# Chemical Mutagenesis of Wheat for Herbicide Resistance

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# Declaration

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# Dedications

*This dissertation is devoted to the commemoration of my dearly loved grandparents, Tshinakaho Rosinah Mukwevho and Matodzi Naome Mukwevho, who regretfully did not live to see this work… and for daring us to live our dreams....*

He set another parable before them, saying, "The Kingdom of Heaven is like a man who sowed good seed in his field, but while people slept, his enemy came and sowed darnel weeds also among the wheat, and went away. But when the blade sprang up and brought forth fruit, then the darnel weeds appeared also. The servants of the householder came and said to him, 'Sir, didn't you sow good seed in your field? Where did this darnel come from?'

"He said to them, 'An enemy has done this.'

"The servants asked him, 'Do you want us to go and gather them up?'

"But he said, 'No, lest perhaps while you gather up the darnel weeds, you root up the wheat with them. Let both grow together until the harvest, and in the harvest time I will tell the reapers, "First, gather up the darnel weeds, and bind them in bundles to burn them; but gather the wheat into my barn."'"

– Matthew 13:24-30

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# Dissertation Abstract

Weed infestation is one of the yield limiting factors in crop production. Weeds have negative effect on crop growth and productivity due to competition, allelopathy or hosting other harmful organisms. For large-scale wheat production, the use of wide spectrum pre-emergence or post-emergence herbicides remains the most valuable weed control tool. In South Africa, annual grass weeds are a major wheat production constraint, which is usually managed through application of pre-emergence herbicides. Due to limited water availability and low soil moisture content, these herbicides can often become ineffective and result into high weed infestations, which then have to be managed by manual cultivation or post-emergence herbicidal applications. However, there are no effective selective post-emergence herbicides available to control grass weeds in wheat. There is also limited option to use broad-spectrum post-emergent herbicides because they non-selectively kill the crop and weeds. Consequently, the use of herbicide resistant crops is a viable weed management system in wheat production. Breeding herbicide resistant crop varieties would allow farmers to safely use post-emergence herbicides without damaging the crop. Subsequently yield and quality losses will be reduced significantly. Thus, the development of herbicide resistant crop varieties through mutation breeding is a novel approach for effective weed management under both small-scale and commercial farmers.

Mutagenesis has been recognized as one of the most efficient method to induce genetic variation in plants. Through induced mutations, development of new variants is possible that could be manipulated in plant breeding programs. Mutation leads to alteration of various traits in crop plants including plant height, improved nutritional quality, shorter growing period, increased tolerance or resistance to abiotic and biotic stresses. Ethylmethanesulphonate (EMS) is one of the most widely used chemical mutagens to induce mutagenesis in crop plants.

The objectives of this study were to: 1) determine the optimum EMS concentration, treatment temperature and duration that would provide desired germination percentage and vigorous and healthy seedlings for effective mutagenesis in wheat, 2) investigate variations in agro-morphological traits in two selected wheat varieties (SST56 and SST875) after EMS mutagenesis and 3) select herbicide resistant wheat germplasm after inducing genetic variation using EMS using two selected wheat varieties (SST56 and SST875). The objectives were achieved through three independent studies as outlined below:

In the first study seeds of four selected wheat varieties (B936, B966, SST387 and SST875) were treated in two replicates with three EMS concentrations (0.3, 0.5, and 0.7%), three temperature regimes (30, 32.5 and 35 °C) at four time durations (0.5, 1, 1.5 and 2 hrs). Results showed highly significant interactions ($P<0.01$) among varieties, EMS concentrations, temperature and exposure time on seedling emergence, germination and seedling height. Seeds treated with the highest EMS dose (0.7%), temperature (35ºC) and long exposure time (2 hr) showed delayed emergence by 18 days. At 30ºC, 0.5hr and 0.3% EMS varieties B936, B966 and SST875 had early emergence (6 days). B936 and SST387 had 50% while B966 and SST875 had 53% and 57% germination, respectively. These results were observed at EMS level of 0.7%, $30^0$C and 1.5 hr exposure time in B936 and EMS at 0.5%, $35^0$C and 1.5 hr in B966. SST387 and SST875 required EMS dose at 0.5%, $32.5^0$C and 2 hr treatment time. Other low or high treatment combinations were invariably ineffective comparedto untreated control.

During the second study two selected varieties (SST56 and SST875) were subjected to EMS mutagenesis using 0.5% v/v EMS at $32.5^o$C for 1 hr. Field trials were carried out at Ukulinga research farm of the University of KwaZulu-Natal in the randomized complete block design with two replicates. Data on nine important agro-morphological traits were collected and analyzed using the analysis of variance (ANOVA), correlation and principal component analysis (PCA) procedures. Significant variations were found among the agro-morphological traits between $M_1$ individuals compared to untreated checks. The mutagenesis significantly reduced seed germination in the field at 40% in both varieties. The treatment significantly delayed days to heading by 8 days and shortened days to maturity by 13 days in both varieties. EMS treatment also significantly reduced plant height at 18 cm in SST56 and 21 cm in SST875 and spike length reduced by ~2.5 cm in both varieties. Plant height had positive and significant correlation with number of tillers, number of seeds per spike, flag leaf length and 100 seed weight. However, it had negative correlation with the number of days to maturity. The PCA revealed that three principal components (PC1, PC2 and PC3) accounted to 57% of the total variations among the agro-morphological traits in both varieties. PC1 alone contributed to 27.7% of the variation which was well-correlated with plant height (0.767), tiller number (0.812), number of seeds per spike (0.599) and seed yield (0.720). PC2 explained 15.6% of the variation and well-correlated with germination percentage (0.784), spike length (0.554) and flag leaf length (0.772). PC3 accounted to 12.4% of the variation and had negative correlation with days to maturity (-0.730).

In the last study, seeds of two selected wheat varieties (SST56 and SST875) were treated with EMS at 0.5% concentration for 2 hr at 32.5ºC. Treated seeds and comparative controls were planted at the experimental farm of the University of KwaZulu-Natal using the randomized complete block design. Four weeks after planting M1 plants and untreated standard checks were sprayed with two herbicides, i.e. metsulfuron-methyl and bromoxynil at three different doses *viz.* 2x, 4x and 8x above the recommended rate of 4 g $ha^{-1}$ and 2 kg $ha^{-1}$, respectively. Two weeks after the treatment herbicide resistance were assessed. Results showed significant difference among varieties, tested herbicides and doses used. The EMS treated wheat lines showed variable degree of herbicide resistance compared to untreated controls.

Overall, the study established the requirement of variety specific EMS dose and treatment temperature and duration that could be used for inducing large-scale mutation to select targeted mutant individuals in wheat. Further, the study found that EMS has the potential to increase agro-morphological variations in wheat to select useful and novel mutants with desired phenotypic traits and herbicide resistance which will be subjected for further selections to identify stable and herbicide resistance lines.

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# ABBREVIATIONS

ANOVA = Analysis of variance

BROMO = Bromoxynil

CEF = Controlled Environment Facility

DMSO = Dimethyl sulfoxide

DTE = Days to 50% emergence

DTM = Days to maturity

EMS = Ethylmethanesulphonate

FAO = Food and Agriculture Organization

FLL = Flag leaf length

GMO = Genetically modified organisms

GPT = Germination percentage

HD = Heading date

HR = Herbicide rating

HRS = Hard red spring

HRW = Hard red winter

HW = Hard winter

MMS = Methylmethanesulphonate

MSM = Metsulfuron methyl

PCA = Principal component analysis

PH = Plant Height

SH = Seedling height

SL = Spike length

SPS = Number of seeds per spikelet

SRW = Smooth red winter

SW = 100 seeds weight

SY = Seed yield

TN = Tiller number

# GENERAL INTRODUCTION

## *Problem statement*

Wheat is an important cereal crop which belongs to the family Poaceae. It is one of the major food security and commodity crops in the world. The two important wheat species, i.e., the common or bread wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) and durum wheat (*Triticum turgidum* L., 2n=4x=28, AABB) are widely grown, consumed and traded in the world (FAO, 2010). In South Africa the hexaploid common wheat ranks number one among the cereal crops and plays an important economic role. In the country wheat and other staple food crops face continued yield and quality losses as a result of recurrent drought, weeds, diseases and pests. Further, global warming is increasingly threatening crop production. Mitigation strategies are required to enhance the genetic potential of wheat and improve its yield potential and quality. Among the biotic constraints, weed infestation negatively influence crop yield. In the country, the annual ryegrass (*Lolium rigidum*) and other grassy weeds limit yield potential of wheat due to their competition, allelopathy and hosting various pests and pathogens. Chemical weed control is efficient in many crop production systems throughout the world (Mulwa and Mwanza, 2006). It was estimated that by the year 2020, world demand for wheat is expected to be 40% higher than currently required (Rosegrant, 1997). Therefore with the ever increasing demand, world population growth and dwindling agricultural land, there is a dire need to improve the productivity and cropping technologies of wheat.

## *Research background*

Induced mutagenesis is an important tool in plant breeding and functional genomics for increasing the frequency of mutations, and therefore, broadening the genetic base of germplasm. Mutagenesis causes small but stable genetic changes within individuals. Typically, these changes involve the mutation of one or few genes through spontaneous substitution of one nucleotide for another within the genome (Weil and Monde, 2007). Artificial mutation helps to induce genetic variation of gene loci controlling economically important traits and/or elimination of undesirable genes from breeding lines (Alcantara *et al.*, 1996). Thus, the technique can be regarded as an efficient option to select novel plants with herbicide resistance in various crops (van Harten, 1998; Pozniak and Hucl, 2004). Mutations can be induced in various ways, such as exposure of plant propagules, including seeds, tissues and organs, to

physical or chemical mutagens (FAO, 2010). Physical mutagens are mostly electromagnetic radiations such as gamma rays, X-rays, UV light and particle radiation, including fast and thermal neutrons as well as beta and alpha particles.

One of the ways in which genetic variation can be enhanced and herbicide resistance developed is through chemical mutagenesis with ethylmethanesulfonate (EMS) (van Harten, 1998; Kodym and Afza, 2003). Chemical mutagenesis using EMS is a powerful tool in inducing random useful genetic mutations in crop plants (Adamu and Aliyu, 2007). For example in sorghum EMS has been successfully used to induce mutation which eventually led to the development of a protocol for herbicide resistance in this crop (Ndung'u, 2009). Mutants showing desirable traits can be developed directly as new cultivars or the novel genes introgressed into candidate varieties.

Optimization of treatment combinations and ideal treatment conditions is necessary for a specific genotype prior to large-scale application of mutagens to develop novel germplasm. Chemical mutagens e.g. ethylmethanesulfonate (EMS), ethidium bromide, and base analogues such as bromouracil are incorporated into DNA during replication in place of the normal bases (Mba *et al.,* 2007). Higher mutagenic doses can produce very drastic effects that may lead to death of the organism. Overall, the effectiveness of EMS treatment in inducing genetic variations in crop plants depend on the genetic constitution of test varieties, concentration, treatment temperature and treatment duration (van Harten, 1998; Mba *et al.,* 2007). Thus, the most favourable treatment combinations should be established for effective mutagenesis and to carry out targeted selection in a mutation breeding program. The term lower and higher is relative and may be different for each crop genotypes. Seedling emergence, 50% survival rate ($LD_{50}$), growth and chromosomal aberration are among the commonly used parameters to select ideal treatment doses in plants (Shah *et al.,* 2008).

Through mutation breeding novel crop plants with herbicide resistance can be developed, i.e., the mutation treatment alters crop response to herbicides, rendering the application of the herbicide ineffective or nontoxic to such target plants. Consequently, the applied herbicide will fail to bind or interact in a key physiological process in plants, such as the acetolactate synthesis

(ALS) pathway. The herbicide becomes selectively active against other plants, i.e., weeds, and not against the mutant cultivar (Aliyu and Adamu, 2007).

Chemical mutagenesis has been used successfully in several crops. Developed mutants are used in crop breeding programs to improve various agro-morphologically important traits (van Harten, 1998). Conventional mutations techniques such as chemical mutagenesis using EMS have been applied successfully to select mutants with improved yield, quality and disease and pest resistance in crops. Many mutant varieties involving more than 100 plant species have been officially released. Especially, in some economically important crops (barley, wheat, and cotton) mutant varieties occupy the majority of cultivated areas (Ahloowalia *et al.,* 2004). Herbicides generally function by disrupting unique and essential processes in plants. Both crops and weeds share these processes. Consequently, breeding crops with herbicide resistance genes through chemical mutagenesis will be more significant in controlling noxious weeds in wheat production. The development of wheat mutants with tolerance to effective bromoxynil and metsulfuron methyl herbicides is one of the important breeding goals in wheat.

## *Research objectives*

This study was conducted with the main objective of carrying out chemical mutagenesis using ethylmethanesulfonate (EMS) to induce beneficial mutations in wheat (*Triticum aestivum* L.) and to develop mutants that could be further screened for herbicide resistance. The specific objectives of this study were to: 1) determine the optimum EMS concentration, treatment temperature and duration that would provide desired germination percentage and vigorous and healthy seedlings for effective mutagenesis in wheat, 2) investigate variations in agro-morphological traits in two selected wheat varieties (SST56 and SST875) after EMS mutagenesis and 3) select herbicide resistant wheat germplasm after inducing genetic variation using EMS in two selected wheat varieties (SST56 and SST875).

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction to wheat and mutation breeding

Wheat (*Triticum aestivum* L.) is one of the most widely adapted cereal crops, supplying one-third of the world population with more than half of the calories and nearly half of the required protein. Wheat is mainly grown as a rainfed crop. About 37% of the area in developing countries consists of semiarid environments in which available moisture constitutes a primary constraint on wheat production. By 2020, world demand for wheat is expected to be 40% higher than its current level (Rosegrant, 1997).

Wheat belongs to the family Poaceae (grass family). It is an important commodity crop in the world grain market. It is one of the first grain crops domesticated by humans. Its cultivation began in the Neolithic period. Bread wheat is known to have been grown in the Nile valley by 5000 B.C. and its cultivation spread to other regions including the Indus and Euphrates valleys by 4000 B.C. Since agriculture began, wheat has been one of the chief sources of bread for sub-Saharan Africa and the Middle East (Persley, 1992).

By means of inducing genetic variations, artificial mutations have been used successfully in several crops such as in wheat, barley, rice, cotton, cowpea and sorghumin the breeding of agronomically important traits (Ahloowalia *et al.*, 2001b). Mutation breeding techniques are mostly used to improve yield, quality, and disease and pest resistance in crops. Many mutant varieties involving more than 100 plant species have been officially released. Especially in some economically important crops (barley, wheat, and cotton) mutant varieties occupy the majority of the cultivated lands globally (Chopra, 2005). The use of accurate selection techniques determine the success of induced variation and selection oftraits of interest in classical breeding.

One way to induce mutation is through the use of chemical mutagens. Ethylmathanesuplhonate (EMS) is one of the most effective chemical mutagen, especially recommended to be used in seed materials, since the application and the monitoring of the outcome of mutations are

relatively easy. In plants, EMS usually causes point mutations but loss of a chromosome segment or deletion can also occur to a lesser extent (Chrispeels and Sadava, 2003). Therefore, EMS has the potential of altering few gene loci of specific interest without inducing large genetic changes detrimental to the organism. This creates an advantage for plant breeders to select novel genes than using exotic or wild germplasm in which a group of linked and unfavorable genes may be present in addition to the genes of interest. The most important parameters for inducing mutation with EMS are concentration, duration of treatment, and solution temperature (Jabeen and Mirza, 2002).

Natural mutations arise due to errors in DNA replication. The error rate during DNA replication is enhanced by artificial means such as seed treatment with mutagens. Thus chemical mutagenesis is an ideal tool for increasing the variation in a plant population. Artificially induced mutant variants are currently accepted by consumers compared to genetically modified plants developed through transformation (Koornneef, 2002). Over 2,250 crop varieties are thus far derived through mutation breeding. The herbicide-resistant wheat variety 'Clearfield' is a well-known example resulted from mutation breeding (Newhouse *et al.,* 1992).

### *1.1.1 Production of wheat in South Africa*

Wheat after maize is the most important staple food crop in South Africa. It is among the common winter cereals in the country along with barley and oats. South Africa is said to be one of the major exporters of wheat owing to the large production (Lynam, 1995; FAO, 2010). The main areas of South Africa which are engaged in wheat production include the Crocodile, Vaal and The Hart rivers. A number of irrigation schemes are available in the country for wheat production. Most wheat production in South Africa comes from the North West Province with a total of 40,000-50,000 hectares of land being cultivated. This is followed by other wheat producing areas such as the Free State, Northern Cape, and Mpumalanga Provinces. The availability of rainfall and soil moisture is the most important factor that determines wheat production in the country and globally (FAO, 2010). Over the past years wheat production is reportedly declined in South Africa due to multiple factors including drought, losses from wheat rusts, weed infestation and low grain prices, among others (Rusike and Dumes, 2006).

### *1.1.2 General uses of wheat*

Various classes of wheat are devised for different purposes since the crop has several industrial uses. The greatest portion of the wheat grain is processed into flour for bread making. Hard wheat types are best suited for this purpose. The wheat flour from soft wheat types, grown in most humid areas is suitable for cakes, crackers, cookies, pastries, and household flours. Unlike the bread wheat flour from durum wheat is used to make pastas, macaroni and couscous. Although most wheat is grown for human food, the wheat industry uses small quantities to produce starch, paste, malt, dextrose, gluten, alcohol, and other products. Inferior and surplus wheats and various milling byproducts are used for livestock feeds (Goesaert *et al.,* 2005).

Wheat products are highly nutritious, concentrated, easily stored and transported. Unlike any other plant derived food, wheat contains gluten protein, which enables leavened dough to rise by forming minute gas cells that hold carbon dioxide during fermentation. This process produces light textured bread. Wheat supplies about 20 percent of the food calories globally. In Eastern Europe and Russia, over 30 percent of the calories consumed come from wheat. The per capita consumption of wheat in the United States exceeds that of any other single food staple. Besides being a high carbohydrate food, wheat contains valuable protein, minerals, and vitamins. Wheat protein, when balanced by other foods that supply certain amino acids such as lysine, is an efficient source of protein. Extensive crop breeding efforts have created modern cultivars that are less susceptible to sprouting than those available in the past (James, 1997).

In summary, wheat is the major ingredient in most breads, rolls, crackers, cookies, biscuits, cakes, doughnuts, muffins, pancakes, waffles, noodles, pie crusts, ice cream cones, macaroni, spaghetti, puddings, pizza, and many prepared hot and cold breakfast foods. It is also used in baby foods, and is a common thickener in soups, gravies, and sauces. Germ, bran, and malt are additional types of wheat products. Much of the wheat used for livestock and poultry feed is a by product of the flour milling industry. Wheat straw is used for livestock bedding. The green forage may be grazed by livestock or used as hay or silage. In many areas of the southern Great Plains, wheat serves a dual purpose by being grazed in the fall and early spring and then harvested as a grain crop. Industrial uses of wheat grain include starch for paste, alcohol, oil,

and gluten. The straw may be used for newsprint, paperboard, and other products (Heyne *et al.*, 1964).

### *1.1.3 Germination and growth of wheat*

Seed germination is the process that initiates seedling growth. Like seeds of other cereals, wheat seeds are dormant and must first be subjected to the appropriate environmental conditions (temperature and moisture) to activate hormones within the germ and initiate growth. These hormones, in turn, regulate the production and release of the enzymes governing the metabolic processes involved in growth (James, 1997). Although germination is the required first step in producing a new wheat crop, germination at inappropriate times results in problems for end users of flour. Wet conditions during a harvest can cause mature seeds to germinate in the field. During germination, enzyme activity, most notably that of α-amylase, increases rapidly. If wheat is harvested after it has germinated, it is called sprouted wheat, and the flour made from it often creates significant problems in product quality. After germination wheat growth is generally divided into four broad stages: tillering, stem extension, heading, and ripening. Tillering involves the production of individual wheat plants. Each plant sends out shoots and creates new plants (i.e., tillers). The stem extension phase is further divided into the jointing and boot sub stages. Joints (i.e., nodes) in the stem become clearly visible as the stem elongates during jointing. Most wheat grown today is semi dwarf wheat that grows to a height of about 61 cm at maturity. Semi dwarf wheat was bred to increase harvest index. After the second and final joint appears, the developing wheat head swells in the stem, creating what is called the boot. The wheat head emerges from the boot to initiate the heading phase. Contained within each wheat head are multiple stamens and pistils (Kumar and Dubey, 1998). Flowering occurs during this stage, when the stamens pollinate the pistils, initiating the development of numerous individual wheat seeds (i.e., kernels) within each head through self-fertilization. Finally, during the ripening stage, the seeds fill, becoming harder and drier. The seeds are mature but not storage stable before they are dry. At the end of the ripening stage, the wheat is ready to harvest. The time from germination to harvest varies based on the variety of wheat and growing conditions, but it is generally about three to five months for spring wheat. It is longer for winter wheat because of its dormancy during the winter months (Carter, 1988).

### *1.1.4 Constraints in wheat production*

Among the various factors limiting wheat yield, weed infestation is one of the foremost and most serious production constraints in the world. Weeds infestation inwheat fieldscan be a serious problem in growth since it creates competition between weeds and wheat for light, water, space and minerals and they are among the major limitations on crop yield. Weeds may also inhibit wheat growth through release of allelopathic chemicals that are toxic to wheat plants. Weeds or weed seeds contaminating harvested grain may reduce quality and health of the wheat plant. The FAO now contends that food security in Africa faces risk not just from floods, droughts, insect pests and diseases, but also from weeds. FAO estimates of food production losses due to weeds are approximated USD 95 billion/year of which more than 70% is lost in poor countries (FAO, 2010). Weed problems become prominent when the 'weed triangle' (Figure 1) described by Schonbeck and McCann (2007) is complete.

