DEVELOPMENT OF LODGING RESISTANT BARLEY *(Hordeum vulgare* L.) LINES USING MUTATION BREEDING

BY

ANTONY KIBET CHERUIYOT

AGR/PGB/03/10

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT BREEDING & BIOTECHNOLOGY OF THE SCHOOL OF AGRICULTURE AND BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

UNIVERSITY OF ELDORET, KENYA.

NOVEMBER, 2013

DECLARATION

DECLARATION BY THE STUDENT

This Thesis is my original work and has not been previously presented in this or any other university for a ward of a degree. No part of this thesis may be reproduced without prior permission from the author/University of Eldoret.

ANTONY KIBET (AGR/PGB/03/10)…..……. Date ……
Department of Biotechnology
School of Agriculture Biotechnology
University of Eldoret, Eldoret, Kenya

DECLARATION BY THE SUPERVISORS

This Thesis has been submitted for examination with our approval as the university supervisors.

Prof. Miriam Kinyua …… Date ……
Department of Biotechnology
School of Agriculture Biotechnology
University of Eldoret, Eldoret, Kenya

Dr. Oliver Kiplagat …… Date ……
Department of Biotechnology
School of Agriculture Biotechnology
University of Eldoret, Eldoret, Kenya

DEDICATION

This work is dedicated to my parents Mr. John Rop, Mrs Janet Rop and my sister Rose Rop for their foundation and support.

ABSTRACT

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops in Kenya and the world's fourth most important cereal grown mainly for fodder and beer production. Major constraints to high yield in barley include pests, diseases, changing climatic conditions, land fragmentation and most importantly lodging which causes yield losses of up to 60% and hence reduced grain yield per unit area due to shading of crop which results in reduced grain size. Kenyan varieties are prone to lodging which inevitably translates into severe yield losses that constrain farmers' earnings hence the need to use mutation breeding to create genetic variability and develop lodging resistant lines. Barley seeds were irradiated at 300GY. A mutant (M1) population was established and two sets of 1000 heads selected. The resultant M2 seeds were planted in Njoro and Mau-Narok where selection was done using lodging scale (1-9), height of the plant, number of seeds per head, stem diameter and head size. Genetic diversity was tested at M2 using SSR markers to determine the presence of SSR marker for Btwd1 gene associated with lodging resistance. Of the 102 selected mutant lines, 61 lines had the band and 41 did not amplify. Data for various traits found significant differences among them. Correlation studies at M3, showed that head size, small stem diameter and height increase the chances of lodging. The results obtained from this study are of great help in future barley research where five resistant lines confirmed to have the presence of SSR markers to *Btwd1* identified will be used in breeding.

TABLE OF CONTENTS

LIST OF PLATES

LIST OF APPENDICES

ACKNOWLEDGEMENT

My sincere gratitude goes to my supervisors Professor Miriam Kinyua, and Dr Oliver Kiplagat who worked tirelessly and gave immense contributions to the completion of this thesis and to the University Of Eldoret for giving me the opportunity to pursue the Master of Science degree in Plant breeding and biotechnology through MU-EABL scholarship, Kenya Agricultural Research Institute (KARI) for facilitating my study at the National Plant Breeding Research Center (NPBC, Njoro). In addition, I appreciate the contribution of the entire staff of the School of Agriculture and Biotechnology who supported and helped me to achieve my academic goals. My sincere gratitude goes to Dr. Sridhar Bhavani and Dr. Peter Njau who encouraged and mentored me during the study. Special gratitude goes to the KARI Njoro Farm Manager Mr. John Rop for allocating the land for the trials.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Barley (*Hordeum vulgare L.*) is one of the most important crops in Kenya and the world's fourth important cereal (FAO, 2004). DNA markers point to the origin of barley being the Fertile Crescent especially the Israel-Jordan area in the southern part of the Fertile Crescent (Badr *et al*., 2000). It is a fast growing, cool season, annual grain crop. The plant has a deep, fibrous root system, a desirable feature for erosion control and soil quality improvement. Barley quickly produces large volumes of biomass for improving the soil organic matter content. It provides weed and insect suppression by helping to break pest life cycles. Barley is drought tolerant and can be used in rain-fed agriculture. Barley reaches 24–48 inches (60–120 cm) in height. It has alternate leaves about 10 inches (25 cm) long, flower spikes are notched on opposite sides, with three spikelet at each notch, each spikelet containing a small, individual flower, or floret, that develops a kernel. Barley roots reach a depth of as much as 6–7 ft (1.8–2.1 m) in deep soils. Barley can be grown on many soil types including well drained, fertile loams and lighter clay soils. It tolerates loamy to heavy soils but will not do well in waterlogged soils. It has very good heat and drought tolerance, making it a valuable plant for semiarid areas. Barley is also the most salt-tolerant among cereal crops. It grows at soil pH between 5.0 and 8.3. It thrives in cool, dry conditions. (Sustainable Agriculture Green Manure Crops Aug. 2002, SA-GM-3).

Barley is one of the crops of Old World agriculture. This is the area that has the highest probability of being the geographical area within which wild barley was domesticated about 8000 B.C. (Zohary and Hopf, 1993). It is grown mainly for fodder and beer production. About 20,000 ha of land compared to a potential 85000 ha of land in Kenya are under barley. Barley production in Kenya is still below its potential, yields of 4.5metric tons on an area of 20000 ha (Belay, 2006) is still below the optimal yields (EABL, 2005).

1.2 Lodging in Small Grains

Lodging is one of the most important constraints to optimal yields in barley. Lodging is defined as the state of permanent displacement of stems from the upright position (Pinthus 1973). Lodging alters plant growth and development. It affects flowering, reduces photosynthetic capabilities of the plant, hence affecting carbohydrate assimilation. Severe lodging interferes with the transport of nutrients and moisture from the soil, and thus with food storage in the developing kernels. Lodging always results in some yield loss, and if permanent lodging occurs shortly after heading, the yield reduction can be as high as 40 per cent. (Easson *et al.,* 1993; Briggs *et al.,* 1999; Pinthus, 1973). Lodging in cereal crops is influenced by morphological (structural) plant traits as well as environmental conditions and also plant factors influence lodging and some of the plant factors are head size, plant height, number of seeds and size of the head.

Plant height is one factor that can be controlled by the introduction of a dwarfing gene *Btwd1* that reduces height hence reduce lodging susceptibility.

Lodging in cereals is often a result of the combined effects of inadequate standing power of the crop and adverse weather conditions, such as rain, wind, or hail and it is a variety

(cultivar) dependent trait. Lodging in barley is the most limiting factor in attaining high yields from increased nitrogen fertilization, especially during humid conditions. There are various improvement techniques used but mutation is better and faster.

1.3 Statement of the Problem

Barley losses due to lodging reach up to 60% hence reducing quality and quantity of barley production. Yield losses due to lodging are attributed to reduced grain yield per unit area due to shading of crop which results in reduced grain size, increased amount of shriveled grains due to poor filling caused by reduced photosynthesis and discolored grains and increases harvesting costs by reducing the amount of crop that can be recovered by the combine harvester at a single pass (Briggs *et al,* 1999).

The varieties grown in Kenya are prone to lodging which inevitably translates into severe yield losses that constrain farmers' earnings (EABL, 2010). Traditional breeding strategies concentrate on development of high yielding varieties but neglect development of a short statured plant with a stronger stem which would resist lodging and bear heavier heads (Berry, 2003). There are no barley varieties resistant to lodging in Kenya hence the need to develop variability through mutation breeding.

1.4 Objectives

1.4.1 General Objective

To develop lodging resistant barley varieties using mutation breeding

1.4.2 Specific Objectives

1. To develop and identify mutant lines resistant to lodging (field screening for mutants)

2. To screen the mutant population using Simple Sequence Repeat markers linked to the dwarfing gene *Btwd1*

1.5 Hypothesis

1. H_{o-} A mutant population cannot be developed through mutation breeding.
2. H_o-There are no mutant lines with the marker linked to *Btwd1*gene.

1.6 Justification

Barley in Kenya is grown in high potential areas where due to many factors among them soil fertility and rainfall cause it to be highly susceptible to lodging. Lodging in barley leads to loss in both quality and quantity of the produced grain. Lodging can be managed culturally by management of crop nutrition, water management and wind protection which exacerbate the problem of lodging (Crook and Ennos, 1995). Genetically, lodging resistance is controlled by genes related to plant stature. Shorter plants with thicker stems are better able to resist lodging agents such as wind, rain/ hail, and heavy heads (Hellewel, 2000). There are limited sources of genes that contribute to shorter plant stature (Mickelson *et al*, 1994) that will translate into lodging resistance in Kenya hence the need for genetic mutation to create variability within the barley population and introduce variability in resistance to lodging. Mutation breeding involves exposing seeds to mutagens, either chemicals or radiation in order to generate mutants of which some may have desirable traits to be bred with other cultivars. In this case, the mutation may

enable the mutant barley to withstand particular environmental stresses better than wild-type by altering the plant morphology. Mutation coupled with careful selection introgresses the desired genes into the barley population and would result in lodging resistant barley thus limit yield losses currently attributed to barley lodging. The primary strategy used in mutation based breeding is to upgrade well adapted plant varieties by altering one or two major traits by subjecting the desired plants to mutation inducing chemicals or radiation. The altered characters could include such characters as plant height, stem diameter, maturity, seed shattering, and disease resistance, which significantly contribute to increased yield potential and enhanced quality traits (Ahloowalia *et al*., 2004).

CHAPTER TWO

LITERATURE REVIEW

2.1 Barley: The Crop

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops in the world. Barley originated in the southern part of the fertile crescent around Israel-Jordan (Badr *et al*, 2000). Zohary and Hopf, (1993) point to this area as having the highest probability of being the geographical area within which wild barley was domesticated about 8000 B.C. More than 50% of global barley production occurs in Europe, where Spain is the third largest European producer of barley, after Germany and France (FAO, 2006). It is the most important cereal grain produced in Kenya after maize, wheat and rice and is used for malting and beer brewing (FAO, 2004). Barley was introduced in Kenya by colonial settler farmers in 1920. They used barley as animal feed until 1929 when they commercialized production and sold it to the Kenya breweries. Barley is now used to make barley malt one of the principle ingredients in beer brewing. The major barley producing areas in Kenya include; Timau, Moiben, Nakuru, the wetter escarpment of Samburu near Maralal town, Molo, and Mau Narok. Three varieties of barley;Sabini, Nguzo and Bima are grown widely grown because they are high yielding. (EABL, 2010).

2.2 The Lodging Problem in Barley

Lodging is a serious problem in cereal crops especially the small grains ones such as wheat, Teff, oats and Barley (Easson *et al.*, 1993). The development of short stature plants reduces the problem to some extent in wheat. In barley, lodging is a primary concern and a lot of effort has gone into the development of short statured varieties to

better withstand the lodging forces. This effort has achieved little success especially with intensive production that puts emphasis on the use of higher levels of fertilizers, irrigation, mechanized harvesting and use of current varieties because of their agronomic superiority. The development of high yielding barley varieties that are resistant to lodging has hit a plateau due to limited variation in lodging resistance in the available germplasm and no further achievement can be made in the search for new genes for lodging resistance unless new technologies are used to create variation within the existing germplasm. (FAO, 2004).

2.3 Lodging in Cereals

Pinthus (1973) described the process by which shoots of cereals are displaced from their vertical orientation as lodging. Lodging has the highest chances of occuring two or three months before harvesting, usually after ear or panicle emergence. The result of this process is that shoots permanently lean or lie horizontally on the ground. There are two forms of lodging that have been described and are recognised: Thomas (1982) described stem buckling as stem lodging whereas Ennos (1991) described displacement of roots within the soil as root lodging. Stem lodging is described as the phenomenon where roots are held firmly in a strong soil while the wind force buckles one of the lower internodes of the shoot. Root lodging on the other hand becomes more likely when the anchorage strength is reduced by weak soil or poorly developed anchorage roots. The resulting effect is a reduction in crop yield by up to 80%, with further losses in grain quality, greater drying costs and an increase in time taken for harvesting. Lodging is not a new agricultural phenomenon, but is a problem that limits cereal productivity in both the developed and developing world (Berry *et al*. 2004).

