Physiological and Molecular Characterization of Kenyan Barley Lines (*Hordeum vulgare* L.) for Abiotic Stress Tolerance and Malting Attributes

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ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich für meine Promotion keine anderen als die angegebenen Hilfsmittel benutzt habe, und dass die inhaltlich und wörtlich aus anderen Werken entnommenen Stellen und Zitate als solche gekennzeichnet sind.

Jayne Jebichii BINOTT

Dedication

To
My son George Kimaru
and Parents Joyce Jesaimo and Peter Kipkwe

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Acronyms and Abbreviations

ABA	Abscisic acid
ADP	Adenosine Diphosphate
AGRA	Alliance for a green revolution in africa
AHC	Agglomerative hierachial clustering
AMG	α- amyloglucosidase
AMY	α-amylase protein
AMY1	Gene encoding α-amylase
AP2/ERF	Apetala 2/ethylene responsive factors
ATP	Adenosine Triphosphate
BADH	Betaine aldehyde dehydrogenase
BAMY	β-amylase
BG	β-glucunase
BGlu	β-glucosidase
bZIP	Leucine zipper
CAN	Calcium Ammonium Nitrate
CBF	Cold binding factors
cDNA	Complementary DNA
CDPK	Calcium dependent kinase
CGIAR	Consultative group on international research
CIMMYT	International Maize and Wheat Improvement Center, Mexico
cm	Centimeter
CMO	Choline monooxygenase
DAP	Diammonium phosphate
DAP	Days after pollination
DEPC	Diethylpyrocarbonate
DF	Dietary fibre
DHN	Dehydrin protein
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DP	Diastatic power
	Dehydration responsive element-binding C-Repeats

DRE/CRE -	responsive elements
DREB	Dehydration Responsive Binding rotein
DTT	Dithiothreitol
EA	East Africa
EAMC	East african malting company
EB	Extraction buffer
EDTA	Ethylene diaminetetraacetic acid
EFα1	Elongation factor, housekeeping gene
F	Forward primer
FAO	Food and Agriculture Organization
FURP	Fertilizer recommendation project
G6PDH	Glucose 6 phosphate dehydrogenase
GA	Giberrellic acid
GB	glycine betaine
GBK	Genebank of Kenya
GBu	Guanidine buffer
GDP	Gross domestic product
GDP	Gross domestic product
Glc	Glucose
HD-ZIP	homeodomain leucine zipper
HK	Hexokinase
HPLC	High performance liquid chromatography
Hsdr4	Hordeum spontaneum dehydration responsive gene
Hsp	Heat shock protein
HvBBD1	Barley cytosolic betain aldehyde dehydrogenase
HvGAMYB	Barley MYB transcription factor I aleurone
IDPs/IUPs	Intrinsically disordered/unstructured proteins
INRES	Institute of Resource Conservation
KALRO	Kenya Agricultural and Livestock research Organization
kDa	kilo dalto
KEPHIS	Kenya Plant Health Inspectorate
kg	Kilo gram

KL	Kilning
LEA	late embryogenesis abundant protein
MAPK	Mitogen activated phosphokinase
MCW	Methanol chloroform water
MDA	Malondialdehyde
MEGA 5.0	Molecular evolutionary genetics analysis
mM	Millimolar
MM	Micromalting
MMLV	Moloney Murine Leukemia Virus
MYB	Myeloblastosis
MYC	Mylocytomatosis
N	Nitrogen
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (oxidised)
NCBI	National Centre of Biotechnology Information
NIR	Nitrite reductase gene
NLS	Nuclear localization signal
NRE	Nitrogen response element
NRT	Nitrate transporter gene
NUE	nutrient use efficiency
OC	Organic carbon
P	Phosphorus
P5CR	pyrroline-5-carboxylate reductase
P5CS	pyrroline-5-carboxylate synthetase
PCI	Phenol chloroform isoamyl alcohol
PDH	Proline dehydrogenase
Pht	Phosphate transporter gene
Pi	Phosphate
QTL	Quantitative trait loci
R	Reverse primer
RCBD	Randomized complete block design
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RSA	Root system analysis
RT-PCR	Reverse transcriptase polymerase chain reaction
RWC	Relative water content
S	Seed
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOSK	Salt overlying sensitive kinase
SRL	Specific root length
SSA	Sub Saharan Africa
ST	Barley grain seed
TBA	Thiobarbutaric
TCA	Trichloacetic acid
TEMED	Tetramethylethylenediamine
TF	Transcription factors
UN	United Nations
UoE	University of Eldoret
μM	Micromolar

Summary

Production of sufficient barley with desired malting properties has been hampered by inadequate and unreliable rainfall, disease incidences, declining soil fertility and fragile environments. Barley breeding programmes are prompted to continuously improve barley for specific or combined end use traits in order to meet the increasing demands from the industry. In this study, based on the priority challenges faced by the barley farming community, three thematic areas are considered. These include abiotic stresses such as drought, salinity agriculturally poor soils, and inappropriate fertilizer usage as factors of production determining yield and quality of barley for various end uses. In order to develop appropriate breeding strategies there is a need to understand the physiological, biochemical and molecular processes that lead to the observed phenotypes. We hypothesized that genetic variability exists among Kenyan barley in regard to abiotic stress tolerance, malting qualities and root system architecture in response to nutrient deprivation. If this hypothesis is true, then the use of physiological and molecular morphological parameters to discriminate cultivar responses to stress factors can allow early detection of important traits and subsequently accelerate crop screening and development.

The main activities of the entire project were performed in three facets;

In CHAPTER 2, the physiological and molecular responses to abiotic stress factors including dehydration and increasing salt in Kenyan barley at seedling stage was evaluated. A laboratory based screening of barley seedlings was necessitated to overcome the challenges of variability under field conditions. 14 barley lines including those used as breeding lines, those in commercial production as well as wild progenitors of barley were targeted for screening for abiotic stress tolerance. For physiological assay, an increasing salt stress for 3 and 6 days was employed in order to monitor changes in important physiological parameters. Reduced ion leakage, MDA and increased chlorophyll proline, sucrose and GB contents are indicated in adaptive responses to stress. The assays sensitivity to discriminate cultivars as tolerant or susceptible varied depending on genotype and the duration of stress. A reconciled physiological screening clustered Nguzo, MN-24, MN-8, MN-3 as tolerant while Karne, MN-12, MN-7, MN-4 and MN-5 as susceptible lines. A narrow selection represented by Nguzo, Morex, MN-24, Karne, MN-8 and MN-12 was targeted for transcript analysis of stress responsive genes in root and leaf tissues subjected to dehydration and salt treatments. Expression profiles of 8 out

of the 13 *Dhns* found in the barley genome indicated that gene induction depends on the genotype, type of stress, level of stress and the type of tissue targeted. Tissue specificity of *Dhn*s may suggest a physiological role during abiotic stress. We observed that *Dhn1* and *Dhn9* were exclusively induced by dehydration stress and could be used as potential markers for screening for drought tolerance depending on the choice of tissue. On the other hand *Dhn3* and *Dhn4* were induced by both dehydration and salt stress. Immunoblot analysis using anti dehydrin anti-sera confirmed that the accumulation of both low and high molecular DHN protein depends on the choice of barley cultivars, type of stress and strength of stress. The existence of allelic forms of DHN in a single cultivar type has been linked to variation in the number and type of *cis* regulatory elements including MYB, MYC, ABRE and DRE in the promoter regions the dehydrins. There was strong correlation of transcript level and accumulation of DHN protein in response to increased salt and dehydration stress.

In CHAPTER 3, genotype variation in key malting attributes as affected by agronomic practices in three malting cultivars namely, Nguzo, HKBL 1385 and HKBL 1512 was determined. The effect of N regimes and seeding rates on yield parameters such as starch content, grain protein, hordein polypetide fractions and hordein transcript profiles were determined. There was genotype variation in yield under field conditions in reponse to N treatment. HKBL 1385 recorded the highest yield of 5.1 ton/ha while Nguzo and HKBL 1512 had 3.7 and 3.8 ton/ha respectively. This data corroborated laboratory analysis of starch where the HKBL 1385 recorded 278 mg/g DW while Nguzo and HKBL 1512 recorded 240 and 224 mg/g DW respectively. In regard to grain protein content, HKBL 1512 was highest (10.4%), while Nguzo and HKBL 1385 recorded 9.7 and 9.8% respectively. These values were within the malting protein specifications of 9-12.5%. The use of N rate at 30-40 kg/ha produced the same yield effect as that of 50 kg/ha depending on genotype. Starch levels was negatively correlated to grain protein content with the highest starch cultivar recording the lowest protein and the vice versa. Additionally, N treatment caused variation in the intensities in the hordein subclasses mainly C and B fractions in a genotype-dependent manner. Increased N treatment caused an effect on the C hordein subclasses in cv HKBL 1512 and HKBL 1385 while Nguzo showed hordein fractions stability across the treatments. Expression analysis of storage protein genes showed that the *D, C* and *B* hordeins genes were down-regulated irrespective of genotype or level of N treatment in the desiccated seeds, while *actin* the house keeping gene was

constitutively expressed. Malting quality can also be determined by analyzing differential abundance of seed protein or transcript levels of genes encoding hydrolytic enzymes such as α- amylase (AMY1), β-amylase (BAM) and β- glucanase (BG1) during the different stages of germination. Expression analysis of *AMY1*, *BAMY1* and *BG1* in the different physiological stages was found to be dependent on germination stage and the genotype. Increased expression of *AMY1* was highest in HKBL 1385 and Nguzo but it was expressed at low levels in HKBL 1512 compared to other genotypes. In Barke *BAMY1* was down-regulated while *BG1* was only expressed between 24-72 hrs of malting but not during steeping like in other cultivars. Analysis of hordein polypeptide fractions during progressive germination showed that C and B hordein polypeptide fractions were degraded after 24 hrs of germination which coincided with accumulation of β-amylase protein between 72-96 hrs of germination in a genotype dependent manner. The hordein storage proteins were degraded after 24 hrs of germination contributing to the energy supply for the germinating seedlings.

In CHAPTER 4, the effect of N and P deprivation on root parameters in pot grown and *in vitro* propagated barley were evaluated in Morex, Nguzo, MN-24, Karne, HKBL 1385 and MN-12. Genotype variations in root morphological parameters like root mass, lateral root length and number of lateral roots in response to N and P deprivation were observed in both, pot-grown and *in vitro* propagated barley seedlings. Genotype and treatment had a significant effect on the evaluated parameters. It was observed that N deficiency promoted root elongation and intensive lateral formation in Nguzo and HKBL 1385. On the other hand, P deficiency promoted lateral root formation in genotype-dependent manner. Root architecture is important in resource acquisition especially under limited nutrient supply. This was the case with HKBL 1385, a high yielding cultivar which displayed extensive root network under N and P deficiency. Exploitation of genotype response to various environmental cues, may aid in identification of cultivars with desired agronomic traits.

General introduction

1.1 Climate change and food security crisis

According to the Food and Agriculture Organization of the United Nations (FAO, 2009), it is projected that the world population will reach nine billion people by 2050. As a result, around four billion people representing about 40% of the projected global population of 9.5 billion from 54 countries will experience scarcity of water (Gardner-Outlaw and Engelman 1997). Several other challenges that affect humanity shall significantly prevail. These will include poverty, food security, and most importantly new and complex challenges emerging due to global warming and climate change (World Bank, 2008). Over the years, climate change and especially global warming has triggered a gradual increase of atmospheric temperature through increasing concentrations of atmospheric carbon dioxide produced by burning biofuels and deforestation accelerating desertification worldwide (Lu et al., 2007). According to Turra et al. (2009) atmospheric temperature will increase by 4 ^{0}C by the year 2080 with concomitant doubling of CO_2 concentration. Consequently, it will adversely have serious effects on both rain-fed and irrigation agriculture. Climate change is therefore a global concern and concerted efforts in setting up means of adapting and mitigating its effects are on the top of the agenda toward development of sustainable agriculture (Downing et al., 1997, Brown and Funk, 2008).

The effects of global warming are more pronounced in the tropics where high temperatures are already being encountered. In Sub Saharan Africa (SSA) agriculture is of great importance economically in creating about 70-80% of employment, about 30% of gross domestic product (GDP) and over 40% of export earnings (Worldbank, 2000, 2010). Agricultural development in SSA has been affected by unreliable and poor distribution of rainfall, low prices, poor infrastructure and lack of irrigation facilities, biotic and abiotic stress factors, inadequate selection of suitable crop varieties adapted to marginalized areas, increased post-harvest losses, limited research-extension linkages, high cost of fertilizers and certified seed and failure of the smallholder farmer to adapt to climate and new technologies (Thomas and Twyman, 2005, Faurès and Santini, 2008, FAO, 2009, AGRA, 2011, Muzari et al., 2012). These limitations have resulted in increased poverty, food shortages and severe malnutrition (Havnevik et al., 2007, Lobell et al., 2008)

In East Africa (EA) agriculture is rain-fed and highly vulnerable to climate change and variability. Rain-fed agriculture is the most significant since it accounts for about 96% of the cropland. Water management is therefore a key determinant for agricultural production in these regions and will become even more important during climate change (Rost et al., 2009). Additionally the decline of food production is linked to declining soil fertility in smallholder farms cause material leaching and soil erosion (IFPRI, 1996, Schnier et al., 1996). It is estimated that 112, 2.5 and 70 kg/ha per year of nitrogen, phosphorus and potassium (NPK) respectively, is lost from agricultural soils in Kenya (Smaling, 1993, Scherr, 1999).

In most cases, the long rainy season within the EA region starting from March to June is delayed and ends earlier (Segele and Lamb, 2005) with prolonged dry spells in between (Seleshi and Camberlin, 2006, Lyon and DeWitt, 2012). Alteration of rainfall patterns affects major crops such as maize, wheat and barley during critical stages of development like flowering and grain filling stages (Hochman, 1982, Çakir, 2004, Dolferus et al., 2011). In view of this, development of crops that can cope with higher temperatures, increased water salinity and denser pest loads are inevitable (Godfray et al., 2010). Many agronomically important traits such as yield, quality and some forms of disease resistance and malting traits are quantitatively controlled. Although the use of classical breeding for crop improvement has been successful to some extent, nonetheless it is faced with serious limitations associated with high cost, intensive labour requirement and long duration of product development (Flowers, 2004). The need to augment classical breeding with tools of biotechnology such as genetic engineering to precisely introduce desired traits provide a sustainable means of developing crop varieties with ameliorative effects towards abiotic stress (Dunwell, 2000). The success and rate of progress depends on the occurrence of genetic variation desirable for crop improvement and availability of suitable methods of gene transfer and selection (Saisho and Takeda, 2011).

1.2 Importance of barley

Barley (*Hordeum vulgare* L) is ranked as the fifth most important cereal crop globally (http://faostat.fao.org/site/370/default.aspx). It is used as a source of malt in brewing and in preparation of non alcoholic beverages (Gupta et al., 2010), as animal feed and for human consumption (von Bothmer et al., 1995) Due to its hardy nature, superior nutritional and medicinal value barley is gaining popularity as a rich source of beta

glucans which play a role in management of diabetes (Gallaher et al., 1993, Frost et al., 1999). It is a highly adaptable cereal crop growing in wide agroecological zones ranging from subarctic to subtropical climates with major production areas in Europe, the Meditereanean regions, East Africa, Russia, China, India, and North Africa (Hartan, 1995). In the history of mankind barley has been an important food source in many parts of the world (Newman and Newman, 2006). In SSA, barley consumed as food is usually cultivated in stressed areas characterized by soil erosion, occasioned by drought or frost limits other crops (Kuma et al., 2011). Barley was first introduced to Kenya as animal feed by the British colonialist in 1912. Since then it has increased in terms of the production area. Diversified utilization of barley products includes cakes, cookies and bread where it usually replaces wheat products.

1.3 Barley as model for genomic research in cereal crops

The exploitation of genetics in plant biology aims at the physiological and molecular characterization of the phenotype trait of interest (Koornneef et al., 1997). Barley has been used as an excellent model plant to study plant genetics and physiology because of some special characteristic: it is true diploid with a high frequency of self fertilization occurrence at 99%. It also exhibits a wide physiological and morphological variation. In addition genetic stocks perpetuated by selfing are available in gene banks. Additionally, barley is a short season crop, which needs a minimum of 2-3 months to complete its life cycle depending on the environmental conditions. Moreover, the genetic map based on morphological, physiological, cytological, protein and molecular markers is available (Forster et al., 1997). The crop is amenable to both, conventional and marker assisted breeding techniques, and displays a remarkably wide ecological adaptability and tolerance to cold, drought and salinity (Saisho and Takeda, 2011). Barley has a large genome size of 5.1 gigabytes distributed in seven chromosomes (Barley genomes consortium, 2012). Therefore barley is recognized as a good genomic model for the Triticeae family, which include wheat (einkorn, durum and bread wheat) rye and their respective wild relatives (Schulte et al., 2009) despite its large genome size. With the availability of the barley genome sequence, several genomic resources are available for exploitation towards gene discovery and genetic manupilation of barley (Varshney et al., 2005, Barley genome consortium, 2012).

1.4 Abiotic stress response in plants

Plants encounter a variety of biotic and abiotic constraints while growing in their native environments (Atkinson and Urwin, 2012). Drought, cold, high salinity, heat and nutrient deficiencies are the major abiotic stresses that negatively affect the survival, biomass production and yields of staple food crops contributing up to up to 70% of yield losses (Ali et al., 2004, Humphreys, 2007, Thakur et al., 2010). The stress factors trigger a series of unfavourable morphological, physiological and molecular alterations in plants subsequently causing low productivity and subsequent death (Wang et al., 2000). Drought, salinity and declining soil fertility are becoming particularly widespread in many regions of the world and may cause upto 50% yield reduction in all arable lands by the year 2050 (Ashraf and Harris, 2004). In SSA, declining soil fertility in the arable lands is the major cause of low agricultural productivity characterized by pronounced deficiencies of major macronutrient mainly N and P (López-Bucio et al., 2003). Abiotic stress tolerance is a multigenic trait in plants and the mechanisms are not fully understood (Cushman and Bohnert, 2000). Moreover, yield trait which is an important component in crop improvement is also quantitatively controlled. It becomes therefore difficult to improve abiotc tress tolerance without compromising yield (Hoisington et al., 1999, Collard and Mackill, 2008).

There is an urgent need to employ tools of biotechnology such as genetic engineering to complement ongoing conventional breeding strategies to develop crop varieties that can cope with adverse abiotic and biotic stresses (Witcombe et al., 2008a). In order to achieve this goal a sound understanding of molecular processes during abiotic stress including signal perception, transduction cascades and regulatory mechanism must be demonstrated (Shinozaki et al., 2003, Ray et al., 2009, Heidarvand and Amiri, 2010). The availability of genomic, proteomic and metabolomic databases and analytic tools have accelerated the dissection of genome and physiology of model plants such as Arabidopsis, extremophiles halophytes (*Halophila subsegenia*) and resurrection plants (*Craterostigma plantageneum* and *Xerophyta viscosa*) and subsequent extrapolation of the knowledge to agronomically important crops (Sanchez et al., 2011). Plant species exhibit variation in their sensitivity and response to the decrease in water potential caused by drought, high salinity or low temperature suggesting plants have encoded genetic capability for stress perception, signalling and response (Bohnert et al., 1995). Additionally, plants respond to nutrient deprivation by modulating their root morphology including root growth, root proliferation

and specific functional responses that depend on the prevailing nutrient status of the plant in order to enhance absorption capability. In response to alterations in growth environments, plants modulate series of adaptive mechanisms which can be grouped into physiological and morphological adaptations, biochemical and molecular alterations.

1.4.1 Morphological and physiological adaptations to salinity and drought stress

Although salt and water stresses are clearly different from each other in their physical nature and each elicits specific plant responses, they also activate some unifying reactions in plants (McCue and Hanson, 1990). Both stresses induce dehydrative elements resulting in reduced viscosity and aggregation of macromolecular components causing various alterations, which can act as signal for triggering adaptation responses (Bohnert et al., 1995). Physiological adaptations include leaf wilting and curling, reduction in leaf surface area, leaf abscission, stomatal closure and stimulation of root growth (Anjum et al., 2011) Other cellular changes include decrease of turgor and changes in the composition of the cell wall or plasma membranes, decrease in relative water content, increased production and accumulation of free radicals such as reactive oxygen species (ROS) which disrupts cellular homeostasis, membrane damage and subsequent plant death (Mittler, 2002, Apel and Hirt, 2004, Nayyar and Gupta, 2006, Gill and Tuteja, 2010). Oxidative stress further causes denaturation of nucleic acids, structural and functional proteins and oxidation of lipids (Gill and Tuteja, 2010). In addition, the reactions invoke common signaling pathways (Zhu, 2001, Shinozaki and Yamaguchi-Shinozaki, 1997) that result in the cellular responses involving up-regulation of stress proteins such as late embryogensis abundant proteins (LEA-DII), increased accumulation of antioxidants and accumulation of compatible solutes, such proline and glycine betaine (GB). The induction of some plant genes is the most widely studied common response (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.4.2 Stress transduction during salt and drought stress

Upon the perception of stress, plants initiate a cascade of reactions via the receptors embedded on the membrane of the plant cells. The transduction of perceived signals involves the integration of networks that convey a variety of internal and external stimuli, leading to activation of nuclear transcription factors to induce the expression of many genes and proteins (Boudsocq and Laurière, 2005). The main challenge is the

understanding of how the various networks are interconnected in plant responses. Perceiving of stress is followed by signal transductions downstream, which results in the generation of second messengers including calcium ions (Ca^{2+}), reactive oxygen species and inositol phosphates. Cytosolic Ca^{2+} levels increase in plant cells in response to environmental cues including pathogen challenge, osmotic stress, water stress, cold and wounding (Bonaventure et al., 2011, Takahashi et al., 2011). The receptor-coupled protein phosphorylation is a common form of signal initiation. Although none of the receptors for cold, drought and salinity in plants has been determined to certainity, current knowledge indicates that receptor-like protein kinases, two-component histidine kinases, as well as G-protein-associated receptors may represent the potential sensors of these signals.

1.5 Changes of gene expression during abiotic stress

Modulation of gene expression with extensive regulation occurring at both the transcriptional and post-transcriptional level has a central role in cellular adaptation to short or long-term environmental changes. The diverse metabolic and molecular responses elicited by environmental extremes are presented in **Fig. 1**.

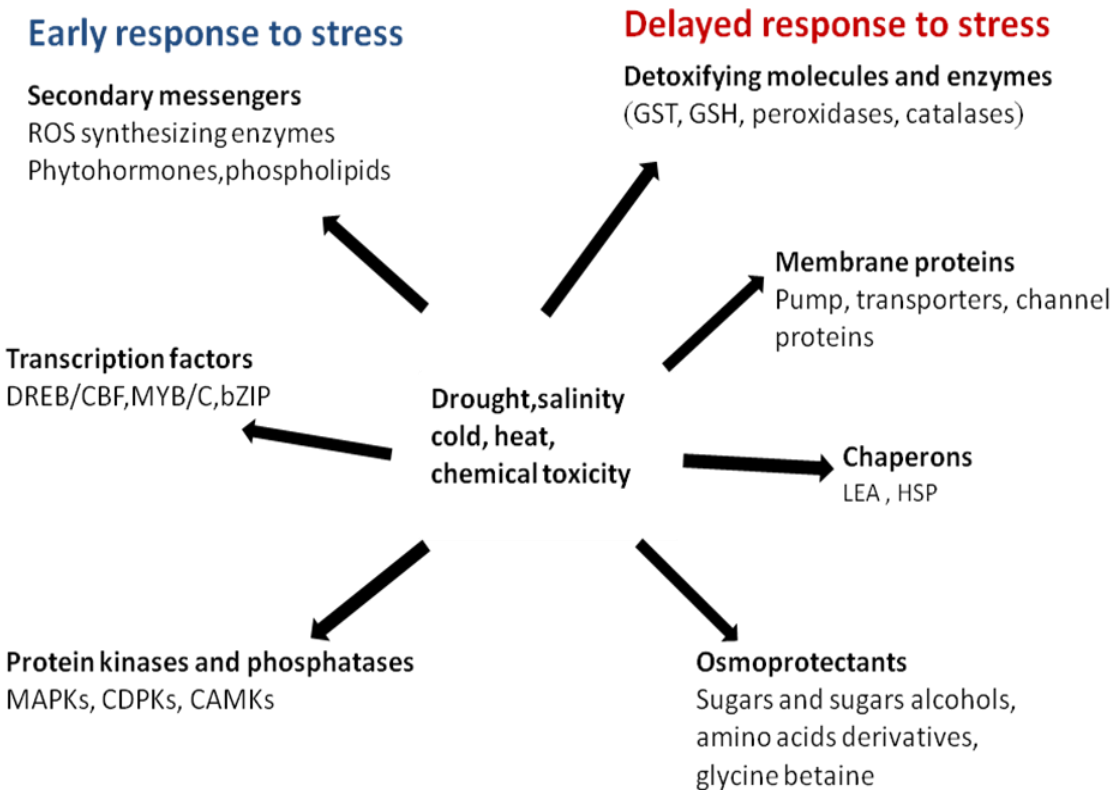

Figure 1. The categories of genes that may be involved in adaptive responses to abiotic stress. Early response genes on the left are induced rapidly in response to abiotic stress and largely regulate the expression of genes with protective roles on the right. Adapted and modified from Wang et al. (2003a).

Most of these gene expression responses are transient and, even with persistent stress, gene expression returns after some time to new steady-state levels that are close to those in unstressed cells. The magnitude of stress response is related to the severity of the perturbation, and different perturbations result in distinct expression signatures. Tolerance to stress is dependent on genotype and the expression patterns of the available stress-regulated genes. However, not all the genes that are up-regulated have adaptive roles in stress tolerance, but may be products of cellular injury (Zhu, 2000) Transcriptome analysis using microarrays revealed that stress-responsive gene expression fall under two groups (Shinozaki and Yamaguchi-Shinozaki, 1997, Bohnert et al., 2001). The various categories of stress responsive genes are illustrated in **Fig. 1**.

The first group on the left side referred to as primary or early response genes are detected within a short period of stress exposure and regulate the expression of other genes at the transcription level through phosphorylation (Zhu, 2002) or methylation (Zhang et al., 2006). Most of these genes are involved in signalling cascades and transcriptional control.

The use of the primary response genes has become the primary target for plant genetic engineering as they trigger rapid up-regulation of downstream genes. These include protein kinases such as mitogen activated protein kinase (MAPK), salt overly sensitive kinase (SOSK), calcium dependent protein kinase (CDPK), phospholipase (Frank et al., 2000) and transcription factors such as dehydration responsive element binding (DREB), MYC (myelocytomatosis), MYB (myeloblastosis) and basic leucine zipper (bZIP) (Shinozaki Yamaguchi-Shinozaki, 2000Agarwal et al., 2006, Dubos et al., 2010, Lata and Prasad, 2011, Mizoi et al., 2012,). The second group on the right constitutes proteins which directly protect proteins and membranes. These include enzymes involved in biosynthesis of osmolytes such as proline, glycine betaine, sugar and sugar alcohols, heat shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA-II), and free-radical scavengers (Ingram and Bartels, 1996, Close, 1997, Cuming, 1999, Chen and Murata, 2002, Wise and Tunnacliffe, 2004, Swindell et al., 2007, Gill and Tuteja, 2010, Luis et al., 2006, Sun and Lin, 2010, Szabados and Savoure, 2010). Transgenic plants with enhanced abiotic stress tolerance have been generated by overexpression of the protective proteins (Cushman and Bohnert, 2000, Davey et al., 2010)

1.5.1 Transcription factors

Initiation of transcription is an important control mechanism in gene expression. Transcription factors (TFs) are proteins that work in a coordinated manner with other transcriptional regulators, including chromatin remodeling/modifying proteins to employ or obstruct RNA polymerases to the DNA template (Udvardi et al., 2007). TFs interact with *cis*-elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes, thus imparting stress tolerance (Agarwal and Jha, 2010). Induction of TFs is tightly regulated by environmental factors, developmental stage and type of tissues (Albert et al., 2011). In Arabidopsis, *cis*-elements and corresponding binding proteins, with distinct type of DNA binding domains, such as AP2/ERF (apetala 2/ ethylene responsive factor), bZIP, HD-ZIP (homeodomain leucine zipper), MYC, MYB and different classes of zinc finger domains, have been elucidated (Shinozaki and Yamaguchi-Shinozaki 2000, Pastori and Foyer, 2002)

1.5.2 The role of ABA in abiotic stress responses

It is well established that abscisic acid (ABA) is a major physiological signalling molecule that is induced in response to abiotic stress (Gómez et al., 1988). This was reported by Xiong et al. (2001) through the demonstration that ABA-deficient mutants are highly sensitive to both drought and salt stress and died upon prolonged exposure to stress. ABA plays a major role in water balance mainly through guard cell regulation. Transcription induction can be regulated through the ABA-dependent or ABA-independent signal transduction pathways thus forming a highly complex gene network (Fowler and Thomashow, 2002, Umezawa et al., 2006). The complex network of cascades involved in abiotic stress regulation is presented in **Fig. 2.**

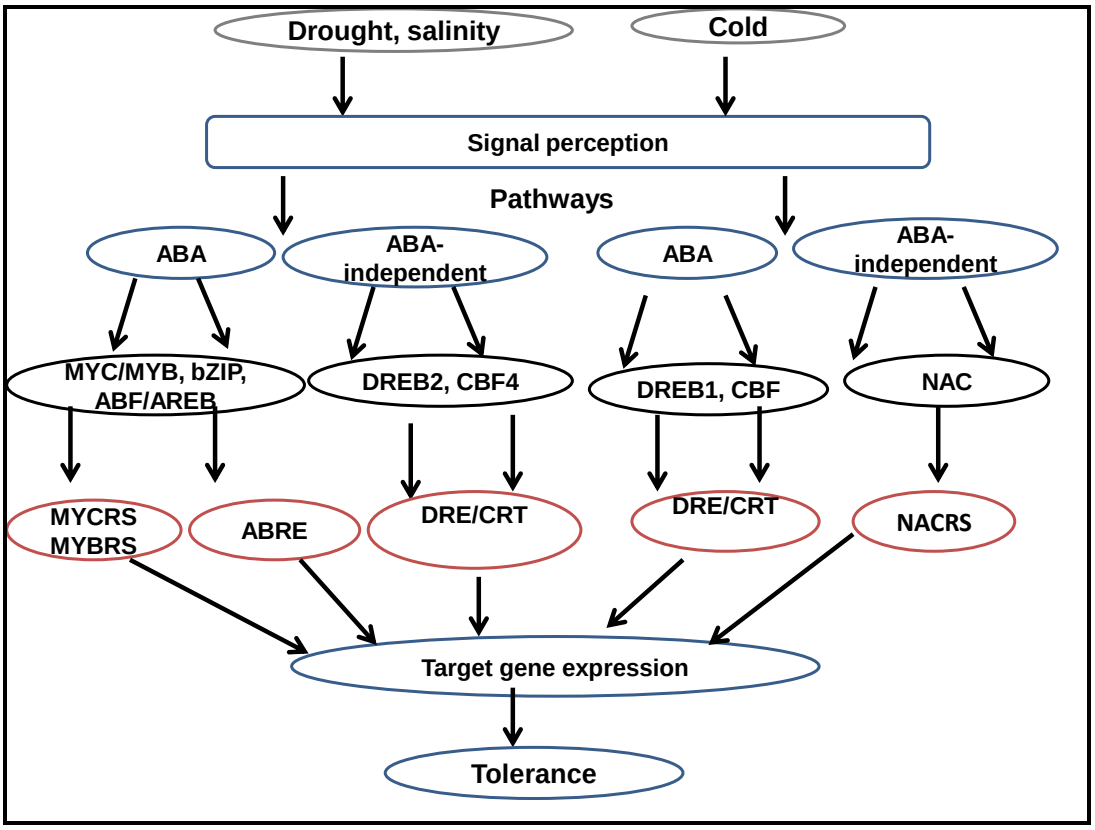

Figure 2. A scheme for cascades leading to gene expression in response to drought, salinity and cold regulated through the ABA-dependent and ABA-independent regulatory pathways. The transcriptional regulators involving the corresponding *cis* regulatory elements have been discussed. The transcription factors are encrypted in black circle, while the *cis* acting elements are in red. This scheme was modified from Agarwal et al. (2006).

ABA is an important plant hormone that plays a regulatory role in many physiological processes in plants, such as embryo maturation, seed development, seed and bud

dormancy, seed germination, root growth, fruit ripening, regulation of stomatal aperture and the activation of stress responsive genes (Agarwal and Jha, 2010). Additionally, ABA acts as a signalling molecule in abiotic stress responses (Grill and Himmelbach, 1998). Increased accumulation of ABA is induced by various abiotic stress cues such as desiccation, salinity and water deficit (Fujita et al., 2006). Induction of several genes in response to dehydration, cold and exogenous ABA treatment has been reported by Zhu. (2002) and Shinozaki et al. (2003). However, there are also many genes that do not respond to such treatments (Yamaguchi-Shinozaki and Shinozaki, 2005) suggesting the existence of both ABA-dependent and ABA-independent signal transduction pathways. The analysis of the expression patterns in promoter regions of the stress-inducible genes have helped to dissect ABA-dependent and ABA-independent regulation pathways (Yamaguchi-Shinozaki and Shinozaki, 1993). Dehydration responsive element-binding (DRE) / C-Repeats responsive elements (CRT) are one of the major *cis*-acting elements, which function in ABA-responsive or non-responsive gene expression during abiotic stresses (Nakashima and Yamaguchi-Shinozaki, 2010). ABA-dependent signaling systems have been described as pathways that mediate adaptation to stress by the activation of at least two different regulons: (i) the AREB/ABF (ABA-responsive element-binding protein/ ABA-binding factor) regulon; characterised by conserved ABA responsive element (PyACGTGGC) in their promoter regions (Grill and Himmelbach, 1998). (ii) the MYC/MYB regulon (Abe et al., 1997, Busk and Jensen, 1997). On the other hand, the ABA-independent regulons are: (i) the CBF/DREB (cold-binding factor/dehydration responsive element binding) regulon and (ii) the NAC and ZF-HD (zinc-finger homeodomain) regulon (Saibo et al., 2009). Additionally, several studies have identified the existence of both ABA-dependent and ABA-independent pathways of stress responses that function through AP2/EREBP (ERF) family members (Yamaguchi-Shinozaki and Shinozaki, 1994, Yamaguchi-Shinozaki and Shinozaki, 1994, Kizis, 2002). Other TF such as WRKY, HARDY, Zinc fingers etc. are important in abiotic stress tolerance responses and play key regulatory roles in plants (Lata et al., 2011). It is strongly envisaged that genetic expression of certain TF can contribute to abiotic stress tolerance in plants. Transcription factor-based technologies are a major target of the next generation of successful biotechnology-derived crop (Century et al., 2008). This was further supported by the studies of Jisha et al. (2015) which demonstrated that overexpression of AP2/ERF type transcription factor OsEREBP1 confers abiotic and

biotic stress in rice. Transformation of rice with the *OsWRKY11* gene under the control of *HSP11* promoter enhance heat and drought tolerance in rice seedling which was attributed with upregulation of two genes encoding raffinose synthase and galactinol synthase involved in the synthesis of raffinose and other sugar alcohols (Wu et al., 2009).

1.6 Accumulation of stress inducible compounds and proteins

One of the most common stress responses in plants is over-production of different types of compatible organic solutes (Serraj and Sinclair, 2002). Compatible solutes also known as osmoprotectants are low molecular weight metabolites that accumulate in the cell at molar concentrations without any toxic effect, stabilize proteins and play a role in survival and growth of plants during stress exposure (Chen and Murata, 2008). Included in this group are amino acids (proline), quaternary ammonium compounds (glycine betaine), polyols and sugars (mannitol, D-ononitol, trehalose, sucrose, fructan) (Nuccio et al., 1999, Bohnert and Jensen, 1996). They are also suggested to act as low molecular-weight chaperones, stabilizing the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Yancey, 2005). It has been demonstrated that engineering osmoprotectant biosynthesis genes are the best strategy to generate stress tolerant plants (Rathinasabapathi, 2000).

1.6.1 Glycine betaine

Glycine betaine (N, N, N-trimethylglycine) is a fully N-methyl-substituted derivative of glycine, that occurs naturally in a wide variety of plants, animals and microorganisms (Rhodes and Hanson, 1993, Chen and Murata, 2008, Chen and Murata, 2011). Accumulation of GB in response to salinity, drought and freezing has been reported in higher plants that naturally accumulate this compound (Gorham, 1995). However, some economically important crops, including rice (*Oryza sativa*), potato (*Solanum tuberosum*), and tomato (*Solanum lycopersicum*), are unable to accumulate GB (Rhodes and Hanson, 1993). GB accumulates in the chloroplasts and plastids and to some extent in the cytosol of many halotolerant plants. *In vitro* experiments have shown that GB stabilizes the structures and activities of proteins and maintains the integrity of membranes against the damaging effects of high salt, heat, cold and freezing (Gorham, 1995). Plants synthesize glycine betaine via a two-step oxidation of choline: Choline→betainealdehyde→ glycine betaine catalyzed by choline monooxygenase (CMO) and betaine aldehyde

dehydrogenase (BADH) (Rhodes and Hanson, 1993). The exogenous application of GB and the introduction via transgenes of the GB-biosynthetic pathway into plants that do not naturally accumulate GB increase the tolerance of such plants to salinity, drought and cold stress. Genetic engineering of glycinebetaine synthesis in tomato was reported to protect seeds, plants, and flowers from damaging effects of chilling (Park et al., 2004). Expression of bacterial Choline oxidase (*CodA*) in transgenic potato chloroplast enhanced oxidative, salt and drought tolerance (Ahmad et al., 2008, Goel et al., 2011). Additionally, Wang et al. (2010) reported increased tolerance to heat and salinity in photosynthetic apparatus overcummulating GB in wheat. Despite its potential benefits, a possible disadvantage of exogenous application of GB is the risk of pathogen attacks on plants applied with GB, as it is a potent growth substrate for certain plant pathogenic fungi (Rhodes and Hanson, 1993).

1.6.3 Proline

The amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to various environmental stresses (Ali et al., 1999, Kishor et al., 2005). The multi-functional roles of proline has been reviewed by Szabados and Savoure. (2010). Proline is indicated in osmotic adjustment, stabilizing sub-cellular structures such as membranes and proteins, scavenging free radicals and buffering cellular redox potential under stress conditions (Smirnoff and Cumbes, 1989, Ketchum et al., 1991, Mohanty and Matysik, 2001). In many plant species accumulation of proline in response to abiotic stress has been shown to be positively correlated to abiotic stress tolerance with remarkably higher levels in stress tolerant than in susceptible genotypes. This has been reported in rice (Lutts et al., 1996) sorghum (de Lacerda et al., 2003), cotton (Parida et al., 2008) and *Agrostis stolonifera* (Ahmad et al., 1981). In plants, the biosynthesis of proline from its precursor glutamic acid occur via two enzymatic steps: pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Delauney and Verma, 1993). Furthermore, regulation of proline is under catabolism and transport with proline dehydrogenase (PDH) responsible for degradation of proline being repressed by abiotic stresses (Nakashima et al., 1998). Transgenic plants overexpressing P5CS exhibited increased accumulation of proline and concomitant tolerance to salinity and drought stresses (Hmida-Sayari et al., 2005).

1.6.4 Late embryogenesis abundant (LEA) proteins

The accumulation of LEA proteins has been described as the most common adaptive mechanism in response to dehydration and cold. LEA proteins were initially isolated from cotton (*Gossypium hirsutum*) during later stages of seed maturation comprising 4% of total proteins (Dure et al., 1981, Galau et al, 1986). Subsequently, proteins homologous to the cotton LEAs have been identified in the seeds of many higher plants (Dure III et al., 1989) and also in vegetative tissues of plants in response to dehydration, salinity, cold and ABA treatments (Ramanjulu and Bartels, 2002). They have been reported in mosses (Ruibal et al., 2012), algae (Li et al., 1998), cyanobacteria (Kim et al., 2012) angiosperms and gymnosperms (Svensson et al., 2002, Close, 1996). Additionally, LEA and LEA-like proteins have been reported in animals, including nematodes (Browne et al., 2004) and arthropod larvae (Kikawada et al., 2006). Currently several entries of different members of LEA superfamily are available in public domain database such as the Genebank (http://www.ncbi.nlm.nih.gov/).

1.6.4.1 Classification of LEA protein

Various methods are available for classification of LEA proteins. Initially LEA proteins were classified according to the conserved short amino acid sequence motif which occur once or several times within the protein sequence (Dure III et al., 1989). With increasing information that describes new members, variations in transcript profiles, descriptions of organisms other than plants using new tools of bioinformatics, classification has been rearranged into superfamilies of LEA (Wise and Tunnacliffe, 2004, Battaglia et al., 2008, Bies-Etheve et al., 2008). According to Battaglia et al. (2008), classification is based on the presence of specific motifs across species which are unique to each family. Based on these characteristics and with consideration of available sequence information from different plant species LEA proteins can be classified into 7 distinct groups. Groups 1 to 4 represent the major groups containing majority of members of LEA proteins described from cotton: group1 (D-19, D-132), group 2 (D-11), group 3 (D-7/D-29), group 4 (D-113). The group 5 is represented by D-34, D-73, and D-95 while group 6 represented by LEA18 (Colmenero-Flores et al., 1999). ASR1 (Rossi et al., 1996) is a member of group 7. Group 1 LEA proteins is characterized by an internal hydrophylic 20-mer sequence TRKEQ [L/M] G[T/E] EGY[Q/K] EMGRKKGG[L/E] and glycine (18%) is glutamate and glutamine rich. The group 2 LEA referred to as dehydrins are the most studied stress

inducible genes (Ingram and Bartels, 1996a, Close, 1997, Saavedra et al., 2006, Choi et al., 1999, The dehydrins are characterized by Y- S- and K- segments. The Y-segment consensus motif T/VDEYGNP is located near the N-terminus and less conserved regions, usually rich in polar amino acids (Allagulova et al., 2003). Group 3 LEA proteins is characterized by a 11 amino acid repeat motif (Dure, 1993). It is predicted that this motif may form an amphipathic α helix with which may form intra- and inter-molecular interactions. Compared to other groups, group 3 LEA proteins are quite diverse. The differences in molecular masses in this group are as a result of the number of 11-mer motifs. The LEA groups 4 are endemic in the plant kingdom including non vascular plants (bradophytes) and vascular plants (angiosperms and gymnosperms). On the other hand group 5 LEA proteins is characterized by high proportions of hydrophobic amino acid residues which are not soluble after boiling suggesting that they adopt globular structures (Cuming, 1999). Group 6 LEA proteins occurs in small size (7-14 kDa) and are highly conserved. Four motifs distinguish this group and two motifs (motif 1 and 2) are highly conserved. The sequence LEDYK present in motif 1 and Pro and Thr present in position 6 and 7 of motif 2 display 100% similarity. Most members belonging to group 7 LEA proteins are small in size (Maskin et al., 2007) and posses 3 highly conserved regions one of them containing a nuclear localisation signal (Silhavy et al., 1995).

1.6.4.2 Dehyrins (LEA-DII) proteins

Dehydrins (DHNs) are a class of highly hydrophilic, thermostable stress proteins enriched number of charged amino acids that belong to Group II LEA family. (Ingram and Bartels, 1996, Kim and Nam, 2010, Yang et al., 2012). They are characterized by wide range of molecular masses from 9–200 kD (Ouellet et al., 1993). Genes encoding DHN proteins are expressed during the late embryogenesis and vegetative tissues exposed to low temperature, dehydration, salinity and application of ABA (Nylander et al., 2001, Xu et al., 2008). They are characterized by the conserved Lys-rich 15 amino acid consensus (EKKGIMDKIKEKLPG) designated K, a track of Ser residues (the S-segment) and consensus motif (T/VDEYGNP) designated the Y-segment usually located near the N-terminus (Close, 1997, Allagulova et al. 2003). The number and order of Y,S, and K segments has been used to define the various DHNs into five structural groups: Kn, SKn, KnS, YnKn and YnSKn (Close, 1996, Svensson et al., 2002).

The wide distribution of dehydrins in the vegetative tissues of plants grown under normal conditions suggests that these proteins may also play an essential role during plant growth.

Existence of dehydrin superfamilies has been reported in many plants species. The Arabidopsis genome revealed the presence of 10 members (Hundertmark and Hincha, 2008, Bies-Etheve et al., 2008) 13 in barley (Choi et al., 1999, Tommasini et al., 2008), 11 members in poplar (Liu et al., 2012), 8 members in rice (Wang et al., 2007), 36 members in soybean (Li et al., 2011), 54 members in wheat (Wang et al., 2014) and 4 members in grapes (Yang et al., 2012).