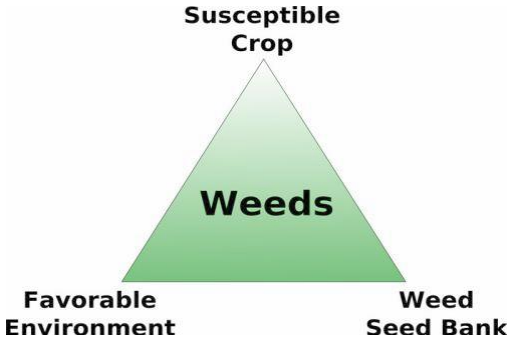


**Figure 1.1.** The weed triangle: (adapted from Schonbeck and McCann, 2007).

Based on this theory of weed triangle, weed problems become evident when the three conditions in the weed triangle are met: first, there is a weed seed bank which include both seeds and vegetative propagules in the environment where the crop is growing particularly in the presence of the soil or water; second, when the environment is favorable for weed growth as a suitable territory; and lastly when a crop which is susceptible to the effects of weed competition is grown in the area.

Wheat production involves planting, fertilizing, controlling weeds and pests, irrigation, and harvesting. Problems arising during the production of wheat can reduce yield and affect characteristics of the flour that are important to the end user. Weather, insect pests, molds, and weeds can all affect the quality and quantity of wheat produced, and the flour produced from such wheat can vary dramatically as a result. A prime example is the well-established relationship that exists between the yield and protein content of the wheat kernel. As more wheat is produced on the same area of land, the protein content of the kernel drops because of the limited available nitrogen in the soil and the number of plants requiring it for growth. Therefore, conditions that affect yield can also affect protein content. Extensive crop breeding efforts have created modern cultivars that are less susceptible to sprouting than those available in the past (Heyne *et al.*, 1964).

When temperatures drop below freezing at critical growth stages (e.g., flowering), yields can be compromised, similarly, low moisture growing conditions often reduce yield. Either or both of these conditions can drastically reduce yield and lead to wheat with a high protein content. Because protein content can have a significant effect on processing or final-product quality, the resultant flour may process poorly or may not produce an optimal product. Conversely, too much moisture during the growing season results in very high yields and depresses protein levels, leading to other, but equally serious, processing and product quality problems. Wet conditions can cause other problems, of which sprouting is the best known. When wheat seed germinates, the activity of many enzymes rises in the germ and aleurone. Some of these enzymes, if present in the flour, can cause problems, such as excessive browning or sticky textures in bread products (Okagaki *et al.*, 1991).

Wet conditions also promote the growth of molds (i.e., fungi). Molds can hinder the development of wheat kernels and in some cases e.g., infection by *Fusarium* produce toxins (e.g. vomitoxin) that render the wheat unacceptable for animal or human consumption. Various insect pests (e.g., weevils, moths, borers, and mites) attack portions of the wheat plant and reduce its ability to develop sound wheat kernels (Loomis, 1974). These pests can also affect wheat in storage by diminishing its inherent quality or by requiring additional processing to remove contamination. Finally, weeds growing in wheat fields compete for nutrients and affect yield. In addition, weed seeds are often carried along when the wheat is harvested and must be

removed before milling. Perhaps the most pressing problems for end users of flour are caused by variation within a wheat crop caused by environmental factors. Rainfall, farming practices, and soil conditions usually vary significantly over any region where wheat is grown, and wheat quality over an entire region may be affected by specific changes in the local environment (Carter, 1988). It is important that the end user understand that flour is a complex biological product and is subject to genetic and environmental influences.

## 1.2 AGRONOMIC REQUIREMENTS OF WHEAT

### *1.2.1 Soil, water and climate requirement*

Wheat grows in a variety of climates and soil. Suitable weather and proper soil are needed to produce a healthy wheat crop. Wheat grows well in a variety of soils; however, loamy to sandy loam is regarded as ideal for optimum growth. Wheat is adversely affected by acidic soils, which are associated with high aluminium ($Al^{3+}$) content, particularly during the early developmental stages of the crop. The soil pH required is 6,0 to 7,5 (Anonymous, 2009).

The water requirement for wheat is about 600 mm per annum. In dry areas where cultivation practices such as zero tillage and minimum tillage are practiced, stubble mulching is recommended for moisture conservation. Frost can damage wheat especially after the formation of ears in spring resulting in low yield. Hail can also result in serious damage on the summer wheat resulting in low yield. Wet weather during harvesting contributes to disease prevalence and quality deterioration of grains. The moisture application under irrigation should be lowered during flowering, increased during pod filling and cease during ripening (Collingwood *et al.*, 1989).

Wheat is adapted to cool environmental conditions. However, it requires different temperatures at different stages of plant growth and development. Temperature requirements may slightly differ from one variety to another at the time of germination, however, general minimum temperature is required from 3.5-5.5°C and optimum 20-25°C and maximum temperature is 35°C. On temperature below or above to optimum, germination of seed decreases slowly. If temperature is more than 30°C at the time of maturity it leads to forced maturity and yield loss.

Winter wheat bears cold waves and frost in a better way in comparison to spring wheat (Hussain and Mudasser, 2004).

# 1.3 CULTURAL PRACTICES IN WHEAT PRODUCTION

## *1.3.1 Soil preparation and planting*

Minimum tillage (75 to 130 mm deep), deep tillage (150 to 300 mm) or no till can be practiced depending on the type of the soil, moisture availability, type of cultivar and the previous crop planted. Firm, smooth, well-drained fields should be selected. The field should be free from weeds, stones and waterlogged conditions. Contour ridges, ridges, field waterways, terraces or windbreaks should be introduced to the field to prevent wind and water erosion. One should not use a field that was planted to wheat the previous year or same year (Saati, 2005). Planting depth should range from 2 to 5 cm; inter-row spacing of 30 to 90 cm is recommended, depending on available water and planting density of 70 to 90 kg seed $ha^{-1}$ under dry land and 100 to 150 kg seed $ha^{-1}$ under irrigation. Planting date for winter wheat is from March to mid May, depending much on vernalisation effect. The irrigated cultivars are planted from early June to mid August. Spring wheat is planted from August to September, depending on soil moisture and on warmer day and night temperatures during growth and reproduction. The seed should be planted evenly and shallowly in a moist, firm seedbed. A no-till planter can be used for seeding or a planter fitted with tines can be used for planting (Smika, 1991).

Planting must be done on a clean field. This will avoid unnecessary weed control during early seedling growth. As soon as the crop has covered the soil surface, the chances of weed to develop are minimal. However, any weeds during seedling stage should be eradicated. Weeds can be controlled chemically, mechanically or organically (Saati, 2005). The main interest of weed control is to avoid reduction of yield by weeds, since they compete highly with the desired crop for soil nutrients, water and light. Furthermore weeds serve as carriers for disease and pests (Smika, 1991).

## *1.3.2 Fertilization and irrigation*

Nitrogen fertilizer is applied through broadcasting directly before or during planting, and should be incorporated lightly into the soil. Nitrogen fertilizer should be reduced in case a legume crop

is followed by wheat. Excessive fertilization with nitrogen should be avoided owing to luxuriant vegetative growth with the resultant lodging. It is advised that nitrogen should be applied at planting (Hussain and Mudasser, 2004). Direct contact between seed and fertilizer should be avoided. For irrigated wheat, the required nitrogen can be broadcasted before or at planting on clayey soil (>25% clay). Split applications are recommended for sandy soils, but all should be applied before growth stage 10–12 (late tillering) for maximum productivity. Potassium deficiencies are seldom observed in the wheat production areas, as the South African soils are relatively rich in potassium. Potassium deficiencies may occur under the following conditions:

• highly leached sandy soils with low levels of soil potassium
• cold and/or wet and/or very dry conditions
• very high magnesium and/or calcium content of soil. Fertilizer applications should be based on the recommendation subsequent to soil sampling.

Irrigation scheduling must be according to evaporation and needs, as per growth stage. It is, however, very important that irrigation is not stopped too early and the last irrigation must be applied when the total plant is almost discolored. This is to ensure an even ripening and to produce grain with a high percentage plumpness and acceptable nitrogen content. Proper irrigation scheduling can also minimize lodging and disease occurrence and optimize yield quality. The method of irrigation will depend on the water availability and the available irrigation equipment (FAO, 2009).

### *1.3.3 Weed, pest and disease control*

Wheat competes well with most weeds, due to its extensive root system and close intra-row spacing. In spite of the fact that weeds are less of a problem with wheat than with many other crops, they do become a serious problem in certain situations. The most difficult weeds to control are those annual grasses that have life cycles similar to wheat and certain deep-rooted perennial species. Some of these can not be controlled successfully under a continuous cropping system. With the life cycle similar to wheat, the grasses reseed themselves until wheat will no longer grow in the infested area (Heyne *et al.,* 1964). Good quality seed may make a more vigorous seedling which will compete more strongly with weeds. Therefore the use of chemical

practices to control weeds is then most crucial to be implemented. Chemical weed control is only a supplement to good farming practices and cultural methods of weed control are to be used in conjunction with chemical control procedures.

Weeds limit grain yields by approximately 20% annually. By alternating crops and changing herbicides, it is possible to control a wider spectrum of weeds. Effective weed control in one crop often means that the following crop can be grown without the need of expensive selective herbicides (Shimizu *et al.*, 2008). Rotating crops and herbicides reduce the potential for herbicide resistance to develop in target species, for example wild oats. This can also reduce the potential for herbicide residue accumulation in the soil.

Wheat is susceptible to various pests that feed on the underground and above ground parts of the plant, negatively affecting the vegetative growth, flowering, grain filling, water and nutrient cycle of the plant. Continuous damage without treatment results in reduced yield or zero yields. Major pests are the American bollworm, aphids (green and brown); black maize beetle, black sand mite, false chinch bug and mite (Powell and Justum, 1993). The following cultural control mechanisms can be used; proper crop rotation, biological control, the use of pest resistant cultivars, weed removal, proper soil preparation, intercropping and avoiding too much moisture or very humid conditions, especially during the flowering or harvesting period.

Several diseases affect various parts of wheat, including roots, stems, leaves and ears. Leaf diseases occur more frequently in the Western Cape relative to the summer rainfall regions. Only a few are listed, Anthracnose, Aschochyta, Bacterial blight, Rust, Schlerotinia (white mould). The control for these includes crop rotation, use of disease-free seeds or certified seeds, scheduled irrigation, use of resistant cultivars, removal of plant debris after harvesting and conservation tillage (Powell and Justum, 1993).

### *1.3.4 Wheat maturity and harvesting*

Wheat grains must be dry before it can be harvested. Wheat is harvested in November/December, however, later harvestings are applicable in case of spring and summer wheat. Only fully ripened grains should be harvested. Harvesting should commence at 16% grain moisture content while lower moisture contents up to 13% are preferred for storage (Chao

*et al.,* 1989). The shattering types must be harvested earlier and dried artificially (Anonymous, 2009).

## 1.4 CONSTRAINTS IN BREEDING APPROACHES AND METHODOLOGIES

### *1.4.1 Classical plant breeding*

Classical plant breeding uses deliberate interbreeding (crossing) of closely or distantly related individuals to produce new crop varieties or lines with desirable properties. Plants are crossbred to introduce traits/genes from one variety or line into a new genetic background. For example, a rust-resistant wheat may be crossed with a high-yielding but susceptible wheat variety, the goal of the cross being to introduce rust resistance without losing the high-yield characteristics. Progeny from the cross would then be crossed with the high-yielding parent repeatedly through back crossing to ensure that the progeny were most like the high-yielding recurrent parent. The progeny from that cross would then be tested for yield and resistance and high-yielding resistant plants would be further developed. Plants may also be continuously selfed to produce inbred varieties for breeding.

Classical breeding relies largely on homologous recombination between chromosomes to generate genetic diversity (Chrispeels and Sadava, 2003). The classical plant breeder may also make use of a number technique such as protoplast fusion, embryo rescue or mutagenesis to generate diversity and produce hybrid plants that would not exist in nature.

Most useful traits that breeders have incorporated into crop plants in the last 100 years include:

1. Increased quality and yield.
2. Increased tolerance to environmental stresses (salinity, harsh temperature and drought).
3. Resistance to viruses, fungi and bacteria.
4. Increased tolerance to insect pests.
5. Increased tolerance of herbicides.

The main factors limiting the world's crop production at present are environmental stresses. Drought, pests, and weeds infestation are some of the most significant abiotic stresses limiting crop production on more than 26% of the world's arable land. The stress factors are the major causes of yield gap globally (Akgun and Tosum, 2004). Almost one-third of the global land surface is categorized as dry, but at the same time more than 10% of the human population of the world lives and attempts agricultural production in this environment. Water shortages are increasingly the major limiting factor for crop production including South Africa. Winter wheat has a long cropping-cycle and reasonably tolerates water shortages quite successfully, but its yield may fluctuate appreciably. Drought occurrence such as evidenced in 2003 in South Africa can cause extensive crop damage (Hussain and Mudasser, 2004).

Plant breeding for herbicide resistance is one of the possible strategies against weed infestation. Under South African conditions, the main goal is the breeding of varieties which can adapt to drought and survive the herbicide application during growth. The South African requirements for drought-tolerant varieties are different from those incontinuously drought-prone regions. A drought-tolerant plant is understood to be one whose relatively high grain yield will not decrease markedly due to drought stress (Jabeen and Mirza 2002). Accordingly the goal is not a genotype which survives extreme conditions or herbicides but a high-yielding variety which will yield economically during water shortage and weed infestation. Among the difficulties in conventional breeding are the facts that drought and herbicide resistance and yield capacity are often negatively correlated to each other (Akgun and Tosum, 2004).

### *1.4.2 The development of biotechnology tools*

Biotechnology is regarded as 'any technique that uses living organisms, or substances from these organisms, to make a modified product or to improve plants or animals, or to develop microorganisms for specific uses' (Persley, 1992). Plant biotechnology techniques have three broad applications: plant tissue culture, genetic engineering and plant molecular markers. These applications range from the simple to the sophisticated and may be appropriate for use in Africa. Investments in and development of plant biotechnological research capacity in Africa is being accomplished in phases (Lynam, 1995). The first phase involves the use of plant tissue culture, which is appropriate for Africa as many of the important food crops such as cassava,

sweet potato; yam and banana are vegetatively propagated. Specific techniques include in vitro mass propagation, the production of disease-free plants as well as regeneration systems for plant transformation. By focusing on tissue culture, the skills necessary to maintain and to manage a biotechnology laboratory can be developed. The second phase is the application of biotechnological tools, which can improve the efficiency of selection and breeding of varieties/cultivars. Techniques include more advanced tissue culture techniques for example anther culture and embryo rescue, as well as molecular marker applications for diagnostics, fingerprinting and marker-assisted breeding (James, 1996).

The successful production of transgenic plants requires an adequate infrastructure, expertise in tissue culture and molecular biology, and a critical mass of researchers with supporting sustainable funding to cover the high cost of such research (Akgun and Tosum, 2004). Only a few laboratories in South Africa, Nigeria, and in Egypt have the capacity to produce transgenic plants, but still lack the ability to "commercialize" the product, or to ensure that these plants reach the end user, i.e., the African farmer. To bridge this gap, it is necessary to form partnerships with either seed companies, producer organisations or government institutions which can ensure that the complicated technology be delivered in the most well known and accepted technology known to farmers, the seed (James, 1996).

### *1.4.3 Wheat breeding*

One of the main objectives of plant breeders is to improve existing cultivars which are deficient in one or more traits by crossing with lines which possess the desired trait or by induced mutagenesis. Wheat is endowed with striking genetic, cytological and molecular versatility. Yet it is also a problem plant in the hands of breeders due to three features which add greatly to the complexity of breeding and selection, 1) wide range of end uses, each with differing but specific quality requirements, 2) the complexity of the polyploid wheat genome, and 3) low level of polymorphism in bread wheat (Langridge *et al.,* 2001.). This has imposed many constraints on wheat breeding programs and has, in many cases, restricted the diversity of germplasm that can be used in a specific breeding program (Liu *et al.,* 1990). As a result, the level of diversity detected between commercial wheat varieties is generally lower than for many

other species. Similarly, low level of polymorphism in wheat necessitates that a larger number of markers needs to be screened than is the case with other cereals (Chao *et al.*, 1989).

Breeding for improved plant cultivars is based on two principles: genetic variation and selection. Both processes require high inputs of intellectual and manual work. However, the development of plant cell and tissue culture over the last 20 years has made it possible to transfer part of the breeding work from field to laboratory conditions. Plant breeding requires genetic variation of useful traits. Often, however, desired variation is lacking. Therefore, due to the lack of desired variation mutagenesis can be applied using mutagenic agents, such as certain chemicals like ethylmethanesulphonate (EMS) to induce mutations and generate genetic variations from which desired mutants may be selected (Akgun and Tosum, 2004).

Mutation induction has become a proven way of creating variation within a crop variety. It offers the possibility of inducing desired attributes that either cannot be found in nature or have been lost during evolution. When no gene, or genes, for resistance to a particular disease, herbicide or for tolerance to stress, can be found in the available gene pool, plant breeders have no alternative but to attempt mutation induction (Akgun and Tosum, 2004).

## 1.5 MUTAGENESIS

Mutagenesis is the process that is used to induce mutation which alters the genetic makeup of plants by artificial means (Koornneef, 2002). Mutants with new alleles and genes are created which enhances genetic variation (Singh and Kole, 2005). Mutagenesis can be performed through physical or chemical mutagens. Production of transmissible gene alterations is an important characteristic of many breeding programmes and breeders use mutations to produce these changes (Neuffer *et al.*, 1997). It is well established that mutation breeding has made significant contribution to plant improvement (Larkin, 1998). Several works on mutagenesis has been accomplished in various crops, such as barley, sorghum, rice, wheat and maize with many mutants of agronomic importance recorded as well-reviewed by Koornneef (2002). More than 2000 mutant plant varieties have been released for cultivation, and faced none of the regulatory restrictions imposed on genetically modified material. Generally, mutation breeding has remained popular for the last 70 years because it is found to be simple, cheap to perform and

applicable to all plant species (Alcantara *et al.,* 1996). Mutation breeding is useful especially today to create genetic variation in crops where the genetic variability is limited. For example, mutation breeding using EMS has been found to be effective in generating much desired variation for certain traits where the genetic variation was deficient (Singh and Kole, 2005).

In *Capsicum*, mutation studies have shown that EMS mutagenesis increases the variation in many characters including leaf area, days to flowering, days to fruiting, and plant height. Such variation is important to breed for desirable characters (Jabeen and Mirza, 2002). In maize, the most efficient means of producing gene mutations has been found to be chemical mutagenesis and a rational protocol of chemical mutagenesis in this crop is well presented by Neuffer *et al.* (1997). Many agro-morphologically important mutations affecting plant and seed characters have been identified, including alteration of seed color, stem rust resistance, and earliness in wheat (Chopra, 2005). In oats, isolation and characterization of novel starch mutants has also been achieved (Verhoeven *et al.,* 2004). The success of mutation breeding in ornamentals and horticultural crops in the Netherlands and India is impressive with mutants commercially released (Chopra, 2005). Mutation breeding makes extensive use of deviations from the norms to improve the characteristics of important crops. However, an efficient genetic improvement of a cultivar depends on the knowledge of mode of gene action, genetic variability, and the interrelationship among important plant characters. Induced mutagenesis is a significant tool to break through the limitations of variability and to create variability in a short period of time (Sharma and Sharmam, 1981).

### *1.5.1 Methods of mutagenesis*

Mutation experiments were started on barley by Herman Nilsson-Ehle and AkeGustaffson in 1928, reporting mutants with a compact head type (Medina *et al.,* 2005). Considerable research has successively been performed and a wide variety of methodologies have been developed. All mutagenic agents may be categorized as either being physical or chemical in nature. Chemical mutagens include: diethyl sulphate; ethyl methane sulphonate; isopropyl methane sulphonate; ethylamine; and sodium azide amongst others (Medina *et al.,* 2005). Physical mutagens include: ultra violet radiation; electromagnetic radiation (eg. x-rays or gamma rays); corpuscular radiation (fast neutrons or beta particles); and ion or electron beams (Medina *et al.,* 2005). In

addition, mutants have been generated through somaclonal variation in callus cultures, and grown out and introduced into breeding programs (Sharma and Sharmam, 1981).

A wide variety of plant tissue has been treated successfully including seeds, pollen, cuttings, bulbs, tubers, corms, stolons, in vitro cultured cells and even whole plants. At a genetic level there are several phenomena which cause mutations, some of which occur naturally without exposure to mutagenic agents (Medina *et al.,* 2005). They can be categorised as spontaneous or induced, somatic or genetic, chromosomal or extra-chromosomal (Medina *et al.,* 2005). The type and frequency of mutation is dependent upon the mutagenic agent, and its dose rate (Medina *et al.,* 2005). Most mutagenic agents used by plant researchers and breeders in the past have caused large genetic changes resulting in high mortalities and the need for large populations for treatment (Micke, 1999). Induced mutations occur more or less randomly in the genome thus only one of the two or more alleles of a locus is affected, resulting in the need for breeders to ensure homozygosity through self-pollination before the trait will be properly expressed (Micke, 1999).