Lodging is often not distributed uniformly throughout an affected field but may be scattered over certain sections or spots. Resistance to root lodging is highly dependent on development and position of plant roots especially crown roots that provide anchorage support for the plant. Stem lodging on the other hand is affected by weather conditions such as wind, rain and mechanical characteristics of the plant stem (Easson *et al*, 1993).

2.4 Causes of Lodging in Cereals

Lodging in cereal crops is influenced by morphological (structural) plant traits as well as environmental conditions. The mechanical characteristics of the plant stem are directly linked to the morphological, anatomic, biochemical and physiological characteristics of each genotype and are under genetic control (Berry *et al*, 2003). Lodging in small cereals is often a result of the combined effects of inadequate standing power of the crop, soil characteristics that affect root anchoring and adverse weather conditions, such as rain, and wind. The tendency for barley and other cereals to lodge may begin as early as the emergence of the ear or panicle and increases as the crop nears harvesting. Winter wheat has been observed to lodge from the emergence of its ear until its grains have matured (Easson et. al., 1993). Lodging occurs in barley as the crop nears maturity because the combined weight of the ear and plant acts on its weak stem to heighten lodging susceptibility. Taller weak stemmed plants are more prone to lodging than shorter plants with stronger straws (Jedel *et al*, 1991)

Root lodging is more common in wet soils. Root lodging in wheat is associated with as little as 4 mm of rainfall, though wind speed plays a more secondary role in both stem and root lodging. Winds above average speed are more likely to cause lodging in small cereals. A crop subjected to wind speeds of 8m/s for five minutes will lodge if the soil is

saturated with moisture (sterling et al., 2003). Field topography and field management practices also affect lodging of barley and other cereals.

2.5 Effects of Lodging on Yield

Cereal lodging, whether caused by the use of tall varieties, poor nitrogen management, poor soil physical conditions or by unfavorable climate conditions is one of the main barriers to the attainment of higher mean yields and an enhanced quality of cereal crops (Floss, 2004). Lodging is a limiting factor to increased yield in cereal production (Berry *et al*, 2004) and especially barley production in both developing and developed countries (Stanca *et al*, 1979). Lodging causes varied decreases in yield depending on country, region and conditions. A 50 % yield loss attributed to lodging in cultivated rice was reported in Japan. Similarly up to 60% yield losses have been reported in barley (Berry *et al*, 2003). Lodging causes yield losses not only in barley and other small cereals such as millet, sorghum, sugarcane but also in sunflower, rapeseed, peas, soya and other crops. Yield losses due to lodging are attributed to reduced grain yield per unit area due to shading of crop which results in reduced grain size, increased amount of shriveled grains due to poor filling caused by reduced photosynthesis and discolored grains (Day, 1957; Pumphrey and Rubenthaler, 1983). Lodging also increases harvesting costs by reducing the amount of crop that can be recovered by the combine harvester at a single pass (Weber and Fehr, 1966).

Mean grain weight (MGW) is an important component of both grain yield and grain quality in barley because grain size is associated with the potential malt extract (Cochrane and Duffus, 1983). Lodging affects barley plants interception of PAR (photosynthetic active radiation) which leads to poor assimilation and therefore smaller

grains and MGW, thus poor quality barley for malting (Bingham *et al*, 2007). A lodged crop has disrupted photosynthesis therefore assimilates very little carbohydrates and high N in the grain which impacts negatively the germination of malting barley and hence affects the malting quality of barley. The small grains and low specific weight indicate that lodging reduces the supply of assimilates to the grains and this increases the concentration of protein. Shriveling of the grain and reduction in test weight is the most common feature due to lodging (Pinthus, 1973). Sprouting in the heads has also been found to occur more frequently in lodged than standing crops due to creation of microclimates in the canopy of lodged crop (Nguyen *et al*., 2004). The high relative humidity in the canopy of lodged crop is responsible for germination of barley grains while still on the spike and its infestation by rot fungi (Baker *et al*, 1998)

2.6 Lodging in Relation to Stage of Occurrence

Pinthus (1973) showed that the magnitude of yield losses in cereal grains occur at different stages of cereal growth. Yield losses in wheat varied with stage of development at which lodging occurred. Yield reductions of 25%, 20% and 12% were reported when lodging occurred at milk stage, soft dough stage and hard dough stage of grain filling respectively (Weibel and Pendleton, 1964). Jedel and Helm (1991) observed 27-40% yield losses were observed to occur at heading stagewhereas 17-39% yield losses were observed to occur when lodging occured 15-20 days after heading. Lodging at milk stage of barley would also cause significant yield losses. Yield losses when lodging occurred during vegetative growth is attributed to reduced grain yield per unit area whereas during grain filling, the yield losses are attributed to reduced average weight of the grains (Fischer and Stapper, 1987).

2.7 Management Options to Reduce Lodging in Barley

There are many factors that contribute to the susceptibility of a barley crop to lodging. Environmental and genotypic factors interact in determining the susceptibility of a barley crop to lodging and the severity of lodging and yield loss. Environmental factors such as temperature, rainfall, soil water potential, wind velocity and light interact with barleys morphological (structural) characteristics which are genetically controlled to affect lodging (Van den Burg, E, 2008). Nitrogen, the most yield limiting nutrient also has a significant effect on barley lodging. Nitrogen is an important part of the compounds that regulate plant growth and development and forms an important part of the plant biomass. Its excessive application or excess residual nitrogen weakens plant stems and causes proliferation of tillers which make the plant heavy and prone to especially root lodging. On the other hand, potassium, plant population and planting time has moderate effect on barley lodging. The management options for barley lodging need to be considered while looking at factors that influence barley lodging such as barley genotype, nutrient management and especially nitrogen and potassium management, soil water management and other cultural management options (Koutna *et al*, 2003)

2.7.1 Management using Method of Planting and Tillage

Conservation tillage has an effect on the resistance of wheat to lodging. Lodging is more pronounced in wheat grown on ploughed land than on a field that has been slit seeded without ploughing first (Hull, 1967). Subsoiling increased lodging of barley over that obtained on a regularly prepared seed bed, whereas rolling after sowing decreased it (Pedersen and Lauer, 2002). The same effect was observed in wheat by Thomason *et al*, (2005) where the lodging index for conventional tillage was 7.1 as opposed to lodging

index of 4.0 and below for no tillage and reduced tillage. Tripathi *et al*, (2005) recommended planting on raised beds as one of the better options to manage lodging. Lodging prone wheat cultivars which are high yielding can be cultivated on raised beds to improve yields. According to Sayre and Hobbs (1998), bed planting reduced the wheat plant height and improved the grain yields by significantly affecting the lodging score. Bed planting is however not suitable for all cultivars in managing lodging. In rice, hill seeding improves the push resistance of rice therefore improves the lodging index of cultivars (Satoshi, 2005).

2.7.2 Management of Lodging using Crop Rotations

Continuous cultivation of cereals utilizes the residual nitrogen in the soil therefore reducing lodging susceptibility of these cereals. Maize (corn) for example had fewer broken plants when grown in a corn-corn rotation as compared to a corn–soybean rotation where the number of broken plants due to lodging increased (Wallace *et al*, 1999). The type of rotation will influence lodging in a cereal crop depending on whether it improves nitrogen content of the soil or mops up the excess nitrogen in the soil and therefore reduce lodging risk in cereals such as barley, wheat and rice.

2.7.3 Managing Barley Lodging by using Crop Nutrition

Crop nutrition plays an important role in the health and development of a barley crop. Nitrogen is essential for cell division and protein synthesis. Nitrogen management in barley and other cereals is a lot more complicated than just adding sufficient nitrogen for yield improvement. Nitrogen is a soluble and mobile element and when added too early, it can result in significant losses of nitrogen through leaching and or conversion to volatile gases and when extra nitrogen is added as insurance the potential for disease and

for lodging are significantly increased (Berry 2004). Lodging as earlier described occurs when leverage force exceeds stem strength or anchorage strength for stem or root lodging respectively. High rates of nitrogen increase lodging by making plants taller and more succulent therefore heavy, the leverage force exerted by the weight of rainwater on the plant shoot that is heavy due to excessive nitrogen fertilization and wind will increase the susceptibility of a barley crop to lodging. Nitrogen also increases the length of lower plant in barley and other cereals while decreasing the length of upper plant internodes therefore reducing the strength of the stem base (Crook and Ennos, 1995). Heavy nitrogen reduces stem diameter and stem wall width thus weakening the strength of stem base and the anchorage system, (Hobbs *et.al.,* 1998). Lush growth associated with heavy nitrogen fertilization causes elongation of lower internodes due to self shading. The timing of nitrogen application also plays a critical role in management of barley lodging by managing nitrogen in the soil. Nitrogen applied at planting results in lodging irrespective of the nitrogen status of the soil. When nitrogen is applied at early booting, it results in lower incidence of lodging in a crop.

Higher rates of nitrogen in the soil restrict development of coronal roots which are used for stabilizing cereal crops and resisting turning moments caused by the wind. Coeonal rot are important in reducing root lodging in cereal crops such as maize, sorghum and play a role in stabilizing barley and wheat. Studies show that application of higher rates of nitrogen to semi dwarf wheat varieties weakened the root anchorage of the crop. Nitrogen is found to increase the shoot: root ratio and lodging because nitrogen has more effect on shoot development as compared to root development which is strengthened by other elements such as phosphates (Pinthus, 1973). Increasing nitrogen increases yield

upto a certain point when the risk of lodging becomes higher, however there is no significant difference in lodging between a crop that receives 240 kg N/Ha and 300 kg N/Ha (Tripathi, *et al,* 2003).

Other elements also play an important role in lodging management though their effect is not as pronounced as the effect of Nitrogen. Wheat plants which are growing on soils deficient in P are weak and have delayed maturity. P deficiency increases the crop's susceptibility to root rots which besides weak stems aggravates lodging of the crop.

Most of the reports cite reduction in lodging due to potassium application. Potassium is needed in large quantities because it is the element that plays an important role in osmoregulation throughout the plant beside controlling cell sap content and maintains the turgor of the plant. K supports movement of materials within the plant including nutrient uptake by roots, transportation in the vascular bundles into the photosynthetic apparatus in the leaves and assimilate movement and partitioning to various plant components within the plant and also protein partitioning. Availability of potassium enhances the development of strong cell walls and therefore stiffer straw (Day *et al,* 1985). Lodging is affected by among other factors, variety, Nitrogen rate and weather; however, low potash levels also increase the risk of lodging with the associated loss of yield and quality in cereals. Potassium sulphate and potassium chloride were ideal for the reduced effect on lodging. Potassium fertilization reduced the disease incidence. Silicon is another element that impacts on the lodging resistance of cereal crops (Kant *et al,*2002). Silicon one of the most abundant elements in the soil, significantly increased the rigidity of rice stalk and this increase was remarkably higher at lower dose of nitrogen (Idris *et al*, 1975).

Srivastava and Kumar, (2003) also reported that root weight was significantly increased by application of silicon.

