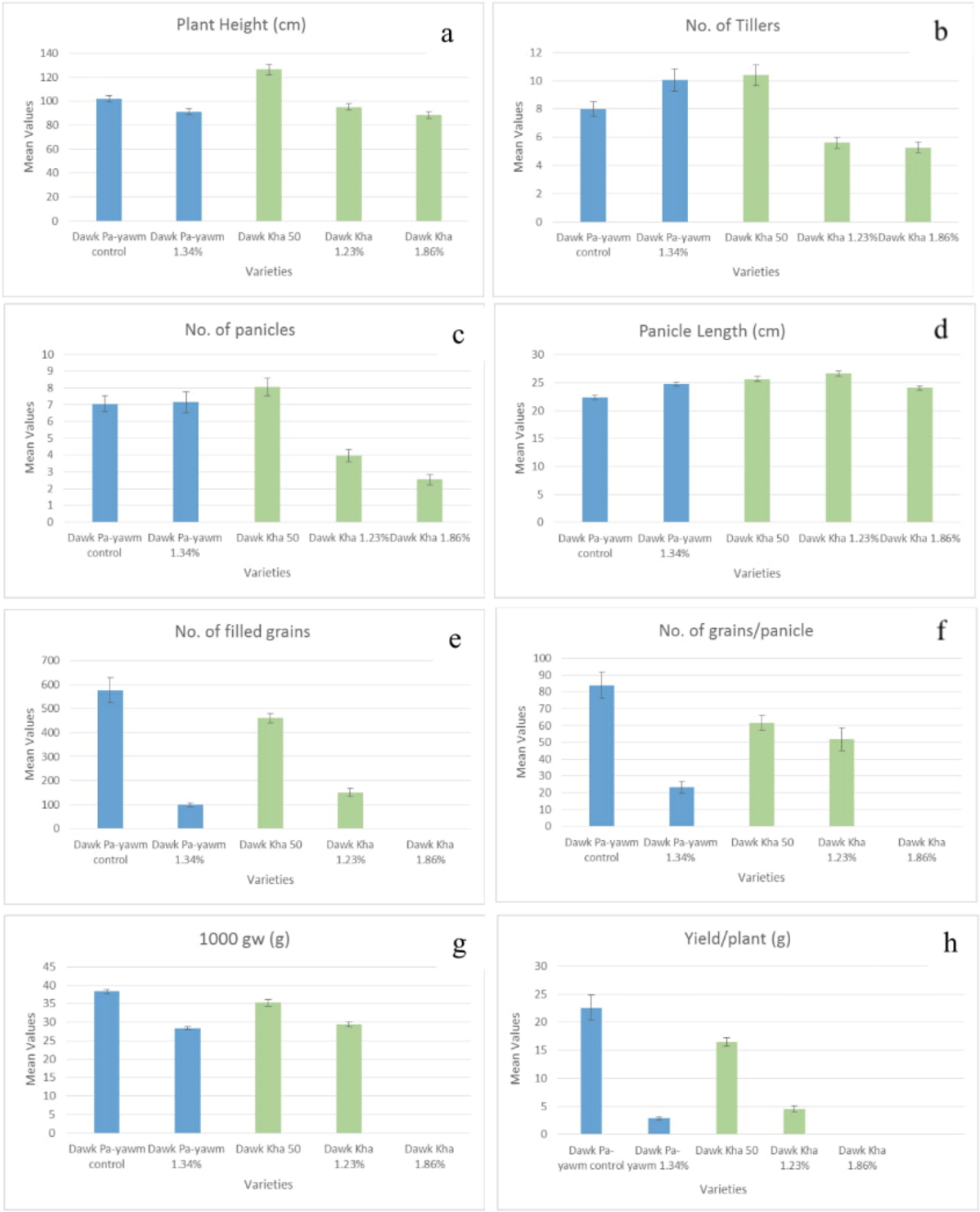

Figure 3: *Mean values comparison of the various studied traits in Dawk Pa- yawm and Dawk kha 50.*