### *1.5.2 Importance of chemical mutagens*

Chemical agents can be useful since they provide high mutation rates and, mostly point mutations. The chemical mutagens which are mostly used for mutation induction belong to the class of alkylating agents such as ethylmethanesulphonate (EMS); diethyl sulphate (dES); ethyleneimine (EI); ethyl nitroso urethane (ENU), ethyl nitroso urea (ENH), methyl nitroso urea (MNH) and azides. The dose assessment for chemical mutagens is determined by varying the concentration, temperature, duration of treatment and the solvent used for example dimethyl sulfoxide (DMSO), or the pH of the solution (Sharma and Sharmam, 1981). Chemical mutagens (EMS, DES, sodium azide) were also used by treating banana shoot tips to produce modifications for tolerance to Fusarium wilt. EMS has been successfully used on chrysanthemum, yielding a frequency of 5.2% mutants. A wide range of variations in petal color (pink-salmon, light-pink, bronze, white, yellow and salmon color) have been recorded. Liu *et al.* (1990) treated sweet potato (*Ipomoea batatas* L.) callus with EMS and obtained salt tolerant lines.

### *1.5.3 Importance of EMS*

EMS is a mutagenic, teratogenic, and possibly carcinogenic organic compound with formula $C_3H_8O_3S$. EMS is a commonly used mutagenic agent in the plant science because of its height level of effectiveness. EMS belongs to the group of alkylating agents which are well known mutation inducers, causing point mutations (like cytosine [C] -to-thymine [T]nucleotide changes) as well as loss of chromosome segments or deletions (Alcantara *et al.,* 1996). A large number of mutations in plants and cultivars have been achieved by the use of EMS, e.g. resistance to herbicides and male sterility (Verhoeven *et al.,* 2004). It produces random mutations in genetic material by nucleotide substitution; mainly by guanine alkylation. This typically produces only point mutations. It can induce mutations at a rate of $5x10^{-4}$ to $5x10^{-2}$ per gene without substantial killing. The ethyl group of EMS reacts with guanine in DNA, forming the abnormal base O-6-ethylguanine. During DNA replication, DNA polymerases that catalyze the process frequently place thymine, instead of cytosine, opposite O-6-ethylguanine. Following subsequent rounds of replication, the original G: C base pair can become an A: T pair. This changes the genetic information and subsequently brings about genetic variations (Khan and Goyal, 2009).

EMS has been used as a mutagen for both animal and plants cells and it was established to be a common, powerful and one of the most effective chemical mutagen, especially recommended using seed materials, since the application and the monitoring of the result of mutations are relatively easy. In plants, EMS usually causes point mutations, on the other hand, loss of a chromosome segment or deletion can also occur to a lesser extent (Ahloowalia *et al.,* 2001a). Therefore, EMS has the potential of altering loci or a candidate gene of specific interest without inducing large deletions. This creates an advantage for the plant breeders to obtain useful alleles than using exotic or wild germplasms in which the group of linked unfavorable alleles may be present. The most important parameters for inducing mutation with EMS are concentration, duration of treatment, and solution temperature (Sharma and Sharmam, 1981).

Thus, prior to the large scale generation of modifications initial studies on induced mutations are usually conducted for finding ideal combinations of these parameters together with the optimum dose to elicit the best response. Both physical and chemical mutagens were tested for

different agronomic characteristics in various crop species such as wheat, barley, rice, tobacco, maize, Brassica, fruit crops and vegetables (Verhoeven *et al.,* 2004). Although any mutagenesis as gamma ray or EMS treatment makes plants vulnerable to negative effects on germination, production of vital seeds, root and seedling lengths during the first mutation generation ($M_1$) and the following generations (Ahloowalia *et al.,* 2001b). Consequently, it is important to optimize the best possible condition for generating large number of mutants having good seed germination for segregation without detrimental genetic damages. Mutations induced through EMS are point mutations resulting in a broad range of allelic variations at a relatively small dose (Verhoeven *et al.,* 2004). Such genetic changes at the nucleotide levels of candidate genes with possible novel gene functions can be generated without losing the genetic homeostasis of the crop.

### *1.5.4 Uses and properties of EMS*

EMS is often used in genetics as a mutagen and the mutations induced by EMS can then be studied in genetic screens or other assays (Kim *et al.,* 2004). EMS is a monofunctional ethylating agent that has been found to be mutagenic in a wide variety of genetic test systems from viruses to mammals and is reported to have recently received much attention as one of the most effective mutagenic agent in higher plants known today. Other studies also revealed that EMS is an effective mutagen and has been used to induce genetic variability in a number of crop plants such as barley, sorghum and wheat (Kumar and Rai, 2005) and in maize (Jabeen and Mirza, 2002).

The stimulatory effect at a lower dose is due to the fact that mutagens at lower concentrations stimulate the role of enzyme and growth hormone responsible for growth and yield while the inhibitory effect is due to the fact that biological damage increased at a faster rate in higher concentrations of mutagens (Verhoeven *et al.,* 2004).

### *1.5.5 EMS treated seeds germination*

The germination of the EMS treated wheat seeds had shown a sharp dose rate relationship, which decreased with the increase in the doses/concentration of mutagenic treatments. Percentage reduction/stimulation in seed germination might have been due to the effect of

mutagens on meristematic tissues of the seed (Ahloowalia *et al.,* 2001a). The decrease in seed germination at higher doses/concentration of the mutagens may be attributed to disturbances at cellular level (caused either at physiological or physical level). Kumar and Mishra (2004) described that in okra germination percentage generally decreased with increasing doses/concentrations of gamma rays and EMS. Reduced germination percentage with increasing doses of gamma radiation has also been reported in Pinus (Thapa, 2004), Rye (Akgun and Tosum, 2004) and Chickpea (Khan *et al.,* 2005 and Toker *et al.,* 2005). Gradual reduction in germination percentage was also observed with an increase in concentration of mutagen, reaching more than 50% lethality at 0.5% EMS in two genotypes of tobacco (Amernath and Prasad, 1998).

## 1.6 HERBICIDE RESISTANCE

Farmers face continued crop losses as a result of weed infestation, recurrent drought, diseases and pests, and low soil fertility. Further, global warming and subsequent climate change presents major risks to crop production in Africa, unless significant mitigation strategies are implemented to generate adapted crops, and improved farming technologies such as developing chemical mutagenesis protocol for herbicide resistance crops (David and Rosamond, 2009). One of these strategies is the development of wheat varieties with herbicide resistance to use post-emergent herbicides.

### *1.6.1 Factors affecting wheat survival and growth*

There are various factors that contribute towards wheat survival and growth, of which some are pre-emergence and some post-emergence and germination problems. The first problem producers might encounter after planting is weed growth that ultimately competes with wheat for light, nutrients, water and other wheat agronomic survival requirements. Weeds may also inhibit growth through release of allelopathic chemicals that are toxic to wheat plants. Weeds or weed seeds contaminating harvested grain may reduce quality (Khan *et al.,* 2004). In addition, weeds may interfere with harvesting or raise the moisture content of the harvested grain, leading to damage from heat and pests in storage.

### *1.6.2 Mutagenesis for herbicide resistance development*

One of the ways in which herbicide resistance can be developed in wheat and other crops is via ethylmethanesulphonate (EMS) mutagenesis. EMS mutagenesis is known to alter genes and produce heritable changes in organisms (Koornneef, 2002). There are no studies that have been done on herbicide resistance development of wheat via mutagenesis using bromoxynil and metsulfuron methyl herbicides. However, this has been achieved in other cereals including sorghum and maize (Newhouse *et al.*, 1992). Herbicide resistance development has also been successful in other crops including soybean, where chlorsulfuron resistant soybean mutants have been developed via seed mutagenesis (Sebastian and Chaleff, 1987).

One of the major advantages of EMS mutagenesis is that it causes small nucleotide changes or point mutations within the genome as opposed to other mutagens which are responsible for deletion of large sections of the genome, thus causing major changes and disrupting most of the characteristics of the variety. With EMS treatment, only small changes are affected, and thus the general characteristics of the variety are maintained (Weil and Monde, 2007). In addition, EMS is generally easy to use and is easily available. Apart from mutagenesis, herbicide resistant crops have also been developed by means of genetic engineering as in the case of barley and tobacco (Shimizu *et al.*, 2008), but this method might not be appropriate to develop herbicide resistance in crops, especially, in some of the African countries, where genetically modified organisms have generally not been accepted.

### *1.6.3 Strategies for effective weed control to ensure sustainability*

Effective long term weed management is based on limiting competitiveness of the weeds that are already in the field and growing with the crop, inhibiting the introduction of new weeds, and preventing the multiplication of the weeds that are already present. To bring about sustainability in crop production, weeds, along with other pests must be adequately controlled. Carter (1988) noted that the concept of agricultural sustainability has social, ecological, economic, and emotional connotations. In Africa several important crops are adapted over years with associated weedspecies in different cropping systems and varied socio-economic situations of the farming communities. Hence, suitable strategies for effective weed control may differ from

one country to another depending on the agricultural set up and socioeconomics of the farming communities. However, solving the weed control problem in Africa would greatly depend on steps taken to address key limitations to improved weed control. Some of these factors are:

### *1.6.4 Herbicide use*

Herbicides are known to have revolutionized agriculture in Europe and North America by providing effective weed control with minimum drudgery. In the continental Africa, on the other hand, the use of herbicides is a recent phenomenon and, until recently, except by large scale producers (Sebastian and Chaleff, 1987). However, more and more countries in Africa are calling for a shift from subsistence to commercial agriculture which is likely to attract elitist farmers who are educated, informed and better placed to access credit to purchase inputs. Diseases such as malaria and HIV/ AIDS are taking a toll on farm labour which is becoming increasingly scarce and or more expensive in Africa. With these prevailing circumstances, weed control by herbicides provides a viable alternative to hand or hoe weeding. There exist potential negative impacts of herbicides if not used properly. However, there are a number of problems related to herbicide use in African agriculture.

The problems range from an inadequate rate of herbicide being applied; the herbicide being applied is not appropriate; and the herbicide is applied too late to be effective on weeds. A major cause of this is likely to be the high cost of herbicides and/or the lack of appropriate information available to the farmer and poor level of understanding amongst herbicide dealers and extension educators. The drive to transform from subsistence to commercial farming will compel farmers to consider herbicides as an alternative to manual systems of weed control for timely and effective weed control at minimum costs. However, there are potential dangers related to herbicide use. Herbicide-resistant weed biotypes are reported to have increased dramatically over the last decade and to date, a total of 273 herbicide-resistant weed biotypes have been identified from 59 different countries.

Smallholder crop productions systems may help create risk areas as herbicides are often not used at appropriate times or dosages, which may hasten the development of resistance. The possibilities of weed population shifts and development of herbicide-resistant biotypes also suggest that adoption of herbicide-based weed control should be accompanied by greater

emphasis on the judicious use of such chemicals (Sebastian and Chaleff, 1987). The lack of appropriate protective equipment and training make poisoning possible through skin absorption, inhalation or even direct ingestion. Information on the use of herbicides for weed control should include clear and emphatic instructions on 'safe use' of herbicides to mitigate unintended effects. Poor standards of herbicide use and disposal pose additional problems of environmental pollution and contribution to the accumulation of obsolete pesticide stockpiles which are now estimated at 27.4 million tons in Africa alone (FAO, 2010).

### *1.6.5 Strategies based on crop improvement*

The development of transgenic crops in which genes that can confer resistance to specific weeds and/or specific herbicides are transferred using marker-assisted breeding and selection has shown promising results and the technique is gaining prominence. ICRISAT (www.icrisat.org), using marker-assisted selection, has been able to identify genes that confer resistance to *Strigahermonthica* and transferred the genes to farmer preferred varieties of sorghum through conventional breeding without jeopardizing other desirable characteristics such as drought tolerance and yield. Five such varieties are currently on initial on-station trials in Kenya, Eritrea, Uganda and Mali and showing initial success in keeping *Striga* away. This development follows failures with potentially effective techniques involving rotation with legumes, weeding, biological and herbicidal control methods which have been evaluated in the past and similar evaluations continue to-date without much success.

## 1.7 DISCUSSION AND OBJECTIVES

This review of the literature highlights the current use and future potential of wheat as a grain crop, and draws attention to its limitations in terms of herbicide resistance, yield and production. A need for the improvement of existing germplasm, as well as generation of new material for breeding, was raised and several methods of achieving this end were discussed.

Out of this, the potential of EMS chemical mutagenesis in introducing new genetic diversity was illuminated. In addition, a need for rapid screening techniques became evident, particularly for screening herbicidal characteristics. The past success and future potential of chemical mutagenesis was discussed in addition to the methodology of reliable calibration development. Accordingly, the objectives of the study reflected in the following chapters were to investigate

the response of wheat varieties to treatment with varying concentrations of EMS so as to develop an EMS protocol for efficient mutagenesis of wheat and to increase the genetic diversity of wheat for various traits and develop a mutant population for use as a source of new pure breeding lines which will be resistant to herbicide.

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# CHAPTER 2

# RESPONSE OF SELECTED WHEAT GENOTYPES TO ETHYL METHANESULPHONATE CONCENTRATION, TREATMENT TEMPERATURE AND DURATION

# Abstract

Genetic variation could be enhanced through various techniques including the use of chemical mutagens such as ethyl methane sulphonate (EMS). Use of EMS was reported in various major crops such as wheat (*Triticum aestivum* L.), rice *(Oryza sativa* L.*),* barley (*Hordeum vulgare* L.), cotton (*Gossypium hirsutum* L.), peanuts *(Arachis hypogea* L.*)* and beans (*Phaseolus vulgaris* L.). Key factors in inducing mutation with EMS include concentration, test varieties, temperature and duration of treatment. The objective of this study was to determine the optimum EMS concentration, treatment temperature and duration that would provide desired germination percentage and vigorous and healthy seedlings for effective mutagenesis in wheat. Seeds of four selected genotypes of wheat (B936, B966, SST387 and SST875) were surface sterilized in sodium hypochlorite and ethanol and soaked in distilled water for 18 hours. Seeds were treated in two replicates using three EMS concentrations (0.3, 0.5, and 0.7%), three temperature regimes (30, 32.5 and 35°C) at four time durations (0.5, 1, 1.5 and 2 hrs). The treated seeds were planted in seedling trays and germinated in a Controlled Environment Facility (CEF) at the University of KwaZulu-Natal, Pietermaritzburg campus, South Africa. Seedling emergence (%), germination (%) and seedling height (mm) were recorded for each treatment combination. Results showed highly significant interactions ($P<0.01$) among varieties, EMS concentrations, temperature and exposure time on emergence, germination and seedling height. Seeds treated with the highest EMS dose (0.7%), temperature (35ºC) and long exposure time (2hr) showed delayed emergence by 18 days. At 30ºC, 0.5 hr and 0.3% EMS varieties B936, B966 and SST875 had early emergence (6 days). B936 and SST387 had 50% while B966 and SST875 had 53% and 57% germination, respectively. These results were observed at EMS level of 0.7%, 30ºC and 1.5 hr exposure time in B936; EMS at 0.5%, 35ºC and 1.5 hr (B966); and EMS at 0.5%, 32.5ºC and 1 hr in SST387. SST875 required EMS dose at 0.5%, 32.5ºC and 2 hr treatment time. Other low or high treatment combinations were invariably ineffective compared to untreated control. All considered traits decreased with increase in EMS dose, temperature and exposure time. The study established the requirement of variety specific EMS dose and treatment temperature and duration that could be used for inducing large scale mutation to select targeted mutant individuals in wheat.



***Keywords***: Ethyl methane sulphonate, mutagenesis, seed germination, *Triticum aestivum* L.

## 2.1. Introduction

Genetic variation in crop improvement can be enhanced through natural or artificial mutations. There are several methods to induce artificial mutation in plants including chemical alkylating (EthylmethaneSulphonate and ethidium bromide) or physical (ionic radiation, X-rays, UV light, gamma rays and neutrons) mutagenic agents (Predieri, 2001). The use of alkylating agents such as Ethylmethanesulphonate (EMS) in inducing mutation is regarded as an efficient option for inducing mutation in wheat genotypes (Leonard, 1967). Induced genetic variations were used successfully in several crops to extract mutants with suitable agronomic traits such as herbicide resistance, early maturity and improved nutrition (Singh *et al.,* 2001).

Mutations are induced in plants by exposure of their propagules, such as seeds and meristematic cells, tissues and organs. Mutation experiments require careful selection of a mutagen with characteristics suited to the tissue source and mutagenesis objective and an appropriate treatment regime (Ahloowalia *et al.,* 2001). Chemical mutagenesis using EMS is known to be a very useful method especially among self fertilized crops such as wheat, and can be used to develop suitable germplasm by mutating a single or few genes controlling a trait that needs improvement (Maluszynski *et al.,* 1995). Chemical mutagenesis is widely applied in major crops such as wheat, rice, barley, cotton, peanuts and beans (Ahloowalia *et al.,* 2001). The method is regarded as an effective option in developing herbicide resistance in various crop plants which is simple, relatively cheap and equally usable on a small or large scale (Siddiqui and Khan, 1999).

Wheat (*Triticum aestivum* L.) is the primary source of staple diet and provides 20% food calories to the world (Anonymous, 2009). In South Africa, wheat is the second most important field crop after maize. South Africa has about 3 800 to 4 000 commercial wheat farmers providing work opportunities to about 28 000 people (Welch *et al.,* 2000). The annual wheat production in South Africa far exceeds that of other Southern African Customs Union (SACU) countries with an estimated 2.4 million tons grain production during 2004 (FAO Food Outlook, 2004). Wheat production faces many challenges such as weed competition, heat stresses, recurrent drought, diseases, poor soil fertility and low yields (Sonada and Amano, 1998). Therefore developing wheat cultivars with increased grain yield potential, superior end use quality, tolerance to biotic and abiotic stresses remains important to boost productivity (Singh *et al.,* 2001). In addition, it is

essential to enhance the genetic resources for plant breeding through techniques such as artificial mutations.

Artificial mutation creates an advantage for the plant breeders to obtain useful alleles, instead of using exotic or wild germplasms in which groups of linked and undesirable alleles may be present. During the past seven decades, more than 2 252 mutant crop varieties were officially released in the world (Maluszynski, *et al*., 2000). A great majority of mutant varieties (64%) were developed using gamma irradiations (Ahloowalia *et al.,* 2004). Among the chemical mutagen, EMS is reported to be the most effective and powerful chemical mutagen (Minocha and Arnason, 1962), especially recommended for use when mutation is used on seed materials, since its application and the monitoring of the outcome of mutations are relatively easy (Sakin, 1998). In plants, EMS usually causes point mutations (Okagaki *et al.,* 1991); Henikoff and Comai, 2003; Khatri *et al.,* (2005) reported that gamma rays and EMS could be fruitfully applied to develop new varieties with high yield and other improved agronomic traits. The use of EMS is reported in capsicum (Jabeen and Mirza, 2002), maize (Neuffer *et al.,* 1997) and sorghum (Sonada and Amano, 1998).

The effectiveness of mutagenic treatment and amount of genetic variation in crops depends on the variety, the mutagen dose (EMS concentration), temperature and the time of exposure to the mutagen (Maluszynski and Khan, 2002; Rupinder and Kole, 2005). This may be observed among other changes such as number of days taken for germination, germination percentage and seedling height, among others that could serves as a criterion in determining the biological effects among mutant plants (Singh *et al.,* 2000). The most favorable treatment combinations should be established for effective mutagenesis in wheat to achieve the desired germination percentage and grow vigorous and healthy seedlings to undertake subsequent selections (Jain, 2005).

Mutation breeding is relatively a quicker method to create genetic variation in crop improvement programs (Ilirjana *et al.,* 2007). Useful mutants were developed for various agronomic characters in different crops through treatment with physical and chemical mutagens (Ram Din *et al.,* 2003). However, the most favorable treatment combinations should be established for effective mutagenesis in wheat to achieve the desired germination percentage and grow vigorous and

healthy seedlings to undertake subsequent selections (Jain, 2005). The objective of this study was to determine the optimum EMS concentration, treatment temperature and duration that would provide desired emergence, germination percentage and vigorous seedlings for effective mutagenesis in wheat (*Triticum aestivum* L.). Results of this study will be useful for large scale use in the selected wheat varieties to create desired genetic diversity and develop mutant populations as potential sources of new breeding lines with herbicide resistance and other suitable agronomic traits.

### *2.2.1. Hypothesis*

The following hypothesis was tested:

The effect of mutagenic treatment and amount of genetic variation in wheat cultivars is independent of the variety, the mutagen dose (EMS concentration), temperature and the exposure time to the mutagen.

## 2.3. Materials and methods

### *2.3.1. Study site and plant materials*

The studies were conducted at the University of KwaZulu-Natal, Pietermaritzburg, South Africa, under controlled environmental conditions.

Seeds of four wheat varieties (SST-387, SST-875, B-936 and B-966) from Sensako Wheat breeding Company, South Africa were used in the study. The varieties were selected based on certain suitable agronomic traits including early maturity and market potential and adaptability to production in the dry semi-arid zones of South Africa.

### *2.3.2. Chemicals and reagents*

Ethyl methanesulfonate (EMS, sigma, formula weight 124.16, and density 1.206, 1M = 108.5 ml/l) was secured from Sigma chemical company, Germany. Absolute Ethanol ($C_2H_5OH$, formula weight 46.07 g/mol, density 0.789 g/cm$^3$) and JIK, Sodium Hypochlorite (NaOCl, 10 - 12.5% m/v), was used for surface sterilization of the wheat seeds before exposure to the chemical mutagen. Dimethyl sulphoxide (DMSO); Sulfinylbis [Methane] (Molecular Weight: 78.13, Chemical Formula: $(CH_3)_2SO$), boiling and freezing point temperatures: 189.0°C and 18.5°C, respectively with a density of 1.100, Assay (GC) 99.5% was used as a carrier agent in solution with EMS. Dimethyl sulphoxide increases absorption and penetration of tissues and also enhances the germination of certain tree seeds (Smale, 1969), because of its phenomenal ability as a biological tissue penetrant.