1.6.4.3 Function of dehydrins (LEA-DII) proteins

There is a strong association in different organisms between expression of LEA proteins and tolerance to water stress, thus suggesting that these proteins represent a common adaptation to water deficit (Garay-Arroyo et al., 2000). Physiological studies focused on plant stress response have reported a positive correlation between the level of accumulation of DHN transcripts or their corresponding proteins and plant stress tolerance (Kosova et al., 2014). Overexpression of DHN in transgenic plants has been found to enhance resistance against various adverse environmental conditions such as drought, cold, salinity and osmotic stress (Puhakainen et al., 2004, Shekhawat et al., 2011, Ochoa-Alfaro et al., 2012), which has created significant interest in their potential application in crop improvement. Cold and chilling tolerance in transgenic lines has been linked to accumulation of LEA proteins (Peng et al., 2008, Yin et al., 2006).

Several other functions have been proposed for DHN proteins. The role of DHN proteins in free radical scavenging and metal binding properties has been demonstrated (Hara et al, 2005). The interaction of dehydrins protein and free metal ions prevents the intracellular compounds from excessive ROS formation since free metal ions act as catalyzers of synthesis of various ROS. The metal binding effect was demonstrated in CuCOR15 and CuCOR19 in *Citrus unshiu* binding to Ca^{2+} or metal ion transporters (Hara et al., 2001, Alsheikh et al., 2003) and further prevented lipid peroxidation (Hara et al., 2003). Additionally the *Citrus unshiu* CuCOR19 dehydrin was shown to protect catalase and lactate dehydrogenase (LDH) against freezing inactivation, and it was more effective than compatible solutes such as sucrose, glycine betaine or proline (Hara et al., 2001a). Interaction of partly hydrated surfaces of DHNs and other proteins or biomembranes

confer protective benefits. The role of DHN, ERD10 and ERD14 isolated from *A. thaliana* was shown to confer protection against thermal aggregation of citrate synthase, firefly luciferase and thermal inactivation of alcohol dehydrogenase (Kovacs et al., 2008) and to stabilize membranes by acting as chaperons.

1.7. Plants responses to nutrient deprivation

Declining soil fertility in agricultural soils is a serious threat to crop productivity and global food security (Roy et al., 2006, Obalum et al., 2012). N and P are essential macroniútrients that determine crop growth and development and their deficiencies are detrimental to crop productivity (Sanchez, 2002). Since water and nutrients are heterogeneously distributed in the soil, the root architecture will markedly affect the ability of plants to secure the soil resources (Lynch, 1995). Roots have a role in sustaining the productivity of agricultural soils, by stabilizing them physically, improving their structure and by driving microbial processes, through substrate inputs, that maintain soil fertility (Hinsinger et al., 2009, Hallett and Bengough, 2013). In response to N and P deficiency an increased root-to-shoot ratio and root surface area has been reported for several plant species (López-Bucio et al., 2003, Gruber et al., 2013). Traditionally, P and N deficiency could be elleviated through the application of N and P fertilizer. This practice is however not sustainable way to solve the problem of nutrient deficiency in soil. Screening or breeding of plant species tolerant to N and P deficiency may be one of the effective alternatives to alleviate nutrient deficiency and to increase the utilization efficiency of N and P fertilizer, since plants exhibit inter-and intra-specific variations in tolerance (Beatty et al., 2010**,** Gupta et al., 2012).

1.7.1 Sensitivity of leaf and root tissue to nitrate

Nitrogen is a major component of chlorophyll, the most essential pigment needed for photosynthesis. It is also a constituent of other biomolecules such as ATP and nucleic acids (Weisany et al., 2013). It constitute the biological compounds involved in photosynthetic activities such as enzymes and vitamins associated with chlorophyll synthesis (Hokmalipour and Darbandi, 2011, Sara et al., 2013). Many metabolic processes in leaves are stimulated by light as a result of a requirement for products of thylakoid electron transport or products of carbon dioxide assimilation. Typical phenotypic characteristics of N deficiency is also associated with symptoms of yellowing,

dropping of leaves, stunted growth, delayed flowering and fruiting (Wu et al., 2006). Ribulose 1, 5 biphosphate carboxylase /oxygenase (Rubisco) the most abundant leaf protein plays a key role in carbon assimilation and is strongly affected by nitrogen limitation. The dual role of Rubisco as a key photosynthetic enzyme and a major nitrogen-containing compound in leaves predetermines its importance for plant productivity and a selection criterion for high yield (Vicente et al., 2011). Although development of transgenic plants overexpression RBSC and Ribulose activase has been achieved, it has not contributed to increased photosynthesis or biomass in rice leaves (Suzuki et al., 2009, Fukayama et al., 2012). Sucrose synthesis and nitrate assimilation are major processes in leaves that are generally coordinated with photosynthesis through the regulation of sucrose phosphate synthase (SPS) and nitrate reductase (NR) activities (Huber et al., 1992, McMichael Jr et al., 1995). The effect of N deficiency was shown to affect plant growth, leaf photosynthesis and hyperspectral reflectance properties in sorghum (Zhao et al., 2005). In related studies, Boussadia et al. (2010) demonstrated that N diminished photosynthesis, carbohydrate status and biomass in two olive cultivars 'Meski' and 'Koroneiki' contrasting in nitrogen use efficiency. N fertilizer, nitrate, (NO_3^-) is highly soluble in the soil and therefore easily lost through leaching or by bacterial denitrification (Vance, 2001). Nitrate from the soils is actively transported in roots through nitrate transporters (NRT) (Gojon et al., 2011). To mitigate the effects of low (<1 mM) or high (>1 mM) nitrate concentrations in soil plant have evolved a variety of nitrate transporters with different localizations and functions: uptake of nitrate by roots, transport in the xylem or phloem, regulation of nitrate content in seeds and transport into the vacuole for storage (Dechorgnat et al., 2011). Nitrogen transporter genes have been most thoroughly characterized in Arabidopsis. The Arabidopsis genome encodes at least 67 nitrate transporters (Forde, 2000), 4 in maize (Quaggiotti et al., 2003). In barley 7 genes encoding high affinity transporters were identified while 4 encoded low affinity transporters (Trueman et al., 1996). Tissue-specific expression of NRT family genes has been observed in several species, e.g. Arabidopsis, rice and cucumber
(Feng et al., 2011, Migocka et al., 2013). Transcriptional regulation of plant nitrogen-related genes is poorly understood, with very limited information available in monocots in particular. In dicots, including *Arabidopsis*, a 43 bp pseudo-palindromic nitrogen response element (NRE) was recently identified in the promoter of a nitrite reductase

(*NiR*) gene and shown to be necessary and sufficient for nitrate-activated gene transcription (Konishi and Yanagisawa, 2010).

1.7.2 Sensitivity of leaf and root tissues to phosphate

Phosphorus is a component of several cellular biomolecules such as ATP, nucleic acids, phospholipids and phosphorylated sugars and therefore plays an important role in carbon metabolism (Huang et al., 2011). It regulates cellular enzyme activities and metabolic pathways activities as well as transport processes. P is absorbed from the soil via the roots in the form of phosphate (Pi) anions but usually occurs in low concentration (1 µM- 5 µM) since most of it occurs in an insoluble form. This insolubility is due to the affinity of P to form compounds (salts) with various divalent and tri-valent cations such as Ca^{2+}, Mg^{2+} and Al^{3+} that are not amenable to plant uptake (Raghothama, 2000). P deficiency induces a wide array of metabolic effects that limit plant growth. Under P-deficient conditions, plants develop numerous morphological, physiological, biochemical and molecular adaptations (Raghothama, 1999a, Vance et al., 2003). Many researchers have reported that P deficiency has a significant influence on photosynthesis and carbon metabolisms in plants (Rao and Pessarakli, 1996). In many studies photosynthesis inhibition due to P deficiency has been explained by diminished Calvin cycle activity in particular limited activity and levels of Rubisco and subsequent regeneration of Ribulose 1, 5 biphospate (RuBP) (Jacob and Lawlor, 1992, Pieters et al., 2001). Usually, inadequate phosphorus slows the processes of carbohydrate utilization, development of a dark green leaf color or plants leaves developing a purple color. Significant decrease in leaf photosynthesis in response to P deficiency has been reported in various plant species including C3 plants such as barley (Foyer and Spencer, 1986) soybean (Fredeen et al., 1989) and C4 plants such as maize (Usuda and Shimogawara, 1992, Usuda and Shimogawara, 1993). Determination of a plant's requirement for P and the ability of the soil to supply this requirement in the form of phosphate (Pi) is crucial in maintaining crop yield and quality as well as and avoidance of excessive Pi entering the environment which contributes to eutrophication (White and Hammond, 2008). Better management of Pi fertiliser applications is one option to improve the efficiency of Pi fertiliser use, without compromising on crop yields (Were and Ochuodho, 2014). Alternatively, diagnostic genes with altered expression under Pi limiting conditions that suggest a physiological requirement for Pi fertilization could be used to manage Pi fertilizer applications and

might be more precise than indirect measurements of soil or tissue samples. The use of transcriptional profiling of phosphate transporters (Pht1) embedded in the plasma membrane is one of the available options (Rae et al., 2003). The expression of these transporters is induced in P-deficient plants, allowing Pi to be effectively taken up against the large concentration gradient that occurs between the soil and internal plant tissues (Smith et al., 2000).

1.8 Malting quality as affected by agronomic practices and abiotic stress

Abiotic stresses including nutrient deficiency not only affect yield and yield attributes, but also yield quality which subsequently influence the quality of seed used in malting processes**.** Starch, protein and cellulose are the major components of barley kernels. (Åman et al., 1985, Åman, and Newman 1986). Important characteristics for malting quality include grain protein content, texture, grain size, malt extract and diastatic power (Henry, 1990, Psota et al., 2007).

1.8.1 Kernel protein content

Grain protein content is dependent on the soil fertility which is directly related to availability of nitrogen and sulphur (Shewry, 1993) that is assimilated through the roots and converted to amino acid and protein in the barley kernel. Early planting and high yield usually reduces the seed protein. Short and dry growing seasons result in higher crude protein and lower starch content (Åman, and Newman 1986). Increase of nitrogen supply in the soil (Therrien et al., 1994, Le Bail and Meynard, 2003), abiotic stresses, including drought or heat, in particular in combination with water stress (Savin et al., 1996) has been shown to increase protein content in barley kernels. Åman et al. (1985) demonstrated that starch is inversely correlated to grain protein content. Moreover high protein content is undesirable and results in lower malt extract for the brewer (Fox et al., 2003). Additionally, increased protein slows down water uptake during steeping, potentially affecting the final malt quality. On the other hand, very low protein content results in limited enzymes necessary to modify the barley kernel and to break down starch during brewing. It further impairs brewing performance by reducing yeast amino acid nutrition. The contribution of environment to alterations of grain protein and subsequent effect on malting quality of barley has been reported (Echart-Almeida and Cavalli-Molina, 2001a, Molina-Cano et al., 2001). Application of excess nitrogenous fertilizer

will increase grain protein but the application of N based on soil analysis may have minimal effect on the grain protein varieties which tend to maintain a moderate protein level under a range of growing conditions. Many storage proteins have been identified with specific functions in regard to barley and malting qualities, whereas some have unknown function yet.

1.8.2 Hordein polypeptides

Grain storage proteins are ubiquitous in all cereal endosperms. Hordeins, the major storage proteins in barley constitute 50% of the total grain protein (Osman et al., 2002). Hordein polypeptides are highly soluble in aqueous alcohol solutions and can be grouped into four distinct fractions, namely D, C, B and A hordeins which differ in their electrophoretic mobility and amino acid composition (Shewry and Miflin, 1985). Since they are highly polymorphic, hordeins have been used in germplasm characterization and determination of malting attributes (Atanassov et al., 2001, Shewry and Halford, 2002, Lee et al., 2010). The D hordeins are found in the 105 kDa range. Synthesis of D hordeins is encoded by the *Hor3* locus located on the long arm of chromosome 1H (5) (Kreis et al., 1984). Chordeins occur between 50–80 kDa and are encoded by the *Hor1* locus. Additionally, the major B-hordeins occur in the range of 35–45 kDa are encoded by the *Hor2* locus. Both the *Hor1 and Hor2* are located on the short arm of chromosome 1H (5) (Shewry et al., 1978, Shewry and Miflin, 1985). During malting process, hordein polypeptides are degraded and utilized as part of the N mobilization source. Using monoclonal antibodies Kauffman et al. (1994) confirmed the presence of B and D hordein and the absence of C hordein components in lager beer respectively.

1.8.3 Physiological and biochemical processes in kernel during germination

Seed development and germination is separated by quiescence period referred to as dormancy. This phenomenon has been observed in higher plants. During seed maturation the cells of the starchy endosperm die, while those of the embryo and aleurone remain metabolically active. In the process of imbibition dormancy is broken and the germination process is initiated. The fundamental cellular activities such as respiration, enzyme activity, RNA and protein synthesis are initiated during seed germination (Potokina et al., 2002). These physiological processes are regulated by two phytohormones, gibberellic acid (GA) and ABA (White et al., 2000). Physiological and molecular mechanisms of

these two hormones have been elucidated (Yamaguchi et al., 2007). During germination, GA is transported to the aleurone and the scutellum layer surrounding the starchy endosperm, where it induces the *de novo* expressions of a number of genes encoding hydrolytic enzymes (hydrolases) such as such α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), limit dextrinase (LD, EC 3.2.1.41), *α*-glucosidase (3.2.1.20), β-glucanase (EC 3.2.1.73) (Manners, 1985, Fincher, 1989, Hayes and Jones, 2000).

The physiological and biochemical processes taking place in the barley grain during germination are illustrated in **Fig. 3.**

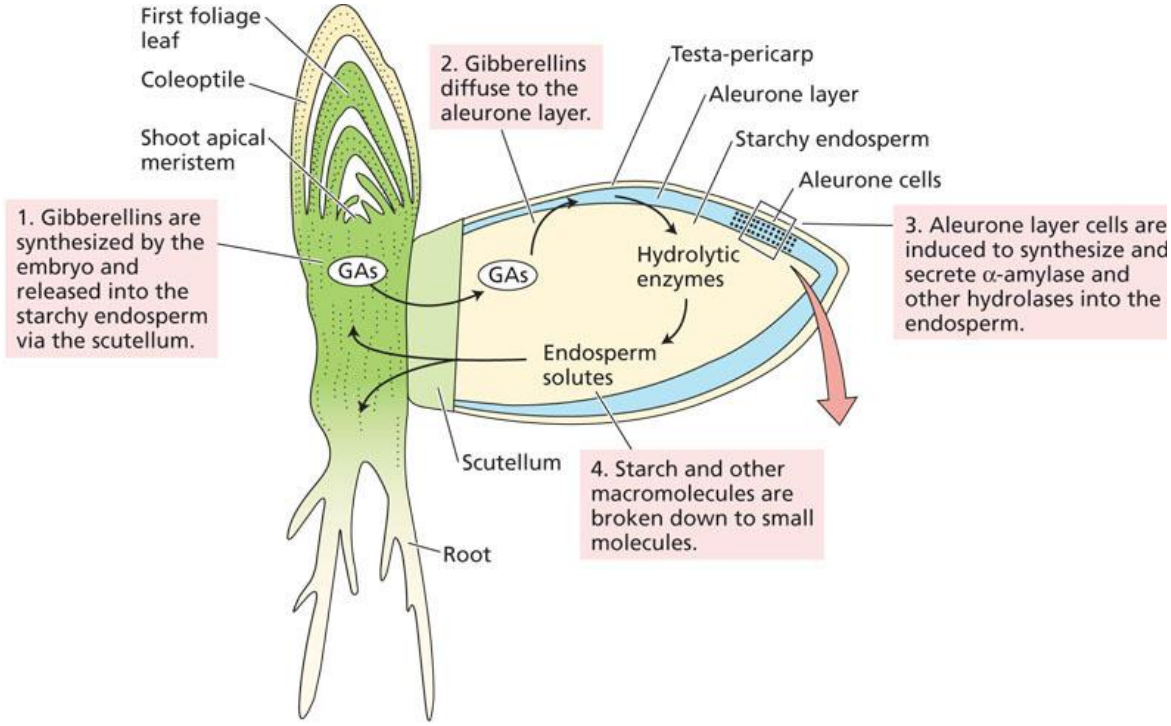

Figure 3. The barley kernel. Physiological and biochemical processes in germinating / malting barley grain. Imbibition triggers production of GA which in turn induces the *de novo* synthesis of hydrolytic enzymes (diastase/hydrolases) in the aleurone and scutellum. The enzymes are then secreted into the endosperm where they progressively degrade biopolymers into simpler forms that can be used by the germinating seedling (Adapted from Jamar et al., 2011).

Many of these hydrolytic enzymes are progressively secreted into the endosperm and mobilize storage biopolymers such as starch, protein and cell wall reserves into simple forms that can be used to support the rapidly growing seedling providing energy in the form C and N as source of energy or in the *de novo* synthesis of hydrolytic enzymes. Promoter analysis of α-amylase identified a 21 bp gibberellin response element (GARE)

containing a conserved TAACAA/GA sequence encoding GA-responsive genes, which is found in the promoters of many of malting enzymes (Skriver et al., 1991). This sequence is similar to the animal and plant MYB binding sites. HvGAMYB was identified as MYB transcription factor expressed in barley aleurone cells in response to gibberellins increase. Mutations introduced to the TAACAA/GA sequence inhibited the GA responsiveness of α-amylase in transient expression analysis. HvGAMYB transactivates a number of GA-responsive genes encoding hydrolytic enzymes in barley aleurone cells including α-amylase, β-glucanase, a cathepsin B-like protease and a cysteine proteinase among others, This suggests that HvGAMYB plays an important role in the GA-response pathway(s) (Gubler et al., 1995, Gubler et al., 2002).

Malting is defined as germination of seed under controlled conditions in order to attain a given physical and biochemical change within the grain and subsequent stabilization by drying. The initial step would involve the modification of the cell wall which is the main barrier restricting the mobility of important external resources such as water. During malting the biopolymers including starch, protein and cell wall glucans are metabolized enzymatically into simpler forms for energy provision during brewing (Jones, 2005). In order to achieve these changes three process steps occur: steeping germination and kilning: 1-steeping to ensure increase of water absorption from 12-42% moisture content; 2-germination to maintain embryo growth and hydrolytic enzymes produced in the aleurone cells (Bamforth and Barclay, 1993) and scutellum (Ranki, 1990, Stanley et al., 2011) and 3-kilning to ensure product stability. In this last step, the malted seed is dried by forcing air of 40-65 ^{0}C and then the temperature is gradually increased to 85 ^{0}C.

1.8.4 Role of hydrolyzing enzymes in malting

The barley grain is made of the starch and non-starch component. Starch is the most abundant component of the endosperm, comprising about 60% of total grain weight (Holtekjølen et al., 2006) while the endoperm cell wall is made of β-glucans which constitute 2-10% of the total barley grain weight (Henry, 1987). 75% of the endosperm cell wall is made of β-D-(1-3) (1-4) glucans and 20% account for minor component identified as arabinoxyglycans. The solubility of β glucans is dependent on the number and arrangement of β-D 1-3 and 1-4 linkages as well as the molecule size (Jeraci and Lewis, 1989). Starch is made of two polymers, amylopectin and amylose (Robyt, 1998). Amylose is a linear polymer made of glucose (Glc) residues linked through the α-(1-4)

glycosidic linkage whereas amylopectin is a highly branched polymer made of α-(1-4) and α-(1-6) glycosidic linkages (Hough and Hough, 1985). Generally, starch comprises 75% and 25% amylopectic and amylose, respectively. A barley breeding target is the development of, either very low amylose (waxy) or high proportion of amylose (non waxy) (Czuchajowska et al., 1998).

Rapid hydrolysis of starch to the fermentable carbohydrates glucose, maltose, and maltotriose is an important aspect of brewing. Extensive research in cereal biochemistry has focused on malting enzymes since they play a critical role in malting, an economically important process in beer industry (Manners, 1985).

A diagrammatic presentation of starch hydrolysis initiated by malting enzymes is described in **Fig. 4**.

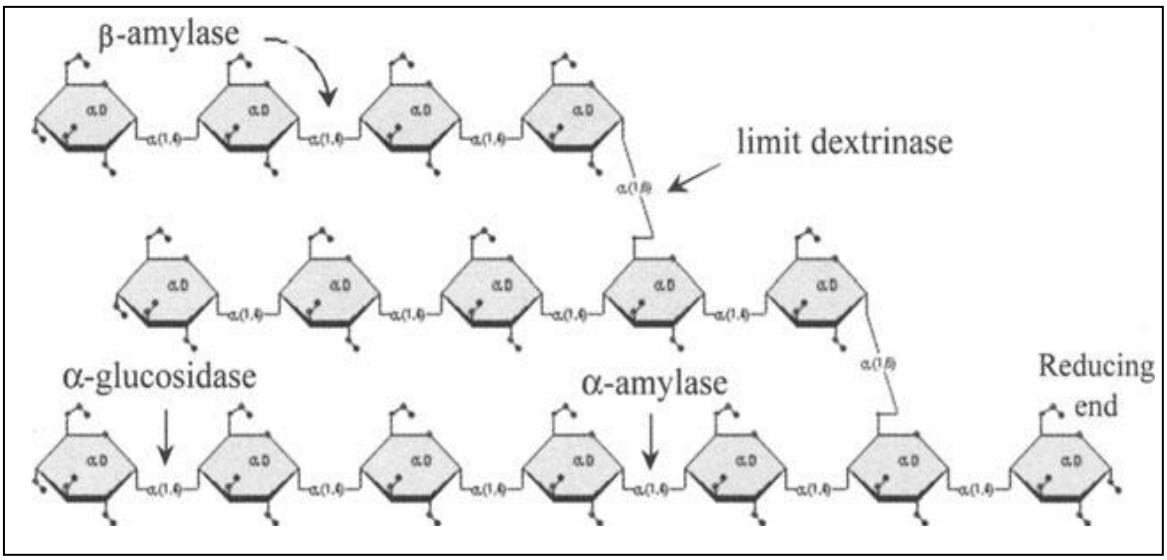

Figure 4. Structure and components of starch. The major hydrolytic (hydrolyases, or diastases) enzymes involved in in the malting process and their specific sites of action Adapted from Tester et al. (2004).

Malted barley is the main source of the diastases or diastatic power (DP) enzymes that hydrolyse starch into fermentable sugars that yeast converts into alcohol. The DP enzymes comprise the combined activity of the malt enzymes shown in **Fig. 4**.

1.9 Molecular basis of malting quality

Malting quality is a complex phenotype involving many interrelated components that display varied and complex hereditary patterns. Understanding the genetic basis of

malting quality remains a key agenda for malting barley breeding programmes (Hayes and Jones, 2000). Using genetic mapping 150 QTLs has been identified, which are associated with 19 malting traits (Zale et al., 2000). During the transition from grain development to germination changes in control of gene expression is tightly regulated. In the seed, the genes encoding storage proteins and enzymes involved in synthesis of other reserve compounds are switched off. On the other hand, the genes encoding enzymes involved in germination, initiation of axial growth and reserve mobilization are turned on. (Potokina et al., 2002, Watson and Henry, 2005, Lapitan et al., 2009).

1.10 Status of barley production and research in Kenya

Barley is grown in high altitude areas of about 3,000 m above sea level (masl) in the central province and mid altitude about 1,400m masl of the rift valley in Kenya. Favourable soil pH ranges between 5.0 and 8.3. Most of the soil requirement by barley is not found in the high and medium altitude zones, where soil pH is acidic (EABL-UoE, 2012). Barley farming injects heavily into the economic grid in Kenya contributing up to 28% of the gross domestic product (GDP) and a further 27% indirectly through its linkages with other economic sectors (EABL, 2005). Currently barley is cultivated on an estimated 40,000 ha with production falling below the expected 4.3 metric tons (Belay, 2006). The barley stakeholders are aiming towards increasing the production per unit to meet the demands albeit the encroaching effects of climate change. The EABL aim to increase production of barley through incentives in the form of contractual agreements with barley farmers by providing financial assistance, certified seeds, fertilizer, insurance services and better market prices in order to expand production areas and to counteract risks of shift to alternative crops that offer competitive disadvantage. As a result, area under cultivation of the crop in the region increased from 3,012 hectares to 21,630 hectares while the production has gone up from 92,434 to 627,705 bags in the seasons. (http://www.businessdailyafrica.com/Corporate-News/Brewery-incentive-stirs-up-barley-output-in-Rift-Valley/). Despite its contribution to economic growth, drought and nutrient deficiency in form of P and N are the major factors constraining barley production (EABL-UoE, 2012). Owing to the significance of barley, there is renewed interest in various stakeholders including farmers, national agricultural research centres, policy makers, plant health regulators and malting industry towards barley research and production to meet the increasing market demands. These include Nation Agricultural

Research Systems (NARS) represented by Universities such as Egerton and Eldoret, National Research Institutes such as Kenya Agricultural and Livestock Research Organization (KALRO), National Gene Bank of Kenya (GBK), Consultative Group on International Research (CGIAR), International Maize and Wheat Improvement Center, Mexico (CIMMYT) East Africa Malting Company, farmers and barley farmers associations, policy makers and plant health regulators such as Kenya Plant Health Inspectorate (KEPHIS) are the major stakeholders.

1.11 Rationale of the study

Production of adequate barley with desired malting specification has been hampered by inadequate and unreliable rainfall, diseases and declining soil fertility and fragile environments. The continuous drought spell has led to decrease in yield per unit area with subsequent compromised grain quality for malting purposes reflected in reduced grain size. Whereas intensive research has focused on improving yield traits and disease resistance in barley under field conditions, little is known about abiotic stress tolerance in at the seedling stage using both physiological and molecular assays in Kenyan barley cultivars. The use of laboratory based screening carried out under controlled conditions to evaluate abiotic stress tolerance in barley seedlings was aimed to overcome challenges of variability under field conditions reflected in disparities in soil pH and nutrient distribution, variability in ambient temperatures and rainfall and prevalence of diseases. A countrywide soil fertility survey, carried out under the auspice of fertilizer use recommendation project (FURP**)** of the World Bank was carried out for food crop production in the smallholder farms in Kenya and data pointed out that the Kenyan soils are characterized by deficiencies in major nutrients mainly N, P and K (Schnier et al., 1996). Additionally, Obura et al. (2010) reported that declining soil fertility is a major cause of food insecurity in sub-Saharan Africa. Whereas N and P fertilizer are important in increasing yield, there is need to determine critical nutrient levels that will neither compromise yield nor cause environmental pollution. The amount of available N in the soil affects the quality of barley grain targeted for brewing but not those targeted for animal feed or human consumption. Defining cultivar sensitivity to varying levels of N fertilizer application and its influence in yield under field conditions and malting attributes such as starch content and grain protein will validate the field and laboratory data and therefore the reproducibility of results.

Identifying nutrient specification of cultivars is an important tool for understanding the physiology, biochemical and molecular mechanism underlying abiotic stress tolerance and nutrient use efficiency. Analyzing gene expression of major starch degrading enzymes is a potential tool to characterize cultivars for suitability of malting especially exploiting the variability of malting enzymes during physiological stages of malting. Currently there is no information on the characterization of malting cultivars based on genotype dependent changes of transcript level of genes encoding key malting enzymes during the seed and progressive germination stages and kilning.

This study therefore seeks to identify physiological and molecular mechanisms that make some barley lines tolerant or susceptible to abiotic stresses and as well defining malting sutability. The findings in this study are novel since this work has not been reported before as far as studies of Kenyan barley are concerned.

1.12 Overall goal

The aim of this research project was to understand physiological and molecular basis of abiotic stress tolerance and malting attributes of Kenyan barley lines used for commercial production and as breeding lines.

1.12.1 Specific objectives

- Evaluation of differential physiological, biochemical and molecular responses to abiotic stress in seedlings of Kenyan barley lines.

- Determination of the effect of N treatment on seed germination, starch level, grain protein and differential expression of hordein polypeptide genes in three Kenyan malting cultivars.

- Determination of molecular and biochemical attributes of genes encoding malting enzymes in seed, during progressive germination and kilning stages in 4 malting barley cultivars.

- Evaluation of changes in morphological parameters of roots from pot-grown and *in vitro* propagated Kenyan barley seedlings in response to N and P deprivation.

1.13 Thesis Profile

The thesis introductory chapter captures themes that are relevant in addressing research for the subsequent chapters. Chapters 2, 3 and 4 are manuscript drafts that were generated from the research. Chapter 5 highlights the general conclusions and the way forward for the research project.

The following are manuscript drafts in preparation for submission:

Chapter 2

Differential physiological and molecular responses to abiotic stress in Kenyan barley seedlings.

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(Manuscript in preparation for submission to the Journal of Plant Physiology)

Chapter3

Biochemical and molecular characterization of malting attributes in response to varying nitrogen treatments and seeding rate in Kenyan malting cultivars

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(Manuscript in preparation to be submitted to the Journal of the Institute of Brewing)

Chapter 4

Analysis of root parameters in response N and P deprivation in pot-grown and *in vitro* propagated Kenyan barley seedlings

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(Manuscript prepared for submission to the African Crops Science Journal)

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2. Differential physiological and molecular response to abiotic stress in Kenyan barley seedlings

Abstract

Genotype variation in selected barley breeding lines was determined by differential profiles of seed hordein proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Consequently, a phylogenetic relationship was generated based on absence or presence of hordein bands using agglomerative hierachial clustering. In this study, physiological and molecular responses to stressors were evaluated in selected Kenyan barley seedlings. For physiological assays 0, 300, 600 mM NaCl was imposed on barley seedlings at the third leaf stage. Variation in physiological parameters including ionic conductance, lipid peroxidation, chlorophyll content, accumulation of proline, glycine betaine (GB), and sucrose was determined. Response to abiotic stress was found to be genotype dependent and varied with the stress magnitude. A low ionic conductance, lipid peroxidation and increased proline, sucrose, GB chlorophyll were associated with stress tolerance. Based on combined phenotype and physiological assays, a primary cluster of putative tolerant and susceptible cultivars was generated. Nguzo, MN-24 and MN-8 were clustered as tolerant while Karne, and Sabini were clustered as susceptiblelines, respectively. Expression profiles of major stress responsive genes following 7 days of exposure to dehydration, 150 mM and 300 mM NaCl treatments were determined in roots and shoots. The genes evaluated included dehydrins (*LEA II*), *Hordeum spontaneum* dehydration responsive (*Hsdr4*) and betaine aldehyde dehydrogenase (*HvBBD).* Expression of dehydrins genes was found to be genotype dependent, tissue specific and was affected by type and duration of stress. Dehydrins *Dhn1* and *Dhn9* genes were exclusively associated with dehydration response while *Dhn3*, *Dhn4* and *Dhn7* were induced by both dehydration and increased salt treatments. *Dhn5*, *HvBBD1* and *Hsdr4* were constitutively expressed. Immunoblot analysis showed that accumulation of DHN proteins was induced by dehydration and 300 mM salt treatments but neither in control nor under 150 mM salt treatments. These screening assays may be potential selection markers to aid rapid screening in breeding programs. The results presented here are the first report on the status of abiotic stress tolerance in Kenyan barley using seedlings and form a basis for further improvement for abiotic stress tolerance using both conventional and biotechnological approaches.

Keywords: Barley (*Hordeum vulgare* L), abiotic stress tolerance, hordein polypeptides, SDS-PAGE, Dehydrins, immunoblot.

2.1 Introduction

Barley is an important cereal crop targeted for animal feed (Jacob and Pescatore, 2012) malting and industrial purposes while 2% is used for human consumption (Baik and Ullrich, 2008). Barley was first introduced to Kenya in 1912 by the British imperialists as animal feed. Since then, barley has not only increased in production area for its demand in the brewing industry but also gained demand as human food due to increased drought prevalence coupled with increased awareness of its associated health benefits. Barley grain is a source of soluble and insoluble dietary fibre (DF) and other bioactive components such as vitamin E and B-complex, minerals and phenolic compounds. (Cavallero et al., 2002, Behall et al., 2004). In Kenya, the crop is grown in mid altitude to high altitude areas of the rift valley and the central provinces under both small and large scale mechanized farming. Despite stimulating growth in agricultural and industrial sector, barley faces many production challenges. These include unreliable and inadequate rainfall, diseases and pest, poor soils, poor infrastructure, low market prices, stiff competition from other crops. Consequently, these constrains have led to reduction in supply of adequate and good quality raw material for malting in brewing industries.

To mitigate some of the on farm production challenges, the barley breeding programs in Kenya continuously aim to develop varieties with a combination of efficient and reliable production characteristics and grain quality attributes targeted towards the diverse end uses. The most sought after agronomic traits include, yield and yield components, malt quality, abiotic and biotic stress tolerance. Determination of malting attributes requires laboratory based analysis of the harvested seeds (Frankel and Brown, 1984). Various methods are available to discriminate barley germplasm including the use of isozymes, hordeins (Naeem et al., 2006) and molecular markers. Analysis of hordein using SDS-PAGE is a powerful tool for determining cultivar identity (Shewry and Halford, 2002). Additionally, continued search for traits related to drought and salinity stress tolerance is an important step in breeding and production of cereals. Utilization of tools that allows early detection of traits will reduce the time taken to develop crop varieties (Ribaut et al., 2002).

Abiotic stresses such as reduced water availability, extreme temperatures (heat or freezing), salinity, deficiency in soil nutrients, increased ionic toxicity, increased light intensity (Verslues et al., 2006), affect root growth and architecture and reduce nutrient acquisition (Langridge, 2006) subsequently diminishing crop productivity worldwide (Boyer, 1982). Exposure of plants to abiotic stress disrupts metabolism of plants and causes a series of unfavorable morphological, physiological, biochemical and molecular changes that impair development (Wang et al., 2003b). Increased salinity or drought leads to reduction in photosynthesis, transpiration and other biochemical processes associated with plant growth, development and productivity (Anjum et al., 2011).

After perception of stress, plants, in order to protect cellular metabolism, initiate complex signaling cascades of survival strategies including physiological and biochemical modifications reflected on the transcript and protein levels (Ingram and Bartels, 1996b). Understanding the mechanisms of signal transduction is not only fundamental in biology, but also will provide the basis of effective engineering strategies for enhanced crop stress tolerance (Cushman and Bohnert, 2000). This calls for effective breeding strategies that combine, both traditional and modern breeding technologies to screen for crop cultivars adapted to the diverse agroecological regimes (Witcombe et al., 2008b).

In the recent years, considerable attention has been directed towards elucidating the molecular basis of plant abiotic stress tolerance (Rejeb et al., 2014). Various stress inducible genes have been characterized in model plants such as *Arabidopsis thaliana* and other agriculturally important crops. The regulation of expression of abiotic stress inducible genes at the transcriptional level have been well characterized and their roles in abiotic stress response elucidated.These include transcription factors including DREB, MYB and bZIP (Agarwal and Jha, 2010). Additionally genes with protective roles targeted on cellular proteins and membranes have been reported. These include heat shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA-II) proteins, osmoprotectants and free-radical scavengers (Ingram and Bartels, 1996b, Close, 1997, Cuming, 1999, Gill and Tuteja, 2010) Other genes such as aquaporins and ion transporters play an important role in water, ion uptake (Hasegawa et al., 2000, Maurel, 2007).

The LEA–DII proteins conventionally referred to as dehydrins are the most studied stress inducible genes (Ingram and Bartels, 1996a, Saavedra et al., 2006). They occur naturally in desiccated seeds and in vegetative tissues in response to salinity, dehydration, low

temperature and application of ABA (Nylander et al., 2001, Kim and Nam, 2010). Dehydrins from other related plant species have been studied and their sequences are available in the NCBI database (**www.ncbi.nlm.hih.gov**). A phylogenetic relationship of DHN protein sequence in barley, rice, Arabidopsis and Brachypodium is illustrated in **Fig. 5**. Dehydrins are defined by conserved amino acid motif designated Y-S- and K segment. The K segment is defined by a Lys-rich 15 amino acid consensus (EKKGIMDKIKEKLPG), S segment has track of Ser residues and consensus motif, and the consensus (T/VDEYGNP) designated the Y-segment usually located near the N-terminus (Close, 1997, Allagulova et al. 2003,). The number and order of Y-,S-, and K segments has been used to define the various DHNs into five structural groups: Kn, SKn, KnS, YnKn and YnSKn (Close, 1996, Svensson et al., 2002).

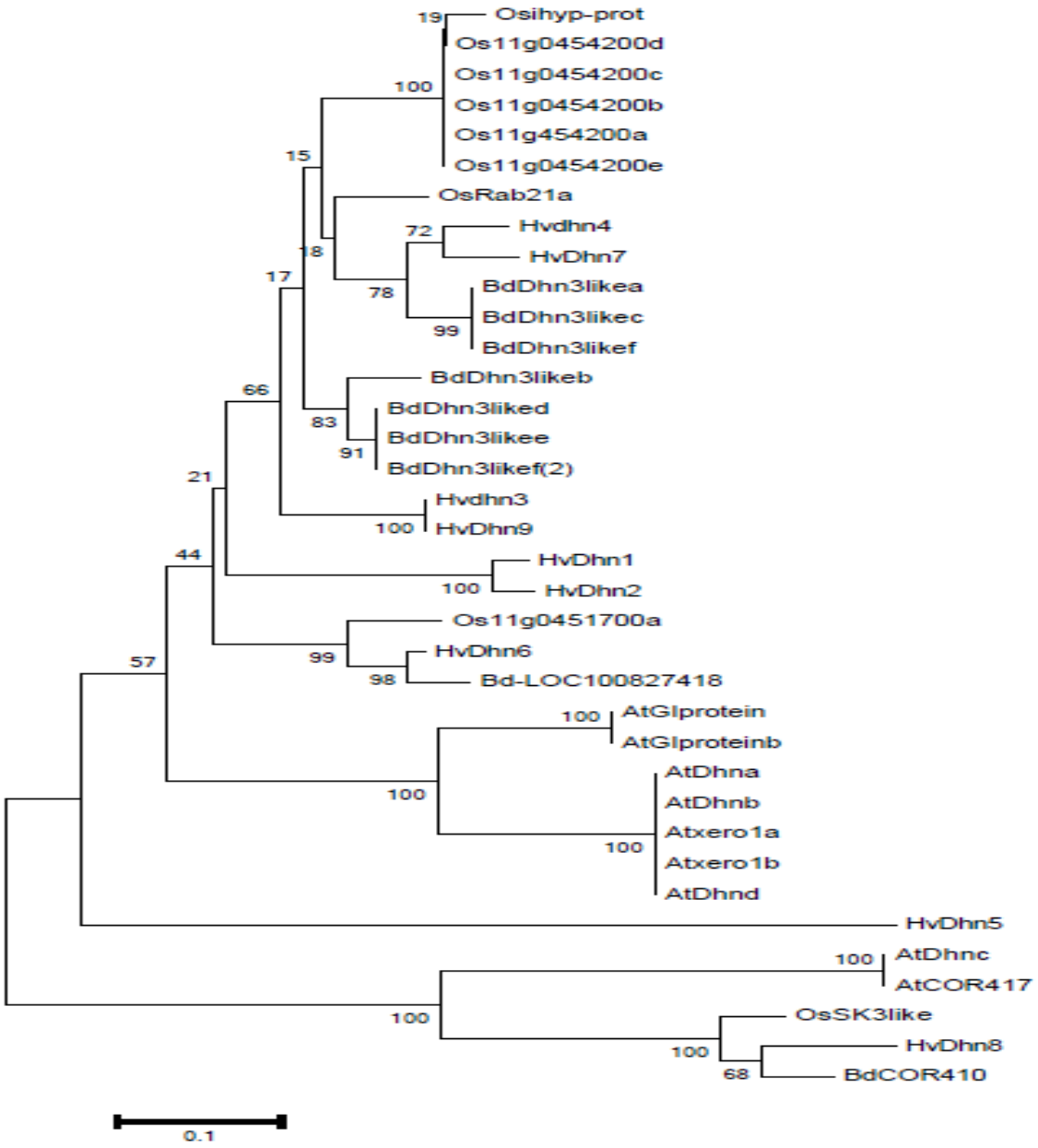


Figure 5. Phylogenetic tree of DHN protein sequences from, barley, rice, Arabidopsis and Brachypodium. The DHNs sequences were downloaded from the NCBI (http://www.ncbi.nlm.nih.gov) and (http://phytozome.jgi.doe.gov/) was analyzed using MEGA 5.0 software.

The structural type of DHN may define its functional role, tissue specificity and distribution (**Table 1**). Nuclear localization signals (NLSs), defined by an RRKK motif, have been found specifically in YSK_n-type DHNs and play a role in nucleus localization (Jensen et al., 1998)

Table 1. An overview of DHN (LEA-2D) protein**s** from different plant species described according to their structural arrangement **(**Close, 1997)

Protein	**Domain**	**Localization**	**Plant species**	**Phenotype**	**Reference**
VyDHN1	S_2K_2	cyt	Grapes	Cold/drgt	(Yang et al., 2012b)
HvDHN5	K_9	**c**yt/nuc	Barley	**c**old/drgt	(Bravo et al., 2003)
WSC120	K_5	Cyt/nuc	Wheat	cold	(Houde et al., 1992)
PV-DHN	K_6	cyt	Pistachio	uknown	(Yakubov et al., 2005)
WDHN3	K_2	**c**yt/nuc	Wheat	cold	(Holková et al., 2009)
HvDHN8	SK_3	mem	Barley	frost	(Choi et al., 1999)
SpDHN1	SK_3	**c**yt/mem	Grass sp	desic	(Yang et al., 2014)
COR47	SK_3	uknown	Arabidopsis	cold	(Nylander et al., 2001b)
CuCOR19	K_3S	mito	Citrus	cold	(Hara et al., 2001b)
HvDHN13	KS	anthers	Barley	chil	(Rodríguez et al., 2005)
Rab17	YSK_2	**c**yt/nuc	Maize	uknown	(Goday et al., 1994)
TeDHN5	YSK_2	**c**yt/nuc	Durum wheat	drgt	(Brini et al., 2007a)
Rab15	YSK_2	**c**yt/nuc	Wheat	drgt	(King et al., 1992)
HvDHN3	YSK_2	cyt	Barley	**s**alt/drgt	(Choi et al., 1999)
HvDHN6	YSK_3	cyto	Barley	Salt/drgt	(Choi et al., 1999)

Abbreviations: Cyt-cytoplasm, Desic- dessication induced, Nuc- nucleus, Mem- membrane Mito-mitochondrion, drgt-drougth, induced, desic-dessication induced; Chil-chilling induced, Salt- salt induced, unknown-undetermined

Breeding for increased yield, malting quality and disease resistance has been the main focus of many decades of barley breeding programmes in Kenya. Evaluation of genotype response to abiotic and biotic stress has been carried out under field conditions. However, due to variability under field conditions in terms of soil fertility parameters such as pH, nutrient distribution, seasonal fluctuations of rainfall and temperature, prevalence of disease incidences, it is difficult to establish a consistent and reproducible abiotic stress tolerance index in barley cultivars. We sought to employ laboratory based screening assays where growth conditions are strictly regulated enabling assessment of the performance of cultivars under various specific stress treatments. The laboratory-based screening assays are fast, sensitive and reproducible. We hypothesize that genotype variation in response to abiotic stresses exist in Kenyan barley cultivars. The use of biochemical, physiological and molecular assays in evaluation of stress response in barley

seedlings will allow early detection of trait of agronomic interest. If this hypothesis is true then these assays can be potential markers for screening genotypes for abiotic stress tolerance consequently accelerating crop improvement in breeding programs. This work is novel, because it is the first attempt to characterize Kenyan barley at seedling stage in response to abiotic stress using physiological and molecular screening assays. So far, there are no documented reports on transcriptional analysis of dehydrin gene superfamily in Kenyan barley in response to abiotic stresses, either under field or laboratory conditions.

2.2 Research goal

The main objective was to characterize Kenyan barley breeding lines using biochemical, physiological and molecular assays in order to accelerate screening for agronomic attributes

2.2.1 Specific objectives

- To identify cultivar variations in the hordein polypeptide fractions using SDS-PAGE.

- To evaluate genotypic and physiological responses following increased salt treatment by monitoring and measuring changes in physiological parameters such as relative water content, electrolyte leakage, lipid peroxidation, chlorophyll, proline, glycine betaine level, sucrose level content in barley seedlings.

- To analyze transcript profiles of selected stress responsive genes with a major focus on dehydrins (*Dhns*) in response to dehydration and two levels of salt (150 mM and 300 mM NaCl) treatments in leaf and root tissues of 6 selected genotypes.

- To perform immunoblot analysis using anti-dehydrin polyclonal antisera in response to dehydration and salt stress (150 mM and 300 mM NaCl) stress in leaf tissues.