2.7.4 Managing Lodging using Plant Population

Low seed rate can increase anchorage strength from an average of 1919±2954 N mm by increasing the spread and depth of the root plate (Berry *et al*., 2000). Plant density has an effect on plant height and depth and spread of root plate. A densely sawn crop has taller plants with small stem diameters which are susceptible to lodging (Bruns and Abbas, 2005). According to Berry *et al*, (2004), there was a gradual increase in the percentage of lodging in wheat as sowing density was increased from 100 to 400 plants/ m^2. This increase in lodging due to increase in plant density was linear. An increase of the inter row and intra row spacing reduces lodging as it reduces inter plant competition resulting in shorter healthier plants as opposed to lanky weaker plants with closer spacing. In their study, establishing fewer plants result in more number of crown roots and better anchorage. Freeze and Bacon (1990) reported that wheat sown at a closer inter row spacing of 4 inches resulted in significantly more lodging in wheat than when the row spacing was increased to either 6 inches or 8 inches. Though higher plant densities resulted in higher yields in corn, it also simultaneously led to a higher percentage of the crop lodging (Pedersen and Lauer, 2002 and William and Thelen, 2002).

2.7.5 Managing Lodging using Sowing date and Depth of sowing

Lodging risk of wheat is almost always reduced by delaying sowing. Late sowing increased anchorage strength by a similar amount to low seed rate as a result of greater root plate spread. A delay of only 2 weeks can reduce the amount of lodging by as much as 30%. Berry *et al*. (2004) showed that sowing winter wheat 6 weeks earlier increased

both root and stem lodging risk by increasing the base bending moment of the shoot by about 30%. Earlier sowing results in greater number of extended internodes as water is abundant (Stapper and Fischer, 1990). Earlier sowing may also increase the prevalence of stem base diseases, which may increase lodging by weakening the stem. Sowing 4 weeks earlier increased the amount of Fusarium foot rot in wheat (Pendleton 1954). Deeper drilling helps in adjusting the depth of crown roots of plants to a depth of 40 cm. Hence, it is better to sow between 4-7 cm, drilling more shallowly than 4 cm may be expected to raise the crown and its structural roots, thus weakening anchorage.

2.7.6 Managing Lodging using Irrigation

Restriction of excessive vegetative growth by delaying or with holding first irrigation reduces the lodging. This indicates possibilities of reducing lodging by delaying or withholding first irrigation. Delaying the first irrigation from 20 DAS to 40 DAS reduced the lodging in wheat from 60% to 10.1%. However, giving irrigation at 30 DAS was found to be optimum with reduced lodging and better yields in wheat under Tarai conditions of Uttar Pradesh, India (Pandey *et. al*, 1997). Excessive moisture especially in the upper soil layer weakens root anchorage and predisposes the barley crop to root lodging. This being the case, it is also worthy to note that dryness of this same upper silt layer hampers development of the coronal roots which are essential in stabilizing the crop against the wind causing lodging. Cereals planted on clay soil may lodge when the soil dries therefore causing cracks in the soil and damaging coronal roots which stabilize the crop against lodging (Hurd, 1964). Water logging causes poor soil aeration that affects root respiration. The changes that occur in metabolism due to poor aeration promote cell elongation and increase a crops susceptibility to lodging. Management of soil water so

that a soil is neither too dry nor too wet is the key to managing lodging using irrigation. Soil aeration and soil structure also affect nitrogen availability, which in turn affects lodging. Managing water so that early vegetative growth and plant height are reduced greatly reduces crop lodging. This suggests that supplement irrigation be withheld for as long as possible without compromising crop development. Managing irrigation is particularly critical during grain development since the crop is particularly susceptible to lodging during that period. The type of irrigation also is critical since studies show that lodging was less promoted by sprinkler irrigation as opposed to furrow irrigation (Pinthus, 1973).

2.7.7 Managing Lodging using Clipping and Grazing

Removal of excessive foliage during elongation of the lower culm internodes by clipping or grazing before sufficient elongation of culm internodes has helped in some cases to control lodging by encouraging development of thicker stems and resulted in more yield. In most cases however, grain yield was reduced following grazing or clipping therefore this method may be more important in reducing lodging than in increasing grain yield (Berry, 2004).

2.7.8 Managing Lodging using Chemicals and Growth regulators

Plant growth regulators are synthetic analogues of plant growth hormones. They are compounds which are used to either increase or decrease cell division and elongation. They can be used to reduce the shoot length of plants by reducing cell elongation, but also by decreasing the rate of cell division. Plant growth regulators (PGR'S) can be used in cereals to reduce lodging by reducing cell division and elongation. The use of plant growth regulators to reduce lodging potential of cereal crops has been prominent in north

and western European countries, Canada and the USA. Some of the most commonly used PGRs are chlormequat chloride and mepiquat chloride. Ethephon is the most commonly used ethylene–releasing compound used on cereals. PGR's applied before the emergence of the ear reduced lodging in almost all the experiments. Application of chlormequat and choline chloride to winter wheat at the beginning of stem extension significantly reduced the percentage area lodged (Herbert, 1982). Plant growth regulators should be applied during internodes extension since they are only effective for a few days and application needs to be timed for effective management of plant height thus reduce crops lodging. Though effective in reducing plant height and therefore increasing lodging resistance, application of PGR such as ethephon (480 g/ha) controlled lodging by reducing plant height but also decreased average grain yield (Tripathi, *et al*, 2003) even though some studies (hobbs *et al*, 1998) indicate that wheat yields of some varieties were also improved by upto 1000 kg/ha by application of ethephon.

2.7.9 Managing Lodging by Managing Diseases

Important diseases like stalk rot in sorghum and Fusarium foot rot in barley predispose cereals to lodging. Mughogho and Pande, (1983) demonstrated that 100% lodging could occur with grain yield losses of 23 to 64% in CSH-6 hybrid, at three locations in India and Sudan due to charcoal rot infection induced by subjecting a crop to drought by withdrawing irrigation at different growth stages.

2.7.10 Management using Host Genotypes

Although environmental factors such as soil structure weather, the amount of available water and nutrients affect lodging, prevention of lodging is possible to some degree using inherent resistance. A large number of genes of the barley plant genome controls the

organization of the shoot and the root system of the barley plant. Further, most agronomic traits of crop plants are genetically controlled. Therefore lodging in barley is largely dependent on the barley genotype. Short statured plants or semi dwarf varieties of wheat and barley have short and stiff straw especially when nitrogen levels in the soil are low, thus having varieties with thick stems. Such varieties are hardier and are often not affected by lodging because they are better placed to withstand lodging agents such as strong winds (Stapper and Fischer, 1990). Most of the lodging resistance genes that control to plant stature, though good for the management of lodging in these crops, are undesirable to most farmers who grow barley and use the straw as animal fodder. These farmers still prefer the taller varieties with taller stature, since the tall varieties are high yielding. The short stature of the barley plant is negatively correlated with yield and quality of the barley yield for malting. (Ennos, 1991).

The depth and spread of root anchorage is also genetically controlled in as much as there is the modifying effect of the environment in the expression of this trait. Depth of anchorage of the roots is important to have erect plants. In sugarcane for example, plants having a depth of 260 mm root anchorage had a very low lodging. Those that had anchorage depth of 120 mm were prone to lodging (Nils and Allan, 2005).

Breeding for lodging resistance as well as improving other desirable traits using conventional breeding in cereal crops such as wheat and barley is sometimes difficult. The addition of genes for specific traits sometimes makes the genotypes susceptible to lodging as the effect of some genes are lost or masked (Tripathi *et al*, 2005). For example, the addition of the *Lr* 19 gene (for leaf rust resistance) to the wheat variety Seri 82 (a lodging resistant wheat cultivar) made susceptible to lodging (Mickelson, 1994).

2.8 Breeding and Genetic Improvement

Lodging in barley can be partially controlled by cultural practices and management of environmental conditions that predispose plants to lodging. However varieties with inherent (genetically determined) lodging resistance are the first choice for lodging control (Mickelson and Rasmusson, (1994). Breeding for yield and plant stature has been underway since domestication of barley. Breeding for lodging resistance involves identification of lodging resistant sources (plants that have lodging resistant traits such as dwarfism or semi dwarfism) these sources could be ancient varieties and wild relatives that express the desired traits. These resistant sources are crossed with desirable but susceptible varieties to generate plant populations that segregate for the traits of the parents. The segregating populations are grown in an environment conducive to lodging and a subsequent selection of the individuals that express resistance to lodging (Ceccarelli *et al*., 2000). There are times when resistance is not found within the existing species or its wild relatives. In such instances, the plants may be subjected to mutagenic agents with a hope to induce variation for the desired trait. Mutation breeding involves exposure of plant seeds, or vegetative propagation parts to mutagenic chemicals or ionizing radiation in the form of x-rays or gamma rays in order to generate mutants (Maluszynski *et al.*, 1995). The primary strategy used in mutation based breeding is to upgrade well adapted plant varieties by altering one or two major traits by subjecting the desired plants to mutation inducing chemicals or radiation. The altered characters could include plant height, stem diameter, maturity, seed shattering, and disease resistance, which significantly contribute to increased yield potential and enhanced quality traits (Ahloowalia *et al*, 2004).

2.9 Molecular Markers

The development of molecular techniques for genetic analysis has led to a great increase in our knowledge of cereal genetics and our understanding of the structure and behavior of cereal genomes. These molecular techniques, in particular the use of molecular markers, have been used to monitor DNA sequence variation in and among the species and create new sources of genetic variation by introducing new and favorable traits from land races and related grass species. Improvement in markers detection systems and in the techniques used to identify markers linked to useful traits has enabled great advances to be made in (Maluszynski *et al.*, 1995).

2.9.1: Simple sequence repeats (SSRs) (microsatellites)

SSR polymorphism is based on variation in the number of co-occurring (tandem) short repeats, generally of mono-, di-, tri- or tetra-nucleotides (e.g., [A]n, [CA]n, [AGC]n, [GACA]n), at a site. These repeat regions have been found to be hyper variable, possibly due to DNA polymerase slippage or mispairing at repeats during the normal replication process. Normally, the more repetitions of a repeat, the more likely it is to be polymorphic. For example, a [CA]10 repeat is more likely to be polymorphic than a [CA]4 repeat. Generally, variation at a single locus only is assessed in a single PCR reaction, although samples are sometimes 'multiplexed' for detection purposes.

Hyper variability means that SSRs are excellent targets when looking for genetic variation. Generally, polymorphism is studied in nuclear DNA, although variation in organellar DNA is sometimes also assessed. Length polymorphisms are generally visualised by running products on polyacrylamide gels. Radioactive, fluorescent, silver staining or other techniques are used for detection (Maluszynski *et al*, 1995). Dwarfing

genes have widely been used in barley breeding program. More than 30 types of dwarfs or semi dwarfs have been reported, but a few have been exploited in barley breeding especially the *Btwd1* gene which is used in malt barley (Xifeng Ren, *et al*., 2010).

2.9.1 Advantages

Nuclear SSRs are co-dominant markers that reveal full genotypic information. This is a great strength in detailed population studies, especially for highly heterozygous organisms such as trees. In addition, nuclear SSRs can show extremely high levels of allelic variation at individual loci. It is not unusual for 20 alleles to be observed at one locus in a single population. High allelic variation makes SSRs the method of choice for studying gene flow, paternity and genetic bottlenecks in populations. The technique can give highly reproducible results, and polymorphisms can be analyzed using automated methods (on sequencing machines).

Since the technique relies on specific primers, it can be used on lower quality DNA than dominant marker procedures. SSR analysis is the basis of modern forensic practice using very small quantities of, often, poor quality DNA.

As the technique relies on specific sequences, analysis can be targeted to different genomes: nuclear, chloroplast or mitochondrial.

2.9.1.2 Disadvantages

Species-specific primer development is relatively expensive and the construction of enriched libraries for the initial detection of SSRs requires technical skill. Sometimes, SSRs are too variable to be useful in comparisons, as there are insufficient common reference points among tested individuals (all differences, no similarities). This has frequently led to misapplication of the approach in cross-population comparisons.

In a single reaction, the SSR technique only assesses variation at a single locus. This is unlike with dominant markers, which can sometimes reveal diversity at very many loci simultaneously. Whether resources are available to carry out sufficient reactions to study sufficient SSR loci to address the question at hand is therefore an important consideration.