### *2.3.3. Seed mutagenesis and planting*

Mutagenesis was performed according to the procedure described by Mba *et al*., (2007). Seeds of the four wheat varieties were subjected to varying EMS concentrations (0.3, 0.5, and 0.7% v/v), four time durations of exposure (0.5, 1, 1.5 and 2 hours) and three temperature regimes (30, 32.5 and 35ºC). This resulted in a total of 144 treatment combinations. Control seeds were only presoaked in water for 18 hours and used for comparison.

Seeds of each variety were placed into 72 mesh bags (30 seeds in each mesh bag) and in two replicates. For surface sterilizing, the seeds were soaked in 70% ethanol for 1 minute and rinsed 3 times and soaked again in 30% JIK (Sodium Hypochlorite (NaOCl, 10 - 12.5% m/v) for 5 minutes and rinsed 3 times. The seeds were then soaked in distilled water for about 16 to 20 hours before EMS treatment. Mesh bags containing different treatment combinations were immersed in their respective EMS dose trays at a specific temperature and for a specific period of time duration. After the EMS treatment, the seeds were washed under running water for about 2 to 3 hours to remove the excess EMS, and to eliminate the mutagen for safe handling. The seeds were dried on a paper towel and then planted in seedling trays and germinated at the Controlled Environmental Facility of the University of KwaZulu-Natal. Treated and untreated (control) seeds were planted at a depth of 1 cm in seedling trays containing a pine bark seedling mix growth media. The media was supplied by National Plant Foods (NPF) and pasteurized in a steam chamber by steam delivery for duration of 4 hours. The trays were irrigated using a watering can immediately after sowing and were then taken to CEF (Figure 1).

### *2.3.4. Experimental design and data collected*

The experiment was set out as a completely randomized design with two replications. For each replicate 40 seeds per treatment were planted. Data collected included number of days to 50% emergence, germination percentage and seedling height (mm). Days to 50% emergence was recorded when 50% of seedlings emerged per variety. Germination percentage was recorded by counting the number of seeds that have germinated and emerged for each treatment. Fourteen days after planting, seedling height of 10 randomly selected plants from each treatment was measured as the length from the base of the plant (from the point of emergence) to the tip of the flag leaf.

**Figure 2.1.** Greenhouse germination trials using the Speedling3 system at the CEF/UKZN.

### *2.3.5. Data analysis*

Data on number of days to 50% emergence, germination percentages and seedling heights was subjected to analysis of variance (ANOVA) using Genstat® Release 12.1. Mean comparisons were conducted using the Fisher's Least Significant Difference Procedure when significant interactions were detected in the ANOVA. The association between emergence, germination percentages and seedling heights was determined using the Pearson correlation test procedure.

## 2.4 RESULTS

The results of this study indicated highly significant interactions (P<0.001) among EMS, temperature, time and variety for days to 50% emergence, germination percentage and seedling height (Table 1).

**Table 2.1.** Analysis of variance on number of days to 50% emergence, germination percentage and seedling height among four wheat varieties when tested using three EMS doses at three temperature regime and four exposure times.

| Source of variation | d.f. | Days to 50% emergence | | Germination (%) | | Seedling height | |
|---|---|---|---|---|---|---|---|
| | | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. |
| EMS | 2 | 337.843 | <.001 | 1115.87 | <.001 | 10632.07 | <.001 |
| Temperature (Temp) | 2 | 155.697 | <.001 | 2365.03 | <.001 | 2554.34 | <.001 |
| Time | 3 | 473.601 | <.001 | 3204.59 | <.001 | 6687.56 | <.001 |
| Variety | 3 | 345.518 | <.001 | 2482.53 | <.001 | 520.42 | <.001 |
| EMS.Temp | 4 | 46.271 | <.001 | 534.12 | <.001 | 405.17 | <.001 |
| EMS.Time | 6 | 93.723 | <.001 | 906.89 | <.001 | 2327.17 | <.001 |
| Temp.Time | 6 | 31.267 | <.001 | 267.51 | <.001 | 473.2 | <.001 |
| EMS.Variety | 6 | 109.644 | <.001 | 694.88 | <.001 | 1475.23 | <.001 |
| Temp.Variety | 6 | 69.679 | <.001 | 251.5 | <.001 | 871.39 | <.001 |
| Time.Variety | 9 | 103.943 | <.001 | 401.12 | <.001 | 516.29 | <.001 |
| EMS.Temp.Time | 12 | 34.717 | <.001 | 273.42 | <.001 | 720.33 | <.001 |
| EMS.Temp.Variety | 12 | 148.778 | <.001 | 442.4 | <.001 | 386.27 | <.001 |
| EMS.Time.Variety | 18 | 70.051 | <.001 | 263.76 | <.001 | 771.38 | <.001 |
| Temp.Time.Variety | 18 | 41.093 | <.001 | 222.83 | <.001 | 244.63 | <.001 |
| EMS.Temp.Time.Variety | 36 | 41.201 | <.001 | 209.93 | <.001 | 360.68 | <.001 |
| Residual | 144 | 2.844 | | 21.65 | | 58.45 | |

d.f. = degrees of freedom; m.s. = mean square; F.pr = Frequency probability

The results suggest that days to 50% emergence, germination percentage and seedling height are significantly influenced by the EMS concentration, variety differences, length of exposure time and temperature. Furthermore, result shows highly significant differences among the four varieties with respect to the traits studied. The mean values for days to 50% emergence are indicated in table 2.

**Table 2.2.** Effect of three doses of EMS (0.3, 0.5 and 0.7%), three treatment temperature (30, 32.5 and 35°C), four exposure time (0.5, 1, 1.5 and 2 hrs) and variety on Days to 50% emergence.

| Treatment conditions | | | Varieties | | | |
|---|---|---|---|---|---|---|
| **EMS (v/v)** | **Temperature ($^0$C)** | **Time (hr)** | **B936** | **B966** | **SST387** | **SST875** |
| 0.3 | 30 | 0.5 | 7 | 7 | 7 | 7 |
| | | 1 | 6 | 8 | 6 | 6 |
| | | 1.5 | 7 | 7 | 6 | 6 |
| | | 2 | 9 | 8 | 6 | 7 |
| | 32.5 | 0.5 | 8 | 7 | 7 | 6 |
| | | 1 | 6 | 8 | 9 | 7 |
| | | 1.5 | 7 | 9 | 10 | 7 |
| | | 2 | 10.5 | 10 | 9 | 8 |
| | 35 | 0.5 | 9 | 10 | 6 | 7 |
| | | 1 | 6 | 7 | 10 | 6 |
| | | 1.5 | 7 | 9 | 9 | 7 |
| | | 2 | 6 | 9 | 8 | 6 |
| 0.5 | 30 | 0.5 | 6 | 7 | 9 | 6 |
| | | 1 | 7 | 8 | 6 | 10 |
| | | 1.5 | 6 | 10 | 8 | 10 |
| | | 2 | 7 | 30 | 6 | 8 |
| | 32.5 | 0.5 | 9 | 8 | 8 | 7 |
| | | 1 | 7 | 8 | 8 | 7 |
| | | 1.5 | 6 | 30 | 9 | 7 |
| | | 2 | 8 | 30 | 6 | 9 |
| | 35 | 0.5 | 6 | 8 | 7 | 7 |
| | | 1 | 7 | 7 | 8 | 7 |
| | | 1.5 | 8 | 11 | 7 | 20.5 |
| | | 2 | 8 | 30 | 8 | 30 |
| 0.7 | 30 | 0.5 | 7 | 6 | 9 | 6 |
| | | 1 | 8 | 7 | 6 | 6 |
| | | 1.5 | 10 | 7 | 7 | 7 |
| | | 2 | 8 | 8 | 30 | 9 |
| | 32.5 | 0.5 | 8 | 9 | 7 | 30 |
| | | 1 | 7 | 7 | 7 | 8 |
| | | 1.5 | 9 | 10 | 8 | 30 |
| | | 2 | 10.8 | 30 | 9 | 30 |
| | 35 | 0.5 | 7 | 6 | 5 | 6 |
| | | 1 | 6 | 30 | 7 | 6 |
| | | 1.5 | 11 | 9 | 7 | 7 |
| | | 2 | 12.1 | 30 | 13 | 19.5 |

**Least Significant Difference (5 % level) = 3.33; Degrees of Freedom =141; Co-efficient of variation % = 17.5

Seeds of the tested varieties treated with the highest EMS concentration, temperature and long duration of exposure i.e. 0.70%, 35ºC, and 2hr, respectively, showed delayed emergence. At 0.3 % EMS, 30ºC and 0.5 hr varieties B936, B966 and SST875 showed early 50% emergence (Table 2) compared to high EMS rate (0.7%). The ideal treatment conditions for early seedling emergence was recorded after 7 days when EMS concentration was 0.5% v/v at a temperature of 35ºC and exposure time of 1hr for all the varieties (Table 2). Increased number of days to emergence shown in table 2 was due to the effect of EMS dose. The mean values of germination % are shown in table 3.

**Table 2.3.** Effect of three doses of EMS (0.3, 0.5 and 0.7%), three treatment temperature (30, 32.5 and 35°C), four exposure time (0.5, 1, 1.5 and 2 hrs) and variety on Germination percentage.

| | Treatment conditions | | | | Varieties | |
|---|---|---|---|---|---|---|
| **EMS (v/v)** | **temperature ($^0$C)** | **Time (hr)** | **B-936** | **B-966** | **SST-387** | **SST-875** |
| | | 0.5 | 71.7 | 75 | 55 | 78.35 |
| | 30 | 1 | 83.35 | 65 | 78.35 | 76.7 |
| | | 1.5 | 78.35 | 70 | 88.35 | 81.65 |
| | | 2 | 58.3 | 68.3 | 78.35 | 83.3 |
| | | 0.5 | 68.3 | 66.65 | 43.3 | 95 |
| 0.3 | 32.5 | 1 | 88.35 | 78.35 | 56.65 | 86.7 |
| | | 1.5 | 80 | 60 | 51.65 | 81.65 |
| | | 2 | 46.7 | 61.65 | 55 | 70 |
| | | 0.5 | 60 | 58.3 | 90 | 88.35 |
| | 35 | 1 | 80 | 76.65 | 55 | 81.65 |
| | | 1.5 | 73.35 | 61.65 | 53.3 | 78.35 |
| | | 2 | 86.7 | 63.35 | 70 | 91.65 |
| | | 0.5 | 85 | 61.65 | 66.7 | 88.35 |
| | 30 | 1 | 83.35 | 63.3 | 73.35 | 73.3 |
| | | 1.5 | 85 | 61.65 | 63.3 | 70 |
| | | 2 | 81.65 | 40 | 85 | 70 |
| | | 0.5 | 65 | 66.65 | 63.35 | 73.35 |
| 0.5 | 32.5 | 1 | 76.7 | 71.65 | 50 | 75 |
| | | 1.5 | 83.3 | 46.7 | 58.3 | 70 |
| | | 2 | 60 | 43.3 | 80 | 56.65 |
| | | 0.5 | 85 | 60 | 45 | 71.65 |
| | 35 | 1 | 78.35 | 63.35 | 61.65 | 70 |
| | | 1.5 | 73.3 | 53.3 | 70 | 48.35 |
| | | 2 | 68.35 | 35 | 56.7 | 46.7 |
| | | 0.5 | 83.3 | 81.65 | 86.65 | 86.7 |
| | 30 | 1 | 71.7 | 78.35 | 78.35 | 95 |
| | | 1.5 | 50 | 73.3 | 71.7 | 81.7 |
| | | 2 | 70 | 40 | 43.35 | 68.35 |
| | | 0.5 | 65 | 65 | 60 | 40 |
| 0.7 | 32.5 | 1 | 81.7 | 66.7 | 73.35 | 76.65 |
| | | 1.5 | 68.35 | 56.65 | 61.7 | 43.3 |
| | | 2 | 26.65 | 40 | 56.65 | 21.65 |
| | | 0.5 | 80 | 78.35 | 95 | 81.65 |
| | 35 | 1 | 86.65 | 31.7 | 61.65 | 93.3 |
| | | 1.5 | 73.35 | 66.65 | 65 | 75 |
| | | 2 | 41.65 | 26.65 | 61.65 | 60 |

**Least Significant Difference (5 % level) = 9.196; Degrees of Freedom =144; Co-efficient of variation % = 6.9

There existed significant differences among treatment combinations with regards to average seed germination percentage (Table 3). The highest % germination was observed on the control

treatment when compared to other treatment conditions and with increasing EMS doses. The mean percent germination varied from 26 (0.70% EMS, 35ºC, 2 hr) to 95% (0.30% EMS, 32.5ºC, 0.5 hr). In the first few days (6 days) there was a delay of germination in the treatment that was exposed to high EMS and treatment conditions (0.70% EMS, 35ºC, and 2 hr). However, in the subsequent days seeds recovered and had significantly higher germination percentages. B936 and SST387 had the required germination at 50% while varieties B966 and SST875 had higher germinations at 53% and 56%, respectively. These was achieved at treatment conditions of EMS at 0.7%, 30°C and 1.5 hr exposure time in B936; EMS at 0.5%, 35°C and 1.5 hr in B966; EMS at 0.5%, 32.5°C and 2 hr in SST387. SST875 required EMS level at 0.5%, 32.5°C and 2 hr treatment.

The average seedling height of varieties per treatment combination is summarized in table 4. Seedling height was significantly affected due to differences among genotypes, EMS dose and treatment conditions.

**Table 2.4.** Effect of three doses of EMS (0.3, 0.5 and 0.7%), three treatment temperature (30, 32.5 and 35°C), four exposure time (0.5, 1, 1.5 and 2 hrs) and variety on seedling height (mm).

| | Treatment conditions | | Varieties | | | |
|---|---|---|---|---|---|---|
| **EMS (v/v)** | **Temperature ($^0$C)** | **Time (hr)** | **B936** | **B966** | **SST-387** | **SST-875** |
| | | 0.5 | 101.7 | 101.1 | 50.25 | 85 |
| | 30 | 1 | 90.15 | 100 | 86.3 | 79.3 |
| | | 1.5 | 98.85 | 111.7 | 101.05 | 87 |
| | | 2 | 75.2 | 105 | 80.2 | 88.1 |
| | | 0.5 | 97.55 | 89.4 | 84.05 | 91.4 |
| 0.3 | 32.5 | 1 | 98.1 | 106.1 | 62.6 | 73.7 |
| | | 1.5 | 92.7 | 90.6 | 85 | 85.3 |
| | | 2 | 37.5 | 102.5 | 64.55 | 61.7 |
| | 35 | 0.5 | 89.65 | 54.6 | 46.55 | 72 |
| | | 1 | 86 | 81.3 | 40.85 | 101.9 |
| | | 1.5 | 109 | 95.85 | 95.1 | 67.4 |
| | | 2 | 87.35 | 89 | 41.8 | 79.1 |
| 0.5 | | 0.5 | 65.6 | 83.4 | 73.5 | 76.7 |
| | 30 | 1 | 100.4 | 86.1 | 82.25 | 78.7 |
| | | 1.5 | 89.15 | 84.8 | 80.1 | 78.4 |
| | | 2 | 59.95 | 95.8 | 76.1 | 78.9 |
| | | 0.5 | 89.4 | 107.9 | 100.25 | 81.3 |
| 0.5 | 32.5 | 1 | 85.95 | 88.3 | 75.9 | 79.4 |
| | | 1.5 | 69.6 | 68.7 | 55.75 | 64.7 |
| | | 2 | 59.2 | 46.4 | 50.2 | 73.3 |
| | | 0.5 | 87.35 | 93.4 | 107.2 | 72.7 |
| | 35 | 1 | 87.95 | 80.7 | 78.25 | 78.3 |
| | | 1.5 | 80.45 | 73.1 | 84.75 | 60.7 |
| | | 2 | 66.35 | 53.9 | 75.95 | 62.25 |
| | | 0.5 | 77.7 | 111.9 | 108.85 | 82.3 |
| | 30 | 1 | 53.6 | 63.5 | 83.4 | 95.2 |
| | | 1.5 | 37.8 | 103.2 | 70.1 | 56.9 |
| | | 2 | 65.9 | 31.1 | 42 | 55.3 |
| | | 0.5 | 53.8 | 100.6 | 55 | 45.8 |
| 0.7 | 32.5 | 1 | 83.65 | 70.4 | 69.75 | 70.6 |
| | | 1.5 | 54.95 | 55.8 | 58.95 | 42.1 |
| | | 2 | 33.4 | 13 | 66.4 | 30.65 |
| | | 0.5 | 95.75 | 86.8 | 98.5 | 92.5 |
| | 35 | 1 | 83.9 | 30.25 | 60.85 | 75.5 |
| | | 1.5 | 40.65 | 37.5 | 60.15 | 56.8 |
| | | 2 | 37.4 | 14 | 15.9 | 32.5 |

**Least Significant Difference = 15.1; Degrees of Freedom =144; Co-efficient of variation % = 10.2

Seedling height decreased with increased EMS dose, high treatment temperature and exposure time. The seedling height varied between 14 mm (0.7% EMS, 35ºC, 2hr) and 111 mm (0.3%

EMS, 30°C, 1.5 hr) (Table 2.4). Seedling height showed significant differences (P< 0.001) at 7th and 14th days after EMS treatment. On both measurements, seedling heights were affected adversely due to the mutagenic treatment. The most common physiological injuries caused by the mutagenic treatment are reduction in seedling height, most frequently used identification of injury in the first generation of mutation (M1). Although all doses of EMS mutagen elicited a reducing effect on seedling height, some of the seedlings at 2 hr treatment, 0.7% EMS concentration and at a temperature of 35°C displayed an increase in height, especially SST-387, which may be associated the mutation in major or minor genes (Kleinhofs *et al.*, 1978a).

**Table 2.5.** Correlation coefficients showing pairwise association between days to 50% emergence, germination percentage and seedling height in wheat.

| Character | DTE | GPT | SH |
|---|---|---|---|
| DTE | 1 | -0.86** | -0.70** |
| GPT | | 1 | 0.96** |
| SH | | | 1 |

**=correlation is significant at P=0.01

DTE = Days to 50% emergence; GPT = Germination percentage; SH = Seedling height

Table 2.5 summarizes the pair-wise correlations among the three traits. There were significant negative associations between days to 50% emergence with germination percentage and seedling height. Thus, early days to emergence could not provide high germination percentage and seedling height in the tested varieties. There was significant positive correlation between germination percentage and seedling height (r=0.96, P<0.01) (Table 2.5).

### *2.4.1. General observations*

Visible differences were noted between seedlings of the control and the EMS treated seedlings, as well as among treated seedlings (Figure 2.2). In Figure 2.2, the differences in sizes between seedlings of the control and EMS treated seeds of the four wheat varieties was clearly evident, although germination percentage was significantly higher in the EMS treated seeds (Figure -2.2), the total survival was not significantly different. The relative survival was significantly lower

than the control (Table 2.2). There was no significant difference among EMS treated seedlings compared with the control (Figure 2.2).

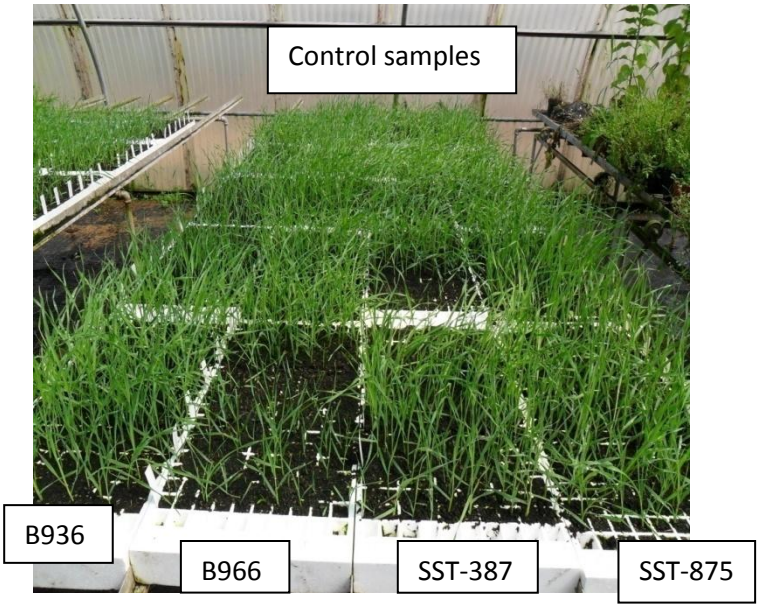


**Figure 2.2.** Treated (0.5% EMS dose, 35°C and 2 hr) and untreated four wheat varieties compared at two weeks after germination.

The reduction in seed germination occurred as a results of high EMS dose on the treated seeds. Although there was reduction or no germination of seeds, only during the first week with the highest dose of EMS, in the following weeks the mutants recovered germination even at the highest EMS treatment dose (0.7%), long exposure time (2hr) and high temperature (35ºC), However after that slow germination process the mutants would struggle to grow taller due to the effective EMS mutagenesis. Highest germination was noticed in the control treatment where the seeds were not exposed to EMS.

## 2.5. DISCUSSION

The results suggest that number of days to 50% emergence, germination percentage and seedling height can be used with equal reliability for estimating the suitable doses of EMS for treatment on a large scale in a mutation induction program. The ultimate aim of a mutagenic treatment is to induce mutations to create variability of seed germplasm that breeders can apply to select economically important traits among mutants. For breeding purposes mutagenic treatments with low physiological effects and strong genetic effects are desirable.

Alkylating agents such as EMS induce modifications, through chemical mutagenesis, to be used for generating breeding lines (Lee *et al*., 2003; Leake, 1967) and are apparent by the results in the current work. Modifications by EMS are mainly due to mispairing and base changes, that is, chemical alterations of nucleotides in a genome (Kim *et al*., 2004). The majority of about 99%, EMS induces C-to-T changes resulting in C/G to T/A substitution (Greene *et al*., 2003; Kovalchuk *et al*., 2000).