2.3 Materials and Methods

2.3.1 Plant material

Germplasm was collected in Kenya and Germany between 2012 and 2013. Three categories of barley namely: two-row, six-row and wild relatives were used. The two-row and six -row barley cultivars were obtained from (KALRO)/(CIMMYT) initiative at the Wheat and Barley Research Centre, Njoro, (Kenya). These are parents of advanced lines used in barley breeding programs. The two- row type from EAMC in Kenya are used for malting purposes and have been improved for yield, lodging tolerance and disease resistance. They are adapted to high and mid altitude agroecological zones. The others were obtained from the National Gene Bank of Kenya (GBK) in Muguga, (Kenya) and the wild relatives of barley Sub spp *Hordeum spontaneum, Hordeum chinensis* from Genbank IPK in Gatersleben, Stadt, (Germany). The lines used as internal controls were obtained from the Institute of Crop Science and Resource Conservation (INRES) at the University of Bonn in Germany. A substantial number of lines from KALRO/CIMMYT that were not viable or had low germination rates were excluded in subsequent experiments. The list of lines used in this study is presented in **Table 1**. All the experimental procedures were conducted under controlled conditions (23 ^{0}C, 16 hrs light and 8 hrs dark) in the growth chamber and greenhouse at the Institute of Plant Molecular Physiology and Biotechnology (IMBIO) at the University of Bonn in Germany.

Table 2. List of barley cultivars and wild progenitors used in this study and their respective sources.

Genotype/	Row type	Sources of material
MN-3	6-row	KALRO/CIMMYT
MN-4	6-row	KALRO/CIMMYT
MN-5	6-row	KALRO/CIMMYT
MN-6	6-row	KALRO/CIMMYT
MN-8	6-row	KALRO/CIMMYT
MN9	6-row	KALRO/CIMMYT
MN-12	6-row	KALRO/CIMMYT
MN-23	6-row	KALRO/CIMMYT
MN24	6-row	KALRO/CIMMYT
Nguzo	2-row	EAMC
Sabini	2-row	EAMC
Karne	2-row	EAMC
GBK 172	6-row	GBK
GBK 171	6-row	GBK
Hs 2699	2-row	IPK-Gatersleben
HSs2698	2-row	IPK-Gatersleben
HOR 2162	2-row	IPK-Gatersleben
HOR 2612	2-row	IPK-Gatersleben
HOR 3301	2-row	IPK-Gatersleben
Barke	2 row	INRES
Morex	6 row	INRES

2.3.2 Biochemical analysis of barley seed hordein polypeptides fractions using SDS-PAGE

The storage protein hordeins have been used to study genotype variations in barley since they are highly polymorphic. In this study we performed hordein profiling using pooled barley seeds to assess cultivar identity. A total of 20 uniform barley seeds from each of the 16 selected barley cultivars was ground in liquid nitrogen to a fine powder and used

for analysis. Hordeins were extracted from 200 mg of seed material in 55% isopropanol and 3% mercaptoethanol water mix as described by Echart-Almeida and Cavalli-Molina, (2001b) and quantified using a Bradford assay (Bradford, 1976). Protein samples were boiled at 95 °C for 5 mins before loading onto the gel (10 cm × 10 cm). Twenty microgram of protein was resolved electrophoretically using SDS discontinuous gel system (Biorad, CA USA). In this system, the proteins were concentrated first in 4% (w/v) acrylamide stacking gel (1.44 ml sterile H2O; 0.27 ml 30% (v/v) acrylamide; 0.25 ml 1 M Tris pH 6.8; 20 µl 10% (w/v) SDS; 20 µl 10% (w/v) APS; 2 µl TEMED in a final volume of 2 ml), before resolving in 12% (w/v) separating gel (1.92 ml sterile H_2O; 2.4 ml 30% (v/v) acrylamide;1.56 ml 1.5 M Tris pH 8.8; 60 µl 10% (w/v) SDS; 60 µl 10% (w/v) APS; 2.4 µl TEMED in final volume of 6 ml). The gels were ran in 1 x SDS buffer (25 mM Tris; 192 mM glycine; 0.1% (w/v) SDS) for 2 hrs at 10 mA until the proteins reached the end of the staking gel, followed by 2 hrs at 20 mA in the separating gel against a standard protein ladder made of proteins of known weight ranging from 14.4-116 kDa (Standard protein marker, Fermantas, Germany). The gel was fixed in 40% (v/v) methanol and 10% (v/v) glacial acetic acid for 1 hr followed by 4 rinses of 10 mins in tap water.

2.3.3 Coomassie blue G-250 staining of SDS PAGE gels

The gel was incubated overnight in Coomassie blue G-250 solution:4 volumes (100 g/l ammonium sulfate; 1% (v/v) phosphoric acid; 0.1% (w/v) Coomassie blue G-250) +1 volume methanol. The gel was destained after several rinses with distilled water and the gel was scanned using Epson Imager III. Variation in banding of hordein polypeptides fractions D, C and B were determined qualitatively based on absence (0) or presence (1) of resolved protein bands. The phylogenetic relationship in cultivars was generated by analyzing the banding frequencies and patterns of hordein protein fractions using agglomerative hierachial clustering (AHC), XLSTAT 13.

2.3.4 Salt stress treatments

A selection of seeds (Table 1) was used to evaluate abiotic stress tolerance. A short term exposure to salt (NaCl) stress was conducted as a preliminary screening step to evaluate abiotic stress response. The seeds were pregerminated in the dark on moist Whatman paper in petri dishes. The seedlings were transferred to 1 L plant pots and grown under

controlled conditions (120-150 μE $m^{-2}s^{-1}$ 23 ^{0}C with 16 and 8 hrs photoperiod regimes). All plants were irrigated with distilled water supplemented with Wuxal nutrient solution (Aglukon, Germany). After 12 days, the seedlings were exposed to salt treatments. One treatment consisted of watering plants regularly with nutrient solution supplemented with 300 mM or 600 mM NaCl for 6 days and the control treatment with only nutrient solution. Leaf samples were collected after 3 and 6 days of treatments. Three fresh fully expanded detached leaf samples were used immediately for ion leakage analysis while the rest was stored at -80 ^{0}C for later analysis of changes in proline, chlorophyll, MDA, glycine betaine and sucrose contents.

2.3.5 Physiological assays

2.3.5.1 Chlorophyll content measurement

Analysis of chlorophyll in leaf tissue was analyzed using the method of Arnon. (1949). 100 mg of ground tissue was homogenized in 2 ml 80% (v/v) acetone. The suspension was incubated in the dark at room temperature (RT) with agitation for 30 mins. It was then centrifuged at 10000 rpm for 5 mins at RT. The absorption of the supernatant was monitored spectrophotometrically at 663 nm and 645 nm. The chlorophyll content (chlorophyll a + chlorophyll b) was estimated using the given formula:

Chlorophyll (mg FW^{-1}) =0.002 x (20.2 x OD_{645} + 8.02 x OD_{663}) /g FW

2.3.5.2 Ion leakage (electrolyte conductance)

Electrolyte conductance was determined as described by Nanjo et al. (1999). Ion leakage test is used to determine the cellular membrane integrity in response to plant tissue damage by measuring the amount of ions that are released to surrounding solutions. In this study, three detached leaves were used from each treatment. A single detached leaf was incubated in 10 ml de-ionised water for 1 hr at RT. The leaf was transferred and incubated in 80 ^{0}C water bath and total conductivity was performed using a conductivity meter (DS15, Horiba, Kyoto, Japan) after 30 mins. The relative conductivity due to leaked ions was determined by the ratio of total ion leakage at RT and after heating. This ratio relates to membrane integrity and reflects tolerance capacity of the leaf membrane.

2.3.5.3 Determination of free proline

The method of Bates et al. (1973) was adopted to estimate the total free proline content in leaf tissues. A standard curve was generated from known proline concentrations. The supernatants extracted from treated and controlled plants were assayed for proline concentration. The total proline was measured by spectrophotometrically at wavelength 520 nm and its concentration was estimated using a using standard curve.

2.3.5.4. Malondialdehyde (MDA) content

The level of beta oxidation of lipids was determined by measuring the amount of the end product of lipid peroxidation, MDA using the thiobarbutaric acid test (TBA) (Kotchoni et al., 2006). 100 mg of finely ground leaf tissue was homogenized in 1 ml pre chilled trichloroacetic acid (TCA) and centrifuged at 13000 rpm for 5 mins at 40 ^{0}C. The supernatant was transferred to another tube containing 0.65% thiobarbituric acid and incubated at 95 ^{0}C in a water bath for 30 mins. The mixture was cooled on ice and centrifuged at 5000 rpm for 10 mins and supernatants were subjected to spectrophotometric analysis at 450 nm, 532 nm and 600 nm. The amount of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$ according to the formula provided by Heath and Packer. (1968):

MDA equivalence (µmol L^{-1}) = 6.45 x (A_{532}- A_{600}) – (0.56 x (A_{450})

MDA equivalents (nmol g^{-1} FW) = MDA equivalents (nmol ml^{-1}) x total volume of the extracts (ml) / g FW.

2.3.6 High performance liquid chromatography (HPLC) for osmolyte measurements

The HPLC system comprises the following components: reservoir for solvents, pump (with two pistons), injection valve for samples, chromatographic column, RI detector (connected to a computer for data analysis), UV detector (connected to a computer for data analysis) and container to collect effluents. The refractive index detector used here enables the quantitative detection of a wide range of different compounds regardless of their absorption properties, such as: betaines, polyols sugars, amino acids and cyclic amino acid derivatives. Samples were separated in isocratic mode on a reversed phase NH2 column using a refractive index detector and a UV detector. The solvent acetonitrile 80 % (v/v) is used at a flow rate of 1 ml/mins. The spectrum of compatible solutes present

in samples was determined by comparing the retention times with those of standards. The area of the signal is used as a measure for the concentration of the particular substance and calculated by a chromatographic software package. By comparing the area values of identified peaks with those of reference compounds of known concentration (standards) the concentration of individual components can be determined.

2.3.6.1 Optimization of HPLC efficiency

The efficiency of HPLC system was determined first by evaluating the recovery of exogenously applied 0.42 µmol g^{-1} of GB (Fluka, Bucks Switzerland) mixed with 100 mg of untreated leaf tissue in a final volume 500 µl of extraction buffer made of methanol, chloroform water mix (MCW) (60: 25: 15). The upper phase was lyophilized for 2 days in order to get rid of chlorophyll pigments since they interfere with the absorbance. The pellet was solubilized and used for HPLC Nucleosil aminopropyl-phase column (Macherey and Nagel, Düren, Germany). Since the recovery rate of GB was 97% then the method was approved for use. The next step was to determine the most appropriate amount of starting material that allowed detectable peak upon sample elution. The use of 500 mg of treated or control yielded low peaks characterized with interference. The use of 2 g of leaf tissue resulted in generation of detectable and distinct peaks.

2.3.6.2 Determination of compatible solutes

The effect of salt treatment on compatible solutes glycine betaine and sucrose concentration leaf tissues of barley genotypes was determined using the HPLC unit. Two g of finely ground leaf tissue derived from control and 300 mM NaCl treatments were extracted in 6 ml ice cold MCW using a modified method of (Park et al., 2004). The homogenate was mixed and shaken in a gyrator at 1000 g for 10 mins. 2 ml of the upper aqueous phase made of methanol and water was transferred to pre cooled 15 ml falcon tube, covered with perforated parafilm and lyophilized for 72 hrs. The recollected dry white pellet was solubilized using acetonitrile/water mix (80:20, (v/v) as a solvent (Galinski and Herzog, 1990). 50 µl of (1:10) dilution of samples and 1 mM GB (Fluka, Germany) and 1 mM sucrose (Roth, Germany) were used as controls. Compounds were monitored using HPLC system coupled with refractive index monitor and UV detector. By comparing the area values of identified peaks with those of reference compounds of

known concentration (standards) the concentration of individual components in the samples was determined.

2.3.7. Determination of leaf relative water content

Relative water content can be used as an indicator of water balance in a plant. Relative water content was determined according Slatyer and Barrs. (1965) where a single detached leaf from each treatment was used in three replications. Leaves were weighed immediately to record fresh weight (FW) then incubated in distilled water for 24 hrs and weighed again to record turgid weight (TW). The leaves were subsequently subjected to oven drying at 80 °C for 24 hrs to record dry weight (DW). The RWC was calculated using the given formula:

$$RWC = (FW - DW) / (TW - DW) \times 100$$

2.3.8 Molecular analysis

2.3.8.1 Dehydration and NaCl stress treatments

Following physiological assays, selected barley cultivars seedlings were subjected to 7 days of chronic dehydration and two levels of NaCl (150 mM and 300 mM) treatments. The leaf and root tissues were harvested and stored at -80 °C for use in expression analysis of selected stress responsive genes. The expression levels of genes encoding dehydrins (*Dhn1, Dhn3, Dhn4, Dhn5, Dhn6, Dhn7* and *Dhn9*), genes encoding betaine aldehyde dehydrogenase (*HvBBD1*) and *Hordeum spontaneum dehydration responsive* gene (*Hsdr4*) were analyzed in root and shoot tissues. The elongation factor (*HvEF1α*) was used as a housekeeping gene. Differential expression of these genes was analyzed using semi quantitative RT-PCR while the quantification was performed using Imagequant 5.3.

2.3.8.2 Total RNA isolation and single strand cDNA synthesis

Total RNA was extracted from 100 mg of ground leaf powder according to Valenzuela-Avendaño et al. (2005). The RNA concentration and purity was determined spectrophotometerically using a Biospec-nano Shimadzu Biotech, Japan followed by electrophoresis on 1% agarose stained with ethidium bromide. RNA was treated with

RN*ase* –free DNas*e* 1 (Thermo Scientific, Germany) for 30 mins at 37 ^{0}C to remove DNA contaminations. First strand cDNA was generated from 2 µg total RNA using universal oligo (dT) 18 primers and 200 U of MMLV reverse transcriptase (RivertAid Fermentas, Germany), incubated at 42 ^{0}C for 1 hr followed by a further incubation at 70 ^{0}C for 5 mins in a 20 µl reaction volume.

2.3.8.3 Semi quantitative RT-PCR and relative expression of stress responsive genes

Single strand cDNA was amplified using Taq DNA polymerase (Thermo Scientific, USA) in the T3000 thermocycler (Biometra, Germany). Gene specific primers were designed based on the available sequences deposited at the National Centre for Biotechnology Information (**http://www.ncbi.nlm.nih.gov/**). Elongation factor EFα1 gene was used as an internal control for the relative amounts of RNA. Each reaction was performed using 1 µl of 1: 20 (v/v) dilution of the first single strand cDNA. Relative expression was analyzed using Imagequant 5.3. All expressions data were normalized to the housekeeping gene, *HvEFα1*. Two technical replicates were performed in this analysis.

Table 3. List of gene specific primers used in semi quantitative RT-PCR analysis

Gene	5'Forward primer 3'	3'R primer 5'
HvDhn1	TCAGCACGGCCACGCCAC	CATGTGTGCCCGCGTACTCG
HvDhn3	GGCAACCAAGATCAACACCACCT	CGGAAGTTTTACTGCATCTCCATC
HvDhn4	GGCAGCGCAAGATGGAGTACCAG	CCCTCCAACAGCCAAGTGAGCTA
HvDhn5	GAGACCACCAGCAGACAGG	AGGCAGCTTGTCCTTGATCT
HvDhn6	GACGTCGTGGCACACACCCTC	CCAGGCCATGTCACAGTACTGC
HvDhn7	GTCATTTCCAGCCGACGAGGAAGG	GGGTCCATACAAGAAGCCATATT
HvDhn9	TGGAGTTCCAAGGGCAGCACGAC	GGCTTCGACGCGTAGCTATGCAA
HvBBD1	CAACATGCACCAATCTCTC	TCAATGGTGGTCAAGTTTGC
Hsdr44	GCCACACTAGGCAGGCTCTTCT	GTATATCCGCAAGATGCAGAGG
HvEF1α	CGTTGCTGTGAAGGATCTGA	GCAAAGGTCTCCACAACCAT

2.3.8.4 Protein extraction from plant tissue

The method of Laemmli. (1970) was used to extract total soluble protein from plant tissue. Approximately 100 mg of control, dehydrated, 150 mM and 300 mM NaCl treated leaf tissues were homogenized with 200 µl of Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2 % SDS (w/v), 0.1% bromophenol blue, 0.7 M mercaptoethanol added freshly) by thorough vortexing in a 1.5 ml Eppendorf tube. The extract was boiled for 5 mins in a heating block for 5 mins, and then centrifuged at 13000 rpm in room temperature and pressure. The supernatant was collected in a fresh tube and stored at -20 °C. Before protein quantification, (Bradford, 1976) assay, SDS interference was removed by mixing 5 µl of protein sample with 100 µl of 0.1 M potassium phosphate buffer pH 6.8 and incubated at RT for 10 mins. Centrifugation was performed at 13,000 rpm for 5 mins at RT and the supernatant was mixed well with 700 µL of water and 200 µl of Bradford reagent. Absorbance was measured at 595 nm after 10 mins of incubation. Samples were heated at 95 °C for 5 min before loading on the gel. 10 µg of crude protein extract was resolved electrophoretically in SDS-PAGE as described for of hordein proteins in **2.3.2**

2.3.8.5 Ponceau S red staining

The SDS-PAGE resolved proteins were transferred onto a nitrocellulose Protran BA-85 membrane (Whatman) using protein transfer chambers (Biorad, CA USA). Transfer was performed at 70 V for 3 hrs using pre-chilled protein transfer buffer (25 mM Tris:192 mM glycine; 20% (v/v, methanol, in 1 L of distilled water- pH 7.5) (Towbin et al., 1979) at RT or at 4 °C in cold room. After protein blotting, the membrane was immersed in Ponceau-S red solution [(0.2% (w/v) Ponceau S in 3% (w/v) Trichloroacetic acid (TCA)] for 10 mins to confirm equal loading of the protein extract. The positions of protein markers were marked and destained with water followed by scanning.

2.3.8 6 Immunoblot analyses

Following protein transfer to the membrane, immunoblot analysis was performed to confirm the accumulation of protein of interest. The distained membrane was blocked for 1 hr or overnight at 4 °C in the blocking solution [(4% (w/v) non-fat dry-milk powder dissolved in TBST (1× TBS + 0.1% (v/v) Tween-20)]. The protein blots were probed with the anti dehydrin antisera in 1: 1,000 dilution in fresh blocking solution corresponding to K segment consensus peptide TGEKKGIMDKIKEKLPGQH (Close et al., 1993). The

membrane was incubated at room temperature for 1 hr and was washed with TBST as follows: 1× rinse, 1× 15 mins and 3× 5 mins. The membrane was further incubated for 45 mins at room temperature with 1: 5,000-fold diluted anti-rabbit IgG peroxidase linked secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). Binding of antibodies was revealed using an ECL plus western blotting detection kit (Amersham, Bioscience, Braunschweig, Germany) and the chemiluminescence signal was detected under a CCD camera (Intelligent Dark Box II, Fujifilm Corporation).

2.3.11 Data analysis

Phylogenetic relationship of seed hordein polypetide fractions was analyzed using agglomerative hierachial clustering. Physiological data including changes in MDA levels, proline content chlorophyll content ion leakage, GB and sucrose in response to stress treatments was analyzed using XLSTAT 2013. Relative expression of stress responsive genes was determined using Imagequant 5.3. Phylogenic relationship in DHN sequence from different members of graminae were

analyzed using MEGA 5.0 software. Plants were grown in randomized block design (RCBD) in pots under control conditions in three replicates. For gene expression analysis, data reported are means generated from plants grown at different times of research period.

2.12 Results

2.12. 1 Biochemical analysis of hordein polypeptide fractions using SDS-PAGE

Variations in isopropanol extracted hordein polypeptides were evaluated in Kenyan barley cultivars used in the breeding programmes. In our study, 20 µg of protein extracts from each genotype was resolved in SDS-PAGE. Hordein polypeptides were resolved into four distinct fractions: D, C and B according to their electrophoretic mobility **(Fig. 6).**

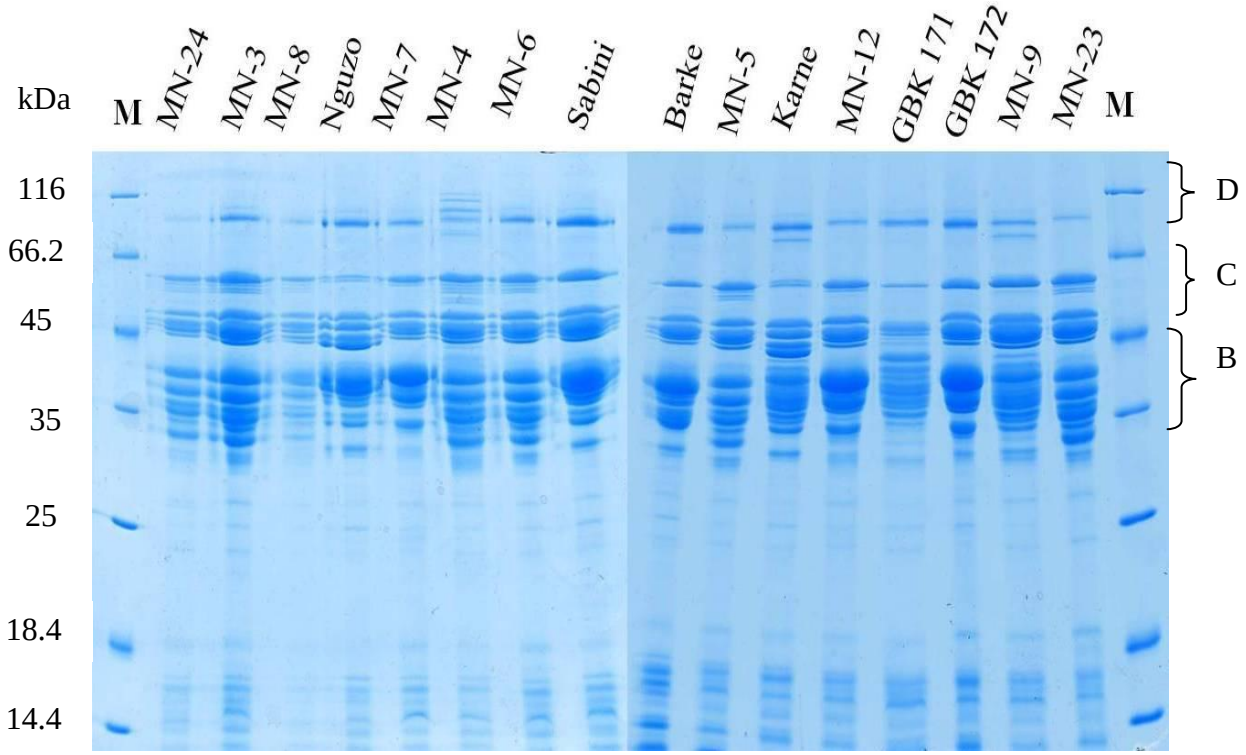


Figure 6. Isopropanol extracted barley seed hordein polypeptide fractions resolved using SDS-PAGE. Protein was extracted from 20 pooled seeds (<2.5mm in size) of barley lines selected from **Table 1.**

The D fractions which migrated slowly in the separating gel occurred in low frequency while the C and B hordein fractions dominated the electrophoregrams. Only one genotype, MN-4 exhibited D hordeins. The resolved protein fractions varied in frequency and intensity between the genotypes. The C and B hordein showed a lot of variation in terms of band size and frequency across the genotypes.

There were a total of 29 hordein bands recorded in all the genotypes. A range of 13-20 bands were detected per genotype in most of the genoytpes with exception of cultivar MN-4 which exhibited 25 protein bands. Using the standard protein ladder 14.4-116 kDa (Fermentas, Germany) the sizes of the calculated hordein fractions ranged from 33-96 kDa. The majority of the hordeins analyzed were found to be between 33-80 kDa. In order to determine whether hordein polypeptides could be used to establish the genetic variations in the cultivars under study, a phylogenetic relationship using agglomerative hierachial clustering (AHC) generated 5 clusters (groups) of hordein banding patterns namely: C4, C3, C2, C5 and C1 respectively (**Fig. 7**).

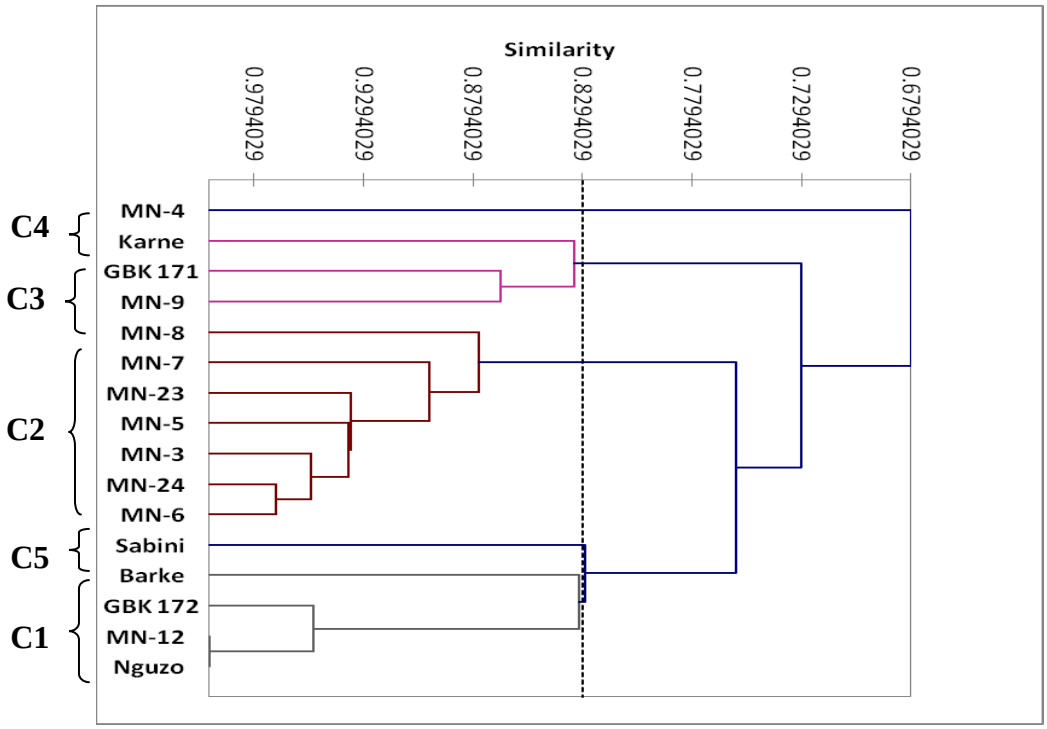


Figure 7. A dendogram showing the phylogenetic relationship between 16 barley lines generated from agglomerative hierachial clustering [(AHC) using (XLSTAT 13)]. Five distinct clusters were generated. The frequency of hordein banding per genotype was scored as absence (0) or presence (1) of the polypeptide band.

Group C4 is represented by MN-4 alone with highly polymorphic D fractions. Group C3 represents Karne, GBK172 and MN-9. The highest number of genotypes namely: MN-3, MN-5 MN-6, MN-7 MN-23 and MN-24 are included in group C2. Cv. Sabini in the only representative of group C5 while group C1 includes Barke, GBK171, MN-12 and Nguzo (**Fig.15**). Although all the varieties are used for malting purposes and fall under either six

row or two row cultivars, the clusters generated groups represent either each row type (homogenenous) or the combined row types (homogeneous or heterogeneous) as detailed in Table 2. For example, C1 was exclusively represented by 2 row malting barley, C2 only 6 row malting type while C3 by both 2 and 6 row barley while C5 is represented by Sabini a 2 row an old variety released in 1970 as the name suggests. C1 represents cultivars with good malting characters which are highly preferred by the malting industry. Cv. Nguzo is a good malting cultivar. Most of the new varieties have a Nguzo genetic background.

2.12.2 Physiological assays

2.12. 2 1 Effect of salt stress on electrolyte conductance

The effect of NaCl stress (0, 300, 600 mM) treatments on electrolyte leakage of 14 barley genotypes was determined during a time course of 3 and 6 days (**Fig. 8**).

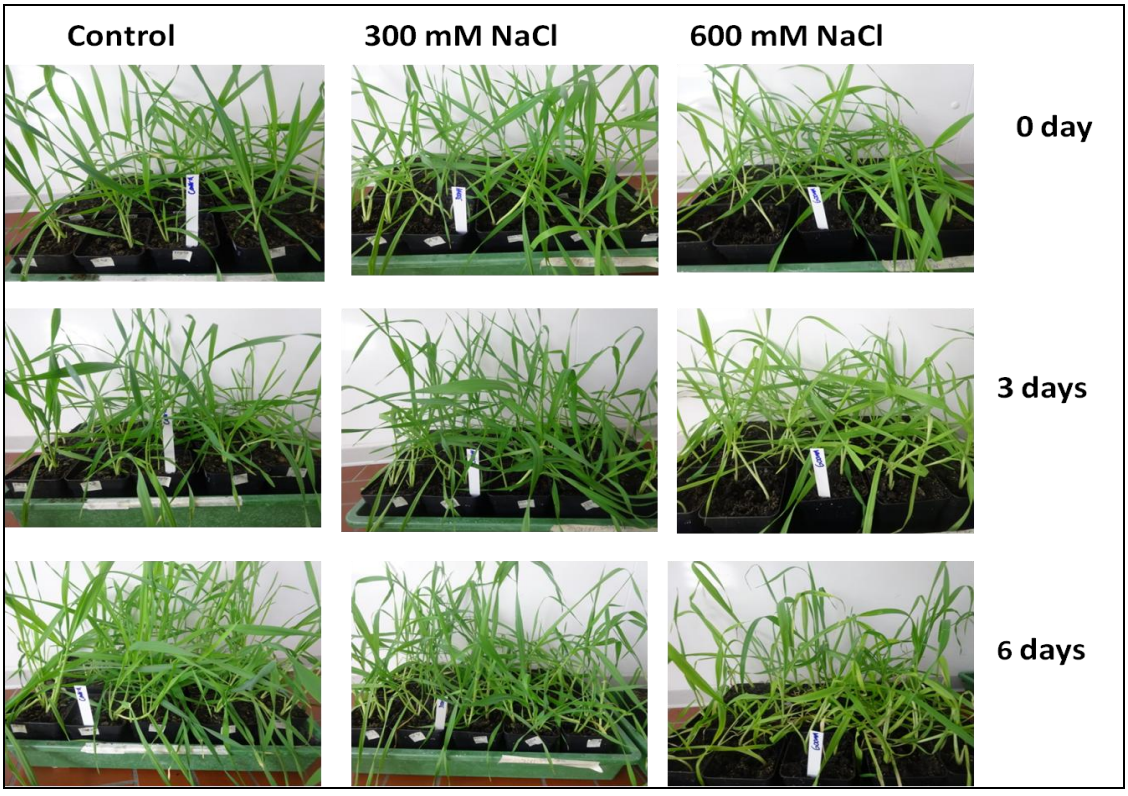


Figure 8. Barley seedlings grown under 300 mM and 600 mM NaCl treatments. Leaf samples were collected after 3 and 6 days of stress treatments.

The changes in electrolyte conductance (µs) were determined in detached leaves in response to salt treatments. Relative ion content analysis revealed variations between treatments and genotypes (**Fig. 9**).

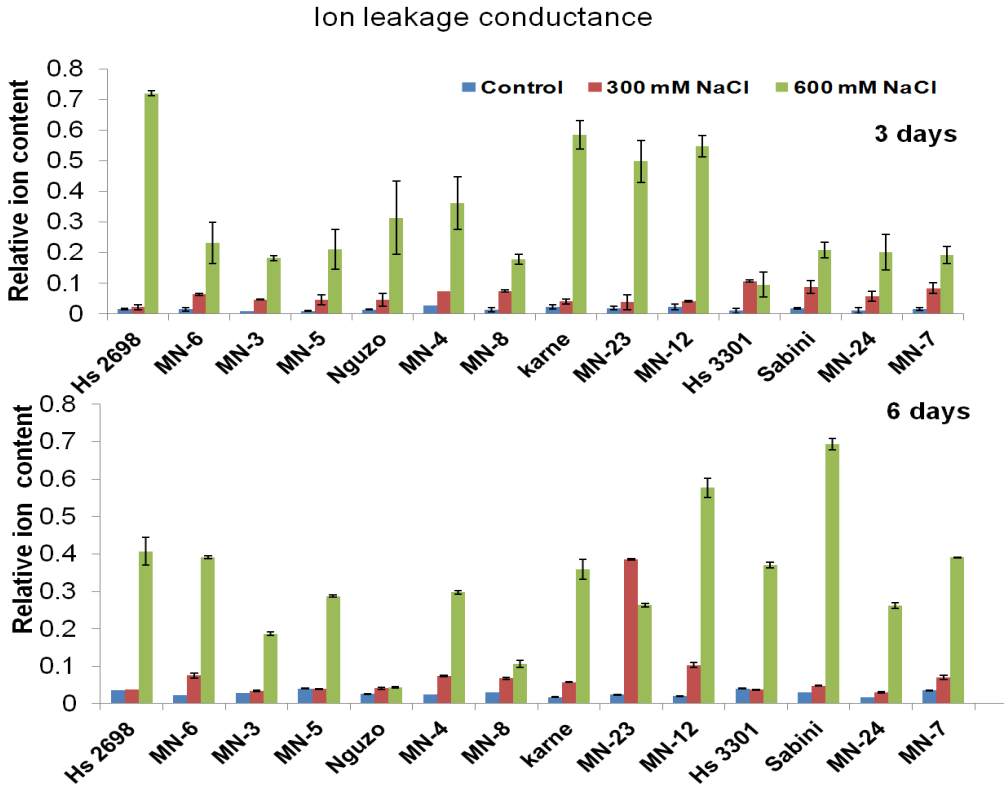


Figure 9. Time course effect of 300 mM and 600 mM NaCl treatment on electrolyte leakage in 14 barley lines after 3 days and 6 days.

After 3 days of incubation the initial conductivity remained relatively low in control plants with minimum increase in 300 mM NaCl treatment across the genotypes. However, there was a marked increase in conductivity in response to 600 mM NaCl treatment. The conductivity varied across the genotypes except in variety HOR 3301, MN-3 and MN-8 which remained comparable in control and 300 mM NaCl treatments. The highest conductance was observed in Hs 2698, Karne, MN-12 and MN-23 with conductance of 0.72, 0.58, 0.54 and 0.49, respectively. The lowest conductance was observed in cultivars HOR 3301, MN-8 and MN-3 with values of 0.09, 0.17 and 0.18, respectively. After 6 days of incubation, control plants maintained a low conductance compared to treated plants while a high genotype dependent increase was observed in 600 mM NaCl treatment. Four categories of genotypes responses were observed in this treatment with

regard to ion conductance ratios. Category A with highest conductance ratio represented by genotypes MN-12 and Sabini (0.7 and 0.58), respectively, category B including Karne, HOR 3301, MN-7 MN-6 and Hs 2698 (0.37-0.4). Category C: MN-5, MN-4, MN-23 and MN-24 (0.25-0.35) and category D with MN-3, MN-8 and Nguzo (0.04- 0.18) which displayed the lowest conductivity. Untreated plants had a minimum cell membrane damage reflected in low conductance values. Increase in conductance varied with genotype and severity of salt treatments. Some peculiar observations were made in Hs 2698 which displayed the highest conductance in response to 300 mM treatment after 3 days of incubation, but reduced after 6 days of incubation in 600 mM NaCl. Using ion leakage assay results from both the 3 and 6 days stress treatments, a reconciled primary susceptibility and tolerance index was generated. Nguzo, MN-8, MN-24, MN-3 and HOR 3301 displayed relatively low ion leakage ratio while Karne, Sabini, MN-12, MN-23 and MN-5 showed increased ion leakage ratios. See **Table 4.**

2.12. 2.2 Effect of NaCl treatment on lipid peroxidation

The study sought to determine the level of membrane damage in 14 barley seedlings following NaCl stress treatments by measuring MDA as the end product of lipid peroxidation. High MDA is associated with susceptibility to stress factors. In this study, the MDA level varied across genotypes and treatments (**Fig. 10**).

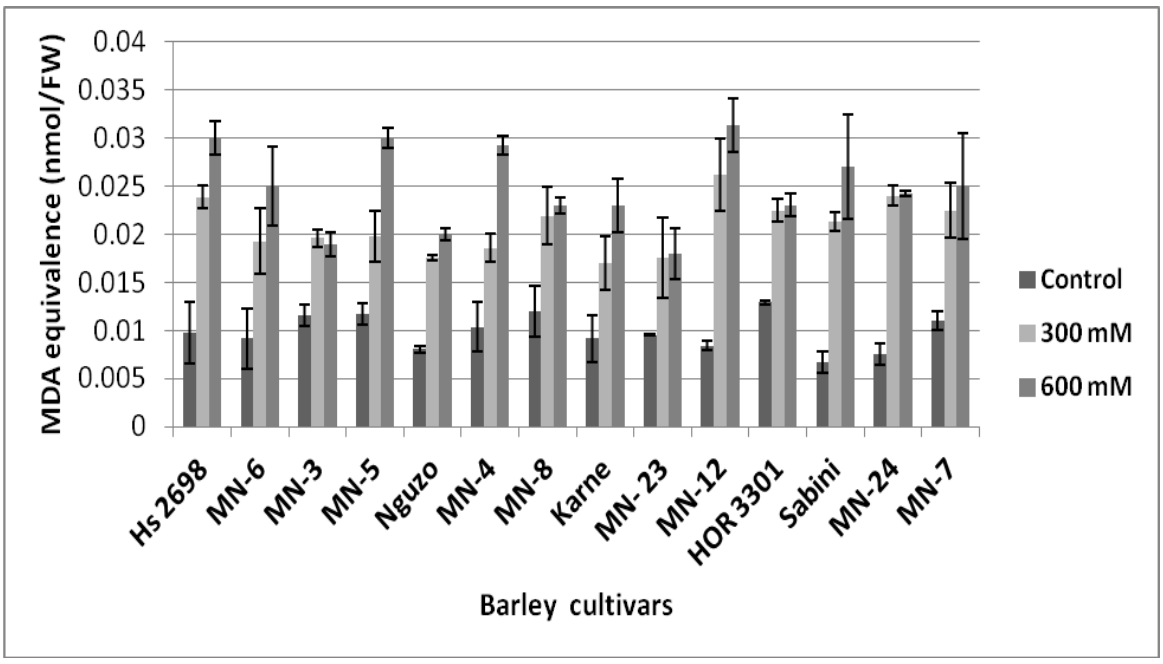


Figure 10. MDA content in seedlings of 14 barley cultivars following 6 days of 300 mM and 600 mM NaCl treatments

After 6 days incubation control plants exhibited a low MDA values compared to treated plants. Following 300 mM NaCl treatment, MDA levels increased 2-3 folds compared to control treatment. Changes in MDA levels were found to be genotype dependent. The highest increase was observed in Hs 2698 and MN-12 while the other genotypes did not display remarkable differences. The 600 mM treatment not only exhibited an up to 3-4 fold increase of MDA compared to control treatments, but also the highest variability among the genotypes. The highest increase was observed in Hs 2698, MN-5, MN-4 and MN-12 and the lowest in MN-3, Nguzo, Karne, and MN-23. Some lines however showed minimum variation between the 300 mM and 600 mM NaCl treatment as shown in the comparable MDA equivalence values. This phenomenon was observed in MN-3, Nguzo, MN-8, Karne, MN-23 and MN-24. From these results, cultivars with remarkably low

MDA levels in both, the 300 mM and 600 mM NaCl. From this assay, we identified in MN-3, Nguzo, Karne, MN-23 and MN-24 as tolerant and in Hs 2698 MN-5 MN-12, MN-4 MN-6 and Sabini as susceptible to salt stress. See **Table 4.**

2.12. 2 3 Effect of salt stress on the proline content

Proline an osmoprotectant which plays a role in stabilizing cellular membranes, protein structures and detoxification induced by abiotic stress (Szabados and Savoure, 2010). The effect of 300 mM and 600 mM salt stress on proline content in seedlings of 14 barley cultivars was determined after 6 days. Analysis showed that the proline content varied within treatments and genotypes (**Fig. 11**).

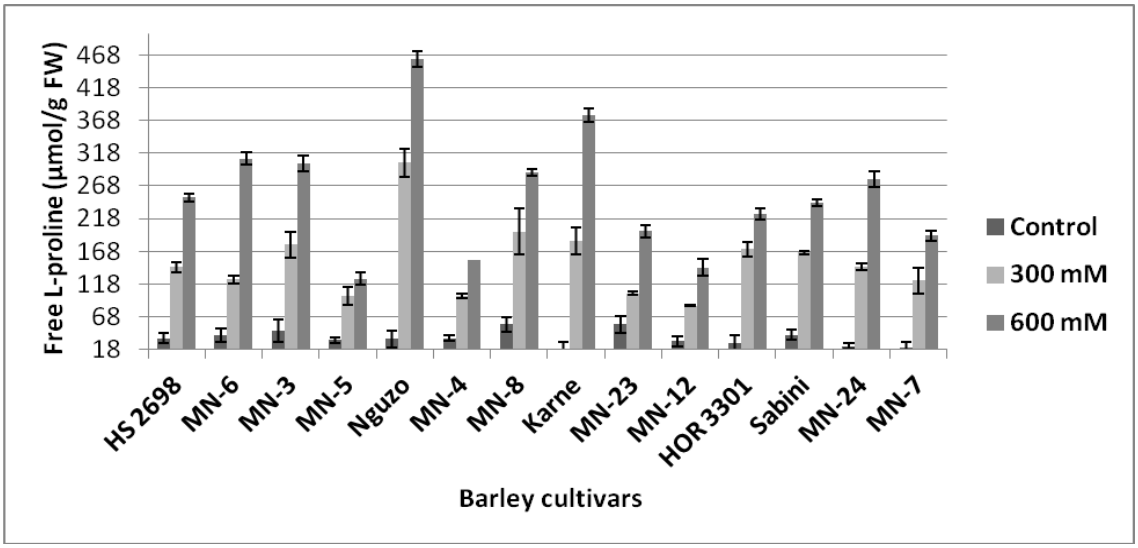


Figure 11. Free proline content in seedlings of 14 barley lines in response to 6 days of 300 mM and 600 mM NaCl treatments.

Plants grown under control conditions showed low proline levels compared to the NaCl treated plants. The proline content increased with salt treatment. In control plants proline was comparable in all the genotypes (approx. 32 µmol g^{-1} FW). In 300 mM NaCl treatment, proline increased 2-4 fold compared to control plants. The highest proline content was observed in Nguzo, MN-3 Karne, Sabini, MN-8 and MN-24 (303, 178, 184, 166, 198 and 145 µmol/g FW), respectively. The lowest accumulators of proline were MN-4, MN-5, MN-12 and MN-23 with values (100, 100, 83 and 103 µmol/ g FW, respectively Following 600 mM NaCl treatment, proline increased 5-14 fold compared to control plants and 5 folds compared to 300 mM NaCl treatment. Variation in proline

content was found to be genotype dependent. The highest level of free proline was observed in Nguzo, Karne, MN, 6, MN-3 and MN-24 (461, 309, 302 and 278 µmol/ g FW) respectively. On the other hand, a low level of proline was recorded in MN-5, MN-4, MN-12 and MN-7 (126, 154, 198 and 142 µmol/g FW respectively). See **Table 4.**

2.12. 2 4 Effect of Salt stress on chlorophyll contents

Chlorophyll or plant greenness is an indicator of the nutritional status correlated to availability of nitrogen as factor of photosynthetic activity. Salt stress causes decreased biosynthesis of chlorophyll and inefficiency of photosynthesis. The effect of NaCl (300 mM and 600 mM) treatment on chlorophyll content was determined in barley seedlings. In this experiment we established that salt stress caused changes in chlorophyll content (**Fig. 12**).

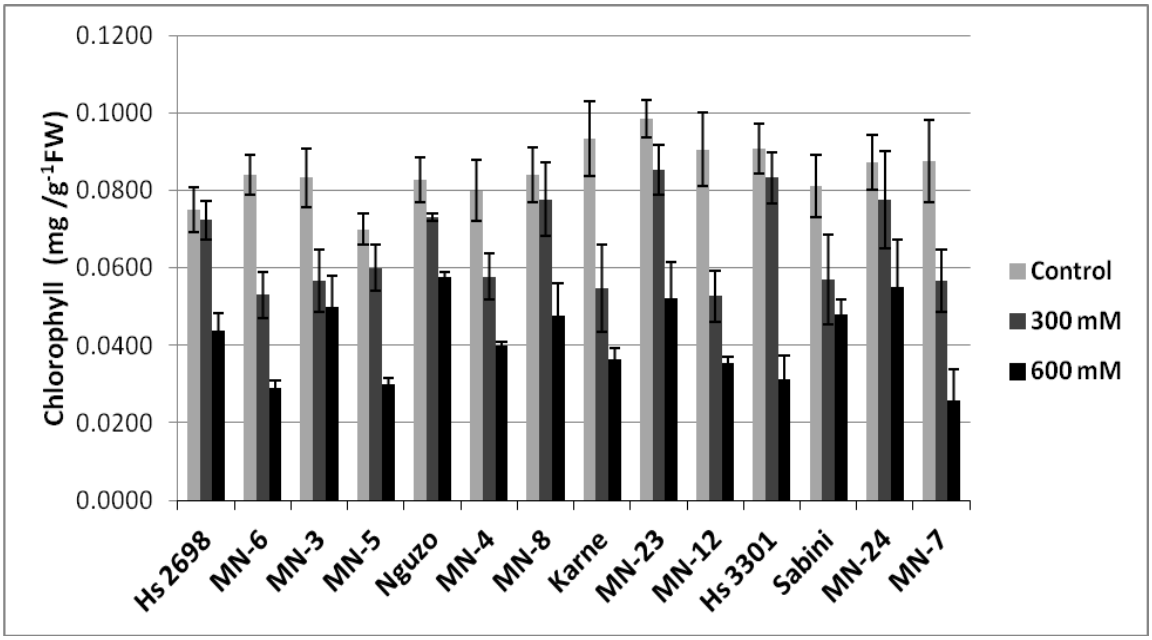


Figure 12. Determination of chlorophyll content in seedlings of 14 barley lines in response to 6 days of 300 mM and 600 mM NaCl treatments.