Although in theory revealing easily interpretable co dominant markers, assessment of SSRs is not always straightforward.

First, ‘stuttering’ often occurs during amplification. This leads to product artefacts and difficulties in accurate sizing. Generally, the smaller the basic repeat, the more problematic is scoring. Second, ‘null’ alleles – in which no amplification of the intended target occurs due to a change in sequence in one of the primer binding sites – are relatively common. This means that what first appears to be a homozygote, with two copies of a particular allele, may in fact be a heterozygote, with one allele amplifying and the other not. ‘Null’ alleles result in biased estimates of allelic and genotypic frequencies in populations, and the underestimation of heterozygosity. ‘Null’ alleles are more likely if using primers originally designed for another species (Mickelson, H.R and Rasmusson, 1994).

2.9.2 Mutation

Mutation is a permanent change in the DNA sequence of a gene. Mutations in a gene's DNA sequence can alter the amino acid sequence of the protein encoded by the gene.The DNA sequence of each gene determines the amino acid sequence for the protein it

encodes. The DNA sequence is interpreted in groups of three nucleotide bases, called codons. Each codon specifies a single amino acid in a protein (IAEA, 2013).

Application of Biotechnology and Mutation Techniques for the Improvement of Crops, Improve local varieties of basic food crops for yield and quality, early maturity, and tolerance to biotic and abiotic stresses. Initiate mutation induction in the local germplasm of neglected crops and promote their collection. Establish protocols for various *in vitro* techniques (such as micropropagation, somatic embryogenesis, haploid production) of basic and neglected food crops. Evaluate performance of mutants and parent varieties for nutritional value and quality traits (protein content, starch, cooking quality, shelf-life). (IAEA, 2013)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site Description

The study was conducted in two sites, Kenya agricultural research institute Njoro and at Mau Narok. The Kenya Agricultural Research Institute, Njoro,(0°20'S 35°56'E), is located in the lower highlands (LH_3), at an altitude of 2185 meters above sea level. The annual mean temperature at the station is 18°C while the average annual rainfall is about 1,000 mm. The soils are deep, well drained, fertile *Vitric Mollic Andosols* (KARI-Njoro meteorological dept 2011). Mau-Narok ($0^o33'S$ $35^o55'E$), is located in the lower highlands (LH_2) at an altitude of 2700 meters above sea level. The annual mean temperature is 14.9^0C, while the average annual rainfall is about 1,000 mm. The soils are deep, well drained, fertile *Vitric Mollic Andosols* (Jaetzold and Schmidt, 1983).

3.2 Plant Materials

The barley genotype Nguzo, a popular commercial variety (grown in most barley growing areas) in Kenya was used to produce the mutants that were included in the study. Nguzo is a high yielder of 18-20 bags/acre with an average height of 78cm, big head size and weak stem which makes it prone to lodging. This variety was sourced from Kenya Malting Limited, Molo a subsidiary of Kenya Breweries Limited and the major source of seed barley for Kenyan barley farmers.

3.3 Irradiation

Two kilograms of Nguzo seed was obtained from East African Maltings in Molo. They were sent to International Atomic Energy Agency (IAEA) lab in Vienna, Austria and

subjected to gamma radiation at an irradiation dose of 300 gy (gray) to obtain M1 (mutated seed that gives rise to the first generation of mutants). The M1 seed was planted at University of Eldoret experimental field for advancement to the next generation (M2) and preliminary evaluation for positive effects of radiation.

3.4 Seed Multiplication and Selection

The land used for seed increase at University of Eldoret, Chepkoilel was disc ploughed and harrowed to fine tilth suitable for barley planting. The irradiated M1 seeds were planted by drilling on a plot measuring 125 m by 40 m. Drills were 5 cm apart and all the agronomic practices like pest, disease and weed control done up to harvest time to ensure good crop establishment. At harvest, a thousand plants were randomly selected and two heads from each of these randomly selected plants harvested. The harvested heads were put in individual envelopes and labeled with corresponding numbers.

One group from the selected plants was planted at KARI Njoro experimental field while the corresponding groups of a thousand ears were planted at Mau-Narok field experimental station. Each of the harvested ears whose seed was designated as M2 was planted at Mau-Narok and at KARI Njoro. Each of the selected set of heads was threshed and planted to form ear to row lines at Njoro and Mau-Narok each.

3.5 Evaluation of Barley Mutants for Lodging Resistance

3.5.1 Planting and Field Management

The M2 seed from each entry was sown in 1m rows in January 2012. The lines were separated by 0.3m and 0.5m wide alleyways within and between the blocks, respectively. Sowing was done at an equivalent seeding rate of 33.33 kg/ ha. At planting time, Di-

ammonium phosphate fertilizer was applied at the rate of 155 kg/ ha in order to supply an equivalent of 27.9 kg N/ ha and 31.5 kg P/ ha. Weed management was effected by applying both pre - and post - emergent herbicides. Stomp® 500 EC (pendimethalin) a broad spectrum, pre-emergent herbicide was applied at a rate of 830g/ ha immediately after sowing and at tillering stage (Zadok's Growth stage 20-29) (Zadok et. al., 1979) the plots were sprayed with Buctril MC (bromoxynil + MCPA) at the rate of 450 g/ ha to control broad-leaved weeds. Hand weeding was done whenever need arose to manage grass weeds. The trial was top dressed with Calcium Ammonium Nitrate (CAN) at stem elongation stage (Zadok's GS 30) at the rate of 100 kg/ ha in order to supply additional 33 kg N/ ha. The crop was watered to eliminate water stress up to maturity.

3.5.2. Preliminary Data collection and Selection at M2

The barley crop was closely monitored during its development. Lines were evaluated for lodging severity at heading (Zadoks GS58) and plant maturity (Zadoks GS70-89). A total of 102 lines were selected according to a lodging scale (table 1), height, stem diameter, head size and number of seeds/head from both Mau-Narok and Njoro where by genetic characterization was done. A total of 19 lines that were lodging resistant and sturdy plant stems together with the parent Nguzo were selected to make 20 lines that were advanced to M3 when resistance for lodging together with other agronomic traits was evaluated in a replicated trial. Lodging was scored on a 1-9 scale (Zuber *et al*, 1999) where 1 was susceptible whereas 9 was resistant as shown in Table 1 below. Rating was done at heading when the susceptible check Nguzo was uniformly lodging.

Table 1 Lodging scale of erect stems

Lodging score	Description	Remarks
9	91 to 100% erect stems	Resistant
7	71 to 90% erect stems	Moderately Resistant
5	51 to 70% erect stems	Moderately Susceptible
3	31 to 50% erect stems	Susceptible
1	0 to 30% erect stems	Very Susceptible

Source: (Zuber *et al*. 1999).

3.5.3 Evaluation of M3 Barley population for Resistance to Lodging

The lines that were selected at M2 for lodging resistance were planted in a Replicated RCBD trial at Njoro in September 2012. Nineteen lines had been selected for high lodging resistance scores together with the parent Nguzo, they were planted in plots measuring 3m×2m and replicated 3 times in randomized complete block design (RCBD) at a spacing of 10 cm per seed. After germination the crop was tended as described previously until it attained heading when scores were taken for lodging resistance measured using the Zuber scale of lodging. Head size (cm), plant height (cm) and stem diameter (mm) were measured. Number of seeds per head was also counted.

3.6 Data Analysis

Data were subjected to analysis of variance (ANOVA) in randomized blocks was done using Genstat discovery edition 4 of 2013. Significant differences in treatment means were separated using Tukeys HSD test at $\alpha = 0.05$ level of significance. Correlation analysis was done to determine how various variables related to each other.

Plate 1: A photo showing the field layout of mutant lines planted in Njoro and Mau-Narok. (Source: Author, 2011)

Statistical model $X_{ijk}=\mu+t_i+\beta_j+e_{ijk}$

Where,

X_{ijk}= observation

μ=overall mean

t_i=treatment effect(mutant lines)

β_j=block effect.

e_{ijk}=experimental error

3.7 Molecular Characterization

3.7.1 DNA Extraction

Three seeds per head of the M2 lines selected from 102 lines were ground to a fine powder with a sterile plastic micro-pestle. Five hundred micro-liter of SDS extraction buffer was added and mixed thoroughly with the help of the micro-pestle. It was allowed

to stand for a few minutes, with occasional inversion to mix contents. The mixture was then centrifuged at 10,000 rpm for 5 min to separate and form DNA pellet. The supernatant was gently discarded by pouring out leaving the DNA pellet in the eppendoff tube. Then 500µl of 70% ethanol was added to the tube to wash the DNA. The tube was gently tapped to dissolve DNA pellet and allowed to stand for a few minutes before centrifuging at 10,000 rpm for 5 min to re-pellet the DNA. The supernatant was discarded by gently pouring out the ethanol and dried using the edge of a clean paper towel by draining away any remaining excess liquid from the lip of the inverted tube. The now upright open tubes were allowed to stand for 30 min for remaining liquid to evaporate and then 100 µl of 1 X TE buffer was added to re-suspend the DNA before using it. The remaining DNA suspension was stored at 4^{o}C.

3.7.2 Polymerase Chain Reaction. (PCR)

A stock solution of PCR mix was made which comprised of PCR buffer, taq polymerase, water and DNA template DNTP mix was used., Two sets of SSR primers Bmac031 and Bmac167 which amplify a fragment of 196 bp which is a marker for the dwarfing gene *Btwd1* and another set of primers Bmag217 and Bmag900 which amplify a fragment of 200 bp which is a marker for the same gene were used. Amplification was performed in a total of 10-ul reaction.The PCR profile amplification was conducted in a thermal cycler (EPPENDORF) using the following temperature profile:

Initial denaturation was at 94^{0}c for 5min followed by Denaturation at 94^{0}c for 30s Annealing temp at 48^{0}c for 30S, Extension at 72^{0}c for 1min and Final extension for 72^{0}c for 5min and then Hold at 4^{o}C .

3.7.3 Gel electrophoresis

A stock solution of 1.0% agarose gel solution containing ethidium bromide was prepared and a mercury thermometer placed in the agarose gel solution while stirring over the magnetic stir plate in order to monitor the temperature. The gel was removed and placed it in the electrophoresis box oriented so that the row of wells are closest to the negative (cathode, black) end of the gel box and filled the box with 1X TAE buffer covering the gel. The samples were loaded on the gel to cast with a pipette and a loading dye. on a 1% agarose gel electrophoresis for 1hour at 100volts on 1xTBE buffer where the gel was removed from the gel tank and taken to a dark room to view it under UV box to view the DNA under UV light

3.7.4 Data scoring

Data analysis was scored for presence or absence of the bands. Positive (+) if the band was present and a negative (-) if no band was present.

CHAPTER FOUR

RESULTS

4.1 Development of a Mutant Population of Barley

Mutation clearly showed variations in terms of plant height, earliness, head types, pigmentation on the glumes and variation in stem color. Plate 2 showed a dwarf mutant line measuring 53 cm in height as compared to the parent which was 78 cm. Besides being short the line also had a distinguished characteristic of earliness and headed in 30 days. The shorter plant stature had a head size of 8 cm with a stem diameter of 0.4 mm. The dwarf line in terms of number of seeds/head has 14, hence making it a poor yielder as the parent which scores 17.