Ethylmethanesulfonate mutagenesis generates randomly distributed mutations throughout the genome as shown by Greene *et al*. (2003) in *Arabidopsis*. As a result, chemical mutagenesis can be used not only to search for loss- or gain- of functional mutants and also to understand the role of specific amino acid residues in protein function (Kim *et al*., 2004). The changes in the mean values of agronomic traits after mutagenic treatments was reported earlier in many pulse crops, which is also in agreement with our studies that include lentil (Singh *et al*., 2000), urdbean (Deepalakshmi and Kumar, 2003; Wani *et al*., 2005; Tah, 2006) and mungbean (Arulabalchandran and Mullainathan, 2009).

### *2.5.1. Seedling emergence*

The result of this work showed that all the variables studied were affected by EMS treatment (Table 2.2). The delay in seedling emergence and reduction in seed germination with increasing mutagen concentration was reported (Adamu *et al*., 2002). Time and rate of seedling emergence are controlled by an array of interacting factors including genetic constitution, seed dormancy, seed vigor, depth of planting, soil impedance and aeration, temperature and water supply (Forcella et *al*., 2000; Samarah and Al-Kofahi, 2008). Accordingly, there was significant

negative correlation between days to 50% emergence with germination percentage and seedling height. However, there was significant positive correlation between germination percentage and seedling height (Table 2.5).

### *2.5.2. Seed germination*

Seed germination percentage was highly significant (P<.001). The mean values on germination percentage in the treated seeds for various mutagenic treatments in all the varieties are given in Table 2.3. Germination percentages decreased with increased mutagen concentration. The highest germination percentage value was observed on the control at 97% while 21% germination was noted when using EMS at 0.70%. In the following days, germination values were very close to each other with high percentage values. Rupinder and Kole (2005) stated that the severe reduction in germination is an indication of effective mutagenesis and Khan *et al.* (2004) also pointed out that mutagenic treatments brought reduction in seed germination. In our assays, such reduction was observed on the first day with the highest dose of EMS i.e. 0.7% v/v. Thus it may be suggested that there is only a single day delay in germination. Slow germination with the highest dose EMS treatment of seeds would be expected (Menda *et al.,* 2004).

Concentration of mutagen is the most critical factor with the results of assays depending to a great extent on the use of optimal concentrations of the mutagen that can be observed in mutants, which could be positive answers as well can results in seedling injury (Mba *et al.*, 2007). As a rule, an increase in the concentration of EMS, for instance, normally results in more mutation events, but these are accompanied by a corresponding greater amount of injury to seedlings and lethality (Mba *et al.,* 2007). Studies by Kleinhofs *et al.,* (1978b), suggested that 0.5% EMS dose increases mutations and reduces germination in barley. Higher doses of EMS can also cause physiological changes and consequent cell death. In our studies it was observed that high EMS dose reduces germination and seedling height and also prolonged the number of days for the seedlings to emerge.

The decrease in germination percentage due to mutagenic treatments observed was also in conformity with the earlier report by Deepalakshmi, (2000) and Thanga Hemavathi, (2002) in black gram. In a similar report by Gaul (1970), damage to the biological material of the seed as

reflected in the study could be considered as an indication of the mutagenic effects (Table 2.3). In the present study considerable reduction in seed germination was noted. In all the cultivars, in comparison to the control, the germination percentage was low in all the EMS treatments. Similar results were also reported by Padavai and Dhanavel (2004). In general, the reduction in germination percentage was associated with the increase in the dose/concentration of mutagens (EMS) (Singh and Kole, 2005).

The decrease in germination at higher doses of mutagens may also be attributed to disturbances at cellular level including chromosomal damages or due to the combined effect of both physical and physiological factors (Ahloowalia *et al.,* 2001). Disturbance in the formation of enzymes involved in the germination process may be one of the physiological effects caused by mutagenic treatments particularly chemical mutagens like EMS leading to decrease in germination. The higher concentration of EMS could have simply lowered the water potential outside the seeds to be more negative and therefore the seeds could not imbibe enough water and could not germinate or germinated slowly (Singh *et al.,* 2000).

Reduced growth due to higher mutagen doses was also explained differently by different researchers. It could be attributed to one or more of the following reasons; the increase in destruction of growth inhibitors, the increase in growth promoters, the sudden increase in metabolic status of seeds at certain levels of mutagen dose or it could be due to the induced chromosomal aberrations (Wang and Yu, 1988: Solanki and Sharma, 1999; Solanki and Sharma, 2002;, Kumar and Selvaraj, 2003; Solanki and Phogat, 2005).

Temperature plays a major role in inducing mutation in wheat through chemical mutagenesis. In chemical mutagenesis temperature has an effect on the half life of the mutagen, high temperature accelerating the reaction. For EMS, the optimal temperature to achieve a half life of 26 h is 30°C (Mba *et al.,* 2007). Another experimental factor affecting the success of mutagenesis is the pH of the environment in chemical mutagenesis, for example EMS is most effective at pH 7.0 (Rupinder and Cole, 2005). Temperature influences the rate of hydrolysis of the mutagenic solution; at low temperatures, hydrolysis rate is decreased, implying that mutagen remains stable for longer period (Mba *et al.,* 2007).

Exposure time to mutagen treatment increases mutation rate (Ahloowalia *et al.,* 2001). The mutagenic treatment duration should be long enough to permit hydration and infusion of the mutagen to target tissue. The relevant seed characteristics that impact on this include seed size, permeability of the seed coat and cell constituents. Additionally, in order to minimize theunintended effects of EMS hydrolysis and maintain the mutagen concentration, the treatment solution should be buffered or renewed with newly prepared EMS solution when the treatment duration is longer than the half life of the mutagen (Mba *et al.,* 2007).

### *2.5.3. Seedling height*

Ethylmethanesulfonate is a strong mutagen and growth of the plant parts are strongly inhibited with increasing EMS concentration and treatment duration. The impact of EMS was observed in barley and it was very effective in inducing mutations with respect to germination capacity, seedling height, seedling survival and yield per plant (Adamu and Aliyu, 2007). Rupinder and Kole (2005) showed that seedling tend to be affected adversely due to mutagenic treatment. Therefore from the results obtained in figure 2, it could be suggested that higher EMS concentration is positively correlated to increased levels of induced genetic variations to the treated seeds. This is in agreement with the results obtained in this study (Figure 2.1).

Seedling height is widely used as a criterion in determining the biological effects of various mutagens in M1 plants (Ahloowalia *et al.,* 2001). The present study showed that seedlings height decreased in all varieties with increasing EMS dose (Figure 2.1). Significant differences ($p<0.01$) among varieties in response to the various doses of EMS were observed for seedling height. This difference is more pronounced with the highest EMS doses.

The effect of different concentration of EMS treatment on seedling height was clearly observed in different crops such as barley, maize, sorghum and rice. In our assay, the lowest seedling height was 14 mm (Table 4) as recorded on the $14^{th}$ day after sowing for the group treatment combination exposed to 0.7% EMS dose for 2 hours at 35ºC. This was in agreement with the report by Ilbas *et al.,* (2005).

### *2.5.4. Conditions for seed treatment with EMS and germination*

From the outcomes of this study it was found that the effect of chemical mutagen, EMS, depends on variety, treatment temperature and duration. To enhance mutagenic effectiveness and efficiency of EMS, specific information is required about the effect of time, temperature of seed soaking and various concentration of EMS. It was also found that EMS dose at the high rate (0.7%) considerably reduced seed germination, seedling height and delayed seedling emergence. However, there are contradictory reports on the mutagenic effects of the EMS at various concentrations in wheat (Mba *et al.,* 2007). This can be probably attributed to the different treatment conditions (presoaking, temperature and a time of exposure) and different varieties used. The various concentration of EMS showed different effects of mutagenesis as mentioned by many authors in literature. Kleinhofs *et al., (*1978b) suggested that 0.5% EMS dose increases mutations and reduce germination and plant height in barley.

Results of other studies suggest that use of chemically induced mutants can also provide useful information for understanding the functions of essential genes by generating weak nonlethal alleles. In the present study the results for the decrease in mean values of various quantitative traits at higher concentrations of EMS is in agreement with the hypothesis that, due to mutagenic treatment, average values are shifted to a direction opposite to selection (Bhatia and Swaminathan, 1962), whereas the increase in mean values could be due to the occurrence of polygenic mutations with cumulative effects (Singh *et al*., 2001).

As the EMS doses increase, the gain tended to sink, but to stabilize at highest doses, exclusively for this character. The behaviour of wheat genotypes subjected to increasing EMS doses is consistent with the results of Donini and Sonnino (1998) on triticale and Rasmussen (1991) on white oat.

### *2.5.5. Conditions to be considered during seed treatment with EMS*

There are some precautionary measures that need to be considered when doing chemical mutagenesis with EMS. The amount of EMS used should be accurate to be more precise and get the correct results, temperature should be kept constant for all the replications and the exposure time should also be the same for all similar treatments.

## 2.6. CONCLUSIONS

The study found that increased EMS doses decreased seedling emergence, germination percentage, and seedling height in wheat. Reduction in seed germination was only at the first few days with the highest dose of EMS, in the following days the mutants germinated even at the highest EMS treatment dose (0.7%). Other treatment combinations resulted into higher germination percentages ranging from 70 to 97% similar to control or severely reduced germination capacity (%) and seedling heights in the harsh conditions (0.7% EMS, 35ºC, and 2 hr). Seedling heights decreased with increase in EMS dose, temperature and exposure time. EMS dose of 0.5% treatment in different durations (1.5-2 hr) and different temperature regimes (32.5 - 35°C) per variety is high enough to produce variability as followed by the other traits under assessments. However, low level of germination was similar to the control at later days. EMS dose treatment (0.5%) at different time durations (1.5 and 2 hr) and in different temperature regimes (32.5 and 35ºC) was observed to be the best treatment combination for effective seedling emergence, as well as seedling development and survival.Therefore ideal conditions for different varieties were observed at EMS level of 0.5%, 35°C and 2 hr exposure time that were ideal in B936; EMS at 0.5%, 35°C and 1.5 hr in B966 and EMS at 0.5%, 32.5°C and 2 hr in SST387. SST875 required EMS dose at 0.5%, 32.5°C and 1 hr treatment. As a result, the study established the requirement of variety specific EMS dose and treatment temperature and duration that could be used for inducing large scale mutation to select targeted mutant individuals in wheat.

## 2.7. Further challenges in mutation breeding in wheat

The foregoing study clearly demonstrated that lower doses of various mutagens, either alone or in combination, induce much more useful variability than higher doses. However, work on mutation breeding of wheat is limited and only a few mutants carrying one or two useful attributes have been obtained so far. Therefore, there is a need to initiate extensive research work at lower doses of various mutagens alone or in combination to induce desirable variability in vitro as well as in vivo in order to exploit the same in breeding for developing early maturing, herbicide resistant, and high seed yielding wheat cultivars.

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# CHAPTER 3

# AGRO-MORPHOLOGICAL VARIATIONS AMONG TWO SELECTED WHEAT VARIETIES AFTER ETHYL METHANESULPHONATE MUTAGENESIS

# Abstract

The study aimed to investigate variations in agro-morphological traits in two selected wheat varietiesafter chemical mutagenesis using ethylmethanesulphonate (EMS). Two varieties (SST56 and SST875) were subjected to EMS mutagenesis using 0.5% v/v EMS at 32.5$^{o}$C for 1 hr. Field trials were carried out at Ukulinga research farm of the University of KwaZulu-Natal in the randomized complete block design with two replications. Data on nine important agro-morphological traits were collected and analyzed using the analysis of variance (ANOVA), correlation and principal component analysis (PCA) procedures. Significant variations were found among theagro-morphological traits between $M_1$ individuals of the varieties after the mutagenesis compared to untreated checks. The mutagenesis significantly reduced seed germination in the field at 40% in both varieties. The treatment significantly delayed days to heading by 8 days and shortened days to maturity by 13 days in both varieties. EMS treatment also significantly reduced plant height at 18 cm in SST56 and 21 cm in SST875 and spike length reduced by ~2.5 cm in both varieties. Hundred seed weight, flag leaf length and seeds per spike significantly increased in the treated seeds of both varieties. Plant height had positive and significant correlation with number of tillers, number of seeds per spike, flag leaf length and 100 seed weight. However, it had negative correlation with the number of days to maturity. PCA revealed that three principal components (PC1, PC2 and PC3) accounted to 57% of the total variations among the agro-morphological traits in both varieties. PC1 alone contributed to 27.7% of the variation which was correlated well with plant height (0.767), tiller number (0.812), number of seeds per spike (0.599) and seed yield (0.720). PC2 explained 15.6% of the variation and well-correlated with germination percentage (0.784), spike length (0.554) and flag leaf length (0.772). PC3 accounted to 12.4% of the variation and had negative correlation with days to maturity (-0.730). The study found that EMS has the potential to increase agro-morphological variations in wheat to select useful and novel mutants with desired phenotypic traits.



***Keywords:*** Agro-morphological traits, Ethylmethanesulphonate, Wheat.

# 3.1. Introduction

Mutation breeding relies on extensive use of genetic variants to improve agro-morphological characteristics in crop plants. However, an efficient genetic improvement of a cultivar depends on the knowledge of mode of gene action, genetic variability and the interrelationships among important agro-morphological attributes. Induced mutagenesis is a significant tool to create variability in a short period of time (Yaqoob and Rashid, 2001; Akgun and Tosun, 2004). Induced mutations lead genetic changes of loci controlling economically important traits and eliminate undesirable genes from elite breeding lines (Ram Din *et al*., 2003).

The technology of mutation induction has become an established tool in plant breeding in order to supplement genetic variations in the existing germplasm and to improve cultivars in specific unique agro-morphological attributes. Improved varieties of many crops have been released to farmers as a result of induced mutation which have been used directly as new cultivars or in cross breeding programs (Njau *et al.,* 2005). Mutations may arise spontaneously or they may be induced by using radioactive or chemical mutagens. Among the chemical mutagens, ethylmethanesulphonate (EMS) induces a vastly higher proportion of point mutations (Jander *et al.,* 2003). Some studies have shown that EMS is an effective chemical mutagen in several species (Alcantara *et al.,*, 1996; Watanabe *et al.,* 2007)

The application of induced mutagenesis has brought several benefits in the modern agricultural production as a method forcrop improvement and addition of new valuable traits into the existing varieties (Ahloowalia *et al.,* 2004).EMS belongs to the group ofalkylating agents which are well known mutation inducers, causing point mutations at nucleotide levels as well as loss ofchromosome segments or deletions (Alcantara *et al.,* 1996). A large number of mutationsin plants (Jander *et al.,* 2003) and crop cultivars (Ilirjana *et al.,* 2007) have beenachieved by the use of EMS e.g. herbicide resistance in maize (Maina *et al.,* 2003).

In mutagenesis, EMS is effective because it form adducts with nucleotides, causing them to mispair with their complementary bases, thus introducing base changes after replication (Greene *et al*., 2003).Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. This will lead in phenotypic changes including plant height, maturity, sterility, yield, chemical composition, pathogen and pest resistance and adaptability to adverse

environmental conditions. The most important parameters for inducing mutation with EMS are concentration, treatment duration and temperature (Alacantara *et al*., 1996). It has also been established that chemical mutagens such as EMS provide opportunities to increase genetic variability in crops of quantitatively inherited characters (Sharma *et al*., 1993).

Seed mutagenesis is employed successfully for early flowering in spring rape (Thurling and Depittayanan, 1992), herbicide tolerance in soybean (Sebastian *et al*., 1989) and male sterility in wheat (Maan and Williams, 1984) and for reduced plant height and high grain rice (Baloch *et al*., 2002). High efficiency of EMS were observed in tomato for creating phenotypic variation such as leaf shapes, reduced fruit size, and maximum disease resistance (Watanabe *et al.*, 2007).

Sustainable yield and enhancement of nutritional value of crops through plant breeding programs using chemical mutagenesis is well recognized worldwide. Increased crop yields, based on the better efficiency in using agricultural inputs (fertilizers, pesticides, herbicides, crop rotation and use of agricultural machinery) would not be possible without varieties designed to meet the specific agro-climatic conditions. Induced mutagenesis is useful in various areas such as in studying gene structure, expression and regulation, and for exploring genomes. Further, many plant breeders and geneticists have started to investigate the use of induced mutations for changing important agro-morphological attributes (FAO, 2008).

The prime strategy in mutation breeding has been to improve the well adapted plant varieties by altering one or two major traits which limit their productivity or enhance their quality. These include characters such as plant height, maturity, seed shattering, and disease resistance, which significantly contribute to increased yield potential and quality traits. However, in many cases, the changed traits had a synergistic effect on the cultivation of the crop, agro-morphological inputs, crop rotation and utilization (Ahloowalia *et al.*, 2004).

Treatment with mutagens alters genes or breaks chromosomes; however gene mutations may occur naturally as errors in DNA replication and most of these errors are repaired but some may pass to the next cell division to become established in the offspring as spontaneous mutations. Gene mutations without phenotypic expressions are usually not recognized and consequently, genetic variation appears rather limited and breeders have to resort to mutation induction (Wani and Khan, 2006).Mutations have played a great role in increasing world food security, since new

food crop varieties embedded with various induced mutations have contributed to the significant increase of crop production and improved yield (Khan and Goyal, 2009). Mutation induction offers the possibility of inducing desired attributes that either cannot be found in nature or have been lost during evaluation. Wheat is an important food crop in South Africa after maize (FAO, 2009). Mutagenesis in this highly self-fertilising crop is beneficial to induce genetic variation with unique agro-morphological attributes (Wani and Khan, 2006).

Chemical mutagenesis is regarded as one of the most effective and important tool in improving the agro-morphological attributes yield and quality characters of crop plants. In general, alkylating agents are very effective mutagens in higher plants. As a result, EMS has become an important tool to enhance unique agro-morphological attributes of crop plants and the role of mutation breeding in increasing the genetic variability using chemical mutagenesis for quantitative and qualitative traits in various crop plants have been reported by a number of scientists (Rachovska and Dimova, 2000; Kumar and Mishra, 2004; Singh *et al*., 2008; Tah, 2006; Bhat *et al*., 2007; Adamu and Aliyu, 2007; Kozgar *et al*., 2011; Mostafa, 2011).

Mutagenesis research in wheat and barley has been reviewed and reported by various workers (Ilbas *et al.,* 2005, Reddy, 1992 and Rachovska, 1998). Wheat (*Triticum aestivum* L.; 2n=6x=42; AABBDD) being a highly self-pollinating polyploid, offers many opportunities for the exploitation of mutations, recombination and of increasing genetic variability in qualitatively or quantitatively inherited characters (Rachovska and Dimova, 2000). The presence of many triplicated and duplicated loci in wheat allows a large number of induced changes to be preserved and transmitted to the next generation. EMS is useful in the induction of directed changes for redesigning ideotypes suitable for various agricultural environments. Induced mutations are also useful when it is desired to improve one or two easily identifiable characters in an otherwise well adapted variety. Thus, the objective of this study was to investigate variations in agro-morphological traits in two selected wheat varieties after chemical mutagenesis using ethylmethanesulphonate (EMS).

## 3.2. Material and methods

### *3.2.1. Plant material*

The present study used two wheat varieties i.e., SST56 and SST875, selected on the basis of their response in the earlier experimental results obtained from the preliminary studies (Chapter 2) after EMS treatment in the controlled environmental facility. The varieties posses suitable agro-morphological traits for comparative mutagenesis studies. The two varieties are recommended for the dry semi-arid zones of South Africa as they are considered early maturing.

### *3.2.2. Seeds treatment and EMS preparation*

An EMS mutagenesis protocol originally proposed by Mba *et al.,* (2007) was customized and applied in treating selected wheat seeds (see chapter 2). Prior to EMS mutagenesis seeds of both varieties were surface sterilized by soaking in 70% ethanol for 1 minute, and then washing three times under running water at room temperature. Subsequently, seeds were soaked in 30% JIK [sodium hypochlorite (NaOCl) at 10 - 12.5% (M/V)] for 5 minutes, and then washed three times under running water for 1 minute. Seeds were then soaked in distilled water for about 18–20 h at 20–22°C. Treated seeds were kept in specially made mesh bags of 11 x 8 cm dimensions for EMS mutagenesis. At the end of the pre-soaking stage, fresh EMS solutionwas prepared according to the desired concentration of EMS 0.5% v/v. DMSO solution 2% (v/v) was prepared into a 1000 ml bottle and autoclaved at 120°C for 15 min at 103.5 kPa (15 psi) and the mixture left to cool at room temperature. 5.0 ml EMS solution was then added to a 1000 ml distilled water containing 2% (v/v) DMSO solution and the solution was shaken vigorously to give a homogeneous mixture. After 18 – 20h at the end of the pre-soaking duration, the bags were removed from distilled water and shaken to remove excess water.With the exception of the control bags, the seeds were soaked in the EMS solutions. Both the SST56 and SST875treatment setswere soaked in 0.5% v/v EMS solution at 32.5°C for 1 hour. The temperatures were maintained in a water bath during the duration of treatment. After treatment, the seeds were washed under running cold tap water for 2–3 hours to remove excess EMS chemical and to make them safe for handling. The mesh bags were shaken off to remove excess water and the seeds placed on a paper towel for blot drying.

### *3.2.3. Trial set up, field arrangement and data assembling*

Fresh treated seeds (45000) and untreated checks per variety were field planted using the randomized complete block design with two replications. Each treatment comprised of 24 rows of 64 $m^2$ (16 m x 4 m) plot size in each replication. Planting was done using an inter-row spacing of 30cm. Other cultural practices were followed as recommended to grow wheat.