The plants grown under control conditions exhibited deep green, healthy and turgid leaves with higher levels of chlorophyll content compared to treated plants. In the 300 mM NaCl treatment, there was a decrease in chlorophyll content compared to control treatments and the decrease was genotype dependent. A change in chlorophyll content compared to control treatment was used to show the extent of chlorophyll degradation or ability to

resist damage. A low % decrease in chlorophyll was observed in Hs 2698, MN-8, HOR 3301 and MN-24 (3, 5, 11, 7.5, 8.2, and 11%, respectively). A high decrease was observed in MN-6, Karne, MN-12 and MN-7 (36.9, 41.4, 41, and 35%, respectively). In response to 600 mM NaCl treatment, a decrease in chlorophyll content was observed relative to 300 mM treated or control treatments. A low % decrease in chlorophyll content compared to control treatment was exhibited by Hs 2698, MN-8, HOR 3301 and MN-24 with 40.7, 30.1 43 and 36%, respectively. The decrease due to increased salt treatment was 2-8 fold depending on genotype. A low % decrease implied that the cultivars could protect their chlorophyll from deleterious effects of salt stress while those with high % change implied they were vulnerable to chlorophyll degradation and therefore, susceptible to salt stress. See **Table 4**.

2.12. 2.5 Glycine betaine and sucrose analysis using the using HPLC

Glyine betaine and sucrose are important compatible solutes that are related in abiotic stress responses. Using 1 mM GB as internal control, HPLC analysis showed that GB was detected at a wavelength of 210 nm with a retention time of about 6 mins. The HPLC output indicated that controlled plants displayed low GB levels compared with salt treatment which had elevated GB to as high as 2-17 fold in all the cultivars. In 65% of the cultivars the increase in > 13 fold compared to control plants (**Fig. 13 A**).

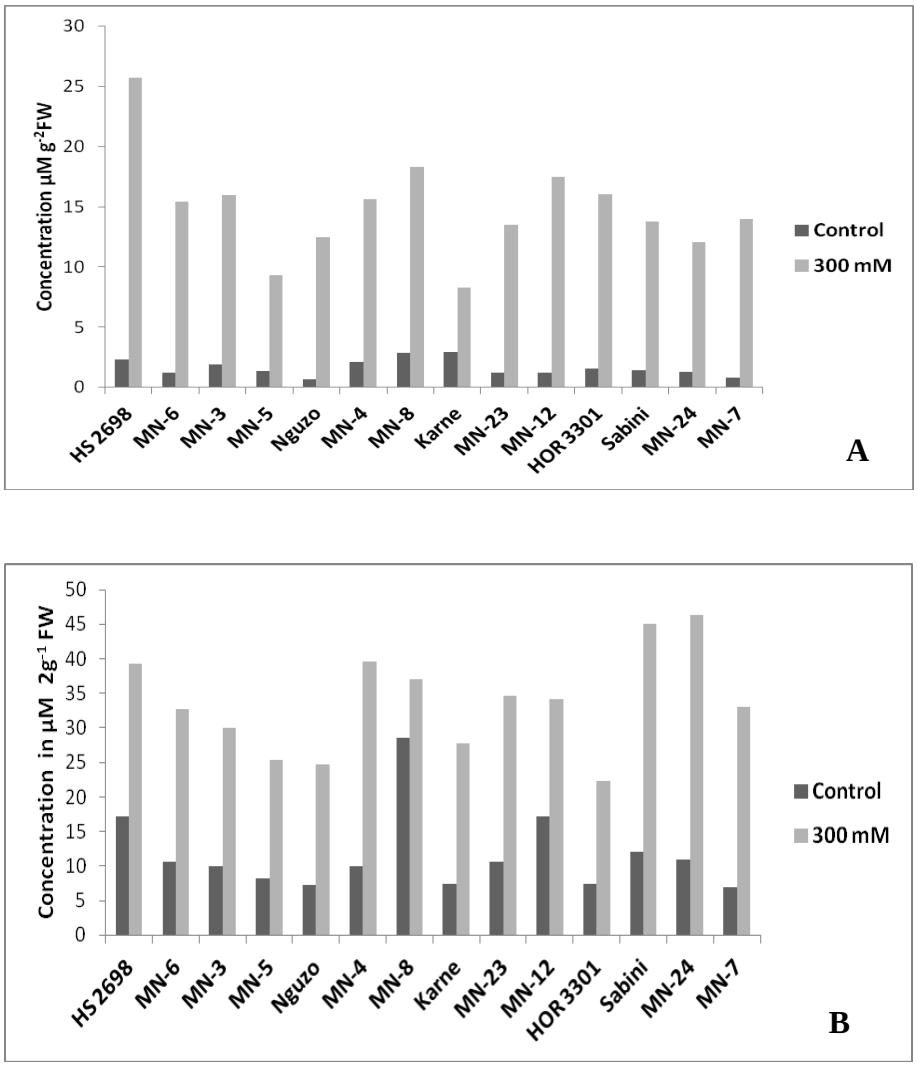


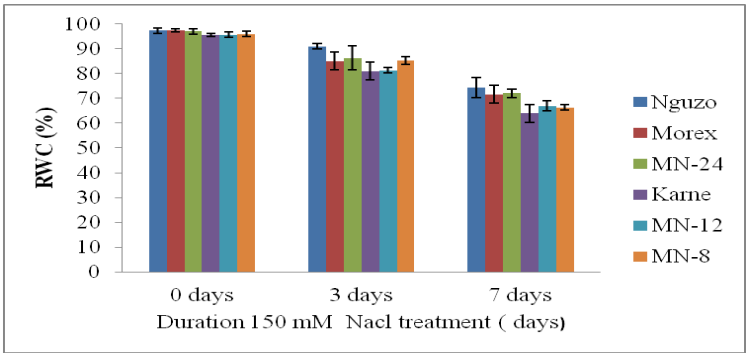
Figure 13. Analylsis of A: Glycine betaine B: Sucrose levels in response to 300 mM NaCl treatement using HPLC

The highest concentration was observed notably in Hs 2698, MN-8, MN-12, MN-3, HOR 3301 and MN-12 corresponding to 25, 18, 17, 16.4 and 16 µmg^{-1} FW, respectively. Hs

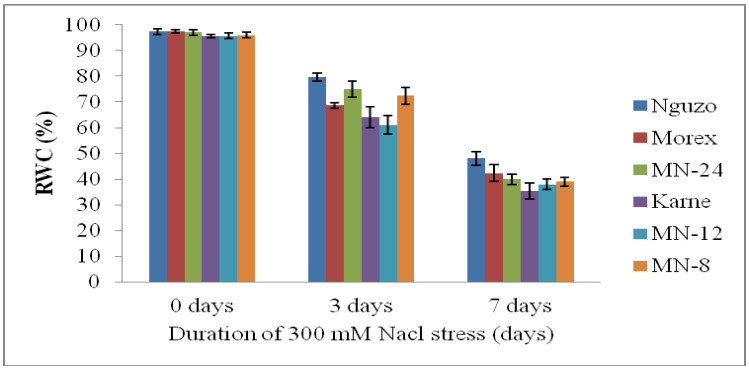
2698 the wild relative of barley exhibited the highest GB concentration. The Lowest GB was observed in Karne and MN-5 (8.25 and 9.33 $\mu Mg^{-1}FW$), respectively. Moderate accumulators were MN-23, Nguzo and MN-4. Additionally, retention time of sucrose in the extract was 18.6 mins in the HPLC unit. This was confirmed by use of 1 mM sucrose as a control. Sucrose concentration was low in the control plants (**Fig 13 B**). However, there was significant increased in sucrose in response to NaCl treatment. This increase was genotype dependent. The increase was 1-4 fold compared to control plants. The highest change was observed in MN-7, Sabini, MN-4 and MN-7 with 4.7, 4.2 and 3.75 μmg^{-1} FW, respectively. In line. MN-8, there was a small change in sucrose concentration between control (28.5 μMg^{-1} FW) and treated (37 μMg^{-1} FW) plants. See **Table 4.**

2.12. 2. 6 Relative water content in detached leaf tissues in six selected barley cultivars

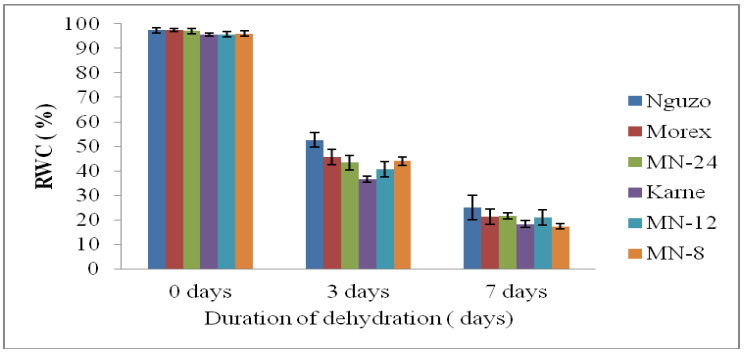
Leaf water content is a useful indicator of plant water balance, since it expresses the relative amount of water present in the plant tissues. In order to evaluate the genotype variations in water status, RWC was determined in 6 selected barley cultivars subjected to 7 days of dehydration as well as 150 mM or 300 mM NaCl treatments. Detached whole leaf samples were collected at three-time point namely: 0, 3 and 7 days post stress treatments (**Fig. 14**).



A



B



C

Figure 14. Changes in leaf relative water content in 6 barley lines in response to A: 150 mM NaCl. B: 300 mM NaCl, C: Dehydration treatments. The detached leaves were analyzed at 3 and 7 days post stress treatments.

From this study, relative water content varied with the type of stress, duration of stress and genotype was not significantly different in the control treatments in all genotypes. However, there was a significant decline in RWC in response to the dehydration and 150 mM, or 300 mM NaCl treatments compared to control treatments. The reduction of RWC was affected by genotype, type of stress and the duration of stress. A gradual decrease in RWC was observed after 3 and 7 days under 150 mM salt stress compared with well watered plants (**Fig. 14 A**). In the 3 and 7 days of stress, a reduction of 80-90% RWC and 60-70% RWC, respectively was observed depending on the genotype. Nguzo maintained a high RWC while Karne showed a decrease in RWC in the different time points. A further increase of salt stress to 300 mM caused a significant reduction of RWC depending on the genotype and duration of stress. The increase in salt stress caused a drastic reduction of 60-80 % RWC and 40-50% RWC in 3 days and 7 days of treated seedlings respectively (**Fig. 14 B**). RWC was high in Nguzo, MN-24 and MN-8 and was the lowest in Karne and Morex within 3 days of stress. Nguzo maintained the highest RWC compared other genotypes after 7 days stress period. Dehydration stress caused the largest reduction in RWC compared to control and 150 mM or 300 mM NaCl treatments (**Fig. 14 C**). This reduction was genotype dependent and was enhanced by duration of the stress. Dehydration caused a reduction of 40-50% RWC and 20% RWC in 3 and 7 days stress period, respectively. Below is a summarized table indicating physiological assays tested for type of stress, duration of stress and genotype response.

Tabe 4. Summary of physiological assays used for stress tolerance evaluation

Physiological assays	Level and duration of NaCl (mM) treatments				**Susceptible lines**	**Tolerant lines**
	3 days		**6 days**			
	300 mM	**600 mM**	**300 mM**	**600 mM**		
Electrolyte conductance	Yes	Yes	Yes	Yes	**Karne** **Sabini,** **MN-12,** **MN-5,** **MN-23**	Nguzo MN-8, MN-24, MN-3, HOR 3301
Lipid peroxidation/MDA (nmol/g FW)			Yes	Yes	**Hs 2698,** **MN-5,** **MN-12,** **MN-4,** **Sabini**	Nguzo MN-24, MN-3, MN-23
Proline content (µmol/g FW)			Yes	Yes	**MN- 4,** **MN-5,** **MN-12** **MN-7**	Nguzo MN-24, MN-3 Karne MN-6,
Chlorophyll content (mg/g FW)			Yes	Yes	**MN-6,** **Karne,** **MN-12,** **MN-7**	Hs 2698, MN-24, MN-8 HOR 3301,
HPLC analysis						
Glycine betaine (µMol/g FW)			Yes		**Karne,** **MN-5**	Hs 2698, MN-3, MN-8, MN-12
Sucrose (µMol/g FW)			Yes		**Karne,** **MN-5 ,** **Nguzo**	MN-4 MN-24, Sabini

2.12.3 Molecular analylsis: Expression characteristics of dehydrins and selected stress responsive genes

Stress responsiveness was determined in roots and shoots of 6 barley lines seedling Nguzo, Morex, MN-24, Karne, MN-8 and MN-12 subjected to dehydration and two levels of salt stress (150 mM and 300 mM) by analyzing transcript levels of genes encoding dehydrins (*Dhn1*, *Dhn3*, *Dhn4*, *Dhn5*, *Dhn6*, *Dhn7* and *Dhn9*), betaine aldehyde dehydrogenase (*HvBBD1*) and *Hordeum spontaneum* dehydration responsive (*Hsdr4*) which encodes a protein of uknown function. This study reports the expression characteristic and tissue specificity of individual stress responsive genes in response to dehydration and increasing salt stress. All the expressions were normalized to the housekeeping gene *HvEF1α*.

2.12.3.1 EFα1

The effect of dehydration and increasing salt on transcript levels of *HvEF1α* was evaluated in leaf and root tissues of barley seedlings. The housekeeping gene was constitutively expressed under all the treatments in leaf (**Fig. 15 A**) and root tissues (**Fig. 15 B**) when analyzed using RT-PCR. The corresponding relative expression was quantified using imagequant**.**

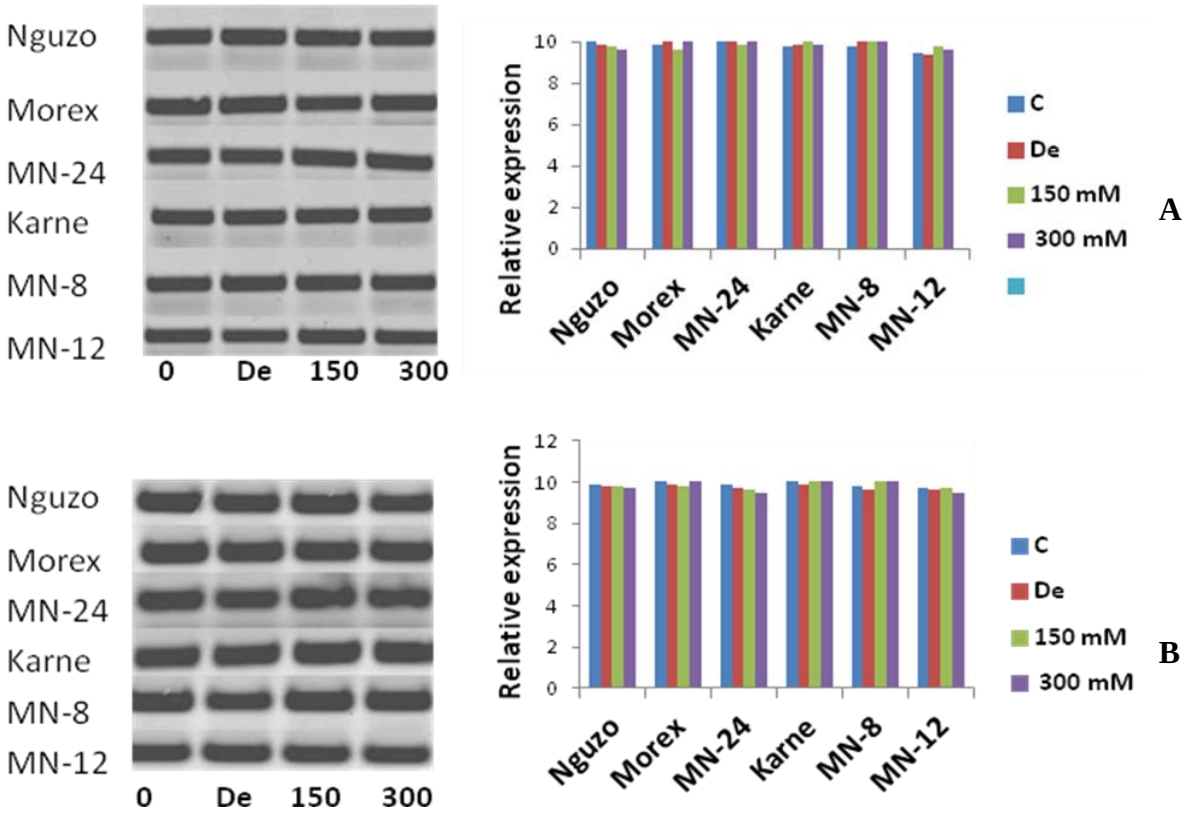


Figure 15. Expression profile of *HvEF1α* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings was analyzed using RT-PCR and Imagequant 5.3.

2.12.3 2 Dhn1

The SK_2 type, *Dhn1* was neither expressed in well watered (control) nor under NaCl treatment in all the genotypes. It was induced exclusively by dehydration stress in leaf tissues (**Fig. 16 A**). The expression was higher in Nguzo and MN-8 compared to other cultivars. Relative expression concurs with RT-PCR analysis. *Dhn1* was however expressed at basal levels in all the treatments in root tissues except in cv. Morex, whose induction was strongly induced by 300 mM salt stress (**Fig. 16 B**). The gel analyses corroborated the relative expression quantified using imagequant.

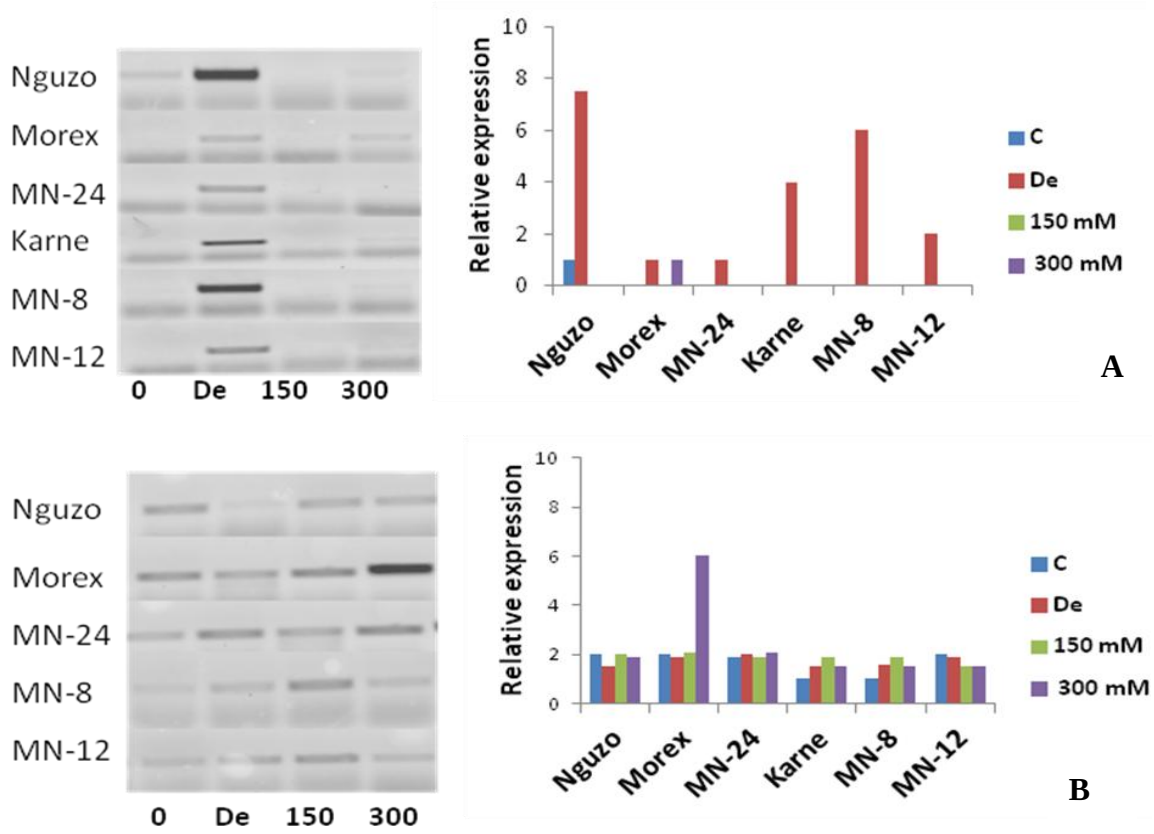


Figure 16. Transcript analysis and relative expression of *Dhn1* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings analyzed using RT-PCR and Imagequant 5.3.

2.12.3.3 Dhn3

Expression analysis showed that YSK_2-type, *Dhn3*, was strongly induced in response to dehydration and 300 mM salt treatments in leaf tissue in all genotypes except in cv. Karne and MN-12 where it was induced exclusively by salt tress (**Fig. 17 A**). It was not expressed in control or low salt stress treatments except in cvs. Morex and MN-12 where a weak expression in response to 150 mM NaCl was observed. Expression of *Dhn3* in root tissues was not induced in control treatments. However, it was strongly induced in response to dehydration and 300 mM salt stress in genotype dependent manner (**Fig. 17 B**). In Morex and MN-12, expression of *Dhn3* was strongly induced in response to dehydration compared to other genotypes. It was strongly downregulated by dehydration and salt stress in Nguzo and induced exclusively by dehydration in Karne. The RT-PCR gel results are validated by the corresponding relative expression analyses using imagequant.

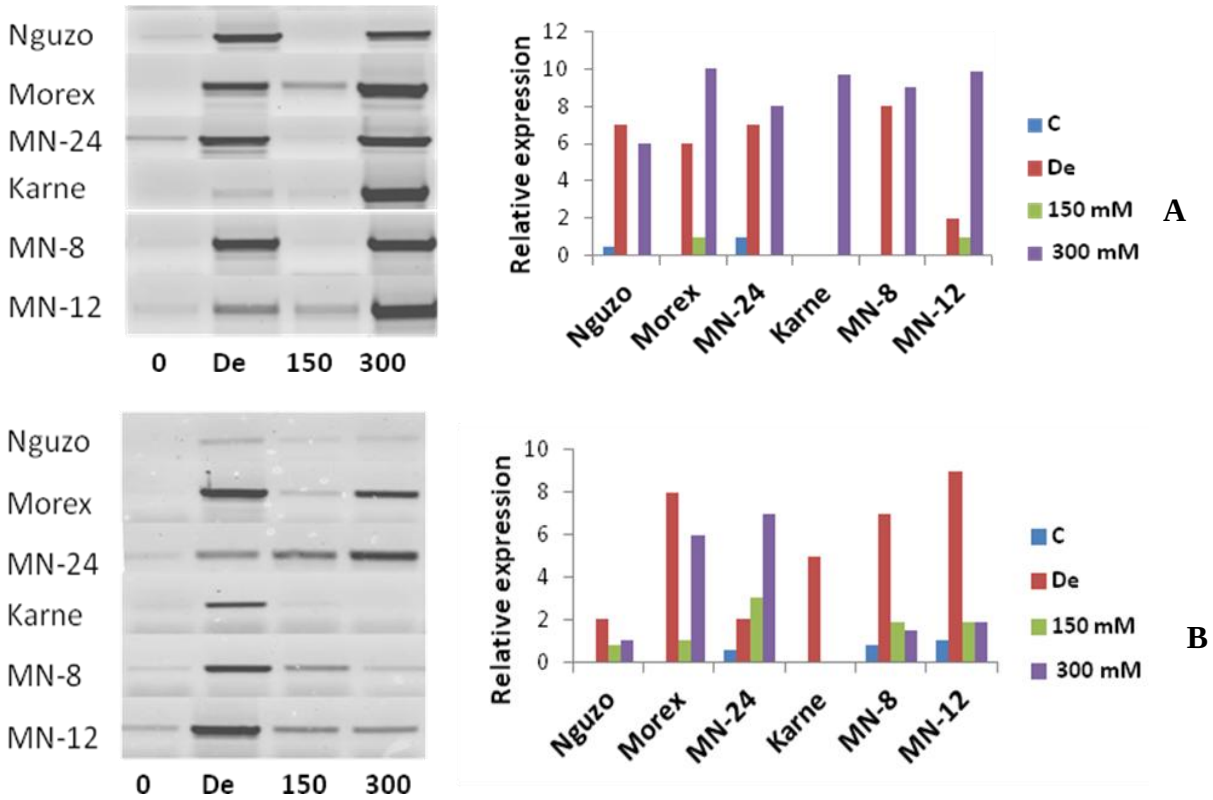


Figure 17. Expression profile of *Dhn3* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings wasanalyzed using RT-PCR and Imagequant 5.3.

2.12.3 4 Dhn4

The YSK_2 type, *Dhn4* was induced strongly in response to dehydration and 300 mM NaCl in all the genotypes except in Morex, which was downregulated by dehydration stress and exclusively induced strongly in response to 300 mM NaCl (**Fig. 18 A**). A strong induction by dehydration stress was exhibited in MN-8 and MN-24. In root tissues the expression of *Dhn4* was induced by dehydration and increased salt stress in a genotype-dependent manner (**Fig. 18 B**). The induction of *Dhn4* in response to dehydration was observed in all genotypes except in Karne. Nguzo, MN-8 and MN-12 had similar expression characterized by high and low expression in response to dehydration and and salt treatments, respectively. Similarly, Nguzp, Karne and MN-12 displayed similar expression levels in response to dehydration and salt treatments. A corresponding relative expression is presented adjacent to the leaf and root tissue gel analyses respectively.

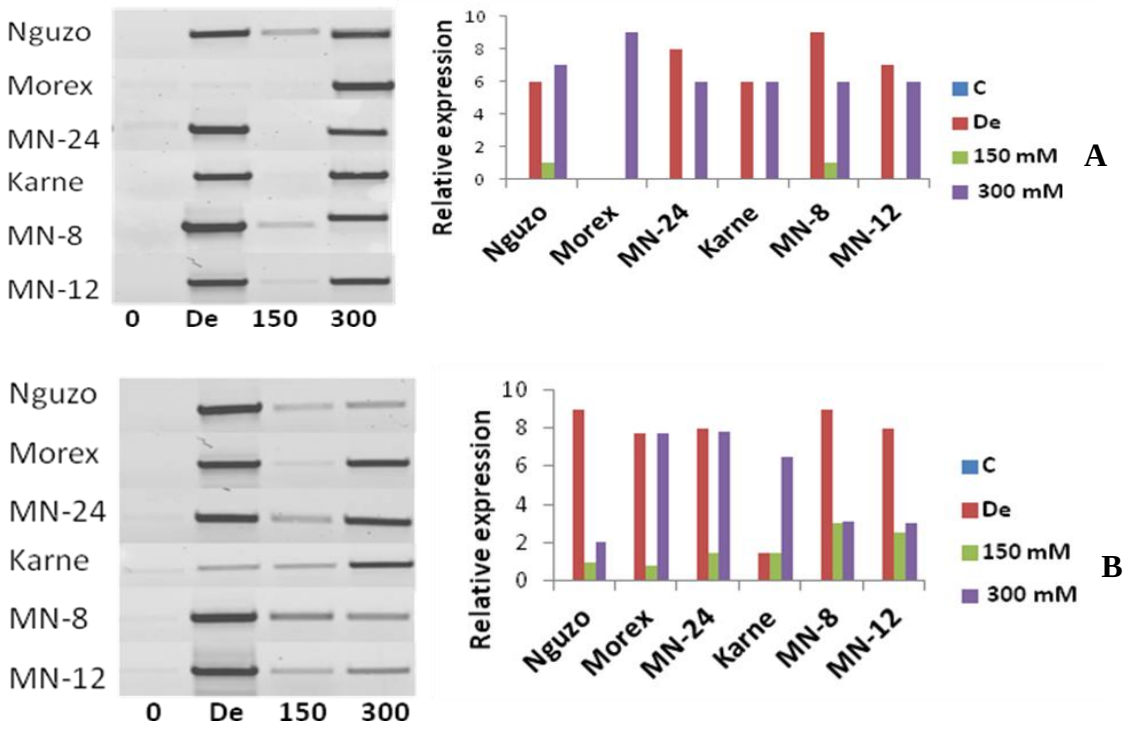


Figure 18. Expression profiles of *Dhn4* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings was analyzed using RT-PCR and Imagequant 5.3.

2.12.3.5 Dhn5

Dhn5, a type K_9 type was expressed at basal levels both in control and treated plants. However, in leaf tissue, there was a strong induction in MN-8 in response to 300 mM NaCl treatment (**Fig. 19 A**). Similarly, a weak constitutive expression was observed in root tissues (**Fig. 19 B**). The RT-PCR is supported by the corresponding relative expression of leaf and leaf gel analyses respectively.

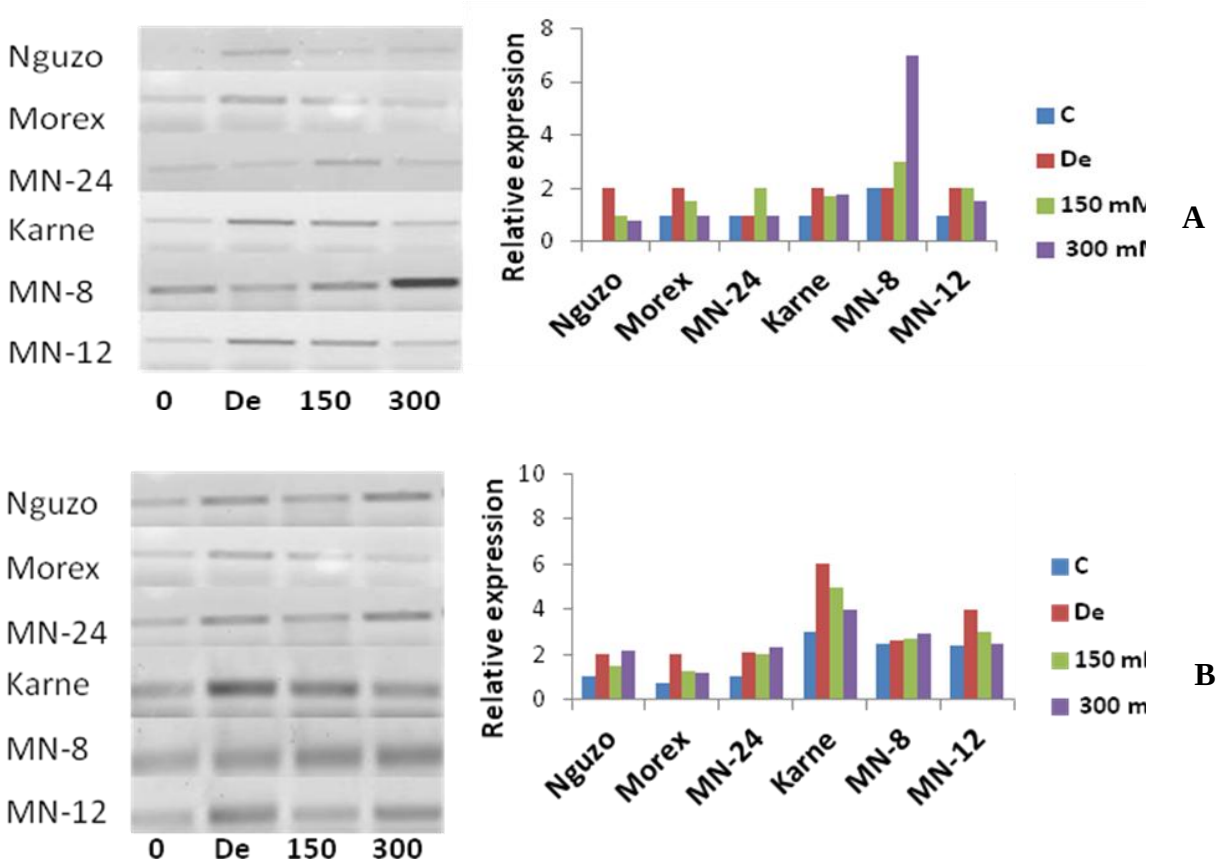


Figure 19. Expression profile of *Dhn5* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings analyzed using RT PCR and Image quant 5.3.

2.12.3 6 Dhn6

Expression analysis of *Dhn6*, a Y_2SK_3 type, was differentially induced in leaves and roots in response to stress in a genotype-dependent manner. In leaf tissues the expression was induced by either dehydration and or high salt treatments (**Fig. 20 A**). In MN-24 and MN-8, the expression was induced by both dehydration and 300 mM salt treatment while in Nguzo induction was exclusively up regulated by dehydration. In Morex and Karne expression was induced weakly by increased salt stress. It was not expressed in Morex or Karne under control and 150 mM NaCl treatment. In root tissue, *Dhn6* was induced in response to dehydration and 300 mM salt treatments only in Nguzo and Karne (**Fig. 20 B**). The expression level was higher under dehydration than in 300 mM NaCl. It was not induced in Morex, MN-24, MN-8 and MN-12 in all treatments. The relative expression confirms the differential expression of *Dhn6* analyzed using RT-PCR.

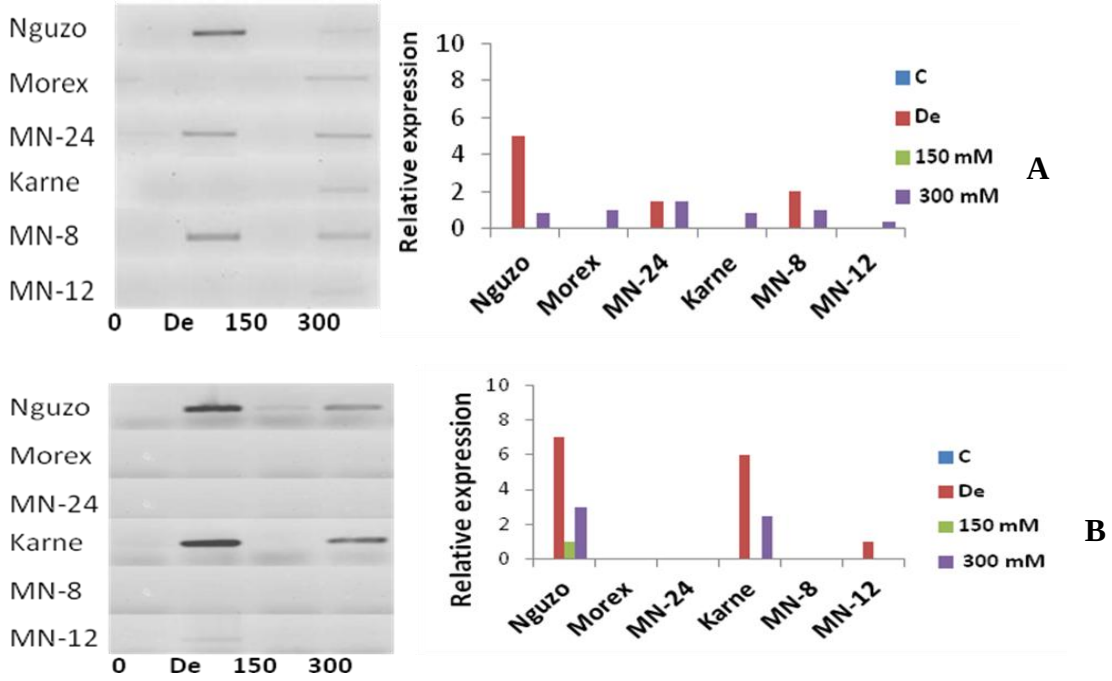


Figure 20. Expression profile of *Dhn6* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings was analyzed using RT-PCR and Image quant 5.3.

2.12.3. 7 Dhn7

The expression of YSK_2 type, *Dhn7* was induced by salt and or dehydration treatments and varied among genotypes. In leaf tissue it was induced in response to dehydration in Nguzo and MN-24 and by 300 mM NaCl treatment in MN-8 and MN-12 **(Fig 21 A**). In cv. Karne it was induced by both dehydration and 300 mM NaCl treatment. The gene was however not expressed in control and low salt treatments. In the root tissues, *Dhn7* was induced in response to dehydration and increased salt only in Nguzo and Karne but not control and low salt treatment (**Fig. 21 B**). It was however not expressed in Morex, MN-24, MN-8 and MN-12 in all the treatments. The genotype response of *Dhn6* and *Dhn7* were comparable in root tissues but differed in the leaf tissues.

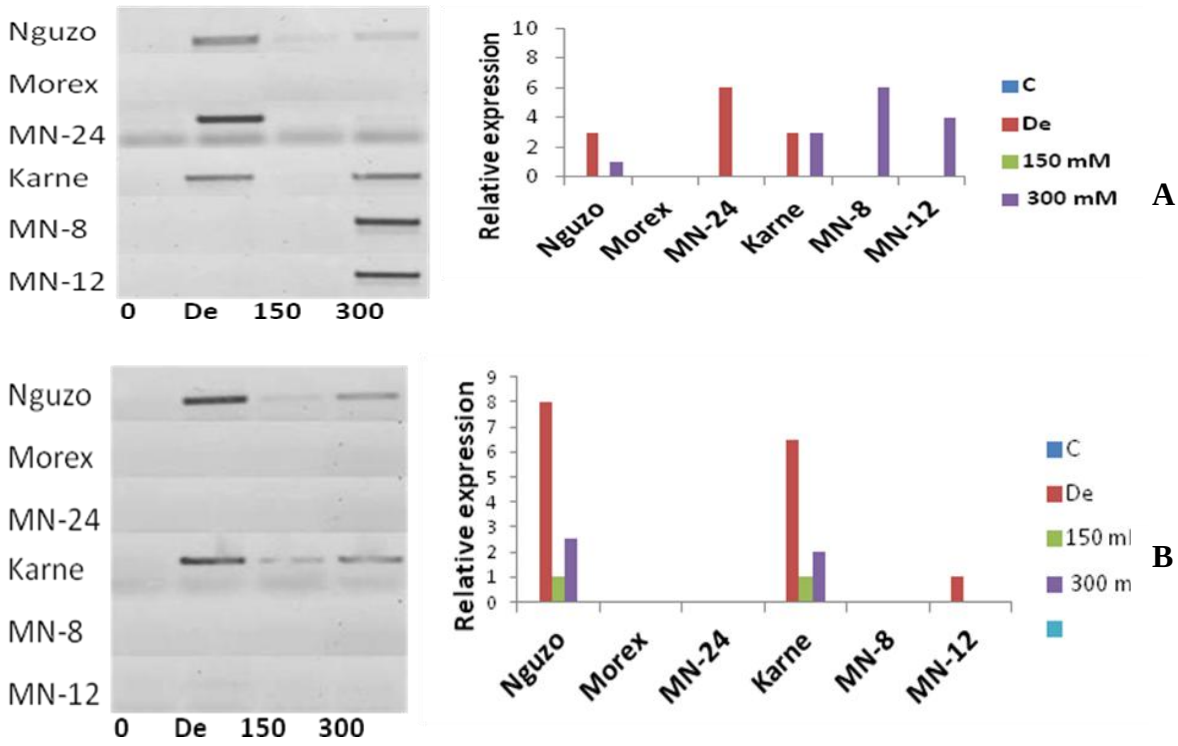


Figure 21. Expression profile of *Dhn7* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A- Leaf. B: Root tissue of 6 barley seedlings was analyzed using RT PCR and Imagequant 5.3

2.12.3.8 Dhn9

The expression of *Dhn9,* a YSK_2 type dehydrin was induced in response dehydration and or 300 mM NaCl treatment in a genotype-dependent manner in leaf tissues (**Fig. 22 A**). Dehydration caused the induction of *Dhn9* in MN-8, MN-24 and MN-12. In Morex and Karne, expression was elicited by both dehydration and salt stress. The expression patters in Nguzo and Karne were comparable in dehydration and salt treatments. In root tissue, expression of *Dhn9* was strongly induced by dehydration in all the genotypes (**Fig. 22 B**). A similar expression pattern was obtained by relative expression analysis.

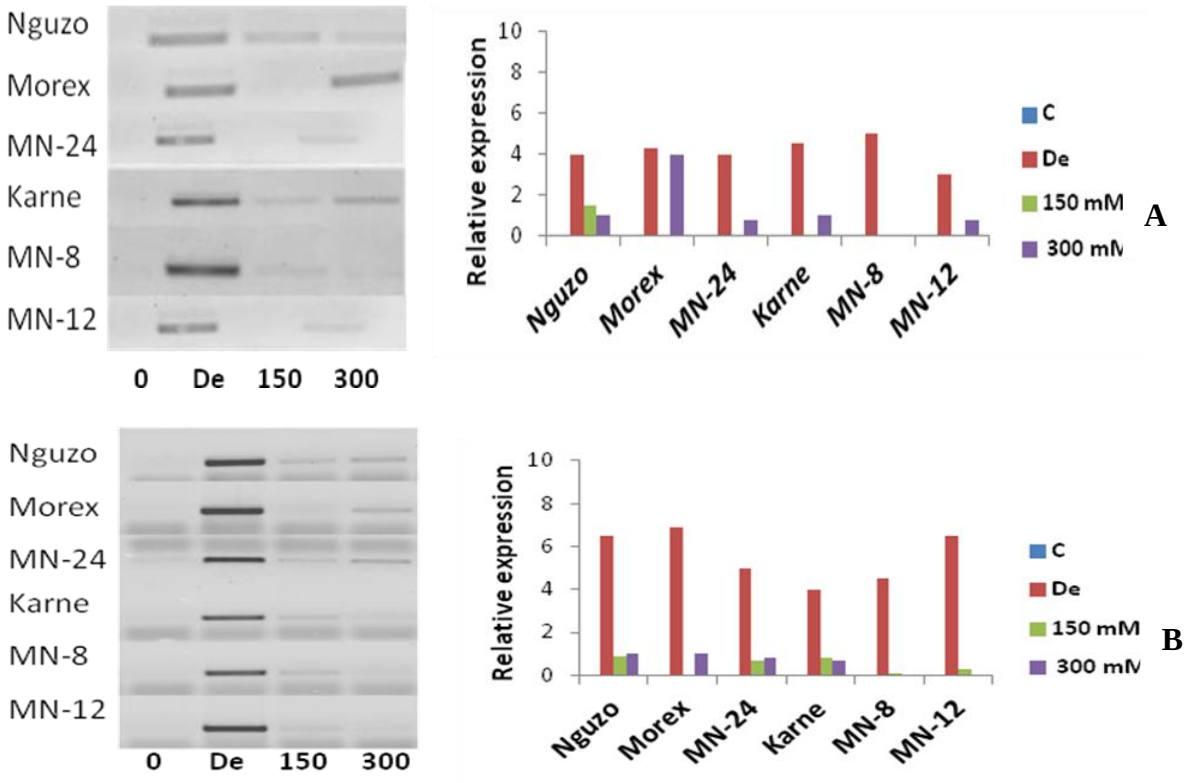


Figure 22. Expression profile of *Dhn9* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B- Root tissue of 6 barley seedlings analyzed using RT-PCR and Imagequant 5.3.

2.12.3.9 HvBBD1

HvBBD1 was strongly and constitutively expressed in leaf tissues in a genotype-dependent manner (**Fig. 23 A**). Karne displayed low expression compared to other genoytypes. There was an equally weak but constitutive expression in roots tissues. (**Fig. 23 B**). The expression of *HvBBD1* was stronger in leaf tissue than in roots. This is confirmed by corresponding relative expression analysis.