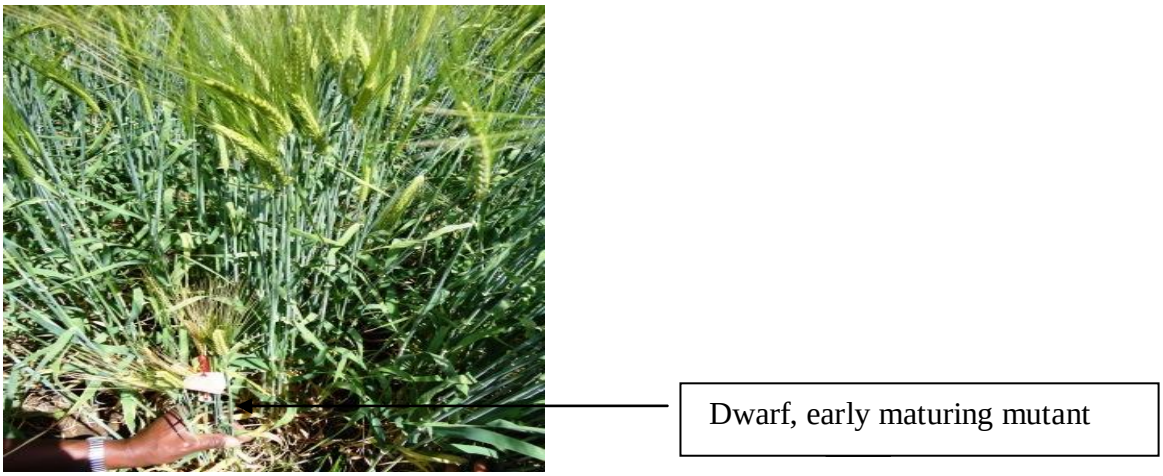


Plate 2: The dwarf mutant line in Njoro

(Source: Author, 2012)

Mutation also caused variation in head size and shape (Plate 3). The parent head (Centre) was uniform and long while the two mutants were club-like. However the numbers of seeds per head were similar in all the three heads. Plate 4 showed a pink coloration on the glumes which was a distinct feature compared to the parent which only had the coloration at the tip of the spikes.

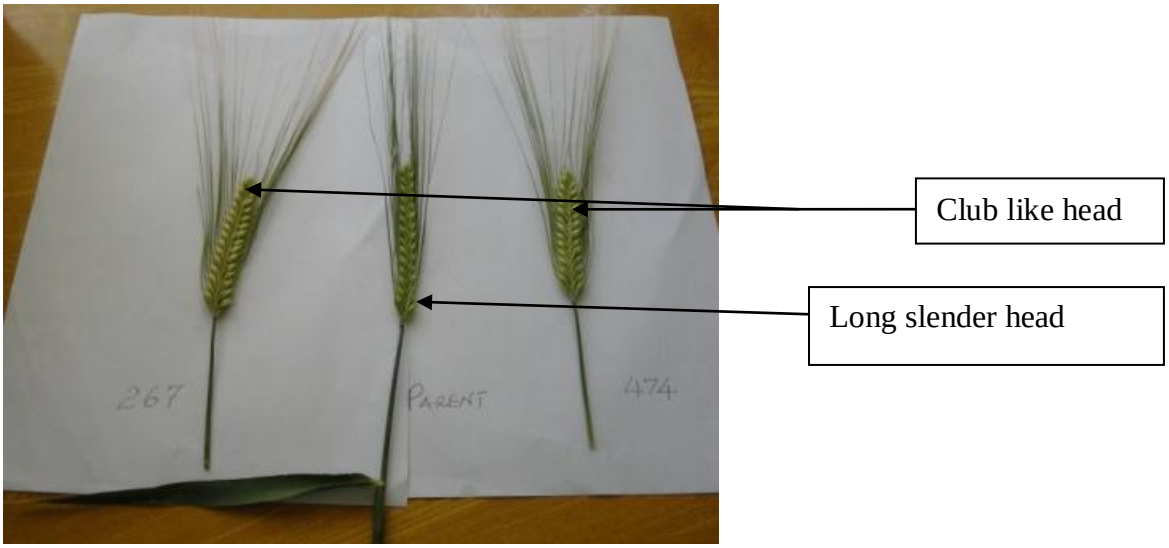


Plate 3: Variation in head types of the mutant lines (Source: Author, 2012)

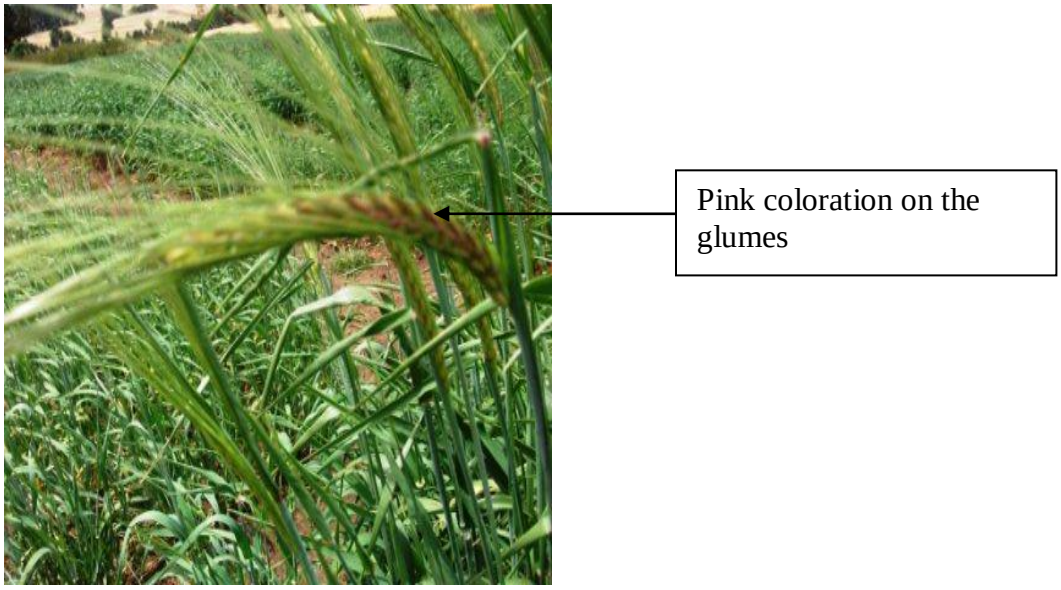


Plate 4: Pink coloration of the glumes of mutant lines (Source: Author, 2012)

A distinct coloration in stem color of the mutants was observed, deep green pigmentation and a lighter green pigmentation. The observable difference in both showed the one having a deep green pigmentation headed early than the one with a lighter pigmentation

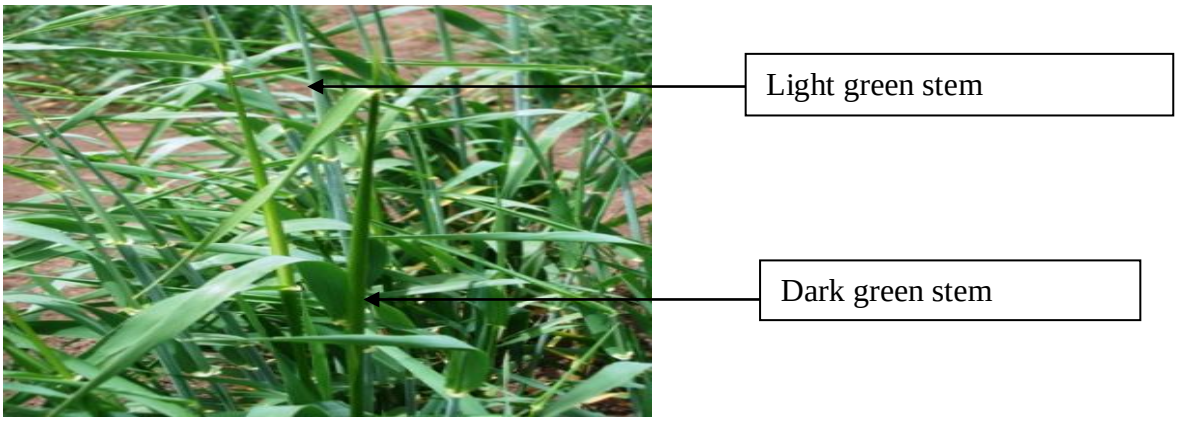


Plate 5: Differences in stem color of Mutant barley plants in Mau-Narok(Source: Author, 2012)

4.2 Evaluation of Mutant lines for Resistance to Lodging

4.2.1 Lodging scale

Lodging scale is the percentage total stems that have fallen and is measured on a scale of 1-9 where 1 denotes highest percent lodging and 9 denotes resistance (Zuber *et al.*, 1999). A total of 28 lines showed resistance in lodging as they scored 9 and 7 except line M3 204, M3 205 and Nguzo which scored 1, 3 and 5 respectively (Table 2). Nguzo was significantly different from all the other lines in terms of height, stem diameter, head size, lodging scale and the appearance of Btwd1 band which was weak. Most of the lines were not significantly different from one another like M3 02, M3 03, M3 04, M3 136, M3 68 which had a score of 9. There were some lines which were moderately resistant like M3 01, M3 06, M3 07, M3 27 and M3 90 which had a score of 7.

4.2.2 Plant height

Height was measured in centimeters and was the distance from the stem just above the soil surface to the tip of the head. Nguzo had a height of 75 cm which was the tallest and M3 136 had a height of 53 cm which was the shortest (Table 2). The height of most of the M3 lines like M3 04, M3 06, M3 07, M3 118, M3 130, M3 136 were significantly reduced compared with the parent which was 75 cm.

4.2.3 Stem diameter

The biggest stem diameter was recorded in line M3 163 measuring 0.7 mm and the smallest stem diameter measuring 0.3 mm in lines M3 90, M3 94, M3 204 and M3 200. Some lines were the same as Nguzo having the same diameter of 0.4 mm, like M3 04, M3 136 and M3 27. Most lines had a diameter of 0.5 mm like M3 03, M3 04, M3 06, M3

07, M3 107, M3 118, M3 130, M3 137, M3 15, M3 205, M3 22, M3 24, M3 39, M3 50, M3 65, M3 66, M3 68, M3 70, M3 84, M3 88 and M3 92.

4.2.4 Head size

This was measured in terms of centimeters and the biggest head size observed was in lines M3 204 measuring 17 cm which was significantly bigger than Nguzo and the smallest head was line M3 136 measuring 8 cm. Nguzo was not significantly different from some of the lines M3 90, M3 68 and M3 22 since they had the same head size of 16 cm.

4.2.5 Number of seeds per head

The highest number of seeds observed was 17 in lines M3 118, M3 130 and Nguzo and the lowest was 13 for lines M3 94, M3 70, M3 205 and M3 04. Most M3 Lines had a total number of 15 that were not significantly different from each other and were M3 92, M3 90, M3 88, M3 84, M3 27, M3 107 and M3 07.

4.2.6 Marker for Btwd1 gene

SSR primers Bmac031 and Bmac167 amplified a band of 196 bp and Bmag217 and Bmag900 amplified a band of 200 bp associated with Btwd1 gene.The presence of the Btwd1 band associated with lodging resistance was evident on several lines like M3 01, M3 02, M3 03 and absent on some lines like M3 94, M3 92, M3 90,(Plate 6). The Btwd1 band in Nguzo was weak compared to most lines that portrayed themselves brightly (Plate 6).