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

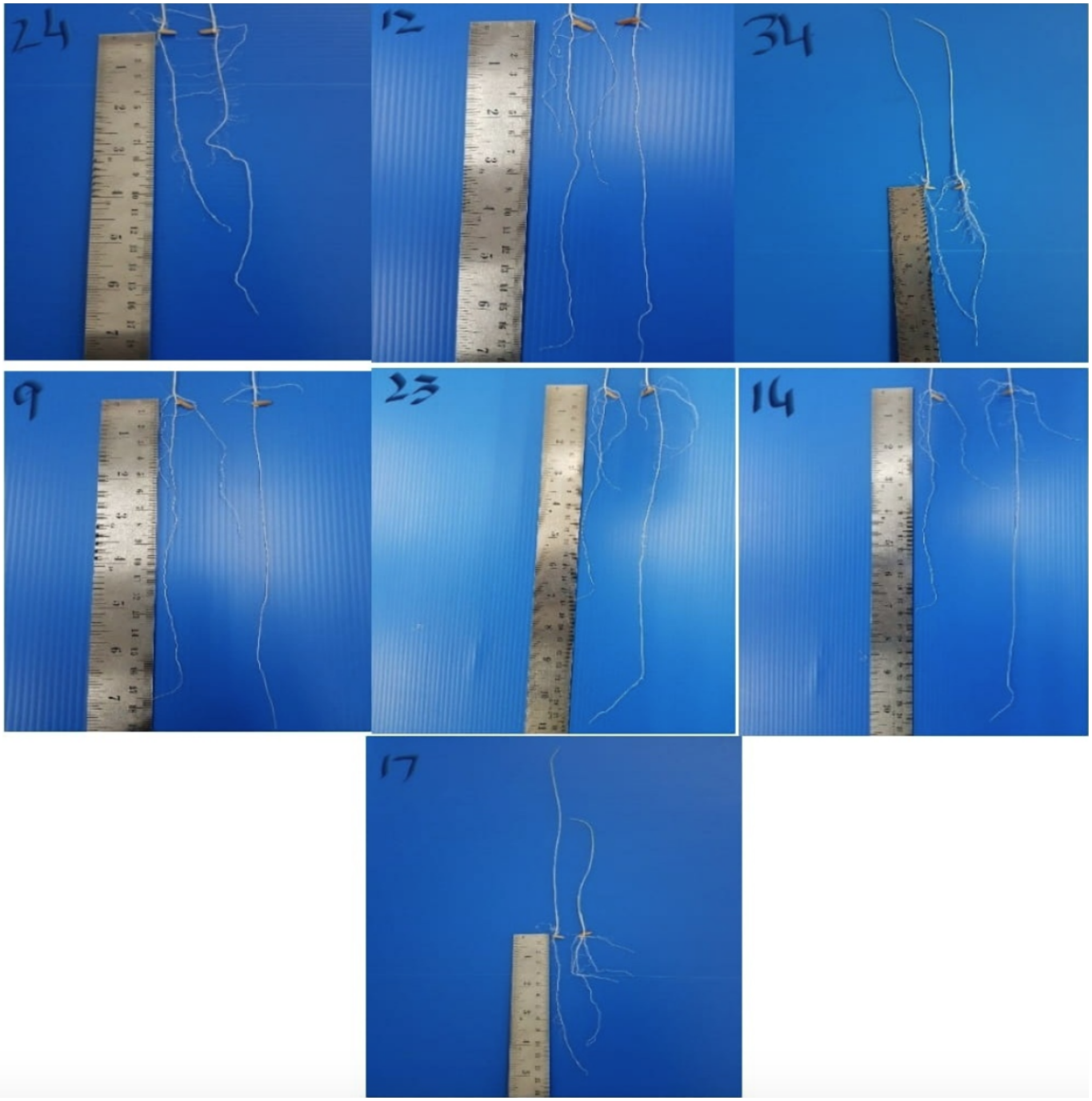

Figure 5: *Representative variation for various root structures in mutagenized population of Dawk Pa-yawn and Dawk Kha 50. (left; Control-Right; Mutants); a): 24, 12, 34 Dawk Pa-yawm mutants; b): 9,23,14,17 Dawk kha 50 mutants.*

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

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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