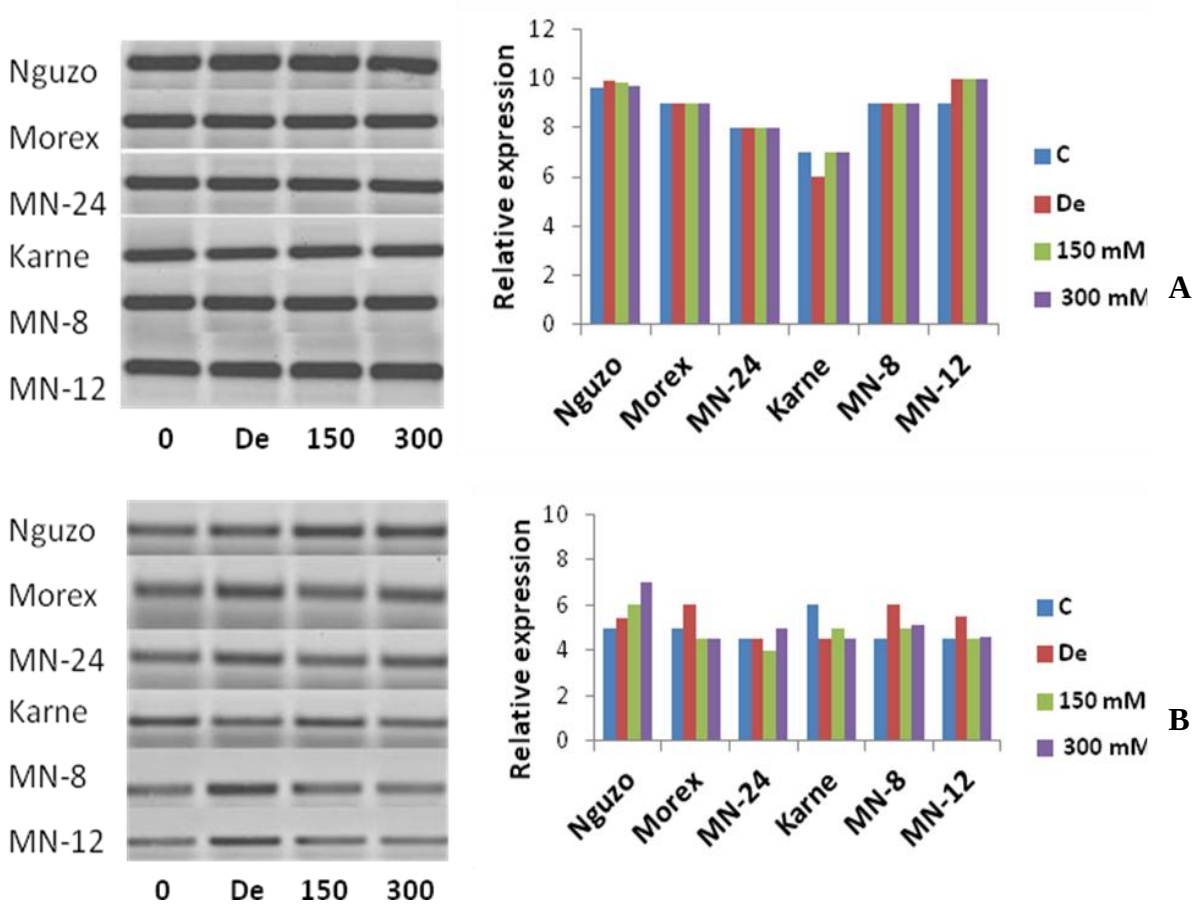


Figure 23. Expression profile of *HvBBDI* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings was analyzed using RT-PCR and Imagequant 5.3.

2.12.3 10 Hsdr4

The expression of the transcription regulator, *Hsdr4* was constitutively expressed in leaf tissue (**Fig. 24 A**) in all genotype except in MN-8 whose expression was induced in response to dehydration and salt treatments. Its expression in root tissues showed a strong constitutive expression in MN-12 and MN-24 and a weak constitutive expression in MN-8 and Karne (**Fig. 24 B**). In Nguzo and Morex, the expression of *Hsdr4* was induced by dehydration and salt stress.

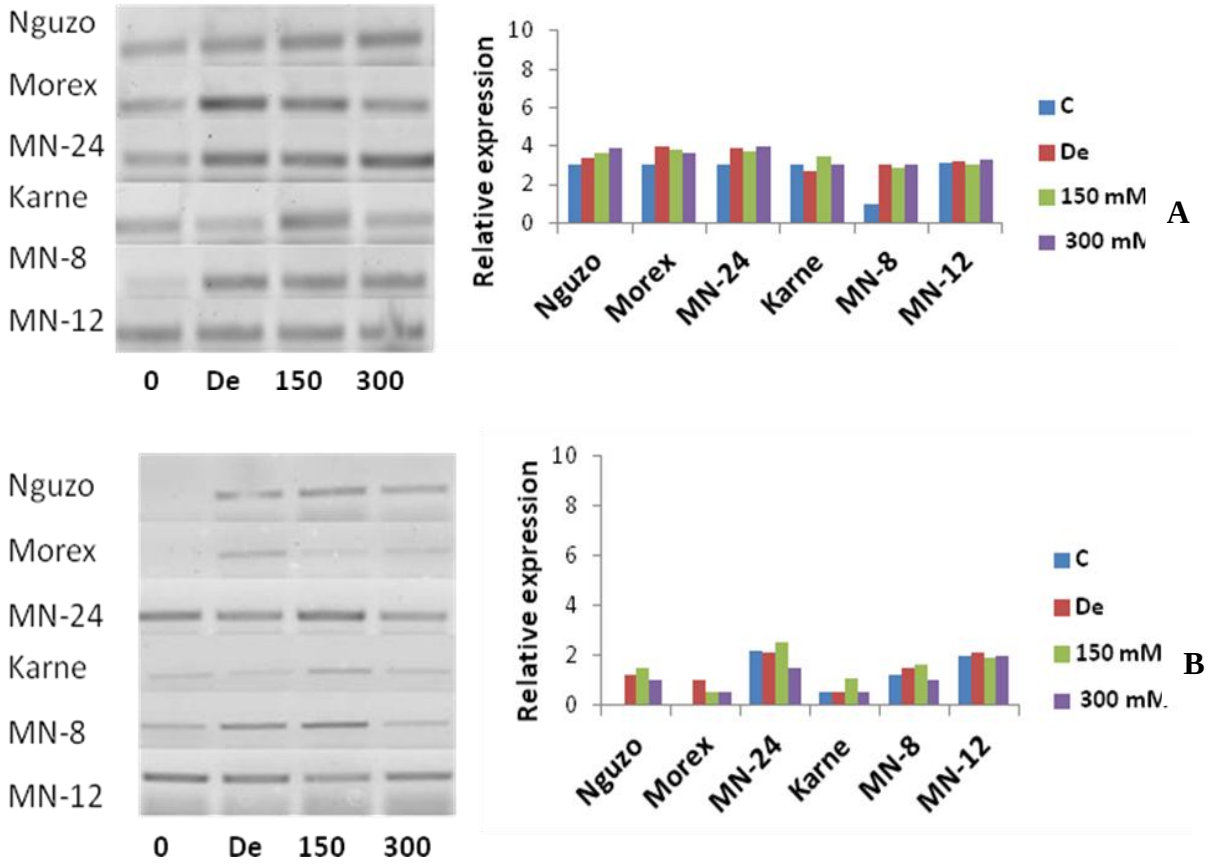


Figure 24. Expression profile of *Hsdr4* in response to dehydration (De), 150 mM and 300 mM NaCl treatments in: A-Leaf. B-Root tissue of 6 barley seedlings analyzed using RT PCR and Imagequant 5.3.

Differences in expression patterns were found in each of the *Dhns* genes depending on genotype, tissue type and severity of stress and duration of stress. We observed that (*Dhn3* and *Dhn4*), (*Dhn6* and *Dhn7*) and (*Dhn1* and *Dhn9*) exhibited similar expression patterns in reponse to dehydration and salts stress. The *Dhn* genes evaluated in these

studies showed that multiple members of *Dhn* superfamily work together in a coordinated manner in adaptive response to dehydration stress.

2.12.4 Tissue specificity in expression of stress responsive genes

To elucidate the physiological functions of different members of the *Dhn* superfamily and other stress responsive genes, their expression patterns in response to various stress treatments was evaluated in both leaf and root tissues. We demonstrated differential expression of different types of dehydrins in both control and stressed plants tissues. We observed that *Dhn1* was strongly expressed in response to dehydration in leaf tissues in all genotypes but was to some extent constitutively expressed in roots (**Fig. 16**). In response to dehydration, *Dhn3* was down-regulated in shoots of Karne and MN-12 but strongly expressed in roots of the same genotypes. Additionally, in Nguzo, a high induction in leaf tissues and a low level of expression was observed in roots following dehydration and increased salt stress (**Fig. 17**). Although *Dhn4* showed a similar expression pattern to *Dhn3* in response dehydration and salt stress, there was notable tissue specificity in its expression (**Fig. 18**). Importantly was the fact that *Dhn4* was down-regulated by dehydration in leaf tissues of Morex. The *Dhn4* induction in MN-8 and MN-12 was comparable to *Dhn3* in both shoot and root tissues. The Expression characteristics of *Dhn6* and *Dhn7* were comparable in response to salt and dehydration stress. In both *Dhn6* and *Dhn7*, expression was down-regulated in roots compared to leaf tissues that varied with genotype (**Fig. 20 A** and **B**, **Fig. 21 A** and **B**). On the other hand, *Dhn1* and *Dhn9* were exclusively induced by dehydration in tissue-specific manner (**Fig. 16** and **Fig. 22**). Additionally, the *Hsdr4* and *HvBBD1* exhibited a strong constitutive expression in leaves than in roots.

2.12.5 Western blots analysis

In order to determine whether induction of *Dhn* mRNA transcripts in response to various stresses was correlated with accumulation of the corresponding DHN proteins, a Western blot analysis was performed using dehydrin polyclonal antisera specific to K segment consensus peptide TGEKKGIMDKIKEKLPGQH (Close et al., 1993). (**Fig. 25 A**, **B, C** and **D**). To verify uniform transfer and equal loading of proteins, the membrane was stained in Ponceau S stain **(Fig. 25 E)**

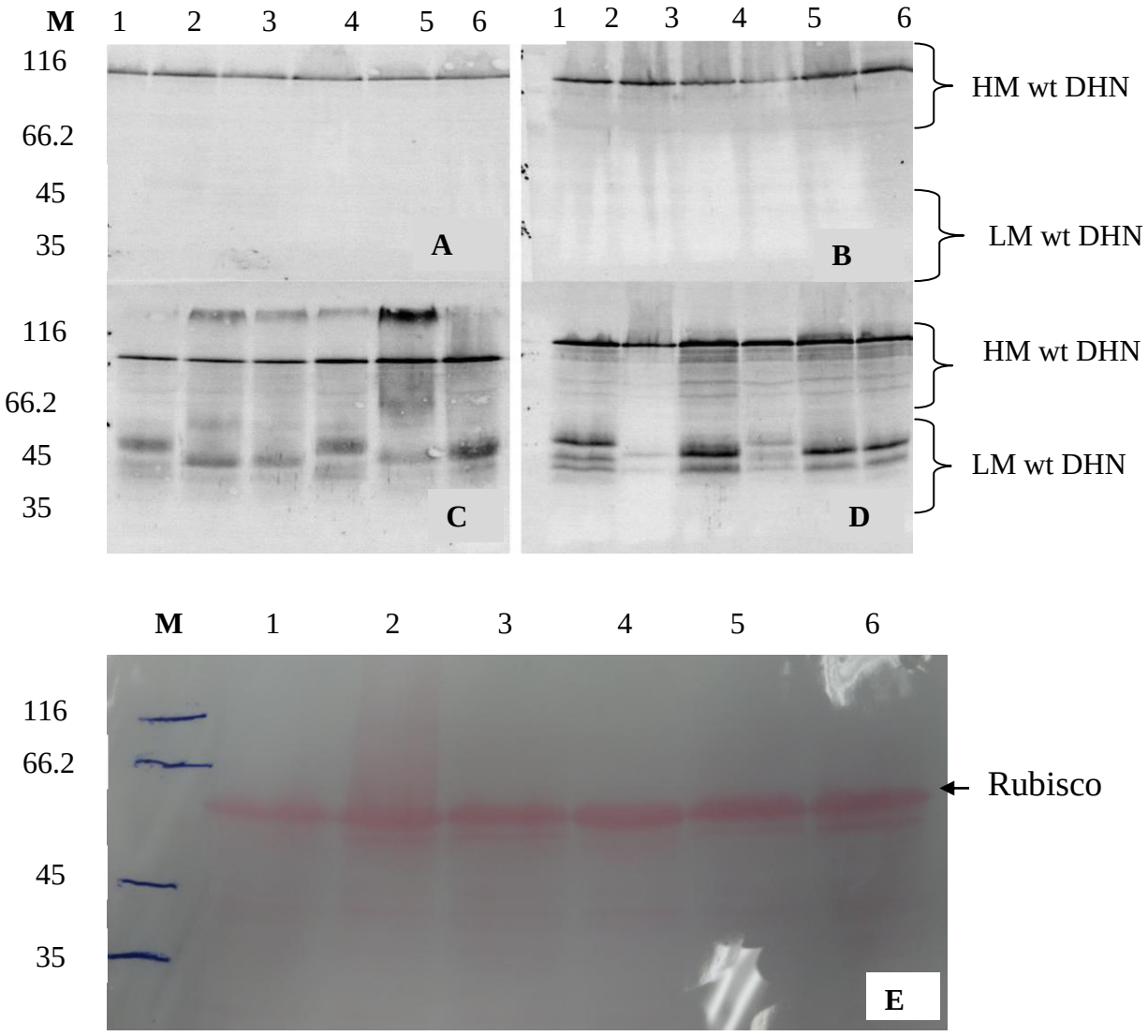


Figure 25. Immunoblots of DHN expression in six barley lines. 1-Nguzo, 2-Morex, 3-MN-24, 4-Karne, 5-MN-12, 6-MN-8 under different stress treatments. A-Control, B-150 mM NaCl, C-Dehydration, D-300 mM NaCl, E-Ponceaus S staining. Total protein (12.5µg) was loaded on 12% SDS-PAGE. Polyclonal antibodies corresponding to K segment were used for protein detection.

Constitutive induction of high molecular weight DHN (HM Wt DHN) corresponding to about 86 kDa was detected in all the treatments (**Fig. 25**). The K segment present in all dehydrins was recognized by anti dehydrin polyclonal antibodies. Accumulation of DHN was not detected in control or 150 mM NaCl treatments (**Fig. 25 A** and **B**). However, dehydration and 300 mM salt stress caused increased induction of low molecular weight DHNs (**Fig. 25 C** and **D**). The occurance of multiple copies of the low molecular weight DHN ranging between 22-50 kDa suggesting that DHN exist as alleles or that more than one member of the DHN superfamily work together to bring the ameliorative effect in abiotic stress (Kosova et al., 2014). In response to 300 mM salt stress, DHN proteins in Morex and Karne was down-regulated (**Fig. 25 D**).

2.11. Discussion

For better exploitation of available genetic resources for breeding purposes, the existence of genetic variability must be demonstrated. Storage proteins in cereal crops have been frequently used to study genetic diversity, identifying genotypes and determining the phylogenetic relationships (Shewry and Halford, 2002, Echart-Almeida and Cavalli-Molina, 2001b, Yin et al., 2003, Leistrumaitė and Paplauskienė, 2007). In this study genotype variation in malting cultivars using SDS-PAGE resolved hordein polypeptide fractions was explored. Hordein polypeptides were separated into D, C and B fractions according to their electrophoretic mobility. The ocurrance of hordein profiles in the electrophoregram was in the range of 33- 96 kDa. The D hordeins were found in the range of 90-96 kDa, C hordeins in 63-88 kDa and B hordeins in 33-50 kDa which followed the pattern that was reported by Shewry et al. (1978) and Radović and Vapa. (1996). Although the extraction method of Shewry et al. (1978) was performed under 60 ^{0}C in the presence of 2% mercaptoethanol, we achieved comparable separation efficiency was achieved in the presence of 3% mercaptoethanol performed under room temperature and pressure conditions. Using acid PAGE (A-PAGE), Pomortsev, (2001) studied polymorphism in 147 local Ethiopian barley accessions and reported a low genetic variability in hordein polypeptides with a genetic distance of 0.138. Consequently, cluster analysis grouped the 20 barley cultivars into 4 groups based on hordein proteins and 3 using agro-morphological traits. In our current study, the barley cultivars and breeding lines examined were distinguished for variability of hordein profiles based on presence or absence of protein bands. It has been shown that the presence or absence of seed protein has been used for cultivars identification since it is not vulnerable to environmental changes. The variability was observed in individual fractions (D, C and B) or combined fractions (D + C+ B) between the genotypes. In this work, the majority of hordeins occurred between 33-88 kDa which represent mainly the C and B hordeins. A total of 29 hordein fractions ranging between 33-96 kDa were scored for the presence or absence of hordein in each cultivar. These findings compared to Lee et al. (2010) who reported that 36 hordeins fractions in the range of 31-97 kDa when 13 local adaptability test (LAT) lines were evaluated. Using agglomerative hierarchical clustering hordein polypetides exhibited specific grouping patterns among the lines under study. AHC analysis generated 5 clusters of barley namely C1, C2, C3, C4 and C5. 50% of the cultivars were clusters in C2 (all 6-row barley). Additionally, C1 cluster (All 2-row barley) represent barley with

good malting qualities usually preferred by the malting industries because of high yielding and low susceptibility to lodging eg Barke and Nguzo. Nguzo is a popular malting variety and most of the new varieties have aNguzo genetic background. Six-rowed barleys have been reported to contain more protein than two-rowed types but, is mainly attributed the the amino acid composition which is probably more dependent on the total protein in the kernel than the row type (Newman and McGuire, 1985). However, the 2-row malting barley is preferred in Kenya because it has low protein content compared to 6-row typewhich is usually preferred for animal feeds.

Plants are exposed various environmental cues such as as drought and salinity during their development and at different growth stages. Since plants are sessile, they initiate a series of physiological, biochemical, metabolic and molecular responses that work in coordinated manner to bring ameliorative effect against stresses (Wang et al., 2003, Bartels and Sunkar, 2005). The exact mechanism of response to either salt or drought is not well understood. However it has been established that stress tolerance is genotype specific and varies with stage of stress (germination, seedling anthesis or grain filling), type of stress and duration of stress (Szira et al., 2008, Rapacz et al., 2010). Comparing physiological changes occurring during the period of stress in closely related genotypes may contribute to the identification of stress resistant and susceptible genotypes (Aboughadareh et al., 2013, Gao et al., 2013). Salt and water dehydration have unifying factors: they impart dehydrative element leading to low water potential (Munns, 2002) and increased accumulation of compatible solutes. Increased intracellular ROS cause chlorophyll degradation, membrane lipid peroxidation, reduced membrane fluidity and selectivity (Moller et al., 2009). The extent of chlorophyll loss, membrane leakage, MDA and proline are regarded as indicators of oxidative damage (Luis et al., 2006, Yasar et al., 2008). Tolerance to salt stress is associated with Na^+ exclusion in the roots and selective mechanism of Na^+ uptake and accumulation in vacuoles therefore minimizing accumulation of toxic ions like Na^+ and Cl^- into in the cytoplasm (Munns and Tester, 2008).

The integrity of cell membranes was evaluated by measuring the extent of membrane damage. This is one of the first targets of many plant stresses and it is generally considered that the maintenance of their integrity and stability under water stress conditions is a major component of abiotic stress. Solute leakage from plant tissue due to membrane damage can easily be estimated through measurements of electrolyte leakage

from the cells (Blum and Ebercon, 1981) usually presented as an index percentage or ratio of total electrolyte present. In our study, we established that control plants showed negligible ion leakage but this increased substantially with increasing salt stress and duration of stress exposure in treated plants. By prolonging the duration of stress from 3 to 6 days, the assay displayed sensitivity in discriminating cultivars that were more vulnerable and tolerant to tissue damage. Worth noting is the fact that some cultivars experience rapid shock in response to short term exposure to salt treatment reflected in increased ionic leakage and reprogrammed protective machinery. Low and high relative conductance was associated with lowest and highest membrane damage respectively. The genotypes Nguzo, MN-8, MN-3, HOR 3301 and MN-24 displayed lowest ionic membrane leakage while Karne, Sabini, MN-12, MN-23 and MN-5 recorded high values. The current results agrees with several bodies of research that demonstrated the potential that electrolyte leakage measurements may be correlated with several physiological and biochemical parameters that trigger the plant responses to environmental conditions such as spectral reflectance (Garty et al., 2000, Zhang et al., 2010) antioxidative enzyme synthesis, (Sreenivasulu et al., 2000, Ashraf and Ali, 2008) membrane acyl lipid concentrations (López-Pérez et al., 2009) water use efficiency (França et al., 2000), transverse relaxation time of leaf water (Nagarajan et al., 2005). It is therefore not surprising that electrolyte leakage has been recommended as a valuable screening method for identification of stress resistant cultivars in several crop species (Bajji et al., 2002, Verslues et al., 2006). These findings could be useful in generation of stress tolerance and susceptibility indices in barley.

Additionally, determination of lipid peroxidation is equally an important physiological assay. Lipid peroxidation indicates the prevalence of free radical reactions in tissues. The amount of MDA is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Smirnoff, 1993, Parthasarathy et al., 2000). In this study, the lipid peroxidation was reduced in control treatment. MDA levels were assayed on leaf tissue exposed to 6 days of NaCl treatment since there was a clear and detectable genotype discriminatory effect in comparison to the 3 days treatment. Hs 2698, MN-5, MN-12, MN-6 and Sabini exhibited higher MDA levels while MN-3, Nguzo, Karne, MN-23, and MN-24 had lower MDA level. Low lipid peroxidation was associated with the ability of plants to mount adequate protective mechanism against ionic and osmotic damage while high levels are associated with susceptible genotypes. These results concur with the

findings in which low MDA was associated with abiotic stress tolerance (Bor et al., 2003, Demiral and Türkan, 2005, de Azevedo Neto et al., 2006). Although Hs 2698 is a wild progenitor of barley, it did not exhibit any level of salt tolerance but instead was highly susceptible to lipid peroxidation as confirmed by high MDA content. The occurrence of lipid peroxidation in biological membranes has been indicated in impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors, proteins and increased unselective permeability of ions such as Ca^{2+} into the cell (Halliwell and Gutteridge, 1984, Gutteridge and Halliwell, 1990, Niki et al., 2005). There is adequate evidence that lipid peroxidation products such as ROS trigger various biological functions *in vivo* such as regulators of gene expression, signaling messengers, activators of receptors and nuclear transcription factors (Ceaser et al., 2004, Zmijewski et al., 2005, Forman et al., 2008). Other studies have shown that lipid peroxidation induce anti-inflamatory reactions.

Additionally, photosynthesis and cell growth are among the primary processes to be affected by drought (Chaves et al., 2009) or by salinity (Munns, 2002). Stomatal closure reduce CO_2 uptake and declines the internal CO_2 concentration in the stressed plants, which causes inhibition of photosynthesis (Cornic, 2000). We confirmed that increased salt stress reduced growth rate, turgidity and chlorophyll pigmentation. The changes of leaf colour from green to yellow are an indication that photosynthesis is impaired with subsequent dismantling of the chloroplast apparatus. On the other hand, control plants were healthy, green and turgid. Chlorophyll is degraded due to stomatal closure limiting CO_2 exchange and degradation of chloroplast enzymes by accumulation of Na^+ in the shoots (Sudakar et al., 1991). In this study, genotype dependent chlorophyll damage due to salt stress suggests that there is an inherent genetic protective mechanism. Cultivars Karne, Sabini, MN-6 and MN-7 exhibited the lowest chlorophyll content. Photosynthetic measurements of chlorophyll as function of photosynthetic activity could be used as a reliable indicator for screening barley genotypes for abiotic stress tolerance (Chaves et al., 2009).

Early biochemical effects of water deficits that involve alterations in photophosphorylation and decrease in the amount of ATP leading to a decreased regeneration of RuBP (Tezara et al., 1999) seem to be dependent on species showing different thresholds for metabolic down-regulation (Lawlor and Cornic, 2002). Increased accumulation of compatible solutes has been indicated in adaptive response to abiotic

stress (Chen et al., 2007). Proline is a well-known osmoprotectants in plants which accumulate under salinity conditions. Proline can also function as a protein stabilizer, a hydroxyl radical scavenger, a source of carbon and nitrogen and a cell membrane stabilizer (Silva-Ortega et al., 2008). In our studies we observed an increase in the proline level in response to increased salt concentration in a genotype dependent manner. Increased proline content was observed in cultivars Nguzo, Karne, MN-6, MN-3 and MN-24 and reduced in MN-5, MN-4, MN-12 and MN-7. Although proline accumulation has been attributed to response to abiotic stress, this relationship is not very clear. This phenomenon of elevated proline was also observed in the cv. Karne which was categorized as susceptible variety using ionic leakage and lipid peroxidation assays. These findings concurred with the works of Widodo et al. (2009) who reported that Clipper a salt sensitive barley accumulated more proline in root than in the salt tolerant cultivar Sahara. This is consistent with earlier suggestions that such elevated accumulation of proline may be correlated with slower growth and/or leaf necrosis rather than being an adaptive response to salinity. We proposed that the increased proline was due to catabolism of nucleic acid and amino acid and may not necessarily confer tolerance against stress. Whereas the stress responses may appropriately protect one plant it can also make another more vulnerable.

The late embryogenesis abundant genes (LEA DII) called dehydrins (Ingram and Bartels, 1996a, Close, 1997) are the most studied stress inducible gene families. They are differentially regulated at the transcriptional level under environmental stress in seeds and vegetative tissues subjected to environmental stresses such as salinity drought and low-temperature and ABA application (Allagulova et al., 2003). The aim of this study was to determine the association of differential expression of dehydrins (*Dhn1, Dhn3, Dhn4, Dhn5, Dhn6, Dhn7* and *Dhn9*), *HvBBD1* and *Hsdr4* and stress response to water dehydration and to two levels of salt stress (150 and 300 mM NaCl) in barley leaf and root tissues. The *Dhns* that we evaluated fall into three categories (SK_n, Y_nSK_n and K_n) out of the possible 5 YSK structural domains (Close, 1997). We illustrated intraspecific variations of *Dhn* and other stress responsive genes in the root and leaf tissues in 6 barley genotypes. We established that gene expression was genotype dependent, tissue specific and dependent on type and level of stress. *Dhn1* and *Dhn9* were exclusively induced by dehydration in leaf tissue while *Dhn3, Dhn4, Dhn6* and *Dhn7* were induced by dehydration and increased salt stress but the expression level varied between tissues.

Dhn1, Dhn3 and *Dhn4* were more strongly expressed in leaves than in the roots. A summary of tissue specificity of *Dhns* is described in detail (2.12.4). Suprunova et al. (2004) reported differential expression of *Dhn1, Dhn3, Dhn6* and *Dhn9* genes in two barley wild genotypes (*Hordeum sponteneum spp*) with contrasting expression in response to water dehydration. Variations in sensitive and tolerant genotypes were detected mainly in the expression of *Dhn1* and *Dhn6* suggesting their potential use as markers for abiotic stress. In related studies, de Mezer et al. (2014) evaluated genotype responses to dehydration by determining expression profiles of dehydrins and transcription factors. It was found that expression of *HvZIP1* and *Hsdr4* genes is linked with barley adaptation to water deficit. In the body of research by Karami et al. (2013) several dehydrins transcript belonging to YnSKn (*Dhn1, Dhn3, Dhn5, Dhn7* and *Dhn9*) were differentially expressed in barley flag leaf exposed to chronic dehydration in two Moroccan barley differing in drought tolerance. In our studies dehydrins *Dhn1* and *Dhn9* could be used as markers for drought tolerance while *Dhn3* and *Dhn4* are potential markers for salt stress tolerance. We established that more than one *Dhn* is expressed in response to abiotic stress but differed in the expression level in different genotypes. Dehydrins work in coordinated manner to bring about the ameliorative effect against abiotic stress. Dehydrins have been associated with cellular orgenelles and their posttranslational modification such as phosphorylations especially in S segment may affect their localization (Goday et al., 1994). Phosphorylation of the S segment may play an important role in stress tolerance (Brini et al., 2007) observed differential phosphorylation pattern of DHN5 in Tunisian Durum wheat (*Triticum durum* Desf) differing in salt and drought tolerance. Variations in stress specificity and spatial distribution suggest the members of the DHN superfamily are functionally specialized.

Many studies have reported a positive correlation between the accumulation dehydrins and tolerance, dehydration and freezing (Rorat, 2006, Kosova et al., 2014). Comparative studies on cultivars showing contrasting stress tolerance have provided evidence of a strong correlation between expression profiles of *Dhns* transcripts or accumulation of corresponding DHN protein and abiotic stress tolerance (Ismail et al., 1999) suggesting a role as molecular markers. The correlation of *Dhn* transcripts level and corresponding protective mechanism has been unraveled by promoter analysis studies. The regulation of expression is controlled by osmotic stress in the presence of transcription factor DRE. The DRE copies in the *cis* regulatory elements of the promoter may vary hence the

variations seen in the genotype response to stress. The presence of putative regulatory elements related with dehydration, ABA and cold response in the 5' flanking regions of all the barley dehydrins suggest they play a role in the expression patterns observed (Choi et al., 1999). These regulatory elements included MYB, MYC, ABRE and DRE could vary in number in dehydrin types (Close, 1997). ABRE and DRE were found in the promoter region of all the genotypes of YSK_2 type. In the current study, immunoblot analysis using polyclonal antibodies specific to K segment of dehydrins revealed increased accumulation of low molecular weight DHN in response dehydration and 300 mM salt treatments. Accumulation of more than one DHN protein within a single genotype could imply that more than one low molecular dehydrin is induced in response to stress. These findings are in consistent with the results Kosova et al. (2014) who demonstrated that cold tolerance in wheat and barley was associated with accumulation of cold induced WCS120 and DHN5, respectively in leaf tissues. Choi et al. (1999) suggested that qualitative differences in accumulated dehydrin proteins may be caused either by accumulation of different low-molecular DHN protein or by allelic variants of the same gene differing in copy number of hydrophilic segments and electrophoretic mobility. But whether the expression of transcript had indication or adaptive response is something that could not be concluded. In general, we found *Dhn*, *Dhn9*, *Dhn3* and *Dhn4* not only act as stress indicators but also as markers for dehydration and salt stress respectively.

A further comparative study of expression changes of homologous of *Hdr4* was evaluated. We observed that the expression of *Hsdr4* was induced strongly in leaves than in roots. In the works of Suprunova et al. (2007), *Hsdr4* was found to be induced in response to severe dehydration in wild barley exhibiting dehydration tolerance. The promoter analysis of *Hsdr4*, showed a new putative miniature inverted-repeat transposable element (MITE), and several potential stress-related binding sites for transcription factors (MYC, MYB, LTREand GT-1), suggesting a role of the *Hsdr4* gene in plant tolerance to dehydration stress.

Many family members of the vascular plants respond to dehydration and salt stress with increased biosynthesis of osmoprotectant betaine (Rhodes and Hanson, 1993). In these members biosynthesis occurs through a two step oxidation of choline via an intermediate betaine aldehyde which are catalyzed by CMO and BADH. Transcripts of BADH have

been shown to increase several fold in responses to stress in sugarbeet (McCue and Hanson, 1992) sorghum (Saneoka et al., 2001) and barley (Ishitani et al., 1995)

Dehydration and salt stress induce accumulation of GB and this accumulation is associated with increased BADH activity. The observed rise was associated with an increase in BADH protein and mRNA transcripts (Ishitani et al., 1995b). In our studies we report a strong constitutive expression of *BBD1* in leaves with a corresponding weak induction in roots in some varieties in response to salt and dehydration treaments. This gene may correspond to BBD2 described by Nakamura et al. (2001) and is responsible for increased GB accumulation in leaves. Nakamura et al. (2001) isolated and characterized two isoforms of BADH in barley (*BBD1* and *BBD2*). Expression of *BBD1* was induced strongly by salt stress, PEG6000 in roots and weakly in leaves. In contrast *BBD2* was slightly induced by salt drought and cold stress but not by PEG6000 and ABA in both leaves and root. *BBD2* was found to be cytosolic while *BBD1* was located in the chloroplast. Although HPLC analysis showed genotype variability and increased GB content in response to salt stress, it did not however correlate with increased BBD1 transcript in the leaf tissue. To continue this study, screening barley under field conditions in the various agroecological zones will strengthen our understanding as the complexities of abiotic stress in relation to changing environments and on how it affects other phenotypes including yield and yield related parameters.

3. Biochemical and molecular characterization of malting attributes in response to varying nitrogen treatments and seeding rate in three field grown Kenyan barley cultivars

Abstract

The production of barley in adequate supply with desired malting quality is the main challenge facing the brewing and malting companies in Kenya. Good malting varieties are associated with high starch content and low protein levels. Although the use of nitrogenous (N) fertilizers is vital for improving yield in the face of declining soil fertility, increased application is correlated with high protein content which reduces the quality of malt extract by lowering the diastatic power. Determining critical N levels suitable for application in cultivated crops without compromised yield coupled with minimum environmental pollution is an integral component towards sustainable agriculture. In order to gain insights into grain quality as affected by agronomic practices, a replicated field experiment was carried out at the University of Eldoret in the growing season of May to September 2012. An evaluation of the effect of N fertilizer levels (0, 30, 40 and 50 kg/ha) seeding rate (150, 200 and 250 seeds m^{-2}) on grain yield, 1000 kernel weight / ha, total grain protein, kernel starch content and hordein (prolamin) fractions and expression profiles of hordein genes were investigated using seeds of three 2 row malting varieties namely Nguzo, HKBL 1512 and HKBL 1385. Further molecular and biochemical analysis were carried out on malting attributes and genes during progressive seed germination and kilning stages. Under field conditions HKBL 1385 had the highest grain yield of 5.1 ton/ha while Nguzo and HKBL1512 recorded 3.7 and 3.8 tons/ha respectively. Enzymatic assay of grain starch indicated that N treatment ($p=0.048$) was and genotype ($p=0.05$) was highly significant. Seeding rate had no effect on grain protein and starch content. The genotype HKBL 1385 displayed the highest starch content of 278 mg/g DW while Nguzo and HKBL 1512 recorded 240 and 225 mg/g DW respectively. Chemical analysis of grain protein showed that the effect of seeding rate was not significant ($p=0.3$). However, genotype ($p=0.025$) and N treatment ($p =0.043$) was significant. Although HKBL 1512 displayed the highest grain protein (10.4%) compared to Nguzo and HKBL1385 (9.7 and 9.8) respectively, the increase was less pronounced across the N treatments. Field data corroborated with laboratory analysis in terms of yield and starch level. Qualitative analysis showed that hordein polypeptides was resolved into B and C fractions and was affected by N treatment and in HKBL1512 and HKBL1385 but

not in Nguzo. Transcript levels of genes encoding malting enzymes: α-amylase, β-amylase and β-glucanase in mature, progressively germinating and kilned seeds showed differential expression which was genotype and germination stage dependent. During this period, hordein proteins were degraded after 24 hrs with concomitant accumulation of β-amylase between 72-96 hrs followed by degradation after kilning. In this study we employed integrated use of phenotypic, molecular and physiological approaches to correlate field and laboratory analysis data to better understand attributes related to malting to rapidly identity cultivars for desired malting qualities.

Key words Nitrogenous fertilizer, starch, protein, hordein polypeptide fractions, germination, malting genes

3.1 Introduction

Barley is a major cereal crop grown primarily for feed and also source of grain used in malting and brewing industries (Abebe, 2010). However there is increasing awareness and demand for barley inclusion in diets due to its potential nutritional and health benefits (Erkan et al., 2006). In Kenya barley is an important crop mainly grown in small and large scale production by smallholder farmers in the high and mid altitudes of central and rift valley provinces in Kenya. It thrives well between 2000-3000 meters above sealevel (masl). The industry faces the challenge of producing good quality malting barley in adequate amounts due to abiotic and abiotic stress factors coupled with low prices and competition from other cereal crops such as maize and wheat. In order to meet local demand for raw material and increase the area under barley production, the malting companies have initiated as incentive contract with the farmers and the use of sorghum (*Sorghum bicolor*. L) as an alternative source of malt (EAMC, 2012). These incentives include access to financial support by providing certified seeds, fertilizer, extension services, transport services, guaranteed ready markets and comprehensive insurance services to the farmers. Breeding barley for improved malting attributes is therefore the main agenda for barley breeding programmes and its stakeholders both in the private and public sector. Major varietal attributes which are considered for barley suitability for malting include starch level, grain protein concentration, hordein polypeptides (Eagles et al, 1995, Echart-Almeida and Cavalli-Molina, 2001). Selection of varieties with a complex range of traits necessary for efficient processing to produce high malting quality

is a difficult process (Hayes and Jones, 2000). The intended end use of barley is defined by quality of barley grain in terms of molecular composition with regard to carbohydrate, proteins, phytochemicals and minerals as well as functional properties in the context of industrial processing (Finnie and Svensson, 2009). Biochemical and molecular assays that predict feed or malt quality are needed to allow rapid screening and development of barley varieties (Ingversen et al, 1989).

The influence of agronomic practices and the prevailing weather conditions during the growing and harvesting seasons has been shown to determine malting quality. High N application rates significantly increases grain yield, kernel weight and plumpness. Major varietal attributes which are considered for barley suitability for malting includes seed nitrogen content, grain size and shape, grain protein content, malt extract and diastatic power (Henry, 1990). Grain protein concentration is dependent on availability of N in soil and plant. Elevated N levels lowers starch and malt extract. Consequently, low protein affects the availability of malting enzymes and amino acids utilized by yeast (Fox et al., 2003). Varietal difference in malting quality is genotype dependent and is influenced by agronomic practices such as N fertilizer application and usually subject to N nutritional status in the soil. The use of optimal N levels of fertilizer rate that allows optimal grain yield without compromising the malting quality and environment is highly recommended in order to achieve sustainable agriculture.

Malting quality is a complex phenotype that combines an array of interrelated parameters each displaying complex inheritance patterns (Lapitan et al., 2009). Unlike other phenotype selections such as abiotic and biotic stress tolerance that requires field evaluations, malting quality determination requires laboratory based analysis of the harvested seed grain. Currently there is no robust method that directly predicts malting quality of barley grain by physical examination. Malting followed by analysis of key determinants of malting quality is prerequisite for selection of breeding materials. During malting, seed is germinated under controlled conditions. Malting is made of three steps: steeping, germination and kilning (Briggs, 1998). During steeping, the barley seed is soaked in water to increase the moisture content from 12-45% for initiation of cellular metabolic activity. In the germination step, central processes include *de novo* synthesis of hydrolytic enzymes triggered by a signal of GA from the embryo to the aleurone and scutellum (Enari and Sopanen, 1986, Jones and Jacobsen, 1991). Subsequently, the hydrolytic enzymes degrade the complex biomolecules including starch, protein and cell

wall components into simpler molecules used as energy reserves by germinating seedlings. Kilning involves drying of the malted seed through alternating series of increased temperature regimes. A good malting quality requires a high conversion of starch to fermentable sugars, high malting extract, optimal protein content, and low haze formation. Diastatic power which represents the starch-degrading ability has close association with the activities of four starch degrading enzymes namely. α-amylase, (β-amylase, limit dextrinase and β-glucosidase (Delcour and Verschaeve, 1987). Degradation of starch into fermentable oligosaccharides and maltose is significantly related to malt extract and fermentability, which in turn determine alcoholic output from barley grains or malt (Agu, 2003). Cultivars have unique malting enzymes characteristics. On the basis of the differential expression of key malting enzyme during malting process, the enzymes have potential use as markers for specific cultivar identification (Lapitan et al., 2009). Although there is progressive release of new varieties of barley from the Kenyan barley breeding programmes, there is no published information on the effects of agronomic practices such as seeding and N rates on yield malting quality parameters such as starch, seed protein and storage proteins hordeins. Furthermore, molecular characterization of Kenyan malting cultivars exploiting expression profiles of starch modifying genes and modification of storage proteins during progressive germination stages has not been documented. The use of biochemical and molecular assays will not only validate the field data ensuring reproducibility of results but also provides a platform for rapid cultivars screening for desired malting attributes.

3.2 Materials and Methods

3.2.1 Choice of seeds

Two row malting barley cvs. HKBL 1512, Nguzo and HKBL 1385 seeds were obtained from the East African Malting Company (EAMC). The cultivars are adapted to high and medium altitude agroecological zones. Their selection was based on good agronomic traits such as high yield, disease resistance, lodging tolerance and superior malting qualities. Phenotypically, Nguzo and HKBL 1385 are related and vulnerable to lodging and tolerant to acidic pH. On the other hand, HKBL1512 is tolerant to lodging. Both the newly released varieties have cv. Nguzo genetic background.

3.2.2 Study area and experimental design

The field experiment was conducted at the School of Agriculture and Biotechnology of the University of Eldoret in the cropping season of 2012. It is located 2,140 masl and lies between longitude 35° 18' E and latitude 0° 30'N with an average annual rainfall 1,124 mm. The average annual temperature is 23 °C with a minimum of 10 °C (Okalebo et al., 1999). The experimental design was randomized complete block design (RCBD) with three replications. It consisted of 108 factorial combinations of three cultivars (HKBL 1512, Nguzo and HKBL 1385), four N regimes (0, 30, 40, 50 kg ha^{-1}) and three seeding rates (150, 200, 250 seeds m^{-2}). Seeding rate was based on pre germination tests conducted in pots under controlled conditions. Prior to planting, a soil analysis was performed to establish the fertility status of the area under study. About 500 g composite soil was taken randomly from top soil (0-20 cm) and analyzed for its content for total nitrogen (N) organic carbon (OC), available phosphorus (P) and pH (**Table 5**).

Table 5. Mean values of important soil parameters analyzed from soil samples before sowing in 2012 at the University of Eldoret

pH	P (mg/Kg)	%N	%C
4.7	8.79	1.15	2.03

At the time of sowing a blanket cover of only phosphorus in form of diammonium phosphate (DAP^{TM}) at the rate of 45 kg ha^{-1} was applied. N was applied as granulated calcium ammonium nitrate (CAN^{TM}) as top dressing at vegetative and tillering stages

applied at different rates. All agronomical practices were observed until the plants reached physiological maturity. The spikes were harvested, dehusked and air dried to eliminate moisture. Yield determination was based on ton/ha and wt /1000 kernels. A small sample (200 g) of seed for each variety from each treatment plot was pooled. Therefore, a mixture from the three plots was used for subsequent analysis. The same seed sample was shipped to Germany for laboratory analysis of total protein, starch content and hordein polypeptides at the Institute of Plant Molecular Physiology and Biotechnology, University of Bonn. One European 6 row malting barley, Barke was used as an internal control in malting/germinations studies.

3.2.3 Determination of yield parameters under field conditions

After physiological maturity and adequate aeration, the effect of N treatment and seeding rate in the three genotypes was evaluated by determination of yield parameter including yield ton/ha and wt (g) 1000 kernels.

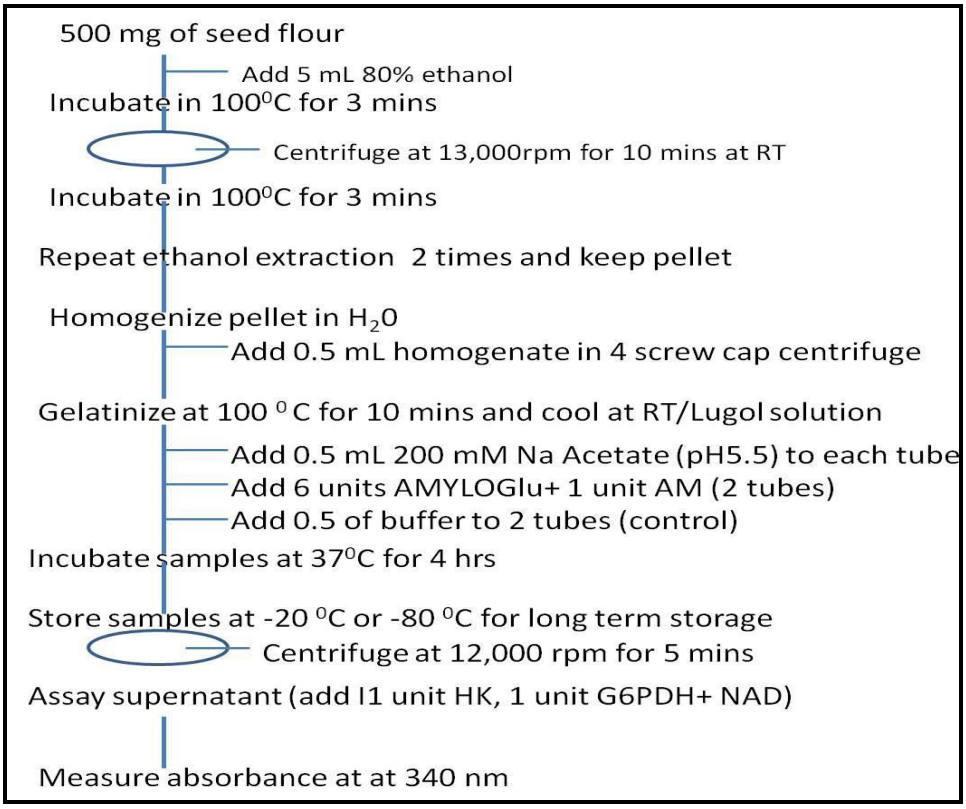
3.2.4 N% and grain protein analysis using Kjeldahl method

About 1000 mg of seeds from each N treatment were dried at 50 ^{0}C in the oven for 48 hrs. It was ground into very fine powder using miller and kept at room temperature for short term storage or used immediately. 300 mg was accurately weighed and used for analysis using a modified Kjeldahl method (1883) as described by the manufacturer. Kjeldahl method is highly automated and it involves the sample digestion with a strong acid under very high temperature of 373 ^{0}C in the presence of potassium sulphate as a reaction catalyst so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the samples using a specific conversion factor 6.25.