Table 2: Characterization of mutant lines

ENTRY	LODGING SCALE (1-9)	PLANT HEIGHT (cm)	STEM DIAMETER (mm)	HEAD SIZE (cm)	NO OF SEEDS (No.)	Marker Btwd1
M3 01	7b	69abc	0.4c	10g	14cd	+
M3 02	8a	70ab	0.6a	10g	16ab	+
M3 03	8a	69abc	0.5b	9i	15bc	+
M3 04	9a	63fg	0.5b	8j	13d	+
M3 06	7b	65def	0.5b	12f	14cd	+
M3 07	7b	61gh	0.5b	13e	14cd	+
M3 107	9a	66cdef	0.5b	12f	15bc	−
M3 118	9a	58hi	0.5b	14d	17a	−
M3 130	9a	57i	0.5b	12f	17a	−
M3 136	9a	53j	0.4c	8j	13d	+
M3 137	9a	67bcde	0.5b	12f	14cd	−
M3 15	9a	68abcd	0.5b	15c	16ab	+
M3 163	9a	65def	0.7a	14d	16ab	−
M3 200	9a	56ij	0.3d	14d	14cd	−
M3 204	1e	71a	0.3d	17a	14cd	+
M3 205	3d	71a	0.5b	15c	13d	+
NGUZO	5c	75	0.4c	16ab	17a	+
M3 22	9a	59hi	0.5b	16b	15bc	−
M3 24	9a	68abcd	0.5b	15c	14cd	−
M3 27	7b	56ij	0.4c	12f	15bc	−
M3 39	9a	68abcd	0.5b	14d	14cd	+
M3 50	9a	65def	0.5b	13e	14cd	−
M3 65	9a	61gh	0.5b	12f	14cd	+
M3 66	8a	68abcd	0.5b	14d	14cd	−
M3 68	9a	67bcde	0.5b	16b	16cb	−
M3 70	9a	64efg	0.5b	14d	13d	−
M3 84	9a	71a	0.5b	13e	15bc	−
M3 88	9a	64efg	0.5b	15c	15bc	−
M3 90	7b	68abcd	0.3d	16b	15bc	−
M3 92	9a	65def	0.5b	12f	15bc	−
M3 94	7b	68abcd	0.3d	16b	13d	−
CV	2.2	1.7	3.8	1.6	2.9	
SE	1.17	1.13	0.01	0.21	0.43	

Mean separation using Tukeys test at α=0.05; means followed by the same letter are not significantly different from each other.

4.3 Correlation of different traits in barley

There was no significant correlation between seed number per head and lodging score of barley (Table 3). On the other hand there was significant positive correlation between stem diameter and lodging score of barley mutants tested. Comparing the lines while considering head size and lodging resistance, the M3 lines that had a head size greater than 16 cm long were more prone to lodging with line M3 204, M3 205 and variety Nguzo having bigger heads and being prone to lodging (Table 2). However, there were a few lines like M3 90 and M3 94 that had bigger heads and smaller stem diameter but were able to better resist lodging despite the larger heads. There was significant negative correlation between lodging scale and plant height, lodging score and head size (Table 3).

Table 3: Correlation between head size (cm), plant height (cm), number of seeds, diameter of the stem (mm) and the lodging scale (0-9) of barley lines tested.

	LODGING SCALE	PLANT HEIGHT(cm)	STEM DIAMETER(mm)	HEAD SIZE(cm)	NO OF SEEDS/HEAD
LODGING SCALE	1				
HEIGHT	-0.3989**	1			
STEM DIAMETER	0.4327**	0.0813	1		
HEAD SIZE	-0.3717**	0.3142	-0.2426	1	
NO OF SEEDS	0.1399*	0.0319*	0.2823	0.1984	1

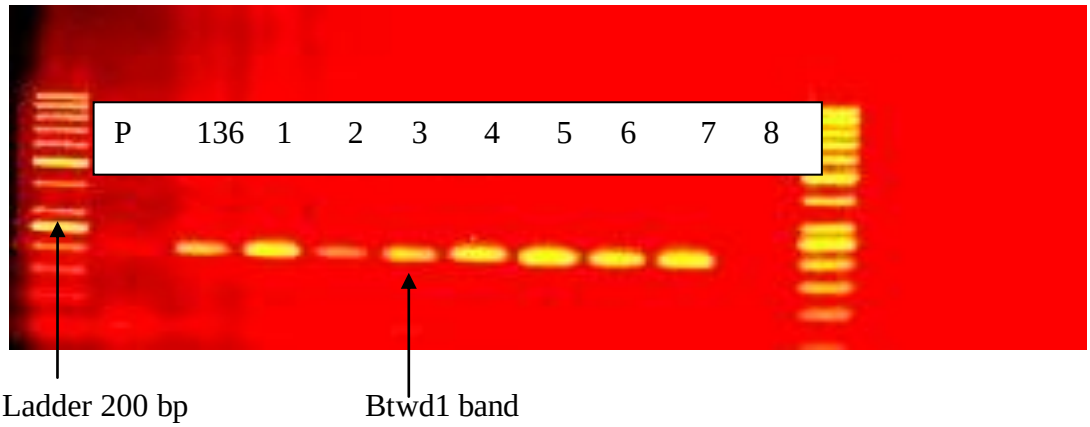
$r_{(0.05,28)}$=0.3610

**= significant p< 0.01, *= significant p<0.05

4.4 Characterization of M2 barley mutants using SSR markers linked to the Btwd1 gene

Genotypic characterization to confirm presence of lodging resistance gene *Btwd1* was done at KARI Njoro. DNA extracted from the barley samples was of good quality and most samples were in the concentration range of the lambda DNA standards used. Four primers were used; the band sizes were 196 to 200 base pairs depending on the primer. The band of interest for Btwd1 was observed in 61 samples while 41 samples did not have the band. Primer Bmac167 and Bmac031 showed the appearance of band at 200 base pairs and two mutant lines did not have the band that is 8 and 14. The band of Nguzo appeared to be weak.

a.



b.

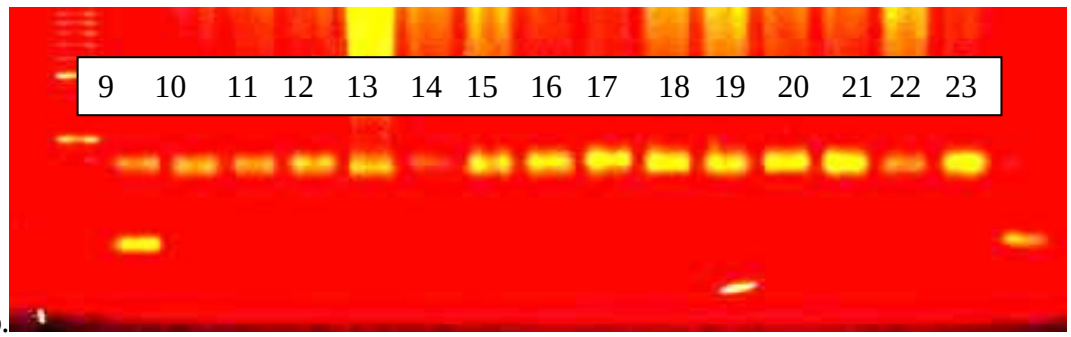
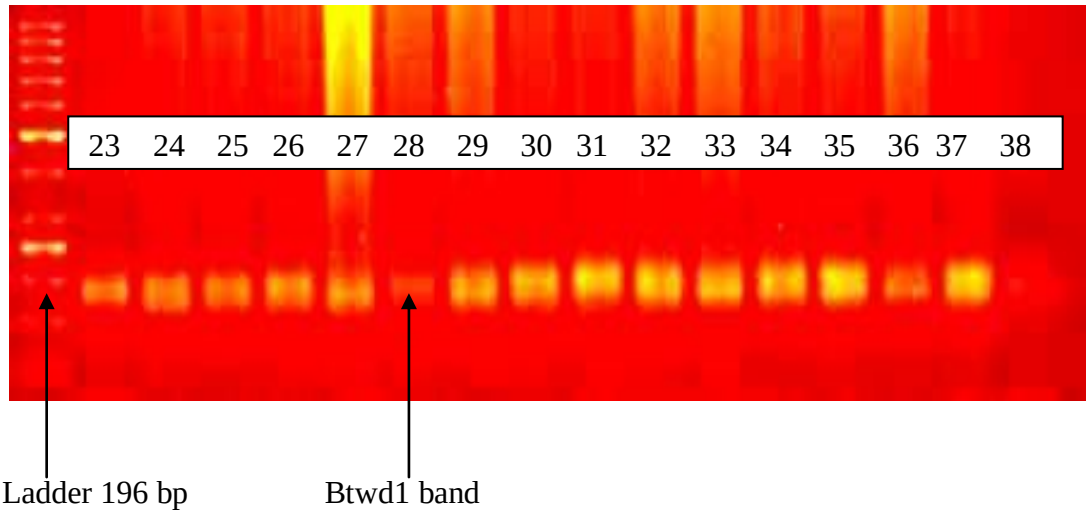


Plate 6: a and b shows the amplified 200 bp band with primers Bmac031 and Bmac167. The first lane is the ladder and the numbers indicate the mutant lines

Bmag217 and Bmag900 primers amplified at 196 base pairs and five mutants did not show the band and are lines 14, 40, 44, 48 and 51.

a.



b.

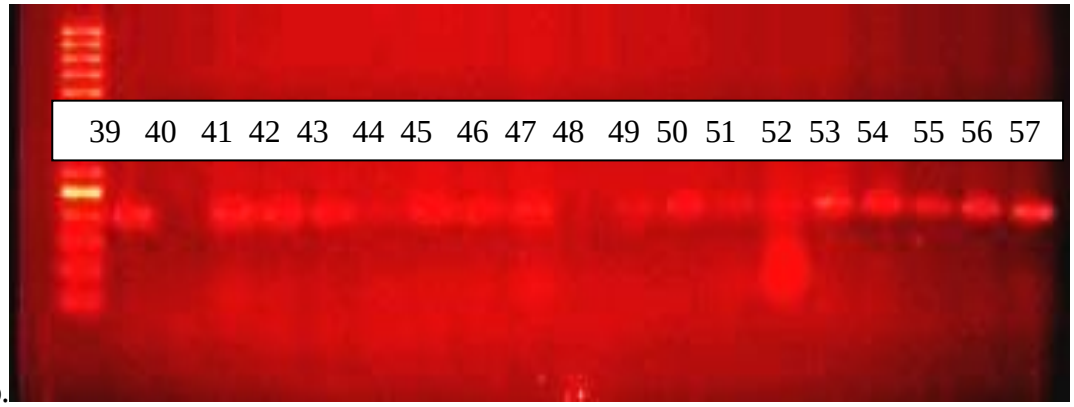


Plate 7: a and b shows the amplified 196 bp band with primerts Bmag900 and Bmag217. The first lane is the ladder and the numbers indicate the mutant lines

CHAPTER FIVE

DISCUSSION

5.1 Effects of Mutation

Generally mutation resulted in a reduction of plant height as all the mutants were shorter than the parent because mutation altered the genetic sequence of the mutant lines.

Besides variation in stem diameter and height of the plant observed in the mutant population developed, there were also other observable variations in the phenotypic characteristics of mutant barley. Some of the more notable characteristics included variation in maturity period of mutant barley, head type, variation in coloration of stems and leaves (Plates 2-5).

5.2 Stem diameter

In general increase in stem diameter translated into resistance to lodging though this was not always the case. Line M3 205 for example had a stem diameter of 0.5 mm whose stem was 0.1 cm thicker than the parent (Nguzo) and most of the resistant lines, yet it was found to be susceptible to lodging with only less than 30% of the stems not lodged. The lines M3 200, M3 90, and M3 94 had significantly smaller stem diameter compared to the parent Nguzo as seen in (Table 2) but scored better than the parent in lodging resistance because they had reduced height and also the number of seeds were less making the lines able to resist lodging.

Ghanbari-Malidarreh *et al*, (2012) in their study on factors that affect lodging in rice reported that a decrease in the stem diameter of rice due to increased nitrogen nutrition

significantly increased rice susceptibility to lodging by buckling of the third and fourth internodes. However, stem diameter alone does not necessarily translate into resistance to lodging in crop plants because there are other intervening factors such as lignification and thickening of cell walls which increases lodging resistance even with small stem diameters (Takayuki *et al*, 2008). The results of this study indicate that there were such intervening factors like lignifications and thickening of cells beside stem diameter that helped determine lodging resistance in some barley lines that had thinner stems compared to Nguzo but scored higher in terms of lodging resistance in mutant barley.

Most of the M3 populations tested had a larger stem diameter as compared to the parent Nguzo. Mutation had a significant effect on stem diameter of barley, mutation either increased or reduced stem diameter although some lines had the same stem diameter as the parent Nguzo.