### *3.2.4. Measurements and data analysis*

The following data were collected: germination percentage (GP) was determined after two weeks of planting in the field; heading date (HD) was recorded as number of days from planting to the date when 50% of the spikes in the line were fully emerged from the flag leaf. Plant height (PH) was measured (in cm) from the base of the plant to the tip of the spike. Flag leaf length (FL) was measured (cm) from the base to the tip of fully expanded flag leaf when 50% of the spikes of the line were fully emerged. Productive tiller numbers (TN) were counted from each plant during harvest. Spike length (SL) was measured from the base to the tip of the spike length of the primary tiller. Seeds per spike (SPS) were counted during harvest from the primary tiller. These measurements were made on primary tiller to provide equal chance of representation and minimize confounding measurement effects from late emerging tillers. Seed weight (SW) in grams was measured from a random sample of 100 seeds. PH, FL, TN, SL and SPS were recorded on single plant basis by randomly taking ten tagged plants from each experimental plot.
To determine significance differences between treatments analysis of variance (ANOVA) was conducted using Genstat®($14^{th}$edition, VSN International, UK) on all the measured characters as proposed by Steel and Torrie (1980). After determining the significant differences, the data were further subjected to mean comparisons and correlation analysis on all the traits according to the method proposed by Searle (1961). A further principal component analyses (PCA) was conducted to identify the principal components and determine the magnitude of the contribution of each component to the total phenotypic variation among the parameters according to Kwon and Torrie (1964).

## 3.3. Results

The analysis of variance for germination percentage, plant height, number of productive tillers, spike length, number of seeds per spike and 100 seeds weight of the varieties SST56 and SST875 is presented in Table 3.1. The results of this study indicated significant interactions ($P<0.05$) among the agronomic traits studied (Table 3.1). The mean squares values and significance levels ($P\leq 0.05$) are indicated intheanalysis of variance (ANOVA) tables to discern significant differences. Corresponding means and ranges, standard errors, least significant differences and co-efficient of variations for various traits are presented in Tables 3.2, 3.3 and 3.5.

### *3.3.1. Response of agro-morphological traits in SST56 and SST875 after EMS mutagenesis*

In SST56 (Table 3.2) EMS treatment significantly enhanced days to heading by 8 days and reduced maturity by 13 days. Germination percentage was significantly reduced; treated seeds germinated at 53% while untreated controlcheck germinated at 93%. Plant height varied from 51-69 cm. The mean seed yield was 118.7 kg per plot. Untreated check of the variety yielded 127.1 kg/plot. EMS has significantly reduced seed yield. EMS treatment increased hundred seed weight; however the increase was not significant. Seeds per spike were significantly increased. There was an increase of seven seeds per spike due to the effect of EMS treatment.

In SST875 (Table 3.3), EMS significantly enhanced days to heading by 11 days and reduced maturity by 13 days. Germination percentage was reduced to 48% in EMS treated plants while 87% germination was observed in the control treatment. Seed yield was highly and significantly reduced. Hundred seed weight was significantly increased, whilst plant height was significantly reduced by 21 cm (from 77 cm to 56 cm). Seeds per spike were significantly reduced (from 6 to 4 seeds). Overall (Table 3.4), the effect ofEMS treatment was significantly pronounced on SST875 treated seeds compared to SST56.

**Table 3.1.** Analysis of variance of agro-morphological traits in M1 generation of wheat varieties SST56 and SST875 tested with and without EMS mutagenesis using two replications.

| Source of variation | d.f. | GPT | | PH | | FLL | | TN | | SL | | HD | | SPS | | SW | | SY | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. | m.s. | F pr. |
| **Replication** | 1 | 48.00 | 0.004 | 168.75 | 0.028 | 366.3 | 0.305 | 3.0000 | 0.184 | 0.1633 | 0.002 | 0.0833 | 0.012 | 4.687 | 0.003 | 0.0003 | 0.231 | 2.5652 | 0.026 |
| **Var.** | 1 | 33.33 | 0.161 | 75 | 0.158 | 5.1 | 0.927 | 0.083 | 0.705 | 0.4408 | 0.23 | 75.2 | <.001 | 93.521 | <.001 | 0.0025 | 0.892 | 1.3333 | 0.097 |
| **EMS** | 1 | 168.75 | 0.003 | 5.33 | 0.701 | 1548.1 | 0.121 | 1.333 | 0.138 | 4.4408 | <.001 | 3.7673 | 0.015 | 247.52 | <.001 | 0 | 0.987 | 40.333 | <.001 |
| **Var.EMS** | 1 | 192 | 0.002 | 24.08 | 0.017 | 1713.6 | 0.104 | 1.033 | 0.108 | 0.0833 | 0.097 | 0.3333 | 0.088 | 63.021 | 0.004 | 0.0896 | 0.018 | 1.3333 | 0.097 |
| **Residual** | 3 | 15.91 | | 35.23 | | 598.3 | | 0.565 | | 0.2894 | | 0.4312 | | 6.166 | | 0.132 | | 0.4457 | |
| **S.E (±)** | | 4.153 | | 6.387 | | 25.26 | | 0.816 | | 0.5331 | | 0.6455 | | 2.471 | | 0.3557 | | 3.876 | |
| **L.S.D.$_{(5\ \%)}$** | | 3.500 | | 1.844 | | 20.44 | | 0.688 | | 0.4492 | | 0.5439 | | 2.082 | | 0.2997 | | 25.3 | |
| **C.V %** | | 11.2 | | 13 | | 18.2 | | 24.8 | | 9.9 | | 1.5 | | 32.5 | | 8 | | 10.3 | |

GPT = Germination percentage, PH = Plant height, FLL = Flag leaf length, TN = Tiller number, SL = Spike length, HD = Heading date, SPS = Number of seeds per spikelet, SW = 100 seeds weight, SY = Seed yield

L.S.D. = least significant difference at 0.05 probability level; CV = co-efficient of variation; S.E= standard error

**Table 3.2.** Mean values of agro-morphological attributes for the wheat variety SST56 when treated with and without EMS**.**

| | Agronomic traits | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Source of variation** | **GPT** | **PH (cm)** | **FLL (cm)** | **TN** | **SL (cm)** | **HD** | **DTM** | **SPS** | **SW (g)** | **SY (kg/ha)** |
| **SST56 + EMS** | 53.17 | 51 | 26.43 | 6.48 | 6.967 | 51 | 112 | 12.42 | 4.884 | 1898.9 |
| **SST56 Control** | 92.6 | 69 | 22.1 | 5.25 | 9.658 | 59 | 125 | 5.58 | 4.319 | 2032.8 |
| **S.E. (±)** | 3.256 | 2.88 | 3.989 | 1.129 | 0.982 | 0.249 | 1.94 | 2.124 | 0.524 | 27.60 |
| **L.S.D.$_{(5\ \%)}$** | 18.35 | 12.658 | 5.689 | 3.122 | 0.924 | 0.515 | 8.129 | 3.312 | 0.618 | 48.67 |
| **C.V. %** | 4.01 | 3.23 | 12.3 | 10.8 | 13.21 | 0.23 | 1.236 | 25.657 | 6.18 | 2.6 |

GPT = Germination percentage, PH = Plant height, FLL = Flag leaf length, TN = Tiller number, SL = Spike length, HD = Heading date, DTM = Days to maturity, SPS = Number of seeds per spikelet, SW = 100 seeds weight, SY = Seed yield

L.S.D. = least significant difference at 0.05 probability level; C.V. = co-efficient of variation; S.E. = standard error

**Table 3.3.** Meanvalues of agronomic traits for the wheat variety SST875when treated with and without EMS.

| Source of variation | Agronomic traits | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **GPT** | **PH (cm)** | **FLL (cm)** | **TN** | **SL (cm)** | **HD** | **DTM** | **SPS** | **SW** | **SY(kg/ha)** |
| **SST875 + EMS** | 48 | 56 | 23.3 | 7.3 | 7.5 | 53 | 114 | 7 | 4.912 | 2149.89 |
| **SST875 Control** | 87 | 77 | 31.86 | 6.67 | 9.3 | 61 | 127 | 6 | 4.35 | 2496.5 |
| **S.E (±)** | 2.253 | 4.18 | 3.91 | 0.712 | 1.253 | 0.5166 | 1.26 | 2.331 | 0.198 | 23.53 |
| **L.S.D.$_{(5\ \%)}$** | 25.286 | 9.18 | 20.66 | 2.135 | 0.849 | 2.236 | 8.157 | 3.256 | 0.513 | 47.86 |
| **C.V %** | 3.414 | 2.37 | 18.4 | 12.38 | 10.1 | 0.18 | 0.73 | 21.12 | 3.158 | 3.1 |

GPT = Germination percentage, PH = Plant height, FLL = Flag leaf length, TN = Tiller number, SL = Spike length, HD = Heading date, DTM = Days to maturity, SPS = Number of seeds per spikelet, SW = 100 seeds weight, SY = Seed yield

L.S.D. = least significant difference at 0.05 probability level; C.V. = co-efficient of variation; S.E. = standard error

**Table 3.4.** Combined mean values of different agro-morphological characteristics among two wheat varieties SST56 and SST875 when tested with and without EMS.

| Source of variation | Agronomic traits | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **GPT** | **PH (cm)** | **FLL (cm)** | **TN** | **SL (cm)** | **HD** | **DTM** | **SPS** | **SW** | **SY** |
| **SST56 + EMS** | 53.17 | 51 | 26.43 | 6.48 | 6.967 | 51 | 112 | 12.42 | 4.884 | 1898.9 |
| **SST56 Control** | 92.6 | 69 | 22.1 | 5.25 | 9.658 | 59 | 125 | 5.58 | 4.319 | 2032.8 |
| **SST875 + EMS** | 48 | 56 | 23.3 | 7.3 | 7.5 | 53 | 114 | 7 | 4.912 | 2149.89 |
| **SST875 Control** | 87 | 77 | 31.86 | 6.67 | 9.3 | 61 | 127 | 6 | 4.35 | 2496.5 |
| **S.E. (±)** | 2.253 | 4.37 | 3.91 | 0.752 | 0.5380 | 0.6566 | 1.67 | 2.483 | 0.21 | 24.56 |
| **L.S.D.$_{(5\ \%)}$** | 30.693 | 9.88 | 20.66 | 0.635 | 0.7869 | 0.5565 | 6.579 | 3.632 | 0.468 | 51.73 |
| **C.V. %** | 3.154 | 2.9 | 18.4 | 22.8 | 9.9 | 1.5 | 0.92 | 32.7 | 3.29 | 2.2 |

GPT = Germination percentage, PH = Plant height, FLL = Flag leaf length, TN = Tiller number, SL = Spike length, HD = Heading date, DTM = Days to maturity, SPS = Number of seeds per spikelet, SW = 100 seeds weight, SY = Seed yield

L.S.D. = least significant difference at 0.05 probability level; C.V. = co-efficient of variation; S.E. = standard error

### *3.3.2. Correlation analysis*

Table 3.5 presents pair-wise correlation coefficients among agro-morphological traits with their levels of significance. Correlation coefficients were separately estimated for EMS treated and untreated groups.In both cases, plant height had positive and significant association with number of tillers, number of seeds per spike, flag leaf length and spike length. Positive correlation of plant height with number of tillers, number of seeds per spike and spike length was also reported by Sandhu and Mangat (1985) and Eunus *et al.*, (1986). However, plant height had negative correlation with the number of days to maturity. Negative correlation of plant height with days to maturity was also reported by Chaudhry *et al.*, (1994) and Akbar *et al.*, (1995). Spike length was significant ($P<0.05$) and positively correlated with number of seeds per spike, 100 seed weight and seed yield. Seed yield was positively and significantly associated with number of tillers, plant height, 100 seed weight and number of seeds per spike. However a correlation with days to heading was significantly negative in both treated and untreated control checks. Heading date had a negative correlation with all the agro-morphological traits in both treated and untreated control checks except for the spike length.

A negative correlation existed between germination percentage with heading date but not significant ($P<0.05$). The length of the flag leaf showed no significant correlation with all the traits except with germination percentage and 100 seed weight in both untreated and treated wheat lines. 100 seed weight ($r=0.012$) and number of seeds per spike ($r=0.009$) had no significant correlation in both treated and untreated control checks (Table 3.5).

**Table 3.5.** Pair-wisephenotypic correlation coefficients of agro-morphologicaltraits among M1 generation of EMS treated and untreated wheat varieties[a, b].

| Traits | GP | PH | HD | FLL | TN | SL | DTM | SPS | SW | SY |
|---|---|---|---|---|---|---|---|---|---|---|
| **GP** | 1 | 0.317 | -0.023 | 0.383 | 0.203 | 0.337 | 0.187 | 0.286 | 0.028 | 0.232 |
| **PH** | 0.214 | 1 | 0.032 | 0.251 | 0.514* | 0.444* | -0.033 | 0.311 | 0.113 | 0.483* |
| **HD** | -0.081 | 0.055 | 1 | -0.121 | -0.072 | 0.038 | -0.254 | -0.235 | -0.055 | -0.724** |
| **FLL** | 0.288 | 0.135 | -0.082 | 1 | 0.294 | 0.452* | 0.175 | -0.054 | 0.159 | 0.063 |
| **TN** | 0.108 | 0.488* | -0.102 | 0.311 | 1 | 0.329 | 0.108 | 0.282 | 0.182 | 0.424* |
| **SL** | 0.223 | 0.365 | 0.028 | 0.293 | 0.524* | 1 | 0.219 | 0.380** | 0.339 | 0.374 |
| **DTM** | 0.098 | -0.018 | -0.031 | 0.225 | 0.129 | 0.187 | 1 | -0.223 | -0.031 | 0.425* |
| **SPS** | 0.122 | 0.425* | -0.354 | -0.052 | 0.352 | 0.587** | -0.025 | 1 | 0.012 | 0.721** |
| **SW** | 0.095 | 0.114 | -0.025 | 0.425* | 0.258 | 0.698** | -0.251 | 0.009 | 1 | 0.734** |
| **SY** | 0.422* | 0.528* | -0.545 | 0.092 | 0.516* | 0.399 | 0.311 | 0.585** | 0.542* | 1 |

*and ** denote significant correlation at the 0.05 and 0.01 probability levels, respectively.

[a]Upper right diagonal = EMS treated; [b]lower left diagonal = EMS untreated check

GP=Germination percentage, PH=Plant height, HD=Heading date, FLL=Flag leaf length, TN=Tiller number, SL=Spike length, DTM=Days to maturity, SPS=Number of seeds per spike, SW=100 seeds weight and SY = Seed yield

### *3.3.3. Principal component analysis*

Principal component analysis was carried out to identify the most influential components and associated traits (Table 3.6). Three principal components i.e. PC1, PC2 and PC3 (Table 3.6) contributed 55.7% of the total variation in the study. PC1 alone contributed 27.7% of the variation which was correlated well with plant height (0.767), tiller number (0.812), number of seeds per spike (0.599) and seed yield. PC2 explained 15.6% of the variation which was well-correlated with germination percentage (0.784), spike length (0.554) and flag leaf length (0.772). Days to maturity had considerably high negative correlation (-0.730) and wasrepresented in PC3 which contributed to 12.4% the total variation in the study. Thus, the traits correlated with the three PCs were relevant to undertake direct or indirect selection in wheat.

**Table 3.6.** Principal component analyses (PCA) with total variances contributed by ten agro-morphological attributes in two wheat germplasm.

| Improved unique agro-morphological Attributes | Principal Components | | |
|---|---|---|---|
| | PC1 | PC2 | PC3 |
| **Germination percentage** | 0.178 | 0.784** | 0.214 |
| **Plant height** | 0.767** | 0.258 | 0.122 |
| **Days to heading** | -0.002 | 0.006 | -0.037 |
| **Flag leaf length** | 0.095 | 0.772** | -0.138 |
| **Number of effective tillers** | 0.812** | 0.088 | -0.180 |
| **Spike length** | 0.494 | 0.554** | 0.044 |
| **Days to maturity** | 0.005 | 0.306 | -0.730** |
| **Number of seeds per spikelet** | 0.599** | -0.016 | 0.499* |
| **100 seeds weight** | 0.119 | 0.069 | 0.086 |
| **Seed yield** | 0.724** | 0.335 | -0.217 |
| **Explained variance** | 5.47 | 2.47 | 1.38 |
| **Proportion of total variance (%)** | 27.7 | 15.6 | 12.4 |
| **Cumulative (%)** | 27.7 | 43.3 | 55.7 |

*and ** denote significant correlation at the 0.05 and 0.01 levels, respectively.

GP = Germination percentage, PH = Plant height, HD = Heading date, FLL = Flag leaf length, TN = Tiller number, SL = Spike length, HR = Herbicide rating, DTM = Days to maturity, SPS = Number of seeds per spikelet, SW = 100 seeds weight, SY = Seed yield

### *3.3.4. Chlorophyll mutation*

The wheat varieties treated with ethylmethanesulphonate showed chlorophyll mutations, as expected as a result of EMS mutagenesis when field grown. The frequencies of chlorophyll mutants were very low and hence not informative in regard to the mutation frequency and for possible associational analysis with theagro-morphological traits. The chlorophyll mutation which was identified in the individuals in this study was albino mutation. The seeds of the survived albino mutants where harvested and stored safe for further analysis in the M2 generation.

## 3.4. Discussion

The present study found significant interactions between varieties and EMS doses on the agro-morphological traits of the two wheat varieties (Table 1). Simple correlation and principal components analysis were utilized to verify the association and consequently to identify the best selection criteria.

Efficient mutagenesis is defined as the production of desirable changes (mutations) free from the usually associated undesirable changes such as chromosomal aberrations, sterility, lethality (FAO, 2008). Alcantara*et al.,* (1996) stated that the highest mutation rates also induce a high degree of lethality, sterility, and other undesirable effects. Ethylmethanesulphonate mutagenic treatment on seeds causes chromosomal aberrations that can adversely affect cell division (Ahloowalia *et al.,* 2004; and Zaka *et al*., 2004), for this reason in our study, delayed heading date and days to maturity of wheat seedlings could have been attributed to a delay in cell division due to the effects of mutagenesis.

From the practical breeding point of view, the mutagenic treatments that induce high mutation rates with the least accompanying deleterious effects are desirable. During the present investigation, through EMS, many agro-morphological attributes changes were induced. The genetic structure of our material was highly affected, favoring new genetic changes in the M1 generation. At phenotypic level number of effective tillers was positively and strongly associated with number of seeds per spike and spike length, while it had significantly negative correlations with heading date (Table 3.5). Similar results have also been obtained by Baser *et al.,* (2000).

The results indicated that number of effective or productive tillers is an important yield contributing factor and it can lead to more number of seeds per spike. These results are in conformation with the findings of Sharma (1993), Singh *et al.,* (1999), Baser *et al.,* (2000) and Shahid *et al.,* (2002). Spike length had positive relationship with number of seeds per spike. These results are supported by the findings of earlier researchers (Eunus *et al.,* 1986; Shah *et al.,* 1988; Gasper and Zama, 1990). Number of seeds per spike had negative correlation with heading date, flag leaf length and days to maturity, while it had positive and significant association with

number of effective tillers and 100 seeds weight. The findings of these results emphasized the role of number of seeds per spike upon ultimate increase of seed yield. These conclusions are in conformity with those of Akbar *et al.,* (1995).

The perusal of both the correlation coefficient results suggested that number of seeds per spike, number of effective tillers and spike length should be given prime importance regarding its contribution to seed yield (Table 3.5). These results are substantiated with those of Singh *et al*., (2008), Aycicek and Yildirim (2006), and Inamullah *et al.*, (2006). The results presented in Table 3.5 indicated that 100 seeds weight was positively and significantly correlated with seed yield at phenotypic levels. It was evident from the results that 100 seed weight had pronounced influence on wheat yield. The present findings are similar to those of Akbar *et al*. (1995), Baser *et al*. (2000), Aycicek and Yildirim (2006) and Inamullah *et al.* (2006), who also observed positive association of 100 seeds weight with seed yield. Days to heading varied between wheat germplasm, ranging from 41 days (SST056) to 48 days (SST875) (Table 3.4).Seed yield was significantly and positively correlated with number of seeds per spike, number of effective tillers and plant height suggesting that plants with more tillers bear plenty of spikes per plant and produce higher seed yield. The above findings agree with earlier reports (Kashif and Khaliq, 2004 and Akram *et al.,* 2008). The negative association of plant height with number of seeds per spike (Table 3.5) is due to the fact that an increase in plant height leads to an increase in biological yield or biomass, thereby, decreasing the number of seeds per spike. Dwarf wheat mutants are, by and large, reported to be good yielders due to increased harvest index (Srivastava *et al.,* 1988). There was a highly significant negative association between days to maturity with number of seeds per spike and flag leaf length with number of seeds per spike, suggesting that indirect selection for earliness alone may not be beneficial to realise optimum yield. Number of seeds per spike showed highly significant and positive association with number of effective tillers and seed yield (Table 3.5). A significant positive association was found between 100 seed weight and seed yield, which is in agreement with the earlier reports of Bekele (1990). Similarly, germination percentage was positively and significantly associated with days to maturity (Table 3.5). Among other characters, spike length showed positive association with plant height, number of seeds per spike, number of effective tillers and seed yield. Flag leaf length was positively and significantly associated with number of effective tillers at phenotypic level (Table 3.5).

Overall, an intensive selection for number of effective tillers, improved germination percentage, number of seeds spike and spike length will automatically improve seed yield in wheat. Since the four traits are correlated among themselves, selection in one of the traits will implicitly result in the improvement of the other traits.Knowledge of correlation alone is often misleading as the correlation observed may not be always true. Two characters may show correlation just because they are correlated with a common third one. In such cases, it becomes necessary to use a method which takes into account the causal relationship between the variables, in addition to the degree of such relationship.