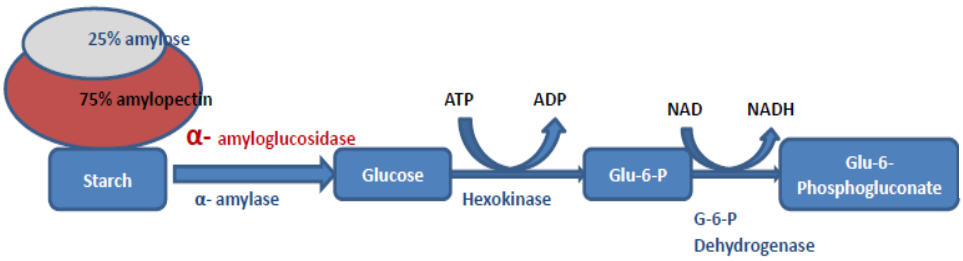
3.2.5 Enzymatic starch analysis

500 mg of finely ground barley was used in methanol extraction of starch. Following centrifugation, the pellet was gelatinized under high temperature of 100 ^{0}C. To ensure that the starch granules were completely modified for them to be accessible to enzyme attack, the sample extract was stained using 5% Lugol solution (in 100 mL volume: 5 g Iodine, 10 g potassium iodide and 85 ml water) for 5 mins then visualized under light microscope (Binocular microscope SMZ-800, Nikon, Düsseldorf, Germany). Photos were capture

before and after gelatinization (**Fig. 27**). Enzymatic degradation of starch to single glucose residues was performed sequentially by additions of α-amyloglucosidase (AMG) and α-amylase (AMY) to the pellet suspension. Following centrifugation, the supernatant was exposed to enzymatic attack that converted starch to glucose residues and subsequently to 6 phosphogluconate through oxidative reduction of NAD to NADH. A schematic flow of the extraction and analysis starch is illustrated in (**Fig. 26**).

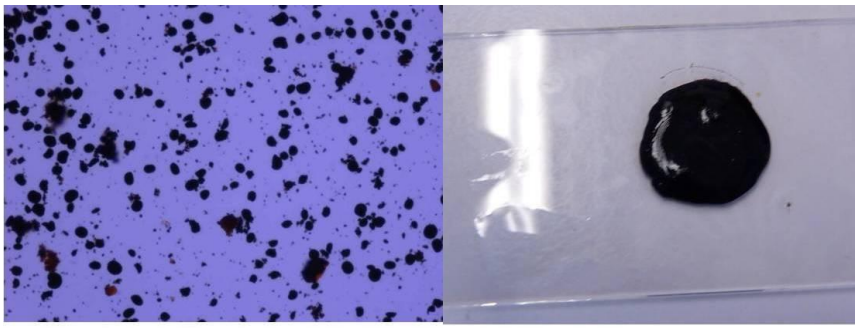


A



B

Figure 26. Indirect enzymatic starch analysis: A-Schematic flow chart of starch extraction and quantification. B-Enzymatic degradation of starch by starch degrading enzymes AMG and AMS followed by phosphorylation of glucose residues by hexokinase and subsequent reduction of G-6-P by G-6-P dehydrogenase NAD coupled reaction as described by Smith and Zeeman (2006).



Before gelatinization After gelatinization

Figure 27. Lugol staining of starch granules before and after gelatinization.

The absorbance recorded at 340 nm was used to calculate the amount of glucose and subsequently conversion to starch. The starch content was determined using the equation as follows

$$\frac{\text{Change in OD}}{6.22} = A$$

Where A is the amount of glucose in the cuvette and 6.22 represents the extinction coefficient of NADH at 340 nm. The mean value of A for control samples (A_c) is subtracted from the mean of A for samples incubated with enzymes (HK and G6PDH) (A_s) to give a net value of (A_e)

Starch content of seed tissue on the basis of dry weight nmol glucose can be calculated as:

$$\frac{A_e}{\text{vol of incubation assayed (between 0.02 and 0.2 ml)}} \times 2 \times \frac{5}{\text{wt of tissue (g)}}$$

This value was converted to starch mg/g starch DW by multiplying with 162 (Mol wt of anhydroglucose).

3.2 6 Qualitative analysis of hordein polypeptide fractions

A total of 20 uniform sized barley seeds from each N treatment cultivar was ground in liquid nitrogen to a fine powder and used for analysis. Hordein was extracted from approximately 200 mg seed using 55% isopropanol water mix in the presence of 3% mercaptoethanol as reducing agent. The sample was incubated for 30 mins and pelleted at 10000 g for 5 mins. The aqueous phase was transferred into new tubes. The Bradford assay (Bradford, 1976) was used to determine relative protein concentration in the extract. Hordein extracts from each genotype and N treatments were resolved electrophoretically on SDS-PAGE as described by Echart-Almeida and Cavalli-Molina (2001). Electrophoresis was carried out in a vertical SDS PAGE discontinuous system with 4% stacking and 12% separating acrylamide gel Laemmli buffer (1x) (62.5 mM Tris-Cl pH 6.8; 10% (v/v) glycerol; 2% SDS (w/v); 0.1% (w/v) bromophenol blue and

0.7 M (≈ 5%) β-mercaptoehanol (add freshly just before use). under a constant current of 20 mA until the tracking dye moved 12cm into the separating gel. The gel was transferred to fixation solution (40% methanol and 10% acetic acid) with agitation for one hour and subsequent overnight staining in coomassie solution (85% phosphoric acid, ammonium sulphate and coomassie brilliant blue G-250). The gel was washed 5 times with normal water to reveal the protein bands and scanned using an Epson scanner. Variations in banding intensity and pattern in hordein polypeptide fractions D, C and B were qualitatively. For genotype profiling only hordein fractions extracted from 0 N treatments were used.

3.2.7 RNA extraction and expression analysis of *D, C* and *B* hordein genes in response to N treatment

Transcript levels of storage protein genes in response to N treatment were evaluated in malting barley. RNA extraction was modified from Singh et al. (2003). Total RNA was extracted from seed ground in liquid nitrogen to a fine powder. 100 mg of this powder was homogenized in extraction buffer (EB) 50 mM Tris-HCl (pH 9), 150 mM NaCl, 1% sarcosyl, 20 mM EDTA, and 5 mM DTT) vortexed and mixed thoroughly with 500 µL of phenol chloroform isoamyl alcohol (PCI) (25:24:1). The homogenate was vortexed and centrifuged at 21,000 g at 4^0 C for 5 mins. 500 µL of the upper aqueous phase was carefully removed and mix with 650 µL of guanidine búffer (GB) (8 M guanidine hydrochloride, 20 mM EDTA, 20 mM MES (pH) 7.0), 200 mM β-mercaptoethanol and 350 µL of PCI and vortexed centrifuged at 21,910 *g* at 4 °C for 5 mins. The upper phase was removed and mixed with 500 µL of chloroform, vortexed and centrifuged at 21,910 g at 4°C for 5 mins. 450 µL of upper phase was transferred to two microcentrifuges and 45 µL of 3 M sodium acetate (pH 5.2) and 900 µL chilled absolute ethanol was added. It was incubated at -80 ^{0}C for 90 mins and pelleted at 21,000 g for 30 mins. The pellet was washed in 70% chilled ethanol and centrifuged at 10,000 g at -4 ^{0}C for 5 mins. The supernatant was discarded; pellet dried at room temperature in 20 µL of DEPC water. The quality of RNA was determined by measuring concentration using a Nanodrop and electrophoresed in 1% agarose gel with ethidium bromide stain. Two µg of total RNA was reverse transcribed to generate first single strand cDNA using universal oligo (dT) 18 primers and 200 U of Moloney Murine Leukemia Virus (MMLV) RevertAid (Fermentas, Germany) incubated at 42 ^{0}C for 1 hr followed by 70 ^{0}C for 5 mins in a 20 µL reaction

volume. Semi quantitative RT-PCR was performed using gene specific primers listed in **Table 6**.

Table 6. The primer sequences of hordein genes used in RT-PCR analysis

Genes	5'F primer 3'	5'R primer 3'	Amplicon size
B-hordein	GCAAGGTATTCCTCCAGCAGC	TAAGTTGTGGCATTCGCACG	300
C-hordein	ATAATTCCCCAGCAACCTCA	GATGGTGCACATCATTTATTCA	273
D-hordein	GACAGTCCACCGAGATGGCTA	CGATTACCGCCACAAAGAGG	253
HvActin	CCCAGCATTGTAGGAAGGC	CCTCGGTGCGACACGGAGC	250

3.2.8 Barley seed samples and micromalting

Seeds from HKBL 1512, Nguzo and HKBL 1385 treated with 50 kg ha^{-1} N fertilizer were used in these experiments. The cultivar Barke obtained from INRES, University of Bonn, Germany was used as an internal control. Mature seeds were sorted out and those greater that 2.5 mm diameter were transferred to room temperature for 1 hr to break the dormancy. Grain samples were micromalted in controlled chambers. Approximately 200 mg of seeds from each variety was transferred into 15 mm petri dishes with wet Whatman paper. Steeping was scheduled as follows: 6: 14: 8: 14: 4 at 15 ^{0}C (wet: dry: wet: dry: wet), germination for 96 hr at 15 ^{0}C, kilning 24 hr at 65 ^{0}C (**Fig. 38**). Seeds that failed germination tests were manually excluded from sample petri dishes. Uniformly germinated seeds were considered. Aliquots of micromalted seeds were picked at different physiological stages: dry seed (S), steeped (ST), 24 hrs, 48 hrs, 72 hrs, 96 hrs of germination and kilning (KL) and used to analyze changes in soluble proteins abundance, hordein polypetide fractions and transcript profiles for genes encoding malting enzymes; α-amylase, β-amylase and β-glucanase. The dry seed was used as nonmalting control. Any phenotype changes during germination steps including: germination viability, uniformity in germination, sprouting density was documented qualitatively and /or quantitatively.

3.2.8.1 Analysis of protein abundance and hordein fractions in dry mature and micromalted seeds

The soluble seed proteins were extracted using a low salt concentration and neutral buffer (5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$) (Finnie and Svensson, 2009). These conditions favored the exclusion of storage proteins which can dominate the one-dimension separation profile and mask the low abundance proteins. 10 g of mature and micromalted seeds (from different germination stages) were ground to fine powder in liquid nitrogen. 4 g of fine powder was extracted in 20 mL volume containing 5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, with 30 mins stirring at 4 ^{0}C. The extract was centrifuged at 17,000 rpm for 30 mins at 4 ^{0}C and the supernatant stored at -20 ^{0}C for immediate use or -80 ^{0}C until use. The protein concentration was determined using the Bradford method Bradford. (1976). 20 µg of protein from seed and micromalted material was resolved using SDS-PAGE. The distributions of protein abundance were compared qualitatively. In parallel, hordein fractions were extraction as described (Echart-Almeida and Cavalli-Molina, 2001). 20 µg of hordein protein extracts from each physiological micromalting stages were resolved electrophoretically using SDS-PAGE. The changes in the hordein polypeptide fractions in the different stages during progressive germination were visualized in coomassie stained gel.

3.2.8.2 Expression profiles of genes encoding malting enzymes

The transcript levels of genes involved in modification of cell wall and degradation of starchy endosperm during progressive germination was analyzed using semi quantitative RT-PCR. These genes encode the proteins α-amylase, β- amylase and β-glucanase. Gene specific primers were designed from available annotation from the NCBI database. The cDNA was amplified using cDNA (1:20 dilution) template, 10 mM dNTP, 10 µM reverse primer, 10 µM forward primers and water in a final volume of 20 µL using the T3000 thermocycler (Biometra, Germany). All gene expressions were normalized to the *HvActin* house keeping gene. Two technical repeats were performed in this experiment. The primer sequences used are listed in **Table 7**.

Table 7. List of genes encoding malting enzymes: α amylase (*HvAMY1*), β-amylase (*HvBAMY1*), β-glucanase (*HvBG*) and gene specific primers used in RT- PCR analyses

Malting Genes	**5'F primer 3'**	**5'R primer 3'**	**Amplicon size (bp)**
HvAMY1	TACCACGTTCGACTTCACCA	ATTTGCAGCTTGCTCTCGTT	339
HvBAMY1	GAAGGGGAGGTTTTTCCTTG	CCTCTCTCCATCCAGCACTC	350
HvBG1	CTCATGGCCAACATCTACCC	TGGTTCTCGTTGAACATGGA	353
HvActin	CCCAGCATTGTAGGAAGGC	CCTCGGTGCGACACGGAGC	250

3.4 Results

3.4 1 Effect of N treatment and seeding rate on yield parameters under field conditions

An effect of N treatment and seeding rate were determined at physiological maturity by measurement of grain yield (ton/ha), wt (gm) /1000 kernels and formation of productive tillers in three HKBL 1512, Nguzo and HKBL 1385 under field conditions. Barley yield increased with increased N treatment. Genotype (p=0.0001) and seeding rate (p=0.0001) caused a significant effect in yield ton/ha in the tested cultivars under field conditions. HKBL 1385 recorded the highest yield of (5.1) Nguzo (3.7) and HKBL 1512 (3.8) ton/ha respectively (**Fig. 28**).

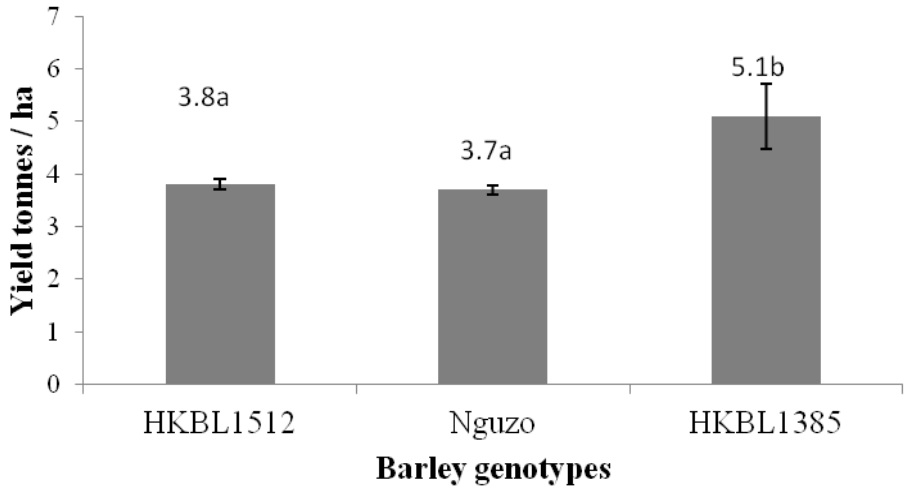


Figure 28. Overall yield performance in ton/ha in three malting cultivars.

There were no significant interactions between seeding rate x nitrogen (p=0.3055) variety x nitrogen (p=0.1123). However, there was a significant interaction between seeding rate x variety (p=0.0018). Evaluation of yield in terms of 1000 kernel weight showed there was a significant difference caused by genotype (p= 0.001) N treatment (p=0.001) and seeding rate (p=0.001). Yield from plots that were not supplied with nitrogen fertilizer recorded the lowest yield. Wt g/1000 kernels increased in response to increased N treatment.

There was no significant difference in kernel weights between 40 and 50 N treatments (**Fig. 29**).

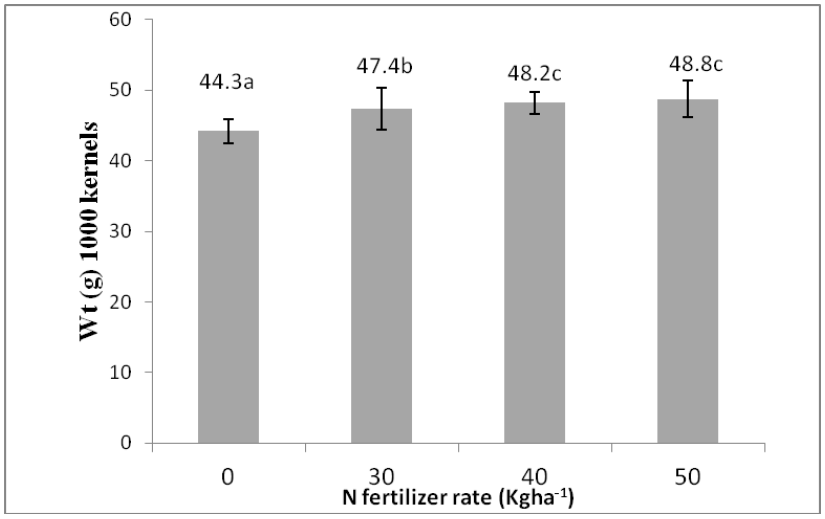


Figure 29. The effect of N treatment in wt (g) in 1000 kernels. Means separated by LDS.Values assigned the same letter are not significantly different from each other ($p<0.05$).

Determination of productive tillers is an important parameter that contributes to yield. There was a significant interaction between seeding rate x nitrogen in the establishment of productive tillers. N treatment caused increase in the number of productive tillers while high number of productive tillers was associated with low seeding rate. Tiller production was greater in 150 seeds m^{-2} than in 200 seeds m^{-2} and diminished in 250 m^{-2} under all N treatments. Maximum tillers were observed in 200 seeds m^{-2} at 40 kg N. treatment.

3.4 2 Determination of grain protein content in kernels

The effect of N treatment and seeding rate on grain protein concentration was determined in three malting cultivars (**Fig. 30**). Single factor anova showed genotype ($p=0.025$) and N treatment ($p=0.0000085$) were highly significant while seeding rate had no effect on grain protein in all the genotypes; HKBL1512 ($p=0.2$), Nguzo ($p=0.900$) and HKBL 1385-13 ($p=0.800$). There was no significant interaction between variety and nitrogen treatment ($p=0.212$). N treatment had an effect on each genotype. In cultivar HKBL 1512 N treatment ($p=0.200$) had no significant effect on grain protein (**Fig. 30 A**) but was highly significant in Nguzo ($p=0.027$) HKBL 1385 ($p=0.01$) (**Fig. 30 B** and **30 C**).

The genotype HKBL 1512 maintained a stable grain protein content across the treatments. Seed derived from untreated plots displayed the lowest protein level compared to

increased N treated seeds. Genotypes displayed fertilizer sensitivity. A comparison of grain protein in response to overall N treatment in the three malting cultivars is illustrated in **Fig. 30 D**. The cv. HKBL 1512 displayed the highest protein content of 10.4 while Nguzo and HKBL 1385 had 9.7 and 9.8 respectively. Although there were variations in protein content in these cultivars, they still met the minimum protein content of 9-12.5% requirement for malting specification.

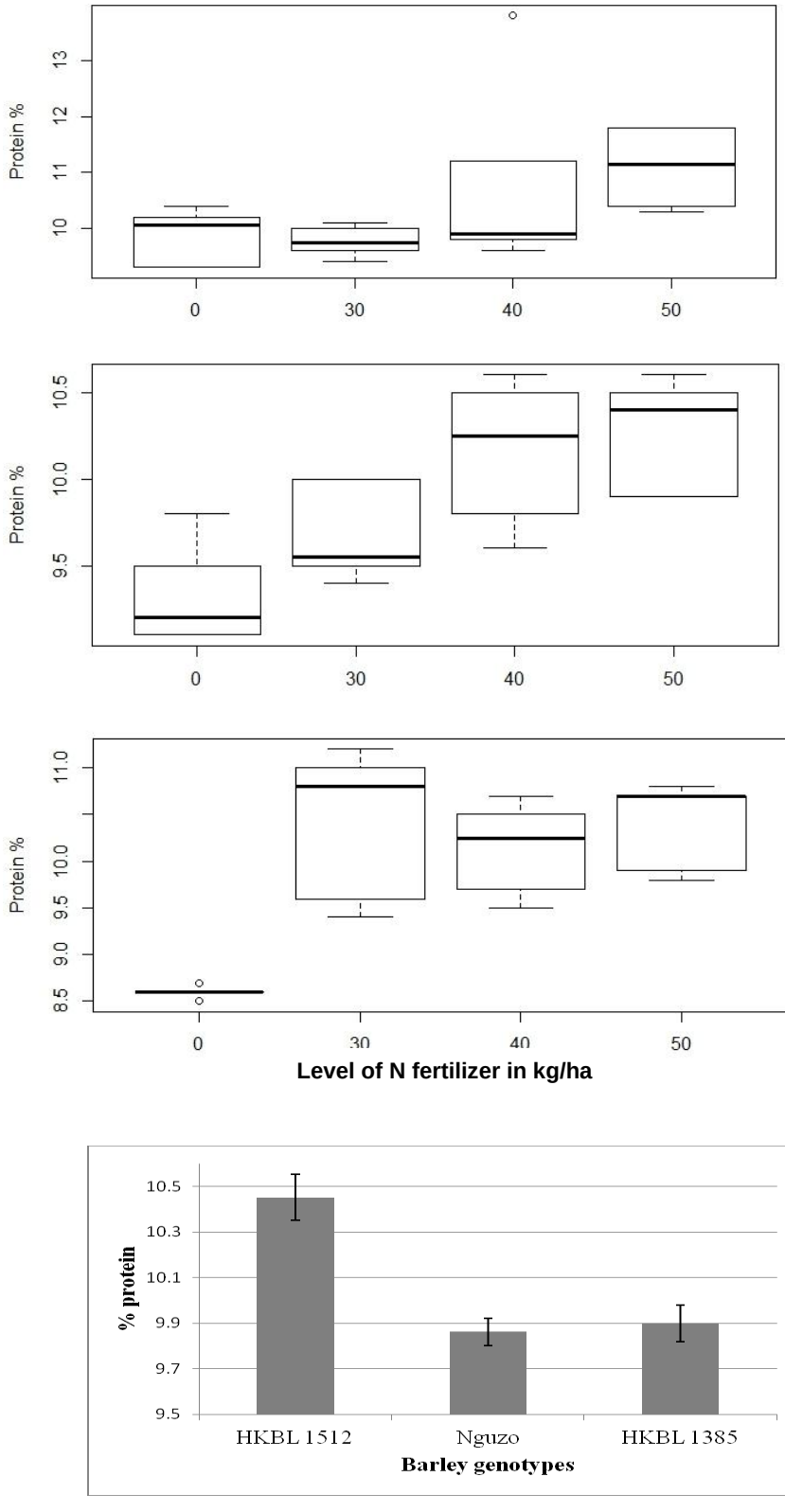


Figure 30. The effect of varying N treatment on grain protein content in three malting cultivars: A: HKBL 1512. B: Nguzo. C: HKBL 1385. D: Comparison of protein content in the three cultivars. One-way ANOVA with Tukey posttests was used to assess differences in mean relative growth. Means with different letters represent significant differences at $^{*}P < 0.05$; $^{**}P < 0.01$.

3.4 3 Starch analysis

The effect of N treatment and seeding rate on starch content was evaluated in three malting cultivars under study (**Fig. 31**) Analysis showed that there was no significant effect of seeding rate on the amount of accumulated starch in the seed across the genotypes. HKBL 1512 (p=0.989), Nguzo (p=0.826) and HKBL 1385 (p=0.966). However, N treatment had a significant effect on the starch content across the genotypes. HKBL 1512-(p=0.001), Nguzo (p=0.048) and HKBL1385 (p=0.016). (**Fig. 31 A, B** and **C)**. The Starch level was lowest in untreated seeds and increased progressively with increased N treatment. However, in the Nguzo, sensitivity to N treatment commenced at 30 kg N but did not change significantly with increased N. There was no significant difference in application of 30, 40 or 50 kg/ha N in the amount of starch accumulated (**Fig. 31 B**).

A cumulative effect of N treatment on grain starch content is illustrated in the three genotypes HKBL 1385, Nguzo and HKBL 1512 in **Fig. 31 D**. The genotype HKBL 1385 displayed the highest starch content of 278 mg/g DW while Nguzo and HKBL 1512 recorded 240 and 225 mg/g DW respectively.

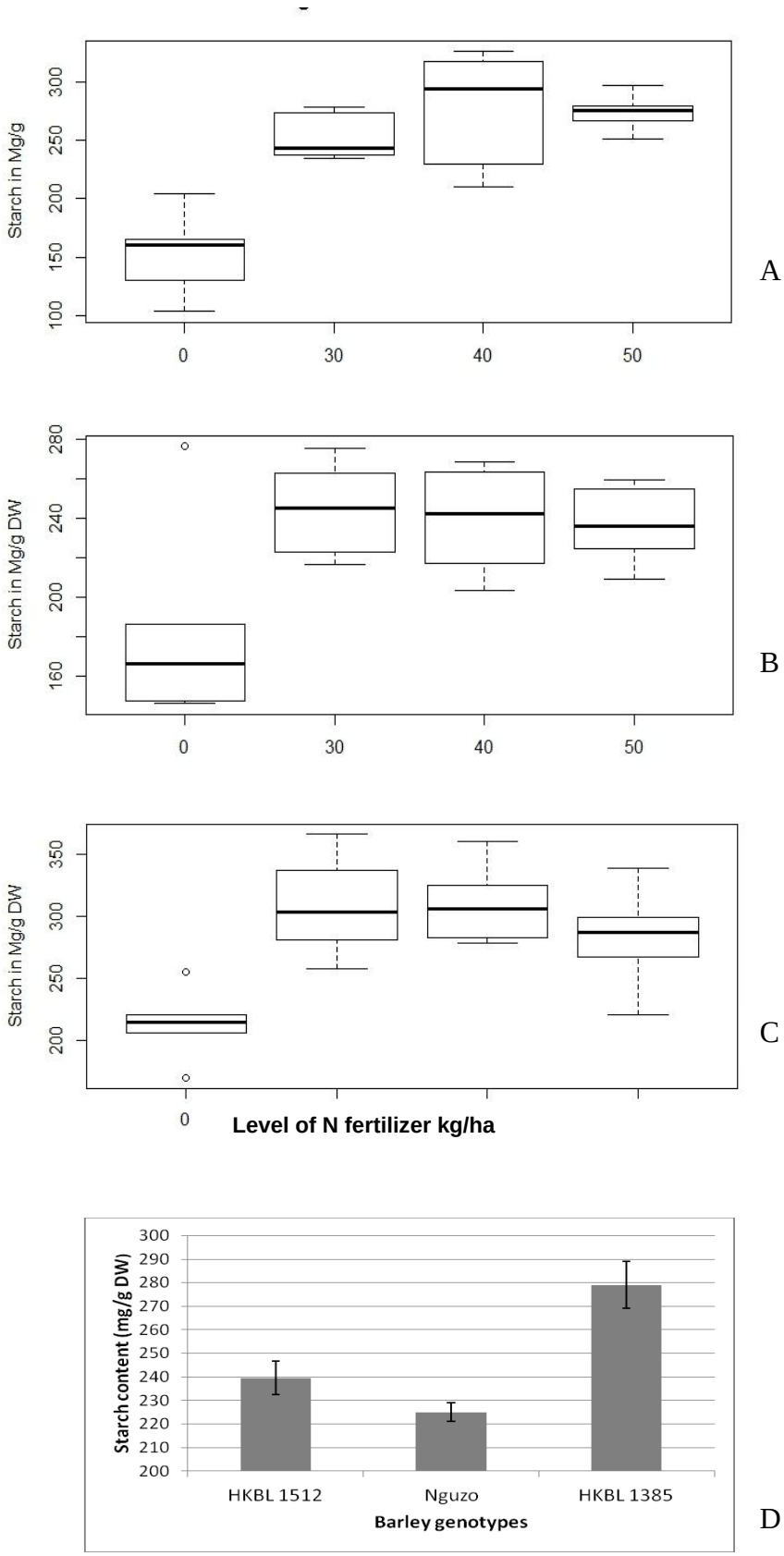


Figure 31. The effect of varying N treatment on grain starch content in three malting cultivars: A-HKBL 1512. B-Nguzo. C-HKBL 1385 and D-Comparison of starch content in the three cultivars. ANOVA with Tukey posttests was used to assess differences in mean relative growth. at $*P <$

0.05; $^{**}P < 0.01$

3.4 4. Evaluation of hordein polypeptide profiles in response to N treatment in three malting cultivars

Variability in the hordein polypeptide fractions has been used as stable marker in cultivar identification and germplasm diversity studies. The effect of N treatment on hordeins was evaluated in three malting cultivars. The changes in seed weight in response to N treatment were determined before use in hordein protein analysis (**Table 8**).

Table 8. Changes in average weight in g/20 seeds in three malting cultivars in response to N treatment

Level of N treatment ($kgha^{-1}$)	HKBL 1512	HKBL Nguzo	HKBL1385
0	1.27	1.31	1.41
30	1.35	1.35	1.49
40	1.43	1.45	1.61
50	1.53	1.64	1.85

Analysis indicated that N treatment caused an increase in average weight of 20 uniform seeds which varied across the genotypes. HKBL 1512, Nguzo and HKBL 1385 had averages of 1.4, 1.44 and 1.61 g/20 seed respectively. This information is extrapolated to the yield data in **Fig. 28.**

3.4 5 Analysis of hordein profiles in three malting cultivars

The hordein proteins of three barley cultivars were resolved electrophoretically using SDS-PAGE (**Fig. 32 A**). Variations in hordein patterns among the cultivars were observed in the C and B fractions. The D hordeins occurred in trace amounts that limited visual score. The point of variations was scored on the absence (0) or presence (1) of hordein bands in the C and B fractions. The hordeins fraction banding patterns were used to establish varietal differences in the three cultivars. A phylogenetic relationship using AHC was generated in the cultivars under study (**Fig. 32 B**). Analysis indicates that Nguzo is closely related to HKBL 1512 and distantly related to HKBL1385. Although under field conditions, HKBL 1385 and Nguzo have phenotype trait similaries such as vulnerability to water lodging and tolerance to acid soils. Available genetic information

indicates that the newly released lines have a cv.Nguzo genetic background (EAM-UOE, 2012)

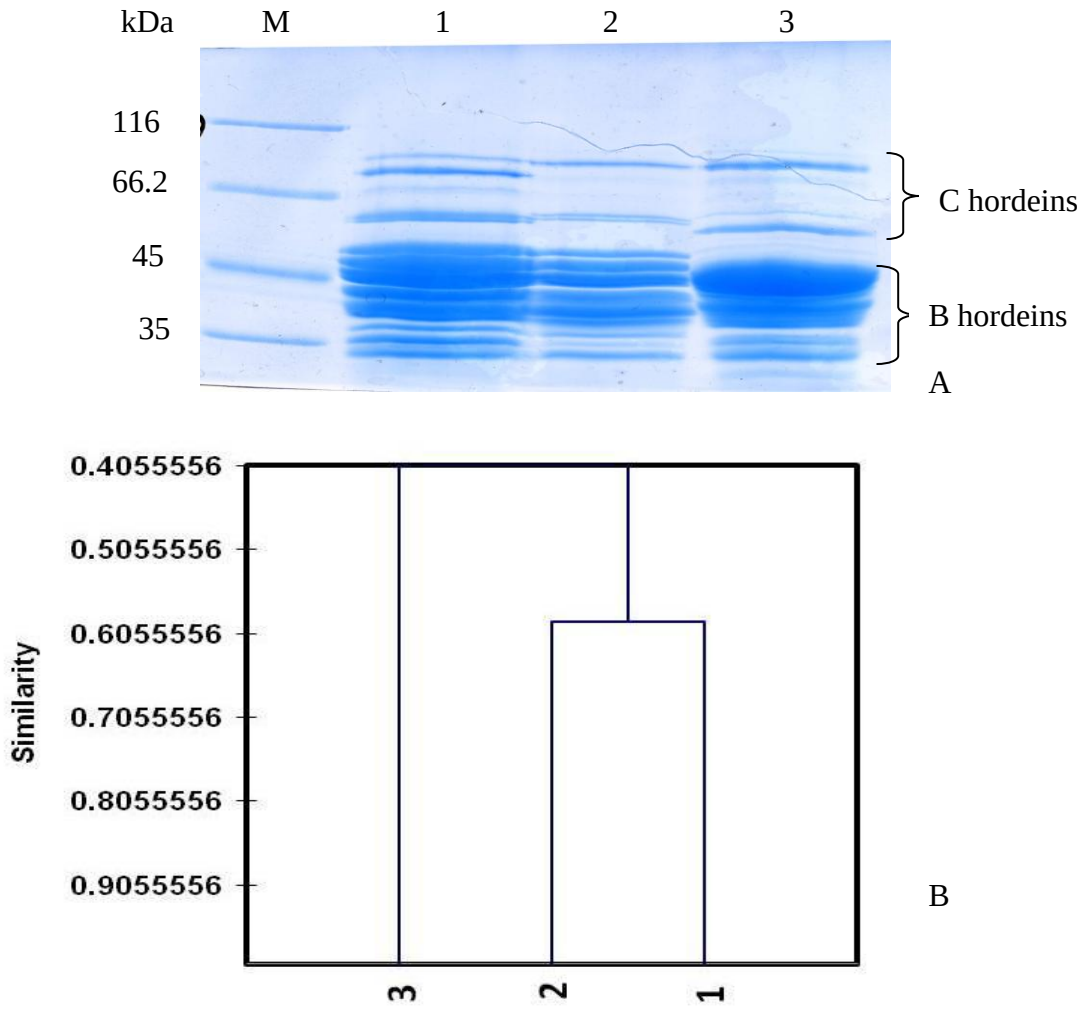


Figure 32. Characterization of malting cultivars: M- Protein marker: 1-HKBL 1512: 2-Nguzo: 3-HKBL 1385 using A: Isopropanol extracted hordein polypetide fractions resolved electrophoretically in SDS PAGE. B: Phylogenetic relationship of the cultivars based on hordein polypetide profiles analyzed using agglomerative hierarchical clustering (AHC), XLSTAT 2013.

3.4 6 Genotype response to N treatment in hordein polypeptide fractions

Hordein proteins isolated from different N (0, 30, 40 and 50 kg/ha) treated seeds in three malting cultivars were resolved using SDS PAGE. The hordein profiles in response to varying N treatments for HKBL 1512 is illustrated in **Fig. 33 A**. From this study, C and B hordein subclasses dominated the electrophoregrams in all the genotypes. The D hordeins occurred in minor amounts and could not be determined. HKBL1512 showed distinct C subclass fractions (C1, C2, C3, C4 and C5). The untreated/control (0 kg) hordein showed

the presence of only C2 form and absence of all the other types while N (30, 40 and 50 N) treatments showed increased intensity of C2. Additionally, intensity of C4 and C5 banding was lowest in control compared to the treated samples. These results are consistent with the grain protein content (**Fig. 30 A**) where untreated seed samples displayed lowest protein content and increased slightly with increasing N levels.

The effect of N treatment on the hordein fraction in cultivar Nguzo is presented in **Fig. 33 B**. There was obvious absence of D fractions and presence of B and C fractions. There was no qualitative difference in the B and C fraction in both the control and treated samples. Nguzo displayed hordein stability under all treatment regimes. In this variety hordein composition is therefore not influenced by environment and can constitute a stable cultivar identification marker. This however does not agree with the results in **Fig. 30 B** which shows N treatment affected overall grain protein.

The effect of N treatment on hordein protein fractions in HKBL 1385 is illustrated in **Fig. 33 C.** The hordein fractions in cv. HKBL 1385 were resolved into C and B fractions in response to N treatment. The cultivar HKBL 1385 displayed a lot of variations in terms of hordein polypeptide fraction banding patterns and intensities in response to N treatments. B fractions were affected by an increase in N fertilizer. Controlled treatment had only B2 whose banding intensity increased with N treatment. These findings agree with the grain protein content which showed increased intensity in response to 30, 40 and 50 N treatments (**Fig. 30 C**).

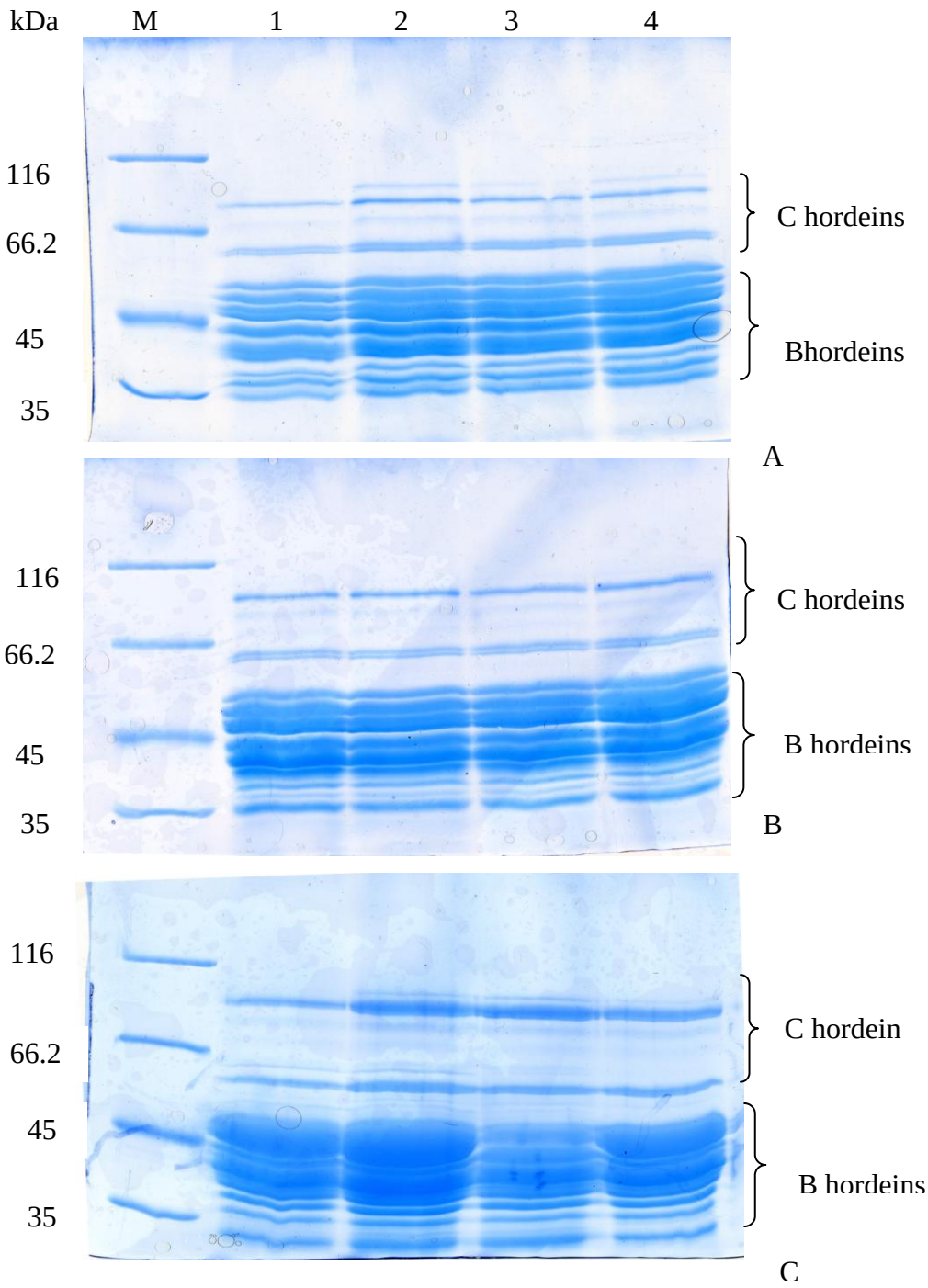


Figure 33. Analysis of isopropanol extracted hordein polypetide fractions resolved in SDS PAGE in three malting cultivars: A-HKBL 1512. B-Nguzo. C-HKBL1385 in response to: 1: M-Protein marker, 1-0 kg/ha, 2-30 kg/ha N, 3-40 kg/ha, 4-50 kg/ha N treatments.

A comparison of hordein protein fractions under two contrasting N treatments were evaluated in the malting cultivars: The effect of 0 and 50 kg/ha N treatments is shown in

Fig. 34. There was increased intensity of C and B hordein subclasses in all the varieties These findings concur with changes observed in hordein protein in response to N treatment in individual genotype (**Fig. 33**)

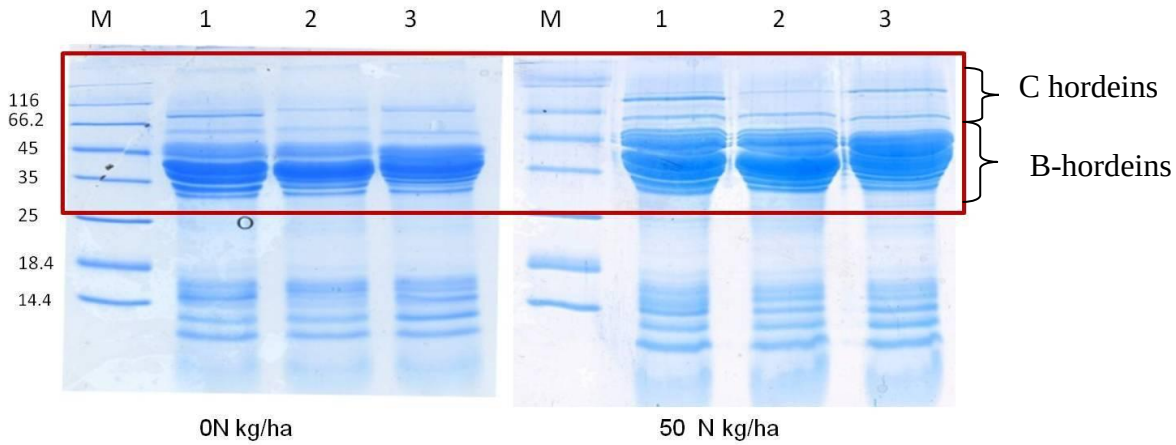


Figure 34. Changes in hordein polypetide profiles under two levels of N treatments (0 and 50 N kg/ha in three malting cultivars: 1-HKBL 1512-2-Nguzo, 3-HKBL 1385. M-Standard protein marker**.**

A comparison of total grain protein and hordein protein in the different N regimes is presented for cultivar HKBL 1512 (**Fig. 35**)

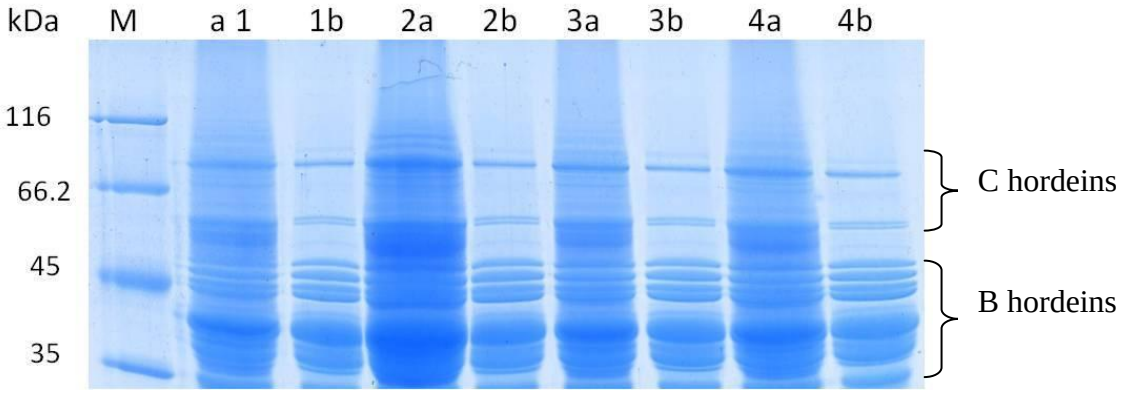


Figure 35. Comparison of total grain protein (GP) and hordeins protein composition (H) in response to N treatments (0, 3 0, 40 and 50 N kg/ha) in cultivar HKBL 1512 1a-GP-0, 1b-H-0: 2a-GP-30, 2b-H-30: 3a-GP-40, 3b-H-40: 4a-GP-50, 4b-H-50: M-Standard protein marker.

Protein comparison was used to validate that the isopropanol method exclusively enriched hordein proteins and excluded all other forms of protein in the barley endosperm. 20 µg of Laemmli extracted total grain protein (Laemmli, 1970) and 20 µg

isopropanol extracted hordeins resolved in 12% SDS-PAGE were compared. The electrophoregram shows that the Laemmli method presents a diverse abundance of protein population that is not soluble in the organic solutions like isopropanol. Most proteins were found between 35–120 kDa.

3.4. 7 Hordein gene expression analysis in barley seeds in response to N treatments

The effect of N treatments (0, 30, 40 and 50 kg/ha) in in expression profiles of of *D, B,* and *C* hordein genes in seeds was evaluated (**Fig. 36**) A single strand cDNA was transcribed from 2 µg RNA as described previously. Semi quantitative RT-PCR was performed using cDNA generated from different N-treated samples and primers specific to *D, B* and *C*-hordein genes. We have established that the housekeeping gene *actin* was constitutively expressed whereas the storage protein genes *B, C* and *D* were completely down-regulated irrespective of treatment or genotype (**Fig. 36 B, C** and **D**). The down-regulation was due to the fact that the desiccated seed is metabolically quiescent and the only genes that are associated with it are the genes involved in desiccation tolerance, housekeeping genes or genes involved in osmotic tolerance. Seeds can only initiate biological activities upon imbibition or during germinations.