5.3 Plant height

The tallest plants including the parent Nguzo which was 75 cm in height were susceptible to lodging as all scored 5 and below except M3 84 that had a an average height of 71 cm but resisted lodging and this was because the M3 84 had a thicker stem diameter of 0,5 mm and a reduced head size of 13 cm compared to the parent which was 16 cm. Mutation had a significant effect on height of M3 barley generations. Mutant barley lines selected had shorter stems compared to the parent Nguzo though in some instances the height difference between Nguzo and some mutant lines was not significant. Looking at the plant height, height affected lodging score in that the bigger the height the more susceptible to lodging it was. Reduction in plant height had an effect on lodging susceptibility of the lines to lodging. The taller lines were more susceptible to lodging in

all instances except M3 84 whose height was not significantly different from the parent Nguzo yet it scored highly for resistance to lodging with only 10% of the plants lodged and was because it had a bigger stem diameter of 0.5 mm compared to the Nguzo which was 0.4 mm. The result indicates that the shorter the plant stature, the better the ability to withstand forces that contribute to lodging. P.M. Berry (2008) reported that wheat breeders have traditionally increased lodging resistance by shortening crop height; however the scope for further reducing crop height appears to be limited because more extreme dwarfing genes have been shown to be incompatible with high yields. Lodging either occurs through buckling of the stem base (stem lodging) or through overturning of the root anchorage system (root lodging). Lodging resistance could therefore be increased by strengthening the stem base and anchorage system. Plant height is one of the most important features of cereal crops that is associated with lodging sensitivity (Crook and Ennos 1994; Berry *et al*., 2000).

5.4 Head size

Mutation had a significant effect on head size with some M3 genotypes having bigger heads compared to the parent Nguzo. It was evident that larger heads which in most cases imply more seeds and therefore a heavy load for the stem to bear will lead to lodging, however head size alone does not determine the susceptibility of barley to lodging. There must be other factors that must intervene to determine the lodging resistance of a barley plant as indicated in the lines M390 and M394. *S. Jezoski*, (1999), evaluated the effects of gene action on the properties determining resistance to lodging in barley and he showed that Genetic analysis was performed on the data for doubled haploids (DH) examined in a 3-year field experiment. Lodging degree and some morphological and physical

characteristics such as stem length, diameter, wall thickness, and elasticity (the Young's modulus) and velocity of the ultrasound flow through the stem were determined.

5.5 Number of seeds per head

There was a significant influence of mutation on number of seeds per head of barley mutants. The number of seeds coupled with seed size would affect the weight of the plant and influence lodging in barley. As the more the number of seeds the heavier the head and the more susceptible it was

5.6 Correlation of different traits in barley

However, looking at the correlation matrix, there is no significant correlation between seed number per head and lodging score of barley. This indicates that in this experiment, lodging was not affected by the number of seed per head of mutant barley. There was significant positive correlation between stem diameter and lodging score of barley mutants. The results indicated that stem diameter could be used to determine the lodging score of barley since it contributes significantly to determination of lodging. There was significant negative correlation between lodging scale and plant height; lodging score and head size. The study showed that three out of the five variables, plant height, and stem diameter and head size were significant variables that affect the lodging of barley positively or negatively. The taller the plant the more likely it is to lodge and the smaller the stem diameter, the more likely the plant is to lodge. Head size is normally correlated with heaviness of the plant.

5.7 Molecular characterization

The SSR primers amplified the bands linked to the *Btwd1* gene and a total of 61 mutant lines showed the presence of the band and 41 did not. Lines M3 204 and M3 205 showed the presence of the band but were susceptible to lodging and this may be attributed to the small stem diameter which was 0.3 mm and a bigger height of 71 cm. According to Tripathi *et al*., (2005), the addition of genes for specific traits sometimes makes the genotypes susceptible to lodging as the effect of some genes are lost or masked. Presence of the band for dwarfism showed that the mutant line was resistant to lodging and it scored a 9 or a 7 however some lines did not have the band for lodging resistance but scored 9.like lines M3 107 and M3 118. This could be attributed to a reduced height of 66 cm and 58 cm respectively

The above findings showed that presence of the *Btwd1* band alone may not contribute to lodging resistance in barley and that there are other factors like plant height and head size which determine if a plant lodges. With this in mind barley breeding should focus on germplasm which contain the *Btwd1* band and have relatively short height as well as suitable head size.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

i. Lodging resistant barley lines were developed through mutation for example lines M3 03, M3 04, M3 06, M3 07, and M3 136.

ii. Mutation was able to significantly reduce plant height in some of the mutant lines therefore making them better adopted to withstand lodging like M3 136 line.

iii. SSR markers were able to detect the markers associated with dwarfing gene *Btwd1* in mutant barley lines. Several mutant lines showed the presence of the btwd1 gene.

6.2 Recommendations

i. The dwarf lines whose average height was of 53cm should be studied further for research as a parent in breeding programs to develop short statured plants for better lodging resistance and also lines better than the parent Nguzo should be advanced. Like lines M3 107 and M3 108.

ii. The mutant lines showing the presence of the *Btwd1* band should also be used for breeding programs to transfer the gene to the varieties lacking this gene.

REFERENCES

Ahloowalia, B.S., Maluszynski, M. and Nichterlein, K. (2004). Global impact of mutation derived varieties. *Euphytica* 135; 187–204.

Badr, A., Muller, K., Schaufer-Pregl, R., El Rabey, H., Effgen, S., Ibrahim, H. H., Pozzi, C., Rohde, W., and Salamini, F. (2000). On the Origin and Domestication History of Barley (*Hordeum vulgare*).*Molecular Biology and Evolution*, 17(4):499–510.

Baker CJ, Berry PM, Spink JH, Sylvester-Bradley R, Clare RW, Scott RK, Griffin JM, (1998). A method for the assessment of the risk of wheat lodging. *Journal of Theoretical Biology* **194**, 587-603.

Belay, G. (2006). Cereals and Pulses in Martin, B (editor) Plant resources of Tropical Africa. Volume 1.PROTA

Berry, P.M., Griffin, J.M., Sylvester-Bradley, R, Scott, R.K., Spink, J.H., Baker, C.J., Clare R.W., (2000). Controlling plant form husbandry to minimize lodging in wheat. *Field Crop Research.***67**, 59-81.

Berry, P.M., Spink, J.H., Sylvester-Bradley, R, Pickett A, Sterling, M, Baker, C, Cameron, N, (2002). Lodging control through variety choice and management. In: Proceedings of the 8th Home-Grown Cereals Association R&D Conference on Cereals and Oilseeds. HGCA, London, pp. 7.1-7.12.

Berry, P.M., Sterling, M, Baker, C.J., Spink, J.H., Sparkes, D.L., (2003). A calibrated model of wheat lodging compared with field measurements. *Agriculture and Forest Meteorology* **119**, 167-180.

Berry, P.M., Sterling, M., Spink, J.H., Baker, C.J., Sylvester-Bradley, R., Mooney, S.J., Tams, A.R., Ennos, A.R., (2004). Understanding and reducing lodging in cereals. *Advances in Agronomy* **84**, 217-271.

Berry, P.M., Spink, J.H., Gay, A.P. and Craigon, J. (2003). A comparison of root and stem lodging risks among winter wheat cultivars. *The Journal of Agricultural Science, 141(2): 191-202.*

Berry, P.M., Sterling, M and Mooney, S.J. (2006). Development of a Model of Lodging for Barley, *Journal of Agronomy and Crop Science*, 192(2):151–158

Berry,P.M.,Sterling,M.,Spink,J.H.,Baker,C.J.,Sylvester-Bradley,R.,Mooney,S.J., Tams,A.R.,Ennos,A.R.,(2004). Understanding and reducing lodging in cereals. Advances in Agronomy, 84:217–271.

Bingham, I.J., Blake, J., Foulkes, M.J. and Spink, J. (2007). Is barley yield in the UK sink limited? II Factors affecting grain size. *Field crops research*, 101:212-220.

Ceccarelli, S. Grando, S., Tutwiler, R., Baha, J., Martini, A.M., Salahieh, H., Goodchild, A. and Michael, M. (2000). A methodological study on participatory barley breeding I. Selection phase. *Euphytica*, 111:91–104

Cochrane, M.P., Duffus, C.M., (1983). Endosperm cell number in cultivars of Barley differing in grain weight. *Annals of Applied Biology,* 102:177–181.

Crook J.M. and Ennos, A.R.(1995). The effects of nitrogen and growth regulators on stem and root characteristics associated with lodging in two cultivars of winter wheat .*Journal of Experimental Botany,* 46(8):931-938.

Day, A.D. (1957). Effect of lodging on yield, test weight and other seed characteristics of barley grown under flood irrigation as a winter annual. Agronomy Journal, 49(10):536-539.

Day, G.E., Paulsen, G.M., Sears, R.G., (1985). Nitrogen relations in winter wheat cultivars differing in grain protein percentage and stature. *Journal of plant nutrition*, 8:555–566.

Dunn, G.J. and Briggs, K.G.(1989). Variation in culm anatomy among barley cultivars differing in lodging resistance. *Canadian journal of botany*, 67(6):1838-1843.

Easson, D.L., Pickles SJ, White, EM, (1993). A study of lodging in cereals. HGCA Report No. 52. HGCA, London.

East African Breweries Ltd, (2005). The Kenyan Beer Industry. Nairobi, Kenya

East African Breweries Ltd, (2010). The Kenyan Beer Industry. Nairobi, Kenya

Ennos AR, (1991). The mechanics of anchorage in wheat (*Triticum aestivum L.)* II. Anchorage of mature wheat against lodging. Journal of Experimental Botany 42, 1607-1613.

FAO. (2004). Meeting of the Technical Subgroup of the Expert Group on International Economic and Social Classifications. United Nations Department of Economic and Social Affairs Statistics Division. New York, U.S.A.

FAO. (2006). Meeting of the Technical Subgroup of the Expert Group on International Economic and Social Classifications. United Nations Department of Economic and Social Affairs Statistics Division. New York, U.S.A.

Fischer, R.A., Stapper, M., (1987). Lodging effects on high-yielding crops of irrigated semi dwarf wheat. *Field Crops Research*, 17:245–258.

Hector Valenzuela1 and Jody Smith2, (2002). Departments of 1Tropical Plant and Soil Sciences and 2Natural Resources and Environmental Management

Hellewell, K.B., D.C. Rasmusson and M.Gallo-Meagher, (2000). Enhancing yield of semi- dwarf barley. Crop sci., 40: 352-358.

IAEA. (2013). Use of mutation breeding and techniques.

Jedel, P.E. and Helm, J.H., (1991). Lodging effects on a semi dwarf and two standard barley cultivars. *Agronomy Journal*, 83:158–161.

Kant, S. and Kafkafi, U. (2002). Potassium and abiotic Stresses in Plants. In 'Role of potassium in nutrient management for sustainable crop production in India'. (Eds NS Pasricha, S.K Bansal) Potash Research Institute of India: Gurgaon, Haryana.

Koutna, K., Cerkal, R. and Zimolka, J. (2003). Modification of crop management and its influence on the structure of yield and quality of spring barley grain. *Plant soil environment*, 49:457-465.

Maluszynski, M., Ahloowalia, B.S. and Sigurbjörnsson, B. (1995). Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica* 85, 303–315.

Mickelson, H.R and Rasmusson, D.C. (1994). Genes for Short Stature in Barley. *Crop Science*, 34(5):1180-1183

Nguyen, V. Q., Pham, S. T., Chu V.H., Pham V D and Zhong, X. (2004). Healthy rice canopy for optimal production and profitability. Omonrice 12: 69-74

Pendleton, J.W.,(1954). The effect of lodging on spring oat yields and test weights. *Agronomy Journal*,46: 265–267.