Principal component analysis showed that agro-morphological traits like plant height, number of effective tillers, number of seeds per spike, yield and 100 seed weight were important traits for selection (Table 3.6). This means that an increase in one of the above traits may directly contribute to seed yield. Similar results were reported by Dhonde *et al*. (2000) and Satya *et al*. (2002). Through principal component analysis Mahesh *et al.*, (2001) also found similar results whereby the effect of number of productive tillers through number of spike per plant was responsible for its significant positive association with seed yield per plant. For flag leaf length, the direct effect was positive, while its association with seed yield was negative, indicating the importance of restricted selection for exploitation of the effect noticed. The positive contribution towards seed yield was exhibited by 100 seed weight through number of seeds per spike; while the maximum negative contribution was exhibited by number of seeds per spike through100 seed weight. The contribution of number of effective tillers was positive throughspike length, number of seeds per spike. Similarly, the contribution of 100 seed weight was positive through germination percentage, plant height and spike length. The present findings are similar to those of Baser *et al*. (2000), Aycicek and Yildirim (2006) and Inamullah *et al.* (2006). The spike length contributed positive effect towards seed yield only through number of seeds per spike. Based on principal component analysis, contribution of plant height; number of seeds spike and number of effective tillers per plant were maximum on seed yield.

## 3.5. Conclusion

The study found that EMS has the potential to increase agro-morphological variations in wheat to select useful and novel mutants with desired phenotypic traits as well as other useful traits such as herbicide resistance.The study provided preliminary evidence on the induction of genetic variability on yield and yield components in wheat. Thus, induced genetic variability can effectively be exploited for eliciting mutant strains possessing desirable attributes and for rectification of simply inherited morphological deficiencies.

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# CHAPTER 4

# SELECTION FOR HERBICIDE RESISTANCE IN WHEAT USING ETHYL METHANESULPHONATE MUTAGENESIS

# Abstract

Development of herbicide resistant wheat has a great economic value since weed control using post emergent herbicides would be possible to achieve high yield and quality produce. The objective of this study was to select herbicide resistant wheat germplasm after inducing genetic variation using the chemical mutagen, ethyl methanesulphonate (EMS). Seeds of two selected wheat varieties (SST56 and SST875) were treated with EMS at 0.5% concentration for 2 hrs at 35ºC. Treated seeds and comparative controls were planted at the experimental farm of the University of KwaZulu-Natal using the randomized complete block design. Four weeks after planting M1 plants and untreated standard checks were sprayed with two herbicides, i.e. metsulfuron-methyl and bromoxynil at three different doses *viz.* 2x, 4x and 8x above the recommended rate of 4 g $ha^{-1}$ and 2 kg $ha^{-1}$, respectively. Two weeks after the treatment herbicide resistance were assessed. Results showed significant difference among varieties, tested herbicides and doses used. The EMS treated wheat lines showed variable degree of herbicide resistance compared to untreated controls. The present study identified herbicide resistance individuals among the M1 plants which will be subjected for further selections to identify stable and herbicide resistance wheat lines.



***Keywords:*** Bromoxynil, EMS, Herbicide resistance, Metsulfuron-methyl, *Triticum aestivum*

# 4.1. Introduction

Crop production is the key to feeding the growing human population. Wheat (*Triticum aestivum* L.) is one of the principal staple and commodity crops in the world (Kangama and Rumei, 2005; FAO, 2006). In South Africa wheat is the second most important cereal crop after maize. However, farmers face continued crop losses due to weed infestation, recurrent drought, diseases and pests, among other factors. Further, global warming and subsequent climate change present widespread risk to crop production. Thus, significant mitigation strategies are required aimed at generating improved farming technologies (David and Rosamond, 2009).

Weed control is fundamental in crop production system to achieve high yield and quality (FAO, 2006). There are different methods of weed control including mechanical, chemical and biological. Mechanical methods include hand pulling, hand hoeing, tillage, crop rotation and burning. Chemical control methods include the use of herbicides, while biological control involves the use of bio-agents (insects or fungus or any other organism) that live on and attack specific weeds (Shahid *et al.,* 2002). The use of novel crop cultivars with herbicide resistance is relatively simple yet effective crop production technology. Use of herbicide resistance in crop production is the cheapest and most effective form of weed control both for small scale and commercial farmers.

Use of herbicides has transformed weed control because they are efficient and cost-effective. Ideally, herbicides should have both a good weed-killing ability and minimal environmental impact. Developing crops with herbicide resistance by either conventional breeding or using GMO technologies is a cheap and effective approach to weed control for small scale and commercial farmers (Sharma, 1993). The use of novel crop cultivars with herbicides resistance can be considered as one of the innovative crop production strategies. This approach is economical and effective for controlling weeds in both small scale and in commercial farmers (Valverde and Gressel, 2006).

Crop resistance to a given herbicide can be a powerful tool to selectively control weeds with efficient chemical use. In some crops, for example, wheat, maize, soybean and canola resistance

to imidazolinone herbicides (trade name Beyond® and Pursuit®) has been selected through mutation breeding and now commercially utilized (Valverde and Gressel, 2006). However, this production system imposes restricted planting only on some crop species due to herbicide carryover limiting options for rotational crops with possible economic loss (Prather *et al.,* 2000). Plant back intervals for Beyond® vary depending on different crops ranging from 3 months for alfalfa up to 26 months for sugar beet. In the case of barley, a common rotational crop following wheat is 9 to18 months' intervals (Duke *et al*., 1991).

Metsulfuron methyl (a sulphonyl urea herbicide) and bromoxynil (a nitrile herbicide) are among the most widely used selective herbicides in wheat production (Faulkner, 1982). These herbicides are toxic to plants and have strong weed-killing characteristics. Metsulfuron methyl is a sulfonyl urea herbicide initially registered by E.I. DuPont in 1986. It is a foliar herbicide registered for use on wheat and barley (EPA, 1986). It is used in the form of water dispersible granules with a formulation of 75% Metsulfuron-methyl active ingredient. Metsulfuron-methyl is among the group of herbicides collectively called acetolactate synthase (ALS) inhibiting herbicides as their mode of action is inhibition of the ALS biosynthesis pathway (Le *et al.,* 2005), the first enzyme that catalyses the biosynthesis of branched-chain amino acids, valine, leucine and isoleucine (Brown, 1990). Metsulfuron-methyl is applied as a selective post-emergence foliar herbicide in wheat at a recommended rate of 4 kg/ha. It has been widely used to control weeds in wheat, barley, rye, and pastures (Eberlein *et al*., 1999). It can be used in combination with other foliar herbicides. The herbicide is typically applied on cereals at 0.04 kg active ingredient/ha, and on non-crop areas at 0.16 kg active ingredient/ha. Metsulfuron-methyl is commercially available in the form of dry flowable formulations (Akbar *et al*., 1995).

Bromoxynil is a nitrile herbicide that is used for post-emergent control of annual broad spectrum of weeds. It is especially effective in the control of weeds in maize, sorghum, onions, flax, mint, turf, and on non-cropland. The compound inhibits cell division in the shoots and roots and photosynthesis, in the target plants (Searle, 1961). In wheat bromoxynil is applied at a recommended rate of 2 l $ha^{-1}$. This herbicide has a moderate to high mobility in the soil profile and is relatively persistent in the environment, especially when applied in winter. These factors would be of concern to the environment (Baser *et al*., 2000). However, metsulfuron-methyl is

biologically effective when applied at a very low rate of 2 g/ha and therefore the amounts which reach the soil are quite low. It is systemic and works rapidly after it is taken up by the plant.

Bromoxynil is an excellent option for the management of herbicide resistant weeds in cereals and pastures and it is effective against many small annual broadleaves (Malik and Singh, 1995). It also controls certain 2,4-D tolerant broadleaf weeds in wheat such as fiddleneck, mayweed chamomile, pineappleweed, speedwell, and wild buckwheat. It is mainly a contact herbicide and therefore is not effective against large weeds and perennials. Because it is not systemic, recovery of the crop is generally rapid with no lasting effect. Frequency and amount of leaf burn may be greater when crops are stressed by abrasive winds, cool to cold evening temperatures, or mechanical injury resulting from hail, sleet, or insects (Anthony *et al.,* 1998). To reduce the potential for temporary leaf burn, application should be made to dry foliage when weather conditions are not extreme.

Mutagenesis has been recognized as one of the most efficient method to induce genetic variation in plants. Through induced mutations, development of new variants is possible that could be manipulated in breeding programs. Mutation leads alteration of various traits in crop plants including plant height, improved nutritional quality, shorter growing period, increased tolerance or resistance to abiotic and biotic stresses in major crops such as wheat, rice, barley, cotton, peanuts, beans and maize (Neuffer *et al.,* 1997; Van Harten, 1998). Mutation induction is a flexible, workable, unregulated, non-hazardous and low-cost alternative to genetically modified organisms (GMOs) (Mba *et al.,* 2007). Initial studies on induced mutations were mainly directed to finding optimum combination of mutagen and dose to elicit the best response (Puchta, 2003). The importance of artificial mutations continues in inducing genetic variation and understanding gene function (Hohn and Puchta, 2003). Mutagenic agents can be divided into physical, chemical, and biological mutagens. Ethylmethanesulphonate (EMS) is one of the most widely used chemical mutagens to induce mutagenesis in crop plants. Most chemical mutagens lead to base pair substitutions, especially GC to AT such as in ethyl-methanesulfonate (EMS).

Weed competition is one of the important constraints, contributing towards low yield of wheat in South Africa. Among the weed control methods, the chemical weed control is one of the recent origins, which is being emphasized in modern agriculture (Taj *et al*. 1986). Different studies are

available on chemical weed control in wheat (Khan *et al.*, 1999; Khan *et al.*, 2004; Hassan *et al.*, 2003) as a better alternative to manual and other weeding methods because it is cheaper, faster and gives better weed control (Chikoye *et al.*, 2005). Thus, development of herbicide resistant wheat is important in wheat breeding through chemical mutagenesis using EMS to achieve high yield and quality wheat. Once herbicide resistant mutants are available, the lines can be used in strategic breeding of wheat.

In view of the importance of weed problem in wheat crop in South Africa, this study was conducted to investigate the effectiveness of different herbicides and different doses for controlling weeds in wheat selections showing mutations under field conditions. The objective of this study was to select herbicide resistant wheat germplasm after inducing genetic change using the chemical mutagen, ethylmethanesulphonate (EMS) and to study the response of the selected mutants to two selected herbicides. Useful mutants could be identified with suitable agronomic traits and herbicide resistance that can be used directly or to introgress their genes into desirable genetic background in wheat.

## 4.2. Materials and Methods

### *4.2.1. Experimental site and plant materials*

Field experiments were conducted at the Ukulinga experimental farm of the University of KwaZulu-Natal in Pietermaritzburg, South Africa in the subtropical hinterland of KwaZulu-Natal province. The farm is characterized by hot dry summers and cool dry winters and situated at 30°24'S, 29°24'E with 775 m altitude above sea level. The mean average daily temperature varies from 14 to 30°C. The farm has sandy loam soil, of the Westleigh form, Soft plinthic B family, which is 31% clay containing 1.2% organic carbon and 2% acid saturation, with the pH ranging from 4.34 - 6. The main rain falls mostly in summer, between October and April. The mean annual rainfall during the study season, 2010/2011, was 970 mm. While the maximum and minimum mean relative humidity for the year were 75.7 and 52.2%, respectively. Occasionally light to moderate frost occurs in winter (May - July). Two varieties of wheat were used for the study, SST56 and SST875, supplied by Sensako, Wheat Seed Company in South Africa. The two varieties were selected based on their suitable agronomic traits and preliminary positive response and high levels of agronomic trait variations after EMS treatment.

### *4.2.2. Field evaluation and selection for herbicide resistance at M1*

### *4.2.3. Mutagenesis*

Seeds of the two wheat varieties were subjected to EMS mutagenesis at 0.5% dose, for 2 hr and at 35ºC. This treatment combination was determined from the preliminary controlled studies conducted in the controlled environmental facility (CEF), Chapter 2. The M1 herbicide resistant plants were selected and seeds planted in the controlled environmental facility for further herbicide evaluation at the M2. A 5 ml EMS volume was used to prepare 1000 ml treatment solution with 2% dimethyl sulfoxide (DMSO) as a career agent.

### *4.2.4. Treatments, herbicides and experimental design*

EMS treated wheat seeds (50,000 seeds per variety) were planted in the randomized complete block design (RCBD) with two replications at Ukulinga experimental farm. The study consisted of two wheat varieties with and without EMS mutagenesis which were subjected to two herbicides i.e. metsulfuron-methyl and bromoxynil each at three different doses *viz.* 2x, 4x and 8x above the recommended rate of 4 g $ha^{-1}$ and 2 kg $ha^{-1}$, respectively. This provided 24 treatment combinations (4 levels of varieties x 2 herbicides x 3 doses) that were randomly allocated per plot. The plot sizes for each treatment comprised 24 rows with 4 m width and 18 m length using two replications. Inter-row spacing was 40 cm; the recommended rate of metsulfuron-methyl is 4 g/ha and bromoxynil 2 kg/ha.

### *4.2.5. Screening for herbicide resistance*

Four weeks after planting both mutagenised and untreated standard check were sprayed with the herbicides using a calibrated handheld garden sprayer (Gardena 864 1.25-Liter Handheld Garden Pressure Sprayer) to contain herbicide drift. The spray contained an amount of spray mix of 4 g/ha for metsulfuron methyl and 2 kg/ha for bromoxynil herbicides concentration, having a nozzle output or flow rate of 1L/30 seconds. Two weeks after treatment, herbicide resistance were assessed on a scale of 0 to 10 (0 = crop death; 3 = severe necrosis; 5 = severe chlorosis, 7 = many necrotic lesions; 8 = moderate foliar chlorosis with small necrotic lesions; 10 = healthy and tolerant plants without any visible damage).

### *4.2.6. Herbicide reaction of selected M1 individuals at the M2*

From herbicide resistant M1 individuals about 800 seeds where available were planted in the controlled environmental facility. 50 seeds were planted in plastic pots of 10L capacity containing a sandy loam soil in 2 replications. The soil was fertilized using 250 kg N.P.K (2:3:4) consisting of $P_2O_5$ as an important source of plant nutrients.

Two weeks after planting, at second leaf stage, plants were sprayed with a calibrated handheld garden pressure sprayercontaining two concentrations viz. 4x and 8x above the recommended rate for both herbicides using a knapsack sprayer. The two rates were chosen to identify the most resistant mutants and for future targeted weed killing including broad leaved ones. If wheat mutants showed resistance at the two levels it was envisaged that selected mutants will show good resistance to the herbicide in the ensuing generations. Very high dose rates are capable of overwhelming even those mutants with some level of resistance.

### *4.2.8. Data analysis*

The scale data were subjected to a non-parametric test analysis using Kruskal-Wallis test procedure to compare the responses of varieties to the different herbicides and doses.

## 4.3. Results

### *4.3.1. Herbicide resistance of wheat varieties at M1*

The result showed significant difference among varieties, tested herbicides and doses applied (Table 4.1). After the EMS treatments both wheat varieties showed variable degree of herbicide resistance compared to the untreated controls. Significant differences ($p<0.01$) among varieties in response to the various doses of herbicide were observed for weed killing potency. This difference is more pronounced with the highest herbicide doses as indicated in Figure 4.1.

**Table 4.1.** Summary of Kruskal-Wallis test on herbicide resistance when testing two wheat varieties with two levels, using two herbicides at three doses.

| **Variable** | **Varieties** | **Herbicides** | **Doses** |
|---|---|---|---|
| Chi-square | 8.939 | 8.105 | 14.394 |
| Df | 3 | 1 | 2 |
| Asymp. Sig. | 0.030 | 0.004 | 0.001 |

* d.f = degrees of freedom; Chi-square = chi-square approximation from Kruskal-Wallis non-parametric test.

### *4.3.2. Assessment of M1 individuals for herbicide resistance*

Treated and untreated group of both SST56 and SST875 had varied responses towards to the two herbicides and three doses (Table 4.2 and Figure 4.1). In both varieties chlorophyll mutation was pronounced on the EMS treated seedlings showing the effectiveness of the mutagen than the untreated controls.

Table 4.2 summarizes herbicide reactions and the number of plants with chlorophyll mutations for all treatment combinations. As expected, EMS untreated treatments of SST56 and SST875 were susceptible for both herbicides especially at the higher doses (8x). At low herbicide doses

the control groups showed a considerable level of herbicide resistance (see treatment combinations at 2x and 4x doses). A higher dose (8x) was very lethal to wheat plants of EMS untreated controls (Table 4.2). Plants in the EMS treated treatment groups of both varieties showed herbicide resistance even at higher doses (8x) with few plants exhibiting chlorophyll mutation (albino phenotypes). This response is more visible in bromoxynil compared to metsulfuron-methyl (Table 4.2).

The action of an herbicide is determined by its chemical composition and physical properties, its effect on plant metabolism, the plant and the environment. Different herbicides could have different effects on different genotypes. In this study bromoxynil was found to be more potent than metsulfuron-methyl (Figure 4.1D). The rate at which bromoxynil kills the weeds was faster than metsulfuron-methyl (Figure 4.1A). As shown in Table 4.2 and Figure 4.2 bromoxynil was the most potent and killed more weeds as from the fourth day after herbicide application (Figure 4.2).

**Table 4.2.** Herbicide resistance rating among two wheat varieties (with and without EMS mutagenesis) tested with two herbicides (Metsulfuron methyl and Bromoxynil) at three doses (2x, 4x and 8x higher above the recommended rates).

| Variety and EMS seed treatment | Herbicide | Dose | Herbicide Resistance Rating (0-10)* | Chlorophyll mutation |
|---|---|---|---|---|
| **SST56, control** | Bromoxynil | 2x | 5 | - |
| | | 4x | 5 | - |
| | | 8x | 3 | - |
| **SST56, control** | Metsulfuron ethyl | 2x | 6 | - |
| | | 4x | 6 | - |
| | | 8x | 5 | - |
| **SST875, control** | Bromoxynil | 2x | 5 | - |
| | | 4x | 3 | - |
| | | 8x | 3 | - |
| **SST875, control** | Metsulfuron ethyl | 2x | 6 | - |
| | | 4x | 5 | - |
| | | 8x | 5 | - |
| **SST56, treated with EMS at 0.5% v/v** | Bromoxynil | 2x | 7 | - |
| | | 4x | 8 | 1 |
| | | 8x | 6 | 3 |
| **SST56, treated with EMS at 0.5% v/v** | Metsulfuron ethyl | 2x | 7 | - |
| | | 4x | 7 | - |
| | | 8x | 8 | 2 |
| **SST875, treated with EMS at 0.5% v/v** | Bromoxynil | 2x | 6 | - |
| | | 4x | 8 | 2 |
| | | 8x | 8 | 1 |
| **SST875, treated with EMS at 0.5% v/v** | Metsulfuron ethyl | 2x | 6 | - |
| | | 4x | 7 | - |
| | | 8x | 8 | 3 |

* 0 to 10 scale (0 = crop death, 3 = severe necrosis, 5 severe chlorosis, 6 = many necrotic lesions, 7 = moderate foliar chlorosis with small necrotic lesions, 8 = less chlorosis and necrotic lesions damage, 10 = no visible damage)

There was a marked difference in herbicide susceptibility or resistance after the EMS mutagenesis among varieties, different herbicides and doses (Figures 4.2 and 4.3). Higher doses (8x) on both herbicides are more potent than low doses (2x and 4x). As the herbicidal dose rate increases, both varieties tended susceptible (Figure 4.2). Figure 4.1C showed the responses of wheat varieties after the application of the low dose (2x) of metsulfuron-methyl that were more resistant. As depicted in Figure 4.1D the test varieties were more susceptible to this herbicide at the higher dose (8x).

In the present study, the application of heavier doses of herbicides bromoxynil and metsulfuron-methyl enabled selection of promising herbicide resistant plants in SST56 and SST875. In SST56, following the EMS mutagenesis, four plants with herbicide rating of 6 and 8 that had chlorophyll mutations was selected showing Bromoxynil resistance (Table 4.2). In this variety, two plants were selected displaying the highest level of Metsulfuron ethyl resistance at scale of 8 (Table 4.2). Whereas, in SST875, three plants were identified with Bromoxynil and Metsulfuron ethyl mutations with a scale of 8 (Table 4.2). The selected mutants of both varieties were advanced to test their herbicide reactions at the M2. These responses were showing moderate foliar chlorosis with small necrotic lesions, and less chlorosis and necrotic lesions damage. The number of chlorophyll mutants in EMS treated groups were observed and counted before herbicide application to relate this with herbicide reactions.

These responses were showing moderate foliar chlorosis with small necrotic lesions, and less chlorosis and necrotic lesions damage. The number of chlorophyll mutants in EMS treated groups were observed and counted before herbicide application to relate this with herbicide reactions.