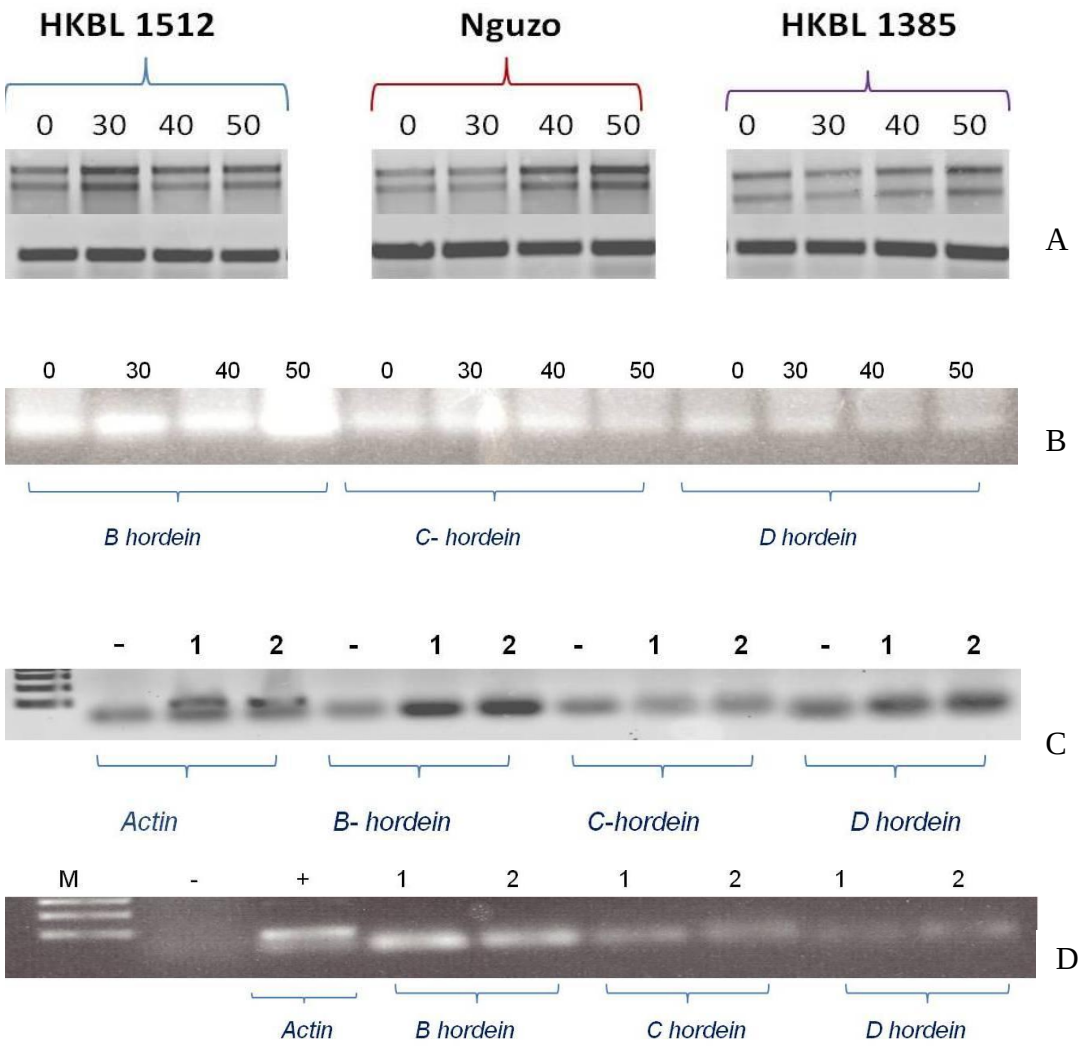


Figure 36. Transcript analysis of hordein genes *B, C* and *D* in response to different N treatments in. HKBL 1512, Nguzo and HKBL. A: Quality of RNA analysed on ethidium bromide stained 1% agarose gel and RT-PCR of *actin*, the house keeping gene. B: RT-PCR from cDNA generated from different N treatments in cv. HKBL 1512 with *B, C and D* hordeins specific primers. C: RT-PCR with *actin, B, C* and *D* hordein specific primers from cDNA. from (1) HKBL 1385 and (2) HKBL 1512 in 0 N kg/ha treatments D: RT-PCR with *actin, B, C* and *D h*ordeins specific primers from (1) HKBL 1385 and (2) HKBL 1512 in 50 N kg/ha treatments.

3.4.8 Analysis of malting attributes in dry mature seed and during progressive germination

In this study, malting attributes including changes in hordein polypetide fractions, protein abundance and transcript analysis of malting genes *AMY1, BAMY1* and *BG1* were evaluated in dry mature seeds, during the progressive malting process and kilned seeds (**Fig. 38**). After 3 days of imbibition, a germination test was evaluated by random picking

20 seeds from each petri dish in a replicated experiment. Analysis showed that all the cultivars met the mandatory 95% germination test (**Fig. 37 A**). After 3 days only micromalted seeds with uniform germination characterized by even sprouting of roots were considered for subsequent analysis (**Fig. 37 B**). Seed that failed to germinate or did not exhibit uniform germination were excluded in the experiment (**Fig. 37 C**).

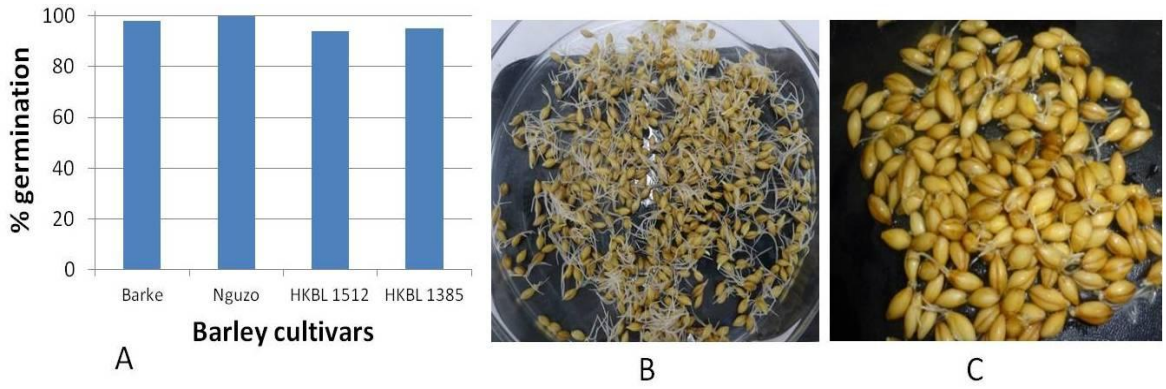


Figure 37. Evaluation of critical malting parameters as primary qualitative screening of seeds grown in control chambers. A: % germination uniformity in four malting cultivars. B Characteristic phenotype for 95% germination uniformity in germinated seed of cv. HKBL 1512. C: Characteristic phenotype of failed germination in cv. HKBL 1512.

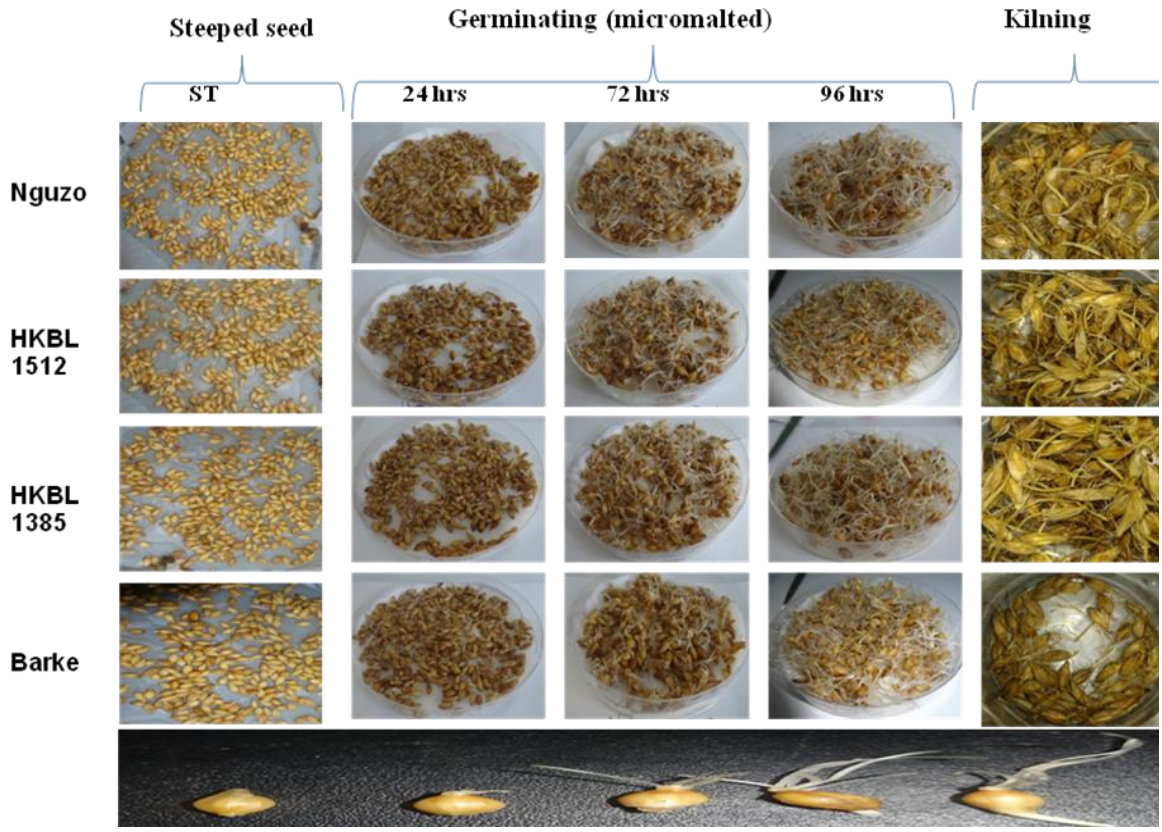


Figure 38. Distinct physiological and developmental stages of barley germination.
A-Progressive phenotype changes in germination of barley seeds under controlled conditions of wet and dry inter phases (6: 14. 8:14: 4) (wet: dry: wet: dry: wet) steeping (ST) at 15 ^{0}C germination at 16 ^{0}C (24 hrs, 72 hrs, 96 hrs) followed by kilning (KL) for 24 hrs at 65 ^{0}C. Steeping was performed to raise RWC from 12- 42% in order to initiate biological processes.
B-Physiological Morphology and time points of germinating barley at each physiological

Morphological changes in the kernels of germinating seeds were characterized with primary root emergence after a steeping period, elongation and sprouting, increase in weight with with characteristic sweet aroma. Different physiological stages kernels seed (S) steeping (ST), 24 hrs, 72 hrs, 96 hrs and kilning (KL) (**Fig. 38 A** and **B**) were targeted for analysis of malting attributes including modification of hordein polypetide fractions, protein abundance and transcript analysis of malting genes.

3.4.8.1 Analysis of relative abundance of seed protein during micromalting stages

Identification of proteins involved in seed development and germination which may influence malting quality is very critical. Following the extraction of seed proteins under low salt buffer (Finnie and Svensson, 2003) that exclude storage protein hordeins, changes in relative abundance of proteins in the dry and micromalted seed were determined by eletrophoretically resolving 20 µg protein extracted using SDS-PAGE in

four malting barley cultivars. There were differential changes in protein profiles derived from seeds compared to micromalted materials. There was increased abundance of protein in seed protein compared to the micromalted material (**Fig. 39**). The abundance of these proteins in seeds is associated with the requirement to maintain the seed in the quiescent. These proteins include the proteins related to desiccation, osmotic, oxidative and salt stress tolerance; protein synthesis, folding and storage; housekeeping enzymes for storage compound degradation. During micromalting, most proteins that dominated the seed were mostly degraded. These proteins are mobilized in metabolism to generate energy in form of N and C needed by the germinating seedling. The proteins that are degraded are marked in **blue spots** while new proteins were further observed is indicated in **red arrow** (**Fig. 39 A**). Increased accumulations of protein bands corresponding to approximately 49 kDa was seen in the micromalted material in all the genotypes as indicated by **black arrows.** Constitutively accumulated proteins are marked by **green arrow** (**Fig. 39 B**). The increased abundance of proteins corresponding to 49 kDa is predicted to be α-amylase (AMY1)) (Jensen et al., 2007) since they dorminate the germination and malting processes.

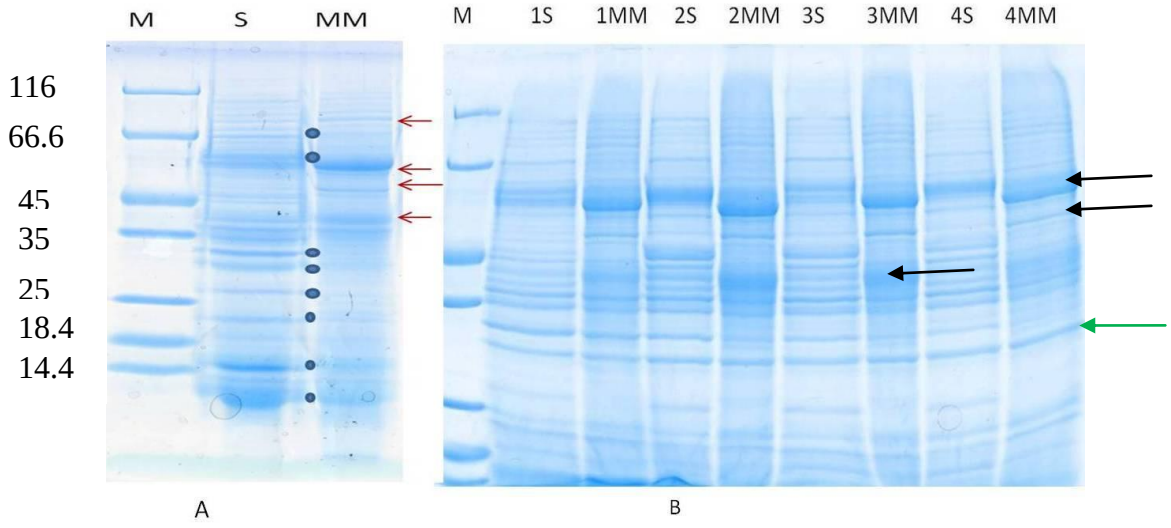


Figure 39. Comparison of protein abundance in the dry mature seeds (S) and micromalted (MM) seeds. M: Protein marker 1: Barke. 2: Nguzo. 3: HKBL 1512. 4: HKBL1385 grown under conditions described in Fig. 42. A: Protein abundance in seed and micromalted material from cv. HKBL1512. B: Protein abundance seed and micromalted material. The proteins marked as follows : ●● degraded ← increased in abundance ← α- amylase and its isoforms and ← constitutive accumulation.

3.4.8.2 Changes of hordein polypeptide fractions during progressive germination

The changes in hordein polypetide profiles during progressive micromalting stages were evaluated in the four malting cultivars in the physiological stages detailed in **Fig. 38**. Analysis showed that there was no difference in C and B hordein fraction derived from the seed, steep and 24 hrs of micromalting in all the genotypes (**Fig. 40**).

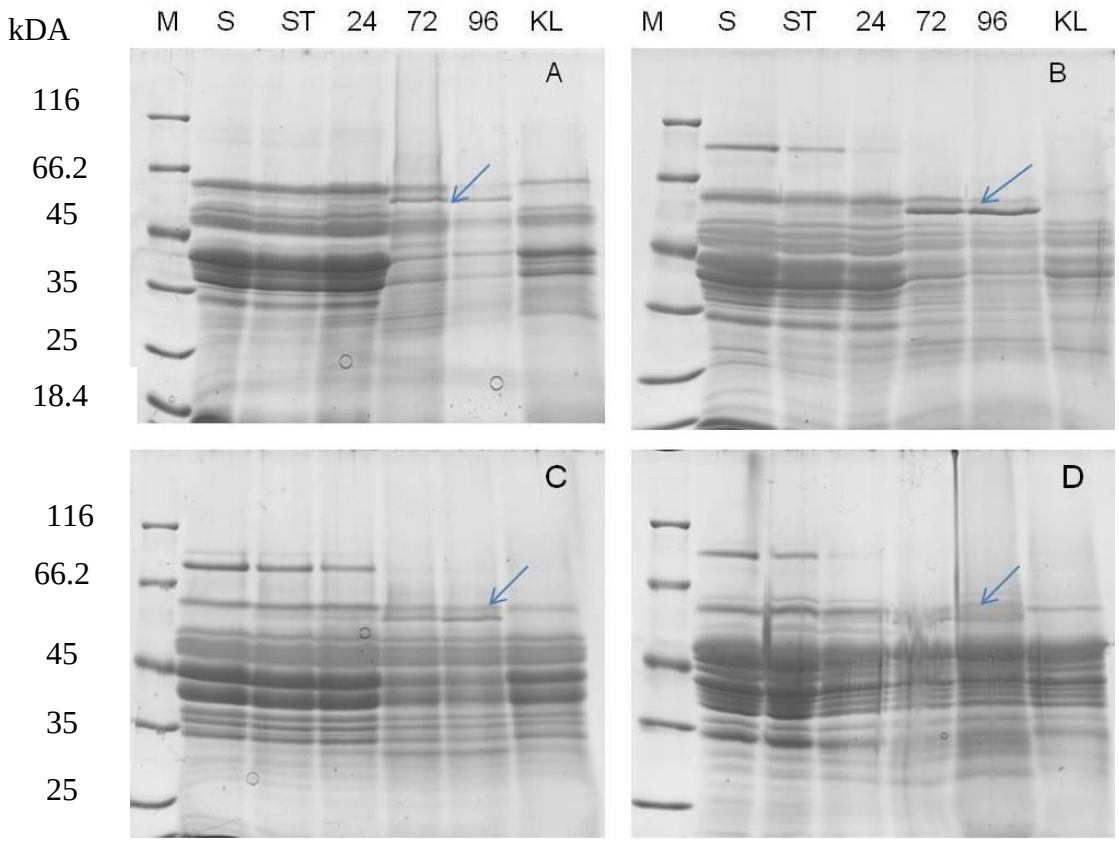


Figure 40. Changes in hordein polypetide fractions at different physiological stages during micro malting (Seed-S, steeping-ST, 24 hrs, 72 hrs, 96 hrs and kilning (KL): A-Barke, B-Nguzo, C-HKBL 1512, D-HKBL 1385. Abundance of new proteins during 72 and 96 hrs is marked by a blue arrow.

The degradation of hordein polypeptides was observed within 72 and 96 hrs and in kilning stages. The degradation of storage proteins facilitates the global mobilization of N which is the main constituent of storage proteins for germination. There was also concomitant accumulation of new proteins within 72 and 96 hrs but they were rapidly degraded during kilning. This protein is identified as β- amylase since it can be mobilized later during germination and is released from its bound inactive state in the presence of

reducing agents such as DTT or β-mercaptaethanol which are constituent of hordein protein extraction buffer. The accumulation of new proteins is marked in the blue arrow in **Fig. 40**. The highest abundance of this was seen in Nguzo. The rate of degradation of C and B hordein subclasses was genotype dependent. In. Nguzo and HKBL1385 degradation of hordein of C subclass occurred after 24 hrs of malting (**Fig. 40 B** and **D**) while in cvs Barke and HKBL 1512 this degradation began after 72 hrs of micromalting (**Fig. 40 A** and **C**). The modification of B subclasses in cv. HKBL 1385 remained steadily minimum compared to other cultivars overruling previous assumptions that in good malting varieties, hordein polypeptides are are rapidly degraded or modified compared to poor malting varieties.

3.4.8.3 Changes in transcripts encoding malting enzymes during progressive germination

Expression profiles of malting genes; *AMY1, BG1 and BAMY1* were evaluated in dry and micromalted seeds at different physiological stages detailed in **Fig. 41**.

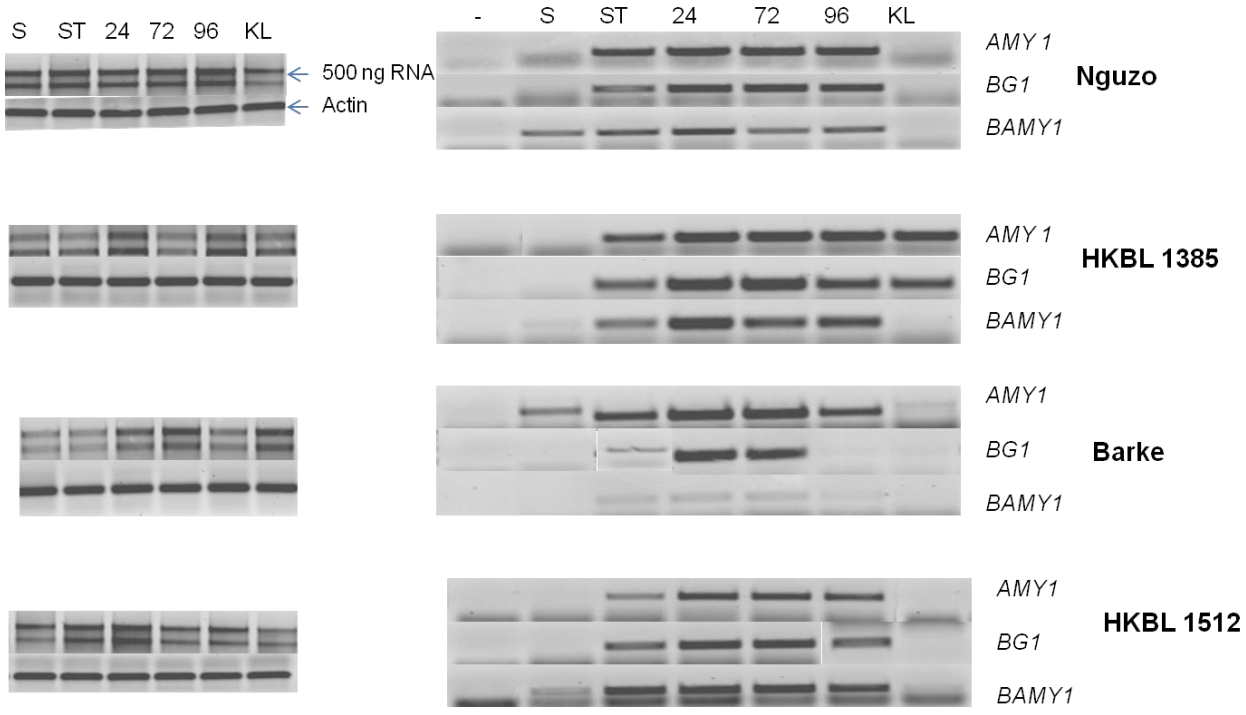


Figure 41. Expression analysis of malting genes: α-amylase (*AMY1*), β-glucanase (*BG1*) and β-amylase (*BAMY*1) in four malting cultivars. Nguzo, HKBL 1385, Barke, HKBL 1512 analyzed at different physiological stages (S), ST-, 24 hrs, 72 hrs and 96 hrs and KL (kilning).

Transcript analysis showed that genes were induced differentially during germination in a genotype dependent manner. With a few exceptional cases, the genes were down-regulated in S (seed) and kilning depending on the genotype. In cv. Nguzo, there was increased induction of *AMY1* and *BG1* during germination but lost during kilning. However, *BAMY1* was induced in seed extracts in all the genotypes. In. HKBL1385 expression profile of both *AMY1* and *BG1* was induced in low levels in steeped tissues and increased consistently through germination and kilning stages. Maximum expression was seen in *BG1* in 24 and 72 hrs of germination. *BAMY1* was induced at a lower level during steeping and maximum at 24 hrs. It was however degraded during kilning. In Barke, *AMY1* was induced in low levels in the seed extract and increased during malting but significantly dropped after 96 hrs of germination and was further degraded during kilning. The expression of *BG1* was induced strongly during 24 and 72 hrs but down regulated in other stages. *BAMY1* was weakly induced in steeped, 24 hrs, 72 hrs and 96 hrs. In HKBL 1512, *AMY1*, *BG1* and *BAMY1* increased during germination. The increase of malting genes during germination is related with degradation of large complex molecules to simpler glucose residues which can be utilized by germinating plant for metabolism. The high expression of malting enzymes genes in HKBL 1385 is tied to the high starch content in the same variety.

3.10. Discussion

Breeding for barley varieties with improved malting attributes is an important aspect in barley breeding programs because of the huge economic impact in the malting and brewing sector. The influence of agronomic practices and the prevailing weather conditions during the growing and harvesting seasons has been shown to determine malting quality and achievement of desired attributes (Fuchs, 1984). In Africa, fertilizer is generally under used which is related to extremely poor soils and limited financial capability in the smallholder farmers. The effect of different fertilizer regimes and seeding rates on grain yield and malting attributes were evaluated in three Kenyan malting varieties grown under field conditions. It was established that N fertilizer and seeding rate caused an effect in grain yield in a genotype -dependent manner. The cv. HKBL 1385 recorded the highest yield of 5.1 ton/ha compared to Nguzo and HKBL 1512 which recorded 3.7 and 3,8 ton/ha, respectively. Seeding rate caused a significant effect on yield ton/ha and formation tillers. The rate of N fertilizer application is critical for cereal crops, but especially for malting barley since N affects tiller number, grain yield and quality (Anderson et al., 2002). Tiller formation was highest in the lowest seeding rate of 150 seeds m^{-2} and significantly reduced in 250 seeds m^{-2}. Maximum tillers were observed at 40 kg N treatment. The reduction has been attributed to increased intrarow competition for nutrients resulting in seedling mortality. Most studies in Northern America have demonstrated that seeding rate had no effect on yield especially at rates above 200 seeds. However, O'Donovan et al. (2012) reported with the seed rate of 300 seeds m^{-2} resulted in maintained or improved yield, decreased protein concentration, increased kernel uniformity and time to seed maturity and decreased tillers.

Malting characteristics is a complex phenotype that combines several interrelated components which display complex inheritance (Burger and LaBerge, 1985, Hayes and Jones, 2000). Malting attributes are not scorable under field conditions like disease resistance and abiotic stress tolerance. Instead, they can be predicted by analyzing the seed in response to defined treatment (Ingversen et al., 1989). Analysis showed the starch content was genotype dependent and was affected by N treatment. The cultivar HKBL 1385 exhibited the highest starch content of 278 mg/g DW while Nguzo and HKBL 1512 recorded 240 and 225 mg/g DW, respectively.

In HKBL 1385 starch was inversely correlated to grain protein level. This inverse relationship between starch and protein has been reported previously by Åman et al.

(1985). Starch being a critical malting attribute is the source of fermentable sugars for malting process. High starch contents is desirable since it result in increased malt extract while increased protein is associated with reduced malt extracts (Fox et al., 2003) and a serious limitation to the steeping process. On the other hand, N treatment did not significantly affect grain protein level in all the genotype since N application rate was based on the soil analysis prior to sowing. The HKBL 1512 recorded the highest grain protein content of 10.4 while Nguzo and HKBL 1385 recorded comparable protein level 9.7 and 9.8 respectively. The protein was within the malting specification of 9-12.5% (Atherton, 1984). Defining cultivar sensitivity and N requirements in a way that does not compromise yield, reduce malt quality and pollute the environmental is critical for sustainable agriculture It was established that cultivars have differing nutrient needs reflecting a component of nutrient use suggesting adaptation to nutrient availability There was specific cultivar nutrient demands reflected in differential starch levels. We observed that Nguzo and HKBL 1385 showed sensitivity to N at 30-40 kg/ha N and did not change much upon increased N while HKBL 1512 was responsive at 40 kg/ha. Under low N application HKBL 1385 recorded relatively high starch content. The microbial community in the root environment may play a role in N remobilization and modulating pH around the roots. The pH under which the crop was established at the University of Eldoret was acidic (pH 4.7). In the works of Liben et al. (2011) fertilizer rate recommendation requirement was based on genotype, malting criterion and geographical suitability was established for three malting cultivars namely HB-52, HB-1533 and Miscale-21 grown in Northwestern Ethiopia. Hordein content is an important malting quality. The variability of hordein fractions has been used to study germplasm diversity as well as their potential use in identification of malting traits (Echart-Almeida and Cavalli-Molina, 2001b, Lee et al., 2010). The results on the effect of N treatment on hordein polypeptides in malting quality have been variable. Analysis of hordein polypeptides in the three malting cultivars revealed that HKBL 1512 and Nguzo are closely related. It is also known that most of the new lines have a Nguzo genetic background. The hordeins were resolved into C and B fractions while D hordeins occurred in low levels that limited visual score. We reported that N treatment had effect on hordein polypeptide fractions mainly on the B and C subclasses depending on the genotype. Low hordein level was observed in control treatment and increased with C and B subclasses bands and intensity. The changes in hordein polypetide accumulation in response to N treatment were only

observed in HKBL 1385 and HKBL 1512 (**Fig. 33 A** and **C**) but not in cv. Nguzo (**Fig. 33 B**). These results were not consistent with grain protein content data. This may suggest that the assimilation mechanism of N towards storage protein may be different from seed protein. The relationship between hordein fractions and malting has been described. Since D fractions were not detected in the electrophoregram, it is most likely that they do not play a great role in malting and the main players are C and B hordeins in the cultivars under study. These findings compared with those of Peltonen et al. (1994) which demonstrated that the B hordeins were correlated with malting quality. However, it contrasted with the works of Howard et al. (1996) which demonstrated a consistent relationship between D hordein and malt extract observed across seasons, treatments and cultivars suggesting a causal relationship between D hordein and malting quality.

Unlike the malting genes which are activated during germination, the storage protein genes are synthesized during the period of rapid growth of endosperm to maturity (Giese et al., 1983). The malting and storage protein genes are however downregulated during seed dormancy. During quiescent stage, the expression of genes encoding storage proteins and enzymes involved in the synthesis of other reserve compounds are downregulated while those involved in germination axial growth and reserve remobilization are upregulated. In their work Hansen et al. (2009) reported marked temporal profiles in storage protein genes of field grown barley in response to inorganic nitrogen fertilizer. The transcript levels of *D, B, C* hordein genes increased steadily from 10 DAP to 18 DAP then declined steadily after 25 DAP.

In the current study we used the dry mature grains from different N treatments to determine hordein transcript levels. We observed constitutive expression of *actin* the house keeping genes while *D, B* and *C* genes were down-regulated in all the treatments irrespective of the genotype. The dry seed is metabolically quiescent and is unable to initiate any biological activity (Potokina et al, 2002). GA and ABA are major phytohormones regulating seed dormancy and germination. GA and ABA play antagonistic roles in regulating seed germination and their ratios govern the maturation versus germination pathways that embryos will take after they complete rudimentary organogenesis (White et al., 2000). Germination in barley seedling growth have been investigated extensively due to its importance in barley agriculture and the brewing industry (MacGregor and Bhatty, 1993). Seed germination is characterized by many distinct metabolic, cellular and physiological changes.

Analysis of the changes in abundance of seed protein in the dessicated seed and during germinations in Barke, HKBL 1512, Nguzo and HKBL 1385 showed that the seed grain harbored diverse and more abundant protein compared with germinating seed. The seed proteins are involved in maintaining many physiological functions in the desiccated state. These proteins include those involved in dessication, oxidative and osmotic tolerance, protein synthesis, housekeeping and storage proteins. Changes in morphology, water up-take, α-amylase activity and loss of seed desiccation tolerance are important characteristics of germinating barley (Biligetu et al., 2011). The transition from seed development to germination requires fundamental changes in control of gene expression within the seed. Germination is tightly regulated at the transcriptional level and depends on developmental stages. Changes in transcript levels of genes encoding malting enzymes *AMY1, BG1* and *BAMY1* in dry seed grain, during progressive germination and kilning was determined in Barke, HKBL 1512, Nguzo and HKBL 1385 Analysis showed that differences in expression levels were dependent on genotype and physiological stage of the germination. The malting genes were down regulated in the seed grain and kilning stages but increased progressively during germination period. *AMY1* was strongly expressed in all genotypes supporting the long held evidence that α- amylase occurs in abundance in germinating barley seeds (Bamforth, 2003). The strong expression of *AMY1* in HKBL1385 may reflect efficiency of starch modification during germination. Transcriptome analyses have been used to examine gene expression during the early stages of germination (Lapitan et al., 2009b). Expression of important germination genes and biological pathways are developmentally regulated over the course of seed germination. Three phases of germination have been previously described: early stage, late stage and post germination stages (Bewley, 1997). Early germination phase represents the first nine-hour germination and preferentially induces genes encoding regulatory components including transcription factors, signaling components and post-translational modification proteins (An and Lin, 2011). In the late germination phase, genes encoding many metabolic pathway enzymes and cellular components including amino acid and nucleotide synthesis, protein degradation, chromatin remodeling and cell division pathways are preferentially up-regulated to provide nutrient and cellular components for cell division and elongation. Post-germination phase mainly represents seedling growth processes after emergence of coleorhiza from the germinating grains. The late germination stage is critically important to the maltesters since the large

molecules compounds including starch and protein and cell wall molecules are degraded into smaller molecules to generate energy reserves for the germinating seedling. In general, a good malting quality requires a high conversion of starch to fermentable sugars, high malting extract, optimal protein content, and low haze formation. Diastatic power which represents the starch-degrading ability, is closely associated with the activities of four starch hydrolytic enzymes, i.e., α-amylase, β-amylase limit dextrinase and β-glucosidase (Delcour and Verschaeve, 1987). Besides being an indicator of overall starch breakdown, the α-amylase concentration in malt can be used as an indicator of the particular breeding lines ability to induce high levels of enzymes, after a certain malting protocol has been followed. On the other hand, β-glucan and protein contents are two other important indicators of malting quality. β-glucanases are responsible for the depolymerisation of the major constituent of the barley endosperm cell wall to low molecular weight. Incomplete degradation of β-glucan results in an elevated content of high molecular weight β-glucan in wort and beer with filtration and clarity problems as a possible result. Additionally high β-glucan content may result in insufficient degradation of cell walls, hamper the diffusion of germination enzymes and kernel reserves, and decrease malt extract (Wang et al., 2004, Bamforth, 1982). Consequently, a low amount of β-glucan in malt will predict a satisfactory modification of the endosperm cell wall while too high protein content will also reduce malt extract. Although malting is basically the germination process carried out under controlled conditions, it results in malts of different quality depending on the barley cultivar, the growth conditions in the field and the storage of grains. During malting, storage proteins undergo degradation like dessication related proteins. Variation in hordein modification has been demonstrated during malting in 2 barley cultivars with contrasting qualities. The malting variety exhibited increased modification in hordein breakdown than the non malting type (Marchylo et al., 1986). In The current study, changes of hordeins occurred 24 hrs after germination with concomitant accumulation of proteins between 72-96 hrs which ispredicted as β - amylase. The research findings in this study provide useful information for developing soil-based N management with the aim of improving both high yield and efficient N use. We attempted to define cultivar sensitivity to N application with respect to overall yield under field conditions, seed starch content, protein concentration and hordein polypeptide fractions. The combination of both field data and laboratory analyses generates a better understanding and reproducibility of results. It will further provide a

bridge in which field phenotypes can be indirectly predicted and validated using both biochemical and molecular analysis as means to rapidly identify malting attribute in barley breeding programmes. Defining cultivar sensitivity to fertilizer application is an important step in fertilizer use recommendation for sustainable agriculture for barley farmers in this agroecological zone since the great rift valley region is not only regarded as the food basket of Kenya but also the most important wheat and barley growing zone.

4. Morphological characterization of pot-grown and *in vitro* propagated Kenyan barley seedlings in response to N and P deprivation

Abstract

A genotype response to N and P nutrient deprivation in pot-grown and *in vitro* propagated Kenyan barley seedlings was evaluated. Changes in growth parameters including root length, lateral root formation, root mass and leaf length were determined in 6 pot grown barley lines (Morex, Nguzo, MN-24, Karne, HKBL 1385 and MN-12) seedlings. Further analysis of root phenotypes was evaluated in *in vitro* propagated barley seeds of Nguzo, Karne and HKBL 1385. ANOVA indicates that the effect of genotype and treatment was highly significant in the traits evaluated under low P and N treatment. Under low N genotype and treatment was significant in all the parameters except for leaf length and the number of lateral roots. Increased availability of N and P repressed root length and lateral root formation. Leaf length was significantly reduced in response to N and P deficiency. P deficiency had serious effects in barley growth parameters compared to N as reflected in suppressed leaf length and leaf chlorosis. The root mass was reduced in Nguzo and Karne under N. There was a positive correlation (r=0.452) between root mass and the number of lateral roots under P deficiency. *In vitro* propagated barley showed diversity in root architecture. N deficiency caused elongation of primary root length. Nguzo and HKBL 1385 displayed a short and dense root network compared to the thin and elongate type seen in Karne under N deprivation. The use of both pot-grown and *in vitro* propagation methods to evaluate plant responses to nutrient deficiencies will not only improve our understanding of nutrient use efficiency but also provide a low input and rapid method to identify barley genotypes with enhanced tolerance to nutrient deficiency.

*Keywords***.** Abiotic stress, barley seedlings, N and P deficiency, *in vitro* propagation, primary root, lateral roots.

4.1 Introduction

The ability of plants to respond appropriately to nutrient availability is of fundamental importance for environmental adaptation (Lynch, 2007). N, P and K are required in relatively large amounts and are considered the most important macronutrients that significantly affect plant growth and development. Deficiencies of these macronutrients are the main factors that limit crop productivity in agricultural soils globally (Sanchez, 2002). P is a constituent of key cellular molecules such as ATP, phospholipids and nucleic acids (Raghothama, 1999, Marschner, 2011). Moreover it is involved in many metabolic processes such as energy transfers, protein activation and carbon metabolism (Wu et al., 2003). P occurs as phosphate (PO_4^{-1}/Pi and occurs in low concentration usually as insoluble salt complexes that are not amenable for uptake into plants (Raghothama, 2000, Vance et al., 2003). Limited P availability is characterised by reduce growth and vigour, reduced tiller production and grain yield, yellowing and death of leaf tips. P deficient plants may also exhibit orange, red or purple pigmentation in stems. On the other hand, N occurs in the form of nitrate and ammonium. It is aso the building block of protein biosynthesis (Masclaux-Daubresse et al, 2010) and plays a role as signaling molecule such as nitric oxide (NO) in many metabolic processes, photosynthesis and growth. Deficiency of N is characterized by reduced activities of enzymes involved in energy metabolism such as respiration and photosynthesis (Scheible et al., 2004). Additionally CO_2 fixation is significantly reduced because more than half of the total leaf N is allocated to the photosynthetic apparatus including chlorophyll, light harvesting complex and rubisco (Makino and Osmond, 1991, Evans and Poorter, 2001, Boussadia et al., 2010). As a result, plant growth is reduced, followed by gradual chlorosis of older leaves and subsequent abscission.

Soil nutrients are depleted due to leaching of nitrogen, fixation of phosphorous, soil erosion and removal by crops (Zobeck et al., 2000, Holmgren and Scheffer, 2001, Holmgren et al., 2006). In intensive agriculture, the nutrient status of the soil has to be maintained through crop rotation, addition of manures or application of inorganic fertilisers (Edmeades, 2003, Vance et al., 2003) except those in which legumes are grown (Epstein and Bloom, 2005). The application of inorganic fertilisers is an important input in any agricultural production. In the past, improvement of crops and agricultural techniques has mainly focused on improving above-ground traits shoot biomass and seed yield (Gonzalez et al., 2009, Xing and Zhang, 2010) with an obvious emphasis on yield.

While breeding efforts have been instrumental for increasing crop production to present capacity, future yield increases are likely to be constrained by lower water and fertilizer inputs, and the use of marginal lands containing nutrient-poor soils (Lynch, 2007, Den Herder et al., 2010). One of the common mechanisms by which plants adjust to an imbalance of exogenous resources is by increasing root to shoot ratio and a specific root length (Hermans et al., 2006). The root architecture is modified to enhance nutrient acquisition under limited environments. These include increased number of lateral root growth for enhanced exploratory effect and formation of root hairs to increase surface area of the meristem and lateral roots (López-Bucio et al., 2003). Since nitrate and phosphate have contrasting mobility in the soils, their effect on the root system architecture may differ. Nitrate migrate three to four folds faster than that of phosphate (Tinker and Nye, 2000). In response to low phosphate, many plants, including Arabidopsis, increase their root hair length and density (Bates and Lynch, 2000, Ma et al., 2001).

Up regulation of transcription factors of the MYB family has been reported (Miyake et al., 2003, Shin, 2011) under nutrient deprivation (Raghothama 2000). Additionally tissue specific expression of nitrogen assimilation enzymes and transporters that are tightly regulated by the presence of nitrate (Dechorgnat et al., 2011). Similarly in response to P deficiency, plant species and genotypes of given species have developed specialized morphological, physiological and molecular responses in root systems in order to increase P uptake and maintain normal growth (Vance et al., 2003, George et al., 2011). These are reflected in alteration of root morphology and establishment of symbiotic relations with arbuscular mycorrhizal fungi to enhance exploration of soils (Péret et al., 2011, Smith and Smith, 2011). Additionally there is enhanced release of organic anions and secretion of phosphatase in the soils to mobilize the release of bound phosphate from organic and inorganic P sources (George and Richardson, 2008) and enhance uptake Pi by high affinity phosphate transporters (Raghothama, 1999b, Schünmann et al., 2004, Teng et al., 2013).

Genotypes tolerant to nutrient stress are able to assimilate and accumulate macronutrients efficiently compared to susceptible genotypes (Jia et al., 2008). Evaluation of root morphology and molecular responses to N and P treatments has been reported in cereal crops grown under field conditions (Beatty et al., 2010, Teng et al., 2013). Additionally laboratory based methods including hydroponics, *in vitro* cultures, aeroponic or pots have

enabled studies on genotype responses to nutrient deprivations (Shinozaki and Yamaguchi-Shinozaki, 2000, Linkohr et al., 2002).

Since N and P are the major limiting macronutrients in the arable soils in Kenya (Obura et al., 2010) there is need to identify cultivars that can thrive under nutrient limitation to reduce the burden of cost to the poor resource farmer at the same time minimizing environmental risks. Identification of genotypes exhibiting tolerance to nutrient deprivation may aid in screening for genotypes relevant to breeding programs for low input agricultural production.

4.2 Specific objectives

- To evaluate genotype response to N and P derivation in 6 pot-grown barley cultivars namely; Morex, Nguzo, MN-24, Karne HKBL 1385 and MN-12 by determining root mass, root length, lateral root formation and leaf length.
- To determine morphological changes in root phenotypes in three selected *in vitro* grown barley genotypes in response to N and P deprivation.

4.3 Materials and methods

4.3.1 Choice of plant material

Six barley genotypes of which 5 had been previously screened for abiotic stress tolerance in **Chapter 2** (Nguzo, Morex, MN-24, Karne and MN-8) alongside a novel malting cultivar HKBL1385 detailed in **Chapter 3** were used to evaluate morphological responses to N and P deficiencies.

4. 3.2 Germination viability tests

Germination efficiency of barley cultivars were determined using pregerminated barley seeds grown under on moist Whatman paper in petri dishes. 20 seeds from each cultivar were grown in two replicates in each petri dish. Germination viability was calculated as a percentage of the total seeds used. This was prompted because of the low viability of *in vitro* propagated seedlings.

4. 3.3 Propagation in pots

The seeds from the selected genotypes were pre-germinated for 3 days in the dark under moist conditions at RT. The seedlings were transferred to sterile clay pebbles in 500 ml pots irrigated with Murashige and Skoog (1962) nutrient solution. The media formulation including concentration for control, N and P deficiencies comprised the following: *Macroelements*; 2.99 mM $CaCl_2$, **1.25 mM KH_2PO_4**, **18.79 mM KNO_3**, 1.50 mM $MgSO_4$, 20.61 mM NH_4NO_3. *Microelements*; 0.11 µM $CoCl_2$. $6H_2O$, 0.1 µM $CuSO_4$. $5H_2O$, 100 µM FeEDTA, 100.27 µM H_3BO_3, 5 µM KI, 100 µM $MnSO_4$.H2O, 1.03 µM $Na_2MoO_4.2H_2O$, 29.91 µM $ZnSO_4.7H_2O$. *Vitamins* (only for *in vitro* propagation apply) 26.64 µM glycine, 554.94 µM myo inositol, 4.06 nicotinic acid, 2.43 µM pyridoxine-HCl, 0.3 µM thiamine-HCl

The solution was replaced every 3 days and phosphate and nitrate deprivation was induced by irrigating with nutrient solution in which **1.25 mM KH_2PO_4** concentration was reduced **to 12.5 µM KH_2PO_4 (-P)** and **KNO_3 (-N)** was replaced by **KCl** respectively (**Table 9**).