Pinthus, M.J., (1973). Lodging in wheat, barley and oats: the phenomenon, its causes and preventive measure. Advances in Agronomy 25: 209-263.

Pumphrey, F. V. And Rubenthaler, G.L. (1983). Lodging effects on yield and quality of soft white wheat. Cereal Chemistry, 60 (4): 268-270.

Shu Q.Y., Forster B.P.Nakagawa. H. (2012). Plant mutation breeding and biotechnology

Setter, T.L.,Laureles, E.V.,Mazaredo,A.M.,(1997). Lodging reduces yield of rice by self shading and reductions in canopy photosynthesis. *Field Crops Research*, 49: 95–106.

Stanca, A.M., Jenkins, G., Hanson, P.R.,(1979). Varietal responses in spring barley to natural and arti"cial lodging and to a growth regulator. J Agric Sci Camb 93 : 449}456

Sterling, M., Baker, C.J., Berry, P.M., Wade, A., (2003). An experimental investigation of the lodging of wheat.*Agriculture and Forest Meteorology* 119, 149-165.

Thomas, W.D., (1982). Plant Growth Regulators. Yield of Cereals Course Papers, 1982. NAC Cereal Unit, RASE, Stoneleigh, UK, pp. 78-95.

Thomason, W. E., Davis, P. A., and Brann, D. E. (2005). Soft red winter wheat cultivar response to tillage in the Mid-Atlantic Coastal Plain. Accessed on 2nd august 2012 at http://www.plantmanagementnetwork.org/pub/cm/research/2005/wheat/

Van den Burg, E., (2008). The expression and inheritance of stem strength in irrigation wheat. Msc Thesis. University of Orange Free State, South Africa.

Weber, C. R. and Fehr, W. R. (1966). Seed yield losses from lodging and combine harvesting in soybeans. *Agronomy Journal*, 58: 287–289

Weibel, R.O.,Pendleton, J.W.,(1964). Effect of artificial lodging on winter wheat grain yield and quality. *Agronomy Journal*,56:487–488.

Xifeng, R., Dongfa, S., Weiwei, G., Genlou, S., Chengdau, L., (2010). Inheritance and identification of molecular markers associated with a novel dwarfing gene in barley

Zadoks, J.C., Chang, T.T., Konzak, C.F., (1979). A decimal code for the growth stages of cereals. Weed Res 14 : 415}421.

Zohary, D., and Hopf, M. (1993). Domestication of plants in the Old World: The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley. Clarendon Press, Oxford, England.

Zuber, U., Winzeler, H., Messmer, M.M., Keller, M., Keller, B., Schmid, J.E., Stamp, P., (1999). Morphological traits associated with lodging resistance of spring wheat (¹riticum aestivumL.). J Agron Crop Sci 182 : 17}24.

APPENDICES

Appendix i: Anova Tables

Variate: LODGING_SCALE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.19355	0.09677	3.21	
Rep.*Units* stratum					
Entry	30	328.00000	10.93333	363.14	<.001
Residual	60	1.80645	0.03011		
Total	92	330.00000			

Variate: DIAMETER_OF_STEM

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.0008602	0.0004301	2.07	
Rep.*Units* stratum					
Entry	30	0.6494624	0.0216487	104.14	<.001
Residual	60	0.0124731	0.0002079		
Total	92	0.6627957			

Variate: HEIGHT

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.452	0.226	0.17	
Rep.*Units* stratum					
Entry	30	2501.613	83.387	64.52	<.001
Residual	60	77.548	1.292		
Total	92	2579.613			

Variate: NO_OF_SEEDS

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.0000	0.0000		
Rep.*Units* stratum					
Entry	30	118.2581	3.9419		
Residual	60	0.0000	0.0000		
Total	92	118.2581			

Appendix ii: Btwd1 primers

Bmac0310

PCR primers

5’CTACCTCTGAGATATCATGCC 3’

5’ ATCTAGTGTGTGTTGCTTCCT 3’

CTATCCTCTGAGATATCATGCC

ATCTAGTGTGTGTTGCTTCCT

Bmac 167

PCR primers

5'CATTTCCACTTCAAAATATCC3'

5' CCAAAGTTTGAGTGCAGAC 3'

CATTTCCACTTCAAAATATCC

CCAAAGTTTGAGTGCAGAC

Bmag217

PCR primers

5'AATGCTCAAATATCTATCATGAA3'

5' GGGGCTGTCACAAGTATATAG 3'

Bmag900

PCR primers

5' AGCCTGTGATACATCAAGATC 3'

5' AGGATGAGGGTATGTAGACG 3'

Appendix iii.Table of means

NEW NO	HEAD SIZE	HEIGHT	LODGING SCALE	NO OF SEEDS	DIAMETER OF STEM
1	10	67	7	14	0.4
2	10	73	9	16	0.6
3	9	70	9	16	0.5
4	8	60	9	15	0.5
5	8	66	7	14	0.4
6	12	65	7	14	0.5
7	13	61	7	14	0.5
8	10	64	7	16	0.5
9	14	73	7	16	0.5
10	13	65	7	15	0.5
11	14	74	7	13	0.4
12	13	65	7	16	0.3
13	13	74	7	16	0.3
14	14	61	9	13	0.4
15	15	68	9	16	0.5
16	15	58	9	13	0.4
17	16	67	9	14	0.4
18	16	53	0	14	0.3
19	16	64	3	16	0.5
20	12	67	9	16	0.4
21	12	68	9	15	0.4
22	16	59	9	15	0.5
23	16	65	9	10	0.4
24	15	68	9	14	0.5
25	15	68	7	14	0.4
26	14	66	7	14	0.5
27	12	56	7	15	0.4
28	14	57	7	12	0.4
29	15	56	7	15	0.4
30	16	56	7	15	0.3
31	16	58	7	15	0.4
32	16	65	7	15	0.4
33	16	71	7	15	0.4
34	15	58	7	15	0.5
35	12	67	7	14	0.5
36	13	53	7	16	0.4
37	14	64	7	15	0.3
38	15	67	9	15	0.4
39	14	68	9	14	0.5
40	12	59	9	15	0.3

41	12	65	9	15	0.4
42	13	68	7	15	0.4
43	16	68	7	17	0.4
44	17	66	7	14	0.5
45	18	56	7	14	0.5
46	14	57	7	15	0.4
47	15	56	7	15	0.4
48	12	56	7	15	0.6
49	14	58	7	14	0.5
50	13	65	9	14	0.5
51	16	71	7	14	0.5
52	17	67	7	16	0.5
53	14	73	7	16	0.3
54	15	70	7	12	0.3
55	12	60	7	14	0.4
56	14	66	3	14	0.3
57	12	65	7	16	0.4
58	12	61	7	15	0.4
59	13	64	7	15	0.3
60	16	73	9	16	0.3
61	17	65	9	14	0.4
62	18	74	9	14	0.4
63	14	65	9	15	0.4
64	15	74	9	16	0.4
65	12	61	9	14	0.5
66	14	68	9	14	0.5
67	13	58	9	14	0.3
68	16	67	9	16	0.5
69	17	53	9	15	0.3
70	14	64	9	13	0.5
71	15	67	7	15	0.5
72	12	68	9	16	0.4
73	10	59	9	17	0.4
74	10	65	9	18	0.5
75	9	68	9	16	0.5
76	8	68	7	15	0.4
77	8	66	7	17	0.4
78	12	56	7	14	0.3
79	13	57	7	15	0.4
80	10	56	7	16	0.4
81	14	56	7	13	0.4
82	13	58	7	15	0.5
83	14	65	7	16	0.5
84	13	71	9	15	0.5
85	13	58	9	15	0.4

86	14	67	7	15	0.4
87	15	53	9	13	0.4
88	15	64	9	15	0.5
89	16	67	9	16	0.4
90	16	68	7	15	0.3
91	16	59	9	17	0.4
92	12	65	9	15	0.5
93	12	68	7	14	0.4
94	16	68	7	13	0.3
95	16	66	9	16	0.4
96	15	56	9	16	0.4
97	15	57	9	15	0.3
98	14	56	7	13	0.4
99	12	56	9	13	0.4
100	14	58	7	14	0.3
101	15	65	9	15	0.3
102	16	71	7	12	0.4
103	16	67	7	16	0.5
104	16	73	7	15	0.4
105	16	70	9	16	0.4
106	15	60	7	17	0.4
107	12	66	9	15	0.5
108	13	65	9	16	0.4
109	14	61	9	15	0.5
110	15	64	9	16	0.3
111	14	73	9	16	0.4
112	12	65	9	16	0.3
113	12	74	9	16	0.4
114	13	65	9	16	0.3
115	16	74	9	16	0.4
116	17	61	9	16	0.4
117	18	68	9	16	0.4
118	14	58	9	17	0.5
119	15	67	9	15	0.4
120	12	53	9	15	0.3
121	14	64	9	15	0.3
122	13	67	9	15	0.4
123	16	68	9	16	0.4
124	17	59	9	17	0.3
125	14	65	9	15	0.3
126	15	68	9	15	0.4
127	12	68	7	14	0.3
128	14	66	7	16	0.4
129	12	56	7	16	0.4
130	12	57	9	17	0.5

131	13	56	7	15	0.4
132	16	56	7	15	0.4
133	17	58	7	16	0.3
134	18	65	7	15	0.3
135	14	71	7	14	0.4
136	8	53	9	14	0.4
137	12	67	9	14	0.5
138	14	53	9	16	0.4
139	13	64	9	14	0.4
140	16	67	7	16	0.4
141	17	68	5	15	0.5
142	14	59	7	14	0.5
143	15	65	1	15	0.4
144	12	68	7	13	0.3
145	10	68	7	15	0.4
146	14	66	9	14	0.3
147	13	56	9	14	0.4
148	14	57	9	14	0.4
149	13	56	9	14	0.3
150	13	56	3	16	0.4
151	14	58	7	13	0.4
152	15	65	7	15	0.4
153	15	71	7	16	0.3
154	16	67	7	16	0.4
155	16	73	9	16	0.4
156	16	70	9	15	0.4
157	12	60	9	15	0.4
158	12	66	9	14	0.3
159	16	65	9	15	0.4
160	16	61	9	13	0.4
161	15	64	9	15	0.4
162	15	73	9	16	0.4
163	14	65	9	16	0.7
164	12	74	9	15	0.4
165	14	65	0	15	0.4
166	15	74	7	15	0.4
167	16	61	7	16	0.5
168	16	68	7	14	0.6
169	16	58	7	13	0.6
170	16	67	7	14	0.4
171	15	53	9	14	0.4
172	12	64	9	15	0.5
173	13	67	7	15	0.4
174	14	68	9	12	0.5
175	15	59	7	16	0.3

176	14	65	7	14	0.5
177	12	68	7	15	0.5
178	12	68	7	13	0.4
179	13	66	9	16	0.5
180	16	56	7	14	0.4
181	17	57	7	15	0.4
182	18	56	9	16	0.3
183	14	56	9	16	0.4
184	15	58	9	15	0.4
185	12	65	7	14	0.4
186	14	71	7	15	0.4
187	13	58	7	16	0.4
188	16	67	7	14	0.4
189	17	53	7	14	0.4
190	14	64	7	17	0.5
191	15	67	7	14	0.3
192	12	68	7	12	0.5
193	14	59	7	16	0.4
194	12	65	7	14	0.4
195	12	68	7	14	0.4
196	13	68	7	16	0.4
197	16	66	7	16	0.4
198	17	56	9	15	0.4
199	18	57	7	16	0.5
200	14	56	9	14	0.3
201	17	56	9	15	0.4
202	17	58	9	14	0.4
203	16	65	0	10	0.3
204	17	71	1	14	0.3
205	15	71	3	13	0.5
206	15	66	3	14	0.4
207	17	68	0	16	0.5
208	16	72	1	16	0.5
209	17	77	5	14	0.4
parent	16	74	5	17	0.4