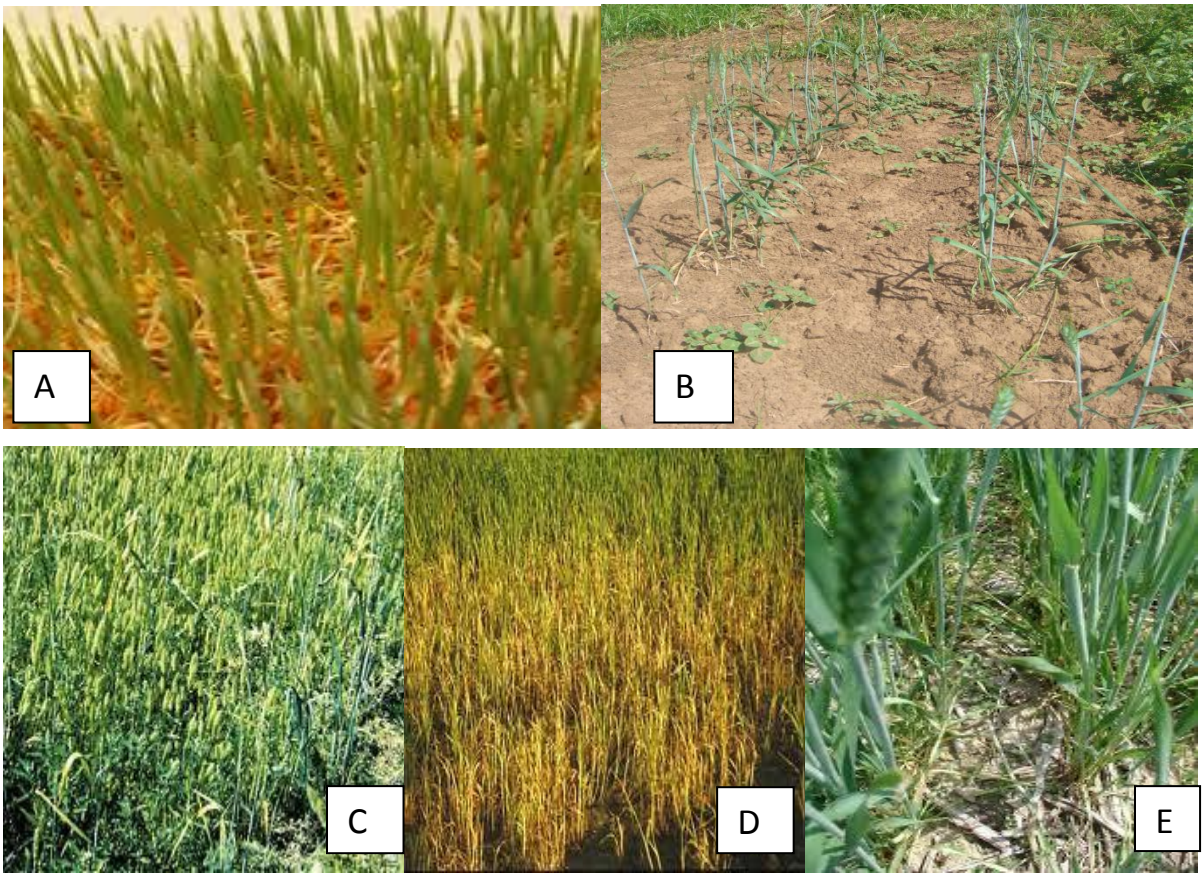


**Figure 4.1. Herbicide reactions of M1 wheats after EMS mutagensis, A (SST875, EMS treated, 4 days after Bromoxynil application at 8x dose), B (SST875, at 8x above the recommended dose, 18 days after Bromoxynil application), C (SST56, control at 2x above the recommended dose after spraying with Metsulfuron-methyl), D (SST875, after 14 days of spraying of check plants with bromoxynil (foreground) and Metsulfuron methyl at 8x the recommended dose (background) and E (SST875, after 25 days of spraying with Bromoxynil at 8x the recommended dose).**

### *4.3.3. Herbicidal effect*

Four days after spraying with the two herbicides the wheat plants commenced expressing the herbicide reaction with the broad leaf weeds showing wilts. Eighteen to 25 days after spraying all the weeds sprayed with a heavy dose (8x the recommended rate) of the two herbicides died off and only few water grasses survived (Figure 4.1E). As summarized above from the wheat population only few individual plants were noted showing herbicide resistance. Figure 4.1B shows the herbicide resistance wheat mutants after a heavy dose (8x) of bromoxynil. The mutant plants showed the herbicide resistance and survived after the herbicide application whereas the entire broad leaf weed and other wheat plants could not survive the herbicide effects. Surviving plants were kept and seeds harvested for M2 analysis.

Unlike EMS untreated checks the wheat varieties treated with EMS showed resistance to the higher doses of herbicides as indicated in Figure 4.1B and Figure 4.1E at 8x the recommended rate of the two herbicides. Where bromoxynil was applied at higher dose almost all the broad leaf weeds died after 18 days. Comparative control plants of both varieties showed severe necrosis, severe chlorosis and many necrotic lesions were observed after herbicides application at higher rates (Table 4.2).

EMS plays a fundamental role in crop breeding as it is widely used to generate genetic changes in crop plants with improved agronomic traits such as increased tolerance or resistance to herbicides, abiotic and biotic stresses, early maturity and reduced plant height, induced sterility and also helps in developing novel mutants by aiming a single or few genes as evidenced in this study. The wheat mutants that were EMS treated showed resistance to herbicides as compared to the EMS untreated comparative controls (Figure 4.2).

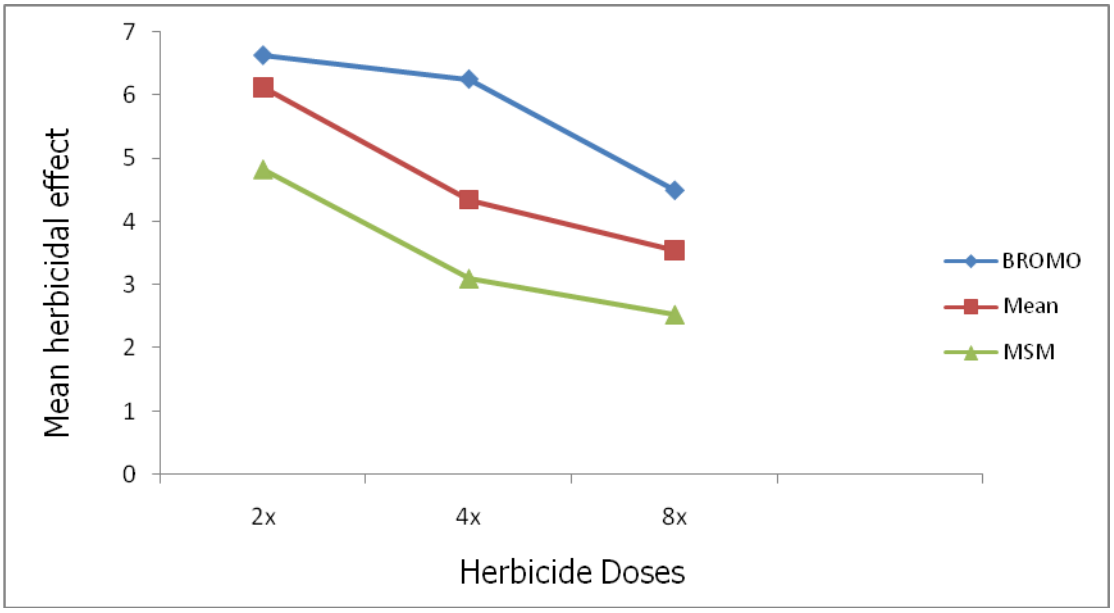


**Figure 4.2.** Mean herbicidal response of EMS treated wheat mutants to three herbicide doses (MSM=metsulfuron-methyl and BROMO=bromoxynil) under field testing.

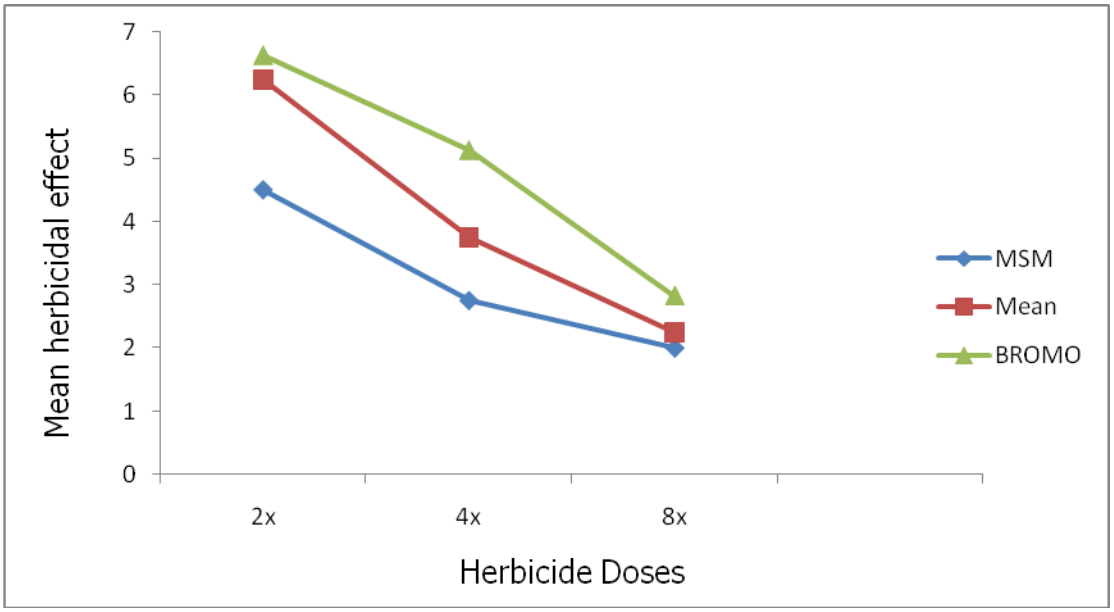


**Figure 4.3.** Mean herbicidal response of EMS untreated control checks of wheat varieties to three herbicide doses (MSM=metsulfuron-methyl and BROMO=bromoxynil) under field testing.

### *4.3.4. Herbicide responses among M2 individuals*

Seeds of M1 plants which showed herbicide resistance and chlorophyll mutation were selected and advanced to the M2 study. Table 4.3 showed the responses of selected wheat M2 individuals the two herbicides at 4x and 8x than the recommended rate.

In SST56 400 seeds were available from the M1 mutants and the seeds were planted in the greenhouse. Four batches of 100 M2 individual plants of the variety were treated with the two herbicides at 4x and 8x above the recommended doses. Whereas in SST875 400 seeds were available and 200 individual M2 plants were sprayed with the two herbicides at 4x and 8x doses. Out of 800 M2 seeds planted in two batches in the greenhouse only 164 plants survived the herbicide treatment at 8x above the recommended dose of bromoxynil and metsulfuron-methyl and 256 plants survived the herbicide treatment at 4x above the recommended dose of bromoxynil without chlorophyll mutation. The seeds of the survived plants with chlorophyll mutation were harvested and kept for further analysis. Out of the 800 M2 plants 7 (6 from SST56 and 1 from SST875) plants were tagged and their seeds were harvested and stored safe for further analysis since they showed herbicide resistance with chlorophyll mutation and having the herbicide resistance rating scale of 8.

**Table 4.3.** Herbicide resistance rating among two wheat varieties at M2 when tested with two herbicides (Metsulfuron methyl and Bromoxynil) at two doses (4x and 8x higher than the recommended rates) in M2 generation.

| Variety | Herbicide | Dose | Herbicide Resistance Rating (0-10)* | Chlorophyll mutation |
|---|---|---|---|---|
| **SST875** | | | | |
| | Bromoxynil | 4x | 6 | - |
| | | 8x | 8 | 1 |
| **SST875** | Metsulfuron ethyl | 4x | 7 | - |
| | | 8x | 8 | - |
| **SST56** | Bromoxynil | 4x | 8 | 1 |
| | | 8x | 8 | 3 |
| **SST56** | Metsulfuron ethyl | 4x | 7 | - |
| | | 8x | 8 | 2 |

* 0 to 10 scale (0 = crop death, 3 = severe necrosis, 5 severe chlorosis, 6 = many necrotic lesions, 7 = moderate foliar chlorosis with small necrotic lesions, 8 = less chlorosis and necrotic lesions damage, 10 no visible damage)

## 4.4. Discussion

The study established that EMS treated wheat mutants showed greater degree of herbicide resistance compared to untreated controls. Non parametric test was conducted using Kruskal-wallis test to evaluate and compare the herbicidal effect among the varieties tested. Significant differences ($p<0.05$) among varieties, dose and herbicide detected for weed killing potency were shown in Table 4.1. This difference is more pronounced with the highest herbicide doses (8x) and it is also in conformity with the study done by Sandhu and Mangat (1985).

Chlorophyll mutation was only observed on the few EMS treated mutants of M1 plants linked with herbicide resistance at high doses (8x). Chlorophyll mutation could have been contributed by the potency of mutagen as it is also in conformity with the study conducted by Newhouse *et al.,* (1991) in maize and Similar results were also reported by Nayar (1969) in *Sesamum*, Marki and Bianu (1970) in flax, Lysshenko and Ulitcheva (1971) in sunflower; Hussein *et al*. (1974) in Peas and Tsukuda *et al*. (1977) in rice.

From both varieties the type of chlorophyll mutation *i.e.*, Albino was observed and noticed linked with herbicide resistance at 8x the recommended rate (1 albino mutants were observed from SST56 at 4x than the recommended dose of bromoxynil, 3 albino mutants at 8x than the recommended dose of bromoxynil and 2 albino mutatnts from SST56 at 8x than the recommended dose of metsulfuron-methyl) at the M1. In SST875 there were 2 albino mutants at 4x than the recommended dose of bromoxynil, 1 albino mutants at 8x than the recommended dose of bromoxynil and 3 albino mutants from SST875 at 8x than the recommended dose of metsulfuron-methyl. Along with chlorophyll mutation frequencies the rate of morphological mutations also increased in both varieties with the increase in dose of mutagens and increase in the herbicide dose which is quite in conformity with results of other researchers on other crops. Bhosle and Kothekar (2010) studied the relative frequency of chlorophyll to morphological and sterility mutations. The maximum total number of chlorophyll mutations (12 plants) was observed in the EMS treated population of SST56 at 8x and SST875 at 4x and 8x the recommended rate all in M1 plants. In the present study a potent chemical mutagen like EMS in either of the mutant generations generated albino mutants, these being extremely low. This

finding is in agreement with the observations from the study done by Das and Kundagramy (2000) in grass pea.

Mutation induction techniques provide tools for the rapid creation and increase of variability in crop species (Inamullah et al., 2006). These techniques apply the principle of alteration of genetic makeup which has been manifested in nature. In our assay this was noticed when comparing the responses of EMS treated and untreated comparative controls to herbicide doses of two wheat varieties SST56 and SST875 (Table 4.2). Mutagenic agents only accelerate the outcome efficiency and magnify frequency (Srivastava *et al*., 1988; Burnside, 1992). These experiments showed that metsufuron-methyl and bromoxynil herbicides could be used to effectively control weed density and subsequent seed production, resulting in wheat seed yield increases.

The most effective herbicide for controlling weed early in the growing season was bromoxynil, thus confirming the results of Code *et al.,* (1978). Mean seed yield increase, however, was greatest with metsulfuron methyl, this could have resulted from greater selectivity as it is known that bromoxynil can significantly reduce yields of weed-free wheat (Elliott *et al.,* 1975), but it was more likely due to control of other weeds present in addition to broad leaved weeds. Both varieties in this study expressed mutations for resistance to the herbicides metsulfuron-methyl and bromoxynil. Following the EMS mutagenesis SST56 had considerable herbicide resistance at 4x and 8x than the recommended rate of bromoxynil and at 8x than the recommended rate of metsulfuron-methyl. SST875 developed considerable herbicide resistance at higher dose (8x) above the recommended rate of bromoxynil in the M2. The seeds harvested from M2 plants were maintained andwill be tested further to check the stability of this trait.

In this study all the tested wheat germplasm treated with 4x and 8x than the recommended dose of bromoxynil and at 8x than the recommended dose in metsulfuron-methyl expressed naturally high levels of resistance to metsulfuron-methyl and bromoxynil at doses 8 times and 4 times the recommended rate, respectively. This suggests that the herbicides metsulfuron-methyl and bromoxynil could be effectively used to control weeds in most wheat germplasm at 8 times and 4 times the recommended rate, respectively, without causing damage (Table 4.3). This also indicates that high doses of herbicide increase the potency of weed killing (Pozniak and Hucl.

2004). These findings show that it is possible to use mutagenesis of wheat for herbicide resistance which will help small and commercial farmers in wheat production. The rate of weed killing was greatest with bromoxynil where weeds died 7 to 10 days after herbicide spraying whereas metsulfuron-methyl gave much slower weed control associated with stunting of the weeds. From the present study it was shown that an increase in herbicide dose results in high potency of weed killing.

## 4.5. Conclusion

Based on the present findings the study concluded that EMS treatment generated herbicide resistant individual plants which will be selected for the next mutation generation using the respective herbicide and doses. Overall, the study showed that EMS mutagenesis is effective in inducing variation in wheat for several traits including herbicide resistance. The mutants developed in this study will be important for wheat breeding and for weed control in wheat.

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**CHAPTER 5**

**GENERAL DISCUSSION**

# 5.1 Introduction

Wheat is the second most important cereal crop in South Africa today. Despite its relative importance especially for the semi-arid zones of the country, production is heavily constrained by weeds among other challenges. Among the comprehensive control method available to manage weeds, herbicide has been shown to offer season long protection against the weeds in maize (Ott *et al.*, 1996), sorghum (Ndung'u, 2009) and barley (Mazur and falco, 1989).

Commercial production of wheat (*Triticum aestivum*) is considerably disadvantaged by weeds invasion. Furthermore, large scale production and industrialisation of wheat is also hindered by lack of mitigation strategies to enhance the genetic potential of wheat and improve farming technologies. Therefore, the use of novel wheat cultivars with herbicide resistance is relatively simple yet effective crop production technology. Use of herbicide resistance in crop production could be one of the cheapest and most effective forms of weed control both for small scale and commercial farmers.

Mutation breeding makes extensive use of deviations from the norm in order to improve characteristics of important crops. Inducing genetic variation in breeding of wheat has been recommended as a reasonable solution to combating challenges encountered in wheat cultivation (Miller *et al.*, 1984; Jabeen and Mirza, 2004). The increasing demand for wheat yield and quality and production rationalization in the food and agriculture industries has led to the development of more novel cultivars and techniques which are environmentally friendly (Kumar and Kumar Rai, 2007). Therefore, optimization of treatment combinations and ideal treatment conditions is necessary for a specific genotype prior to large-scale applications to develop novel germplasm through mutation induction. Mutations can be induced in various ways, such as exposure of plant propagules, including seeds, tissues and organs, to physical or chemical mutagens (Berenschot *et al.*, 2009). Ethylmethanesulfonate (EMS) has been widely applied in inducing genetic variation in plant breeding programs. Therefore, the main objective of this study was to develop herbicide resistant wheat mutants, through ethylmethanesulfonate mutagenesis, that could be used

subsequently to generate new cultivars with improved agro-morphological traits in breeding programs with the following specific objectives:

## 5.2 Summary of the research findings

### 5.2.1 Herbicide resistant wheat

There were significant herbicide concentration effects with increasing herbicide rate resulting in reduced weed density. Though increase in herbicide concentration also resulted in related decrease in wheat plant biomass, there was evidence that herbicide effect was effective in reducing weed infestation in treatments where seed of herbicide resistant mutant cultivars were mutagenised with EMS. However an increase in resistance to the herbicide in the mutants can minimize host damage and allow for use of higher rates that would be more effective in weed control.

EMS treated and untreated seeds of varieties SST56 and SST875 were sprayed with two herbicides, i.e. metsulfuron-methyl and bromoxynil at three different doses *viz.* 2x, 4x and 8x above the recommended rate of 4 g $ha^{-1}$ and 2 kg $ha^{-1}$, respectively. Two weeks after the treatment herbicide resistance were assessed. Results showed significant difference among varieties, tested herbicides and doses used. The EMS treated wheat lines showed variable degree of herbicide resistance compared to untreated controls at 4x and 8x above the recommended rate of both bromoxynil and metsulfuron-methyl herbicides.

### 5.2.2 Implications for wheat breeding

This study has shown that mutation breeding through EMS mutagenesis could be useful in generating valuable genetic variability and new varieties. In chapters three and four, valuable genetic variation for such characteristics as spike length, plant height, flag leaf length and overall yield increment in mutant lines SST56 and SST875 for example, gave the indication that there exists a niche for mutation breeding in wheat improvement. Generally most of herbicide resistant crops have been developed via genetic transformation. This study has shown that genes for herbicide resistance can also be easily developed via chemical mutagenesis, through EMS.

## 5.3 Conclusion

The research presented in this dissertation has highlighted the potential of EMS mutagenesis and mutation in inducing genetic variation in wheat. This study has also provided evidence on the induction of genetic variability on agro-morphological traits in wheat. Hence, induced genetic variability could effectively be exploited for developing mutant strains with required attributes and for developing herbicide resistance wheat mutants. There was evidence that EMS has the potential to increase agro-morphological variations in wheat for the selection of useful and novel mutants with desired phenotypic traits. Mutants generated in this study will be available to breeders for wheat improvement and further selections with farmer-preferred characteristics.

## 5.4 Recommendations

- There is a special need for wheat improvement through induced mutation, therefore special attention of mutants screening and selection for agro-morphological traits and herbicide resistance wheat mutants development is important.
- There is need for more work to optimize the dose requirement for effective weed control. In this regard, the possibility of employing slow release inventions of herbicide that ensure adequate protection against weeds throughout the season needs to be explored.
- Following the development of wheat resistances to many selective herbicides and theprohibitive expense and difficulty associated with the development of new herbicides, a need has arisen to seek alternatives toaddress these challenges.
- Improving wheat cultivars for herbicide resistance and weed control in South Africa will depend on availability of well trained personnel who can translate weed control strategies into workable programmes and also in the knowledge of mutagenesis and herbicide doses selection. However, this would require commitment by relevant sectors in South African government and non-government sectors to allocate financial, human and physical resources to crop improvement and weed science in order to train researchers, extension educators as well as farmers.

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