Table 9: Low N and P MS media

Low N media A

Macronutrient	**Mg/L(X100)**	**500mL (mg)**	**500 mL(g)**
$CaCl_2$	33202	16600	16.6
KH_2PO_4	17000	8500	8.5
KCl	**65**	**32.5**	**0.0325**
MgSO4	18054	9027	9.07
NH_4NO_3	165000	82250	82.25

Low P media B

Macronutrient	**Mg/L(X100)**	**500mL (mg)**	**500 mL(g)**
$CaCl_2$	33202	16600	16.6
KH_2PO_4	**6000**	**3000**	**3.0**
KNO_3	190000	95000	95
$MgSO_4$	18054	9027	9.07
NH_4NO_3	165000	82250	82.25

Plants were grown under controlled conditions (120-150 μE m-2s-1 23 ^{0}C with 16 hrs light and 8 hrs dark regimes). At 16 days post germination the seedlings were evaluated for traits which include biometric measurements including coleoptiles length in (cm), the longest root length (cm), number of lateral roots, root mass (mg), leaf length and qualitative leaf pigmentation in pot-grown barley.

4. 3.4 *In vitro* propagation of barley seedlings

4.3.4.1 Surface sterilization

In vitro propagation of barley was faced with the challenge of reduced seed viability characterized by failed germination and necrosis. Only cultivars that displayed over 75% germination efficiency during pregermination in the petri plates considered as illustrated in **Fig. 46**. In view of this, only three barley cultivars were evaluated namely, Nguzo HKBL 1385 and Karne. The seeds were soaked for 10 mins in sterile distilled water followed by careful dehusking with a special scalpel to remove the testa. The dehusked

seeds were surface sterilized using 70% ethanol followed by 10 mins incubation in 35% hydrogen peroxide (H_2O_2). The seeds were rinsed at least 3 times in sterile water before transfer to petri plates (agar 8 g/l, pH 5.8) under sterile hood (Heraeus, Germany). The sterile seeds were placed embryo-side up and sealed with millipore tape (Parafilm, USA) and grown under control conditions as described above.

4.3.4.2. Evaluation of morphological characteristics in response N and P deprivation of in vitro grown barley plants

After 2 days of incubation in agar plates, the pre-germinated seeds with emerged radical of about 2 mm length (**Fig 5**) were transferred under sterile conditions to previously sterilized 500 ml solid MS media in 1 L sterile glass jars made of control treatments (MS macronutrient, MS micronutrients and MS vitamins), N deprivation treatment (NH_4NO_3 omitted from MS macronutrients and KNO_3 replaced by KCl) while P deprivation treatment (KH_2PO_4 reduced from 1.25 mM reduced to 1.25 μM). The jars were covered with sterile foil. After 16 days of incubation in control, N and P deprived MS media the qualitative and quantitative root system architecture was evaluated. Images of roots under the different treatments were captured at the end of each stress treatment using a Digitalcamera (Canon EOS 550D).

4.4 Results

4.4.1.Seed germination viability

Cultivars displaying contamination and abnormalities in the radical and/or plumule were eliminated. The barley lines Morex, MN-24 and MN-12 displayed reduced germination efficiency while cvs. Nguzo, Karne and HKBL 1385 had a germination efficiency of more than 75% (**Fig. 42**).

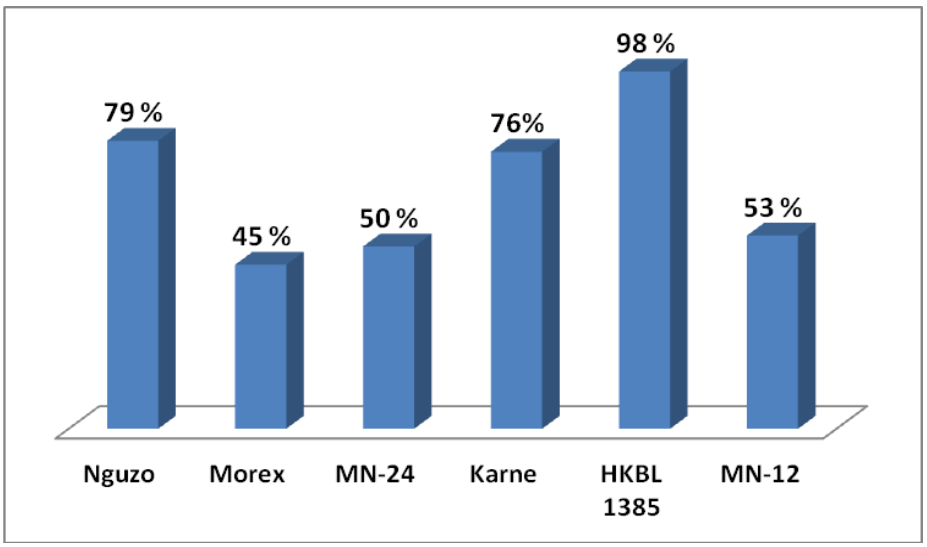


Figure 42. Germination efficiency in barley glines based barley seeds pregerminated on moist Whatmann paper in petri plates.

4.4.2 Effect on N and P deprivation in pot-grown barley

The effect of N and P deprivation was evaluated by monitoring phenotypic changes in morphological parameters such as root architecture (lateral roots, lateral root length, root mass) and length length in 6 pot-grown barleylines.

The ANOVA output for ech treatment are presented in **Table 10** and **Table 11** respecively.

Table 10: Analysis of variance of growth parameters in response to low N treatments (p=0.05)

Traits	Genotype	Treatment	Geno*Treat
	Low N treatment …….		
Leaf length	44.283**	1.33 ns	23.433ns
No. lateral roots	12.383**	10.083ns	7.58 ns
Root length	13.338**	99.188**	2.6n
Root mass	0.12630 **	1.85653**	0.044

Table 11: Analysis of variance of on growth parameters in response to low P treatments (p=0.05)

Traits	Genotype	Treatment	Geno*Treat
	Low P treatment		
Leaf length	97.82**	204.19***	11.3 ns
No. lateral roots	16.08**	4.233*	0.25ns
Root length	9.833*	33.67**	1.383ns
Root mass	0.34859*	1.09505**	0.09799ns

4.4.2.1 Changes in the number of lateral roots in response to N and P deficiency

Variations in the number of lateral roots under N and P deficiency were determined. ANOVA (**Table 10**) output showed that genotype caused highly significant effects (p=0.009**) while treatment had no effects (p=0.088) on the number of lateral roots formed in response to low N. Under low P genotype (p=0.005**) and treatment (p= 0.001**) caused significant effects in the number of lateral roots formed (**Table 11**). The changes in the number of lateral roots in response to low N and P low treatments in the genotypes under study are shown in **Fig. 43**. The formation of the lateral roots ranged between 4-8 in all the treatments. In the control treatment the number of lateral roots was reduced in most of the genotypes. Increased formation of lateral roots was observed in response to low and P treatment in most of the genotypes. However, in Karne and MN-

12, low N treatment caused a reduction in the number of lateral roots. Higher number of lateral roots was seen in Morex, Nguzo and HKBL 1385. These results show that cultivars can initiate root formation in response to P and N availability.

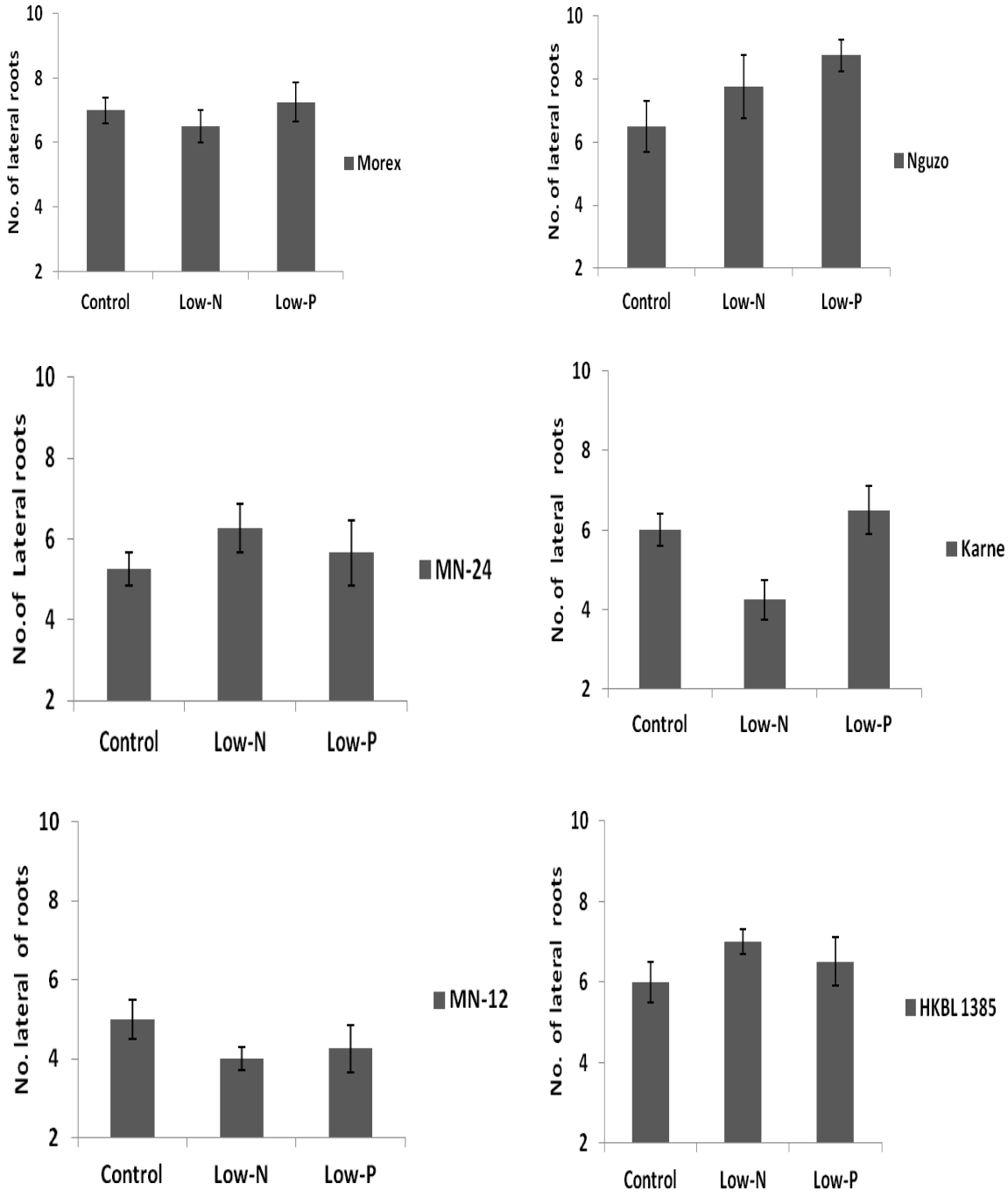


Figure 43. The effect of N and P deprivation on the number of lateral roots in pot-grown barley seedlings

4.4.2.2. Effects of changes in N and P deprivation on root mass

Changes in the root mass in response to N and P deficiency was determined. Under low N a significant effect was caused by genotype (p=0.005**) and treatment (p=0.001**). Similary a significant effect was caused by genotype (p=0 .001**) and treatment (p=0.001**) under low P treatment. The contrasting root masses in response to N and P deprivation are presented in **Fig. 44**.

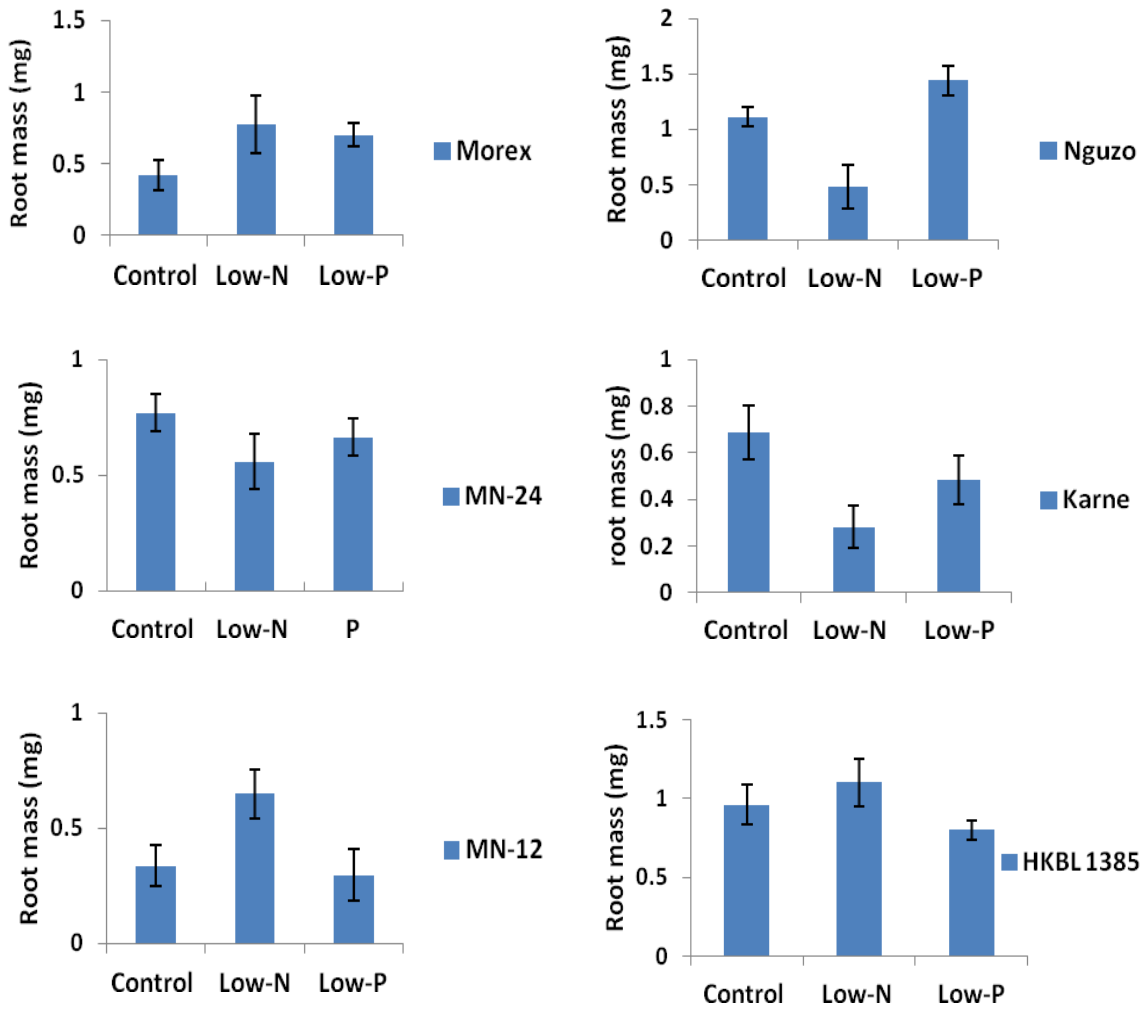


Figure 44. The effect of N and P deprivation on root mass (g) in pot-grown barley seedlings.

Compared to control treatment, N limitation caused a remarkable reduction in root fresh mass in Nguzo, MN-24 and Karne. In contrast, it caused an increase in root mass in Morex, HKBL 1385 and MN-12. HKBL 1385 recorded the higest root mass of 1.1 g in response to N deficiency. Compared to control treatment root mass was diminished in Karne and MN 24 in response to P deficiency. However, it resulted in increased root

mass in Nguzo and Morex. Nguzo recorded the highest mass of 1.44 g in response to P deprivation. In Morex and Nguzo root mass hardly increased under P limitation.

4.4.2.3 Changes in lateral root length in response N and P deprivation

The effect of N and P deficiency was monitored by determining changes in lateral root length. Analysis indicated both genotype (p=0.001**) and treatment (p=0.001**) had significant effects on the lateral root length under low P. In response to low N, there was significant effect caused by genotype (p=0.016*) and treatment (p=0.002**). Genotype variation in lateral root length is shown in **Fig. 45**.

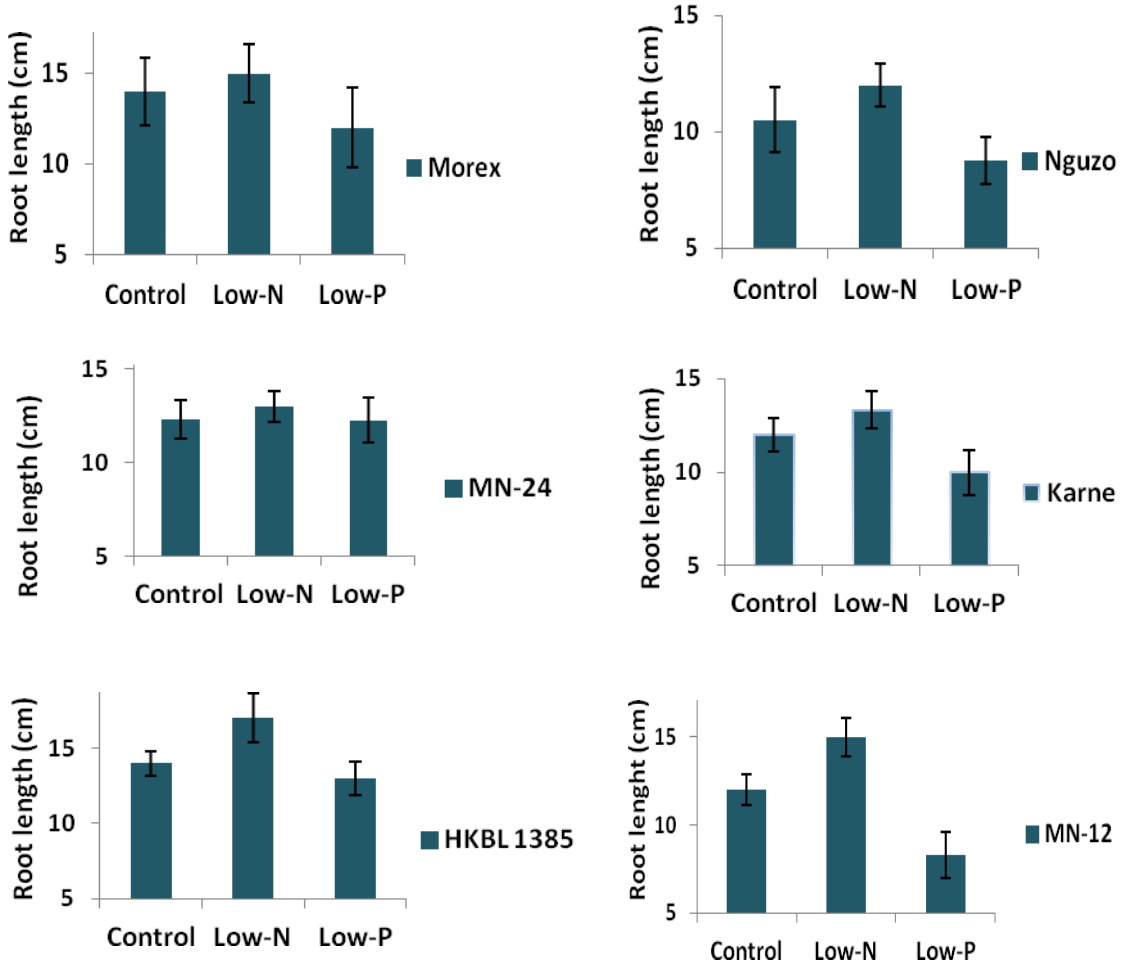


Figure 45. The effect of N and P deprivation on lateral root length (cm) in pot-grown barley seedlings.

Under adequate N supply, root elongation was inhibited in control treatments in all the genotypes but enhanced under low N. Low N treatment further stimulated root elongation in genotype-dependent manner. Highest increase in root length was observed

in HKBL 1385 and MN-12 in response to N deprivation. Low P diminished root length. MN-24 did not exhibit any changes in root lengths across the treatments.

4.4.2.4. Changes in leaf length in response to N and P deprivation in pot grown barley seedlings

Evaluation of above ground organs including leaf length has proved to be very important in evaluation of tissue responses to environmental cues including nutrient deficiency. The analysis showed that genotype effect was highly significant (p=0.003**) while treatment had no effect (p=0.716ns) under low N. However, under P deprivation both genotype (p=0.001**) and treatment (p=0.001**) were highly significant.

Variation in leaf lengths under N and P deprivation in the growth media is shown in **Fig. 46**.

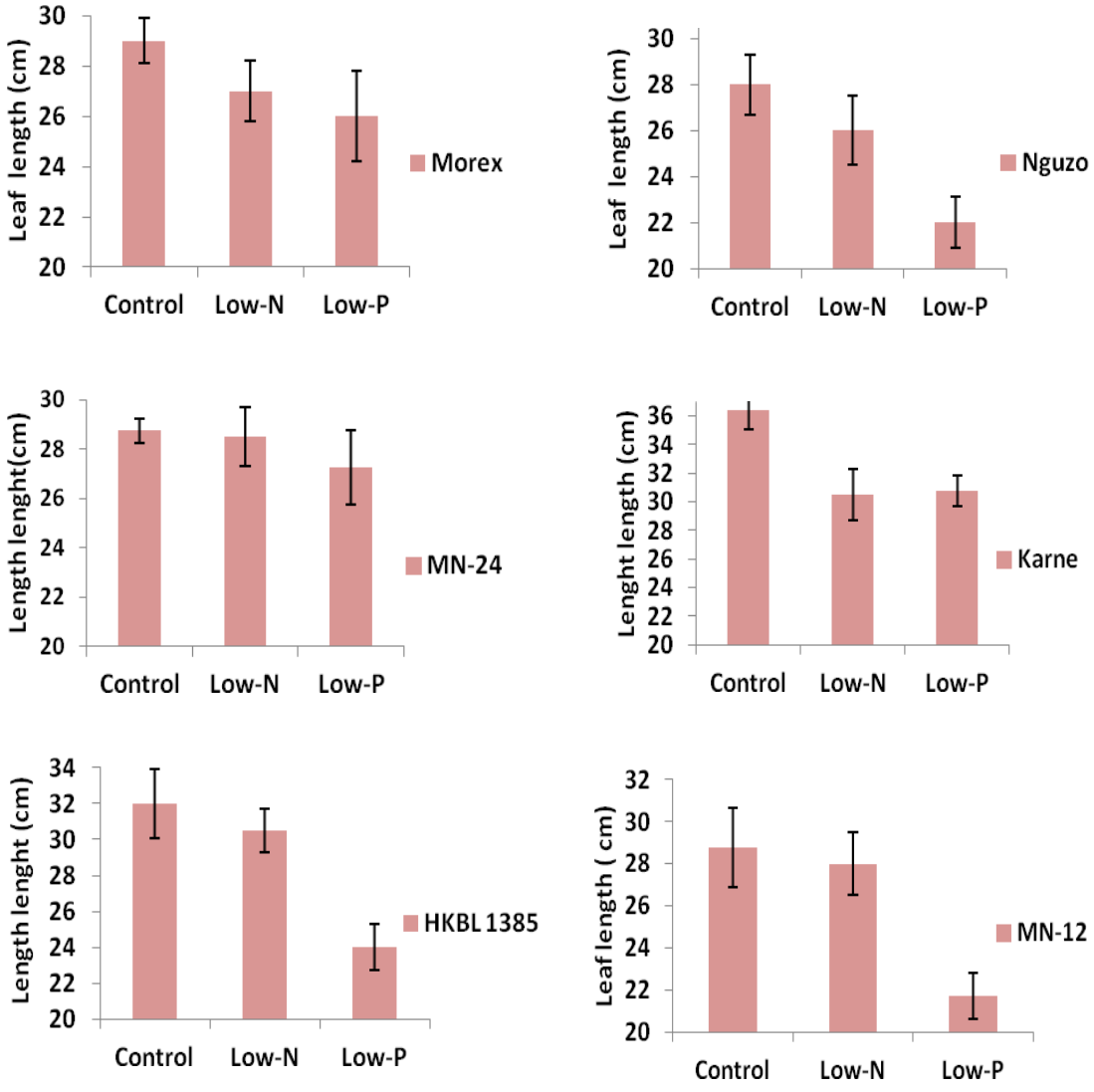


Figure 46. Effect of N and P deprivation on leaf length (cm) in pot-grown barleyseedlings

N and P deprivation caused a reduction the leaf length in a genotype-dependent manner. Highest leaf length was recorded under adequate nutrient supply. The leaf length reduction was inversely related to root length elongation. MN-24 displayed comparable leaf length in all treatments. P deprivation had more reductive effect on leaf length than N deprivation in all the genotypes evaluated. The effect of P deprivation was most pronounced in cv. Nguzo, MN-12 and HKBL 1385. It was observed that the effect of low P caused a severe chlorophyll damage compared to control or N deprived plants (**Fig. 47**).

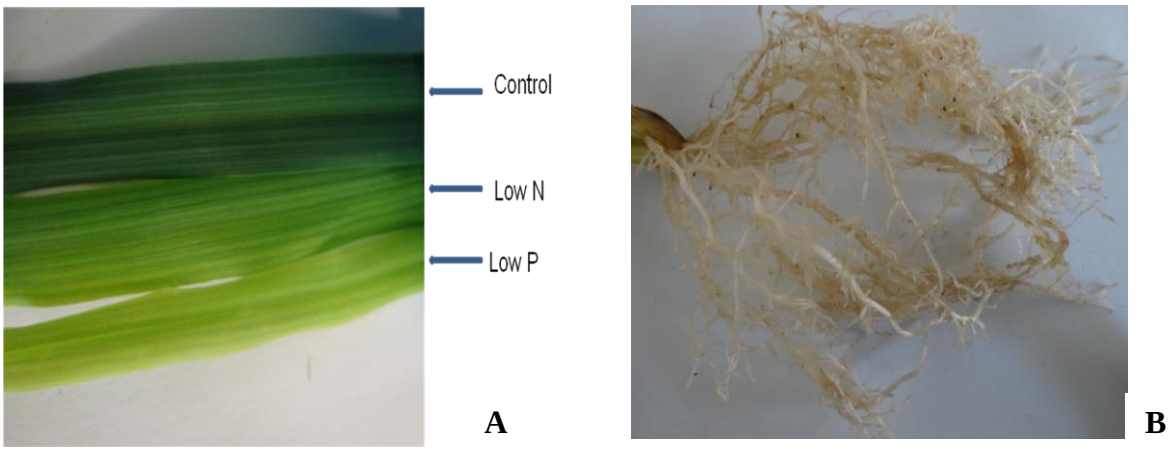


Figure 47. Effect of nutrient deficiency. A-leaf pigmentation in barley leaves grown under control, N and P treament. B -root phenotype under und P deficiency in pot–grown Nguzo.

4.4.3. *In vitro* propagation of barley seedlings

The germination *in vitro* was extremely low resulting in successful propagation of only three genotypes namel Karne, Nguzo and KHBL 1385. The procedures for *in vitro* propagation are presented schematically in **Fig. 48**.

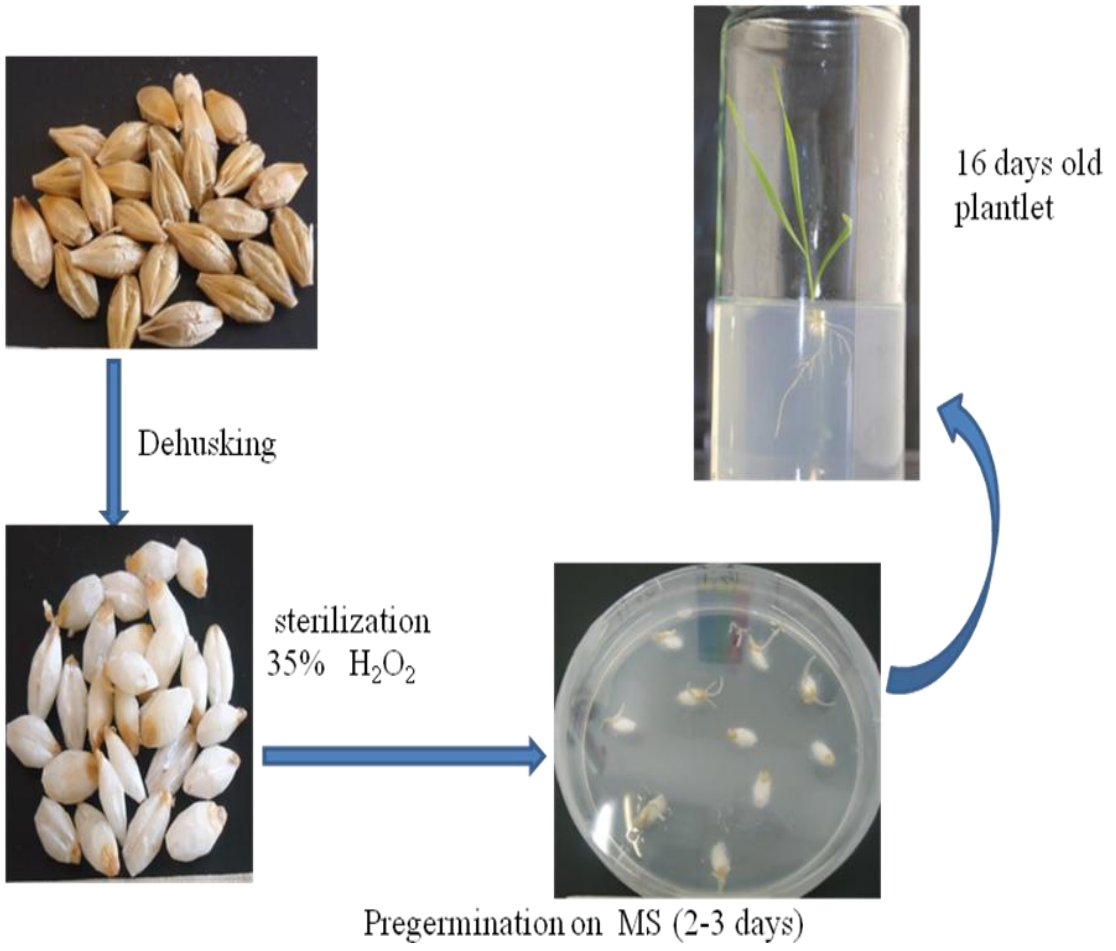


Figure 48. Schematic presentation of *in vitro* propagation of barley seedlings using mature seeds as explants. A: Seeds are dehusked, and sterilized in hydrogen peroxide for 10 mins followed by 3 times wash with SDW B: Dehusked seeds are incubated in 8% agar medium for 2-3 days. C: Transfer and culture in MS media supplemented with appropriate adequate nutrient supply or deprivation for 16 days.

4.4.4 Qualitative analysis of root phenotypes in response to N and P treatments in *in vitro* propagated barley seedlings

In an attempt to support the findings of the nutrient response in pot-grown barley seedlings, an *in vitro* evaluation was carried out. Genotype responses to N and P nutrient deficiencies are reflected in the diversity in root phenotypes characterized by alterations of root architechture including root length, root network and the number lateral roots observed in barley seedlings cultured in *in vitro* in glass jars barley (**Fig. 48**). Additionally, root phenotypes were determined under high magnification light microspcope are described for each treatment. Only three lines rnamely, Karne, Nguzo and HKBL 1385 were targeted for the root phenotype analyses. The Qualitative description of root phenotype for each genotype propagated under control N and P deficiency is illustrated in **Fig. 49 A**, **Fig. 50 A** and.**Fig. 51 A** representing Karne, Nguzo and HKBL 1385, respectively.

4.4.4.1 Root phenotype response to N and P deprivation in cv. Karne

Cultivar Karne, displayed a conspicuous genotype and treatment-dependent root phenotype

(**Fig 49**). Under control treatment, lateral roots twere short and thick. However, when subjected to N deprivation, the lateral roots became thin and elongated in length reaching the bottom of the glass jar at the end of experimental period. In response to P deprivation the lateral roots increased in number compared to control treatment, became thicker with a characteristic increase of the root network and slight increase in lateral root length.

The qualitative differences in root network are illustrated from each treatment in **Fig. 49. B** which shows high magnification microscopy.

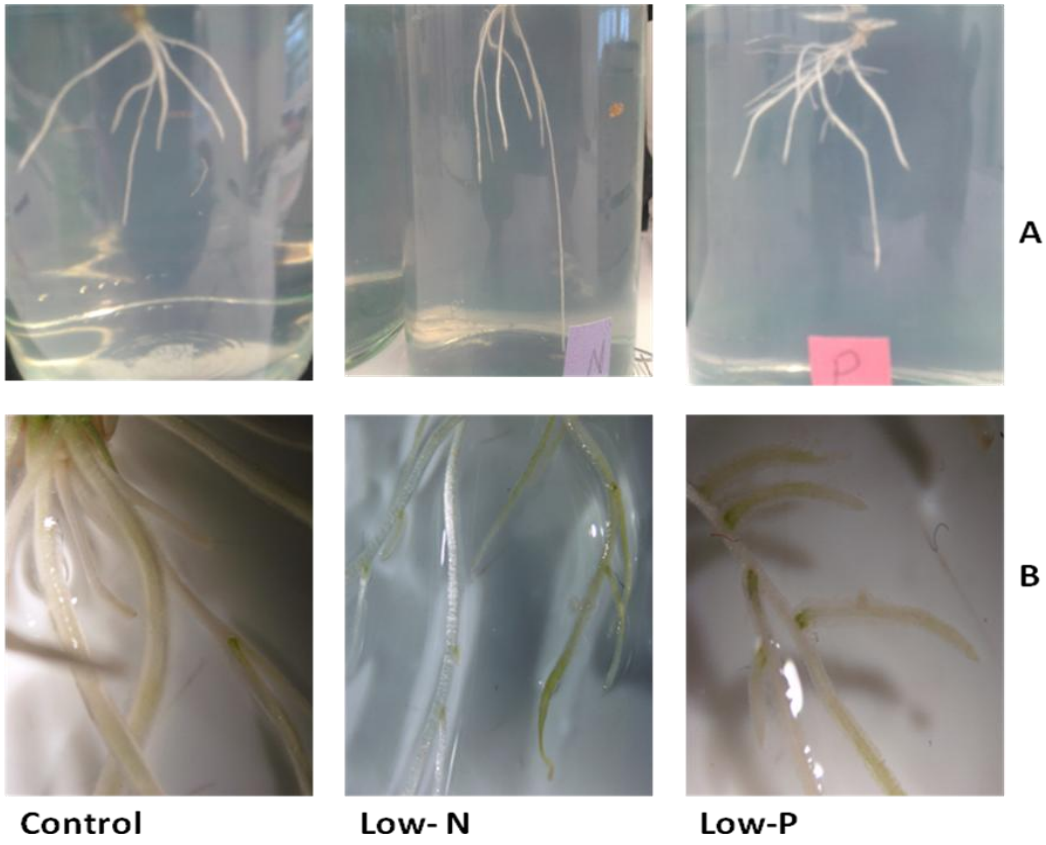


Figure 49. Analysis of root phenotypes propagated *in vitro* under N and P deprivation MS media in cv Karne A. Effect of N and P deficiency in root morphology grown in MS media in glass jars. B. Qualitative analysis of the root network was visualized under high magnification x12 light microscope.The red tape denotes low P and the purple is low N.

4..4.2 Root phenotype response to N and P deprivation in cv. Nguzo

Evaluation of cv. Nguzo root nework in response to N and P deficiency is shown in **Fig. 50** A. Under adequate nutrient supply Nguzo exhibited thin and elongated primary roots with moderate lateral root formation. Deficiency of N caused an increase in the number and elongation of primary and lateral roots. The lateral roots were also highly branched. A decrease in P in the media moderately repressed primary root elongation. The lateral roots were short and more branched compared to control treatment. The root network visualized under high magnification microscopy for each treatment is presented in **Fig. 50 B**.

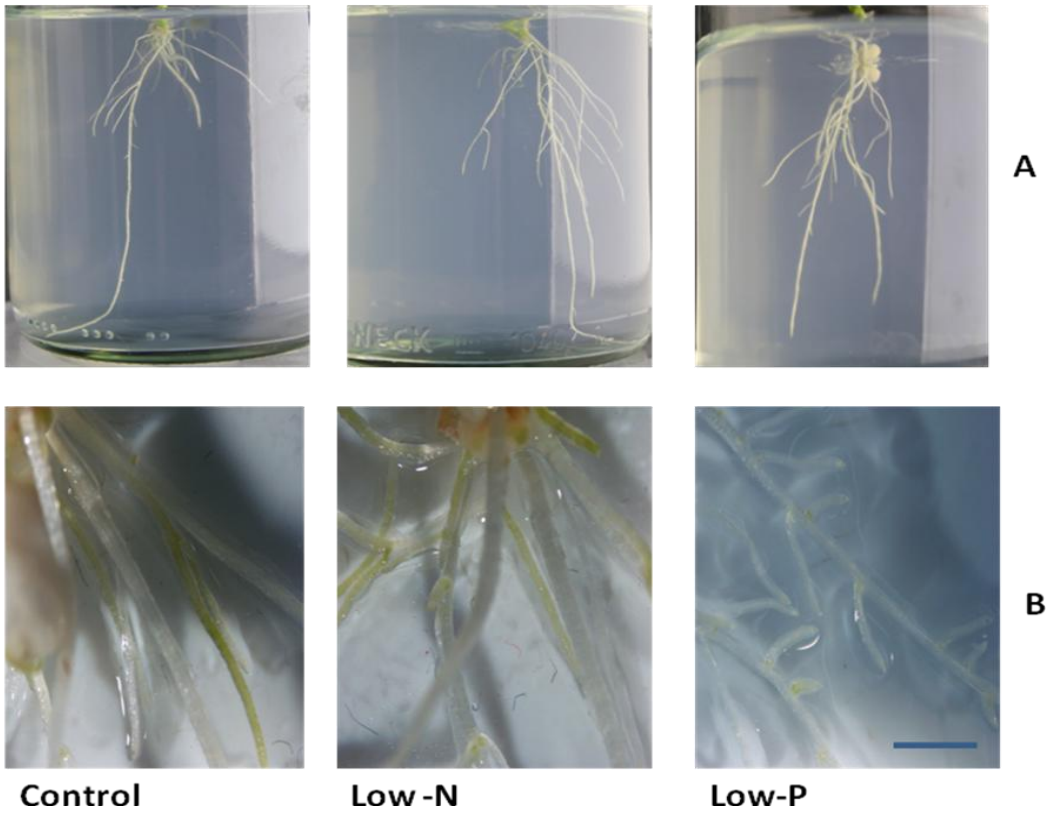


Figure 50. Analysis of root phenotypes propagated *in vitro* under control N and P deprivation MS media in cv Nguzo A. Effect of N and P deficiency in root morphology grown in MS media in glass jars. B. Qualitative analysis of the root network was visualized under high magnification x12 light microscope.

4..4.4.3 Root phenotype response to N and P deprivation in cv. HKBL 1385

In addition, cv. HKBL 1385 exhibited a lot of diversity in root system architecture in response the three treatments evaluated (**Fig. 51 A**). Under adequate nutrient supply the primary root decreased in length, lateral roots increased in number, were short and thick. However, under diminished N, the roots increased became thin elongated and a highly branched compared to the control treatment. A less intensive branching was observed under P deficiency. A qualitative assessment of root phenotypes under high magnification view is shown in **Fig. 51 B**.

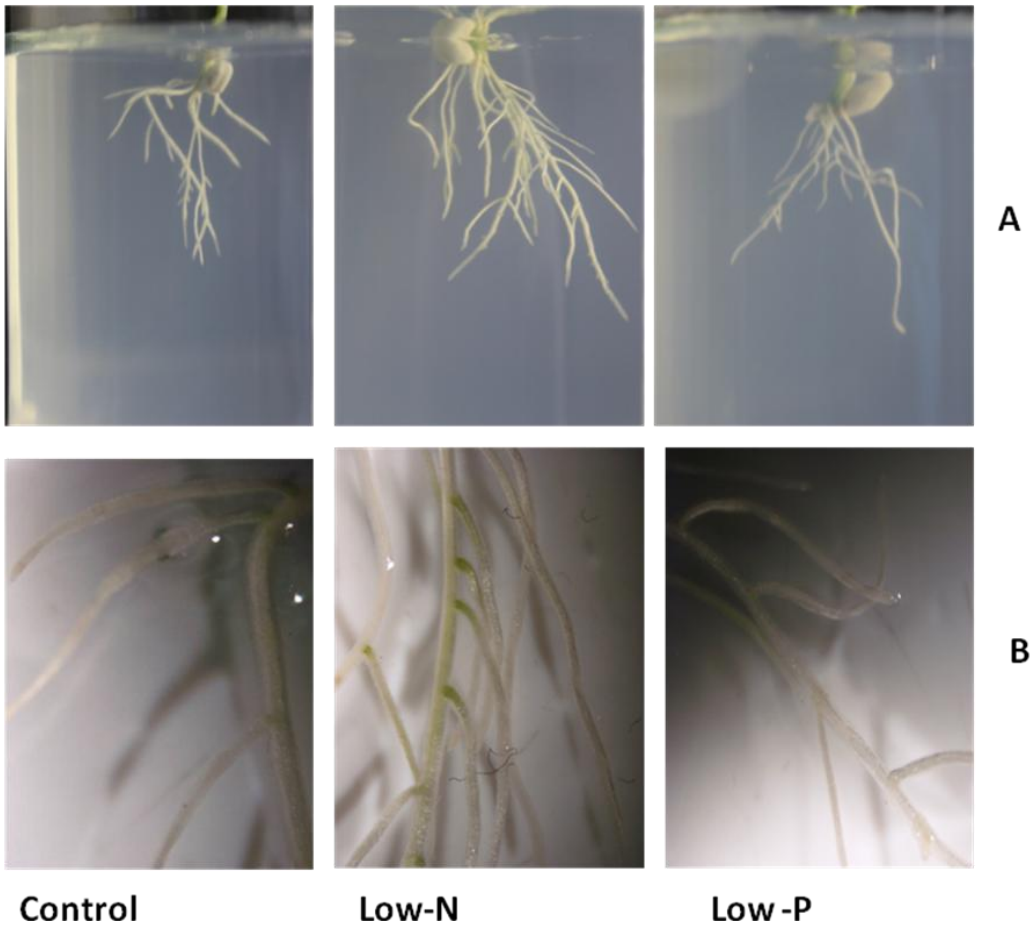


Figure 51. Analysis of root phenotypes propagated *in vitro* under control, N and P deprivation media in cv HKBL1385. A. Effect of N and P deficiency in root morphology grown in MS media in glass jars. B. Qualitative analysis of the root network was visualized under high magnification x12 light microscope.

4.6 Discussion

One of the mechanisms by which plants adjust to an imbalance of exogenous resources is by allocating new biomass to the organs that are involved in acquiring the resources that are limited (Marschner, 2011). These include indefinite proliferation of meristems cells, lateral root growth for enhance exploratory effect of root system and formation of root hairs to increase surface area of the meristem and lateral roots (Hermans et al., 2006).

In this study genotype response to N and P deprivation was evaluated in selected barley genotypes in two facets; pot-grown genotypes represented by Morex, Nguzo, MN-24, Karne, MN-12 and HKBL 1385 and *in vitro* propagated seedlings respresented by Karne, Nguzo and HKBL 1385. The root morphology traits in pot-grown seedlings were influenced by both genotype and treatment. Increased lateral root formation is an adaptive response to increase exploration capability of roots under limited nutrient availability. Lateral root formation was reduced under increased N in cvs. HKBL 1385, MN-12 and MN-24 but reduced under low P treatment in the same in the same genotypes. The alteration of the root-to-shoot growth ratio is a general adaptive response of plants to changes in nutrient availability. Formation of lateral roots is an adaptive response to enhance explorative capacity of roots under limiting nutrient supply (Forde and Lorenzo, 2001).

We report reduced lateral root length under conditions of both P and N abundance but enhanced under N and P deficiency. Increased elongation of lateral roots due to N deficiency was enhanced compared to P deficient treatment in both pot-grown (**Fig. 48 B**) and *in vitro* propagated barley seedlings in **Fig 52, 53** and **54**. This report concur with the findings of Linkohr et al. (2002) who observed lateral root elongation was suppressed by both high nitrate and high phosphate availability reflected at the variability in root phenotypes in Arabidopsis. Additionally Zhang and Forde. (1998) illustrated that that the lateral roots in Arabidopsis exhibited two contrasting responses. Under uniform high nitrate (10 mM) lateral root elongation was reduced throughout the root system whereas in plants grown on a low nitrate concentration (10 μM), exposure of a section of the primary root to high nitrate induced a local stimulation of lateral root elongation. In her recent studies Schaprian, (2015) reported that root elongation was inhibited under high N and low P concentration but highly increased under reduced N treatments in *in vitro* propagated European malting cultivars analyzed using the Software GiA Roots (Duke University, Durham NC, USA). Concomitantly with primary root growth inhibition,

lateral root formation and growth is enhanced by P starvation. However, the link between the two developmental responses is not clearly understood. It is postulated that the fluxes of phytohormone auxin involved in lateral roots development is altered during primary root growth arrest and as a result, more auxin could be made available to induce lateral root formation (Nacry et al., 2005, Péret et al., 2009). In plant nutrition root hairs are extensions of the root vital for nutrient and water uptake (Peterson and Stevens, 2000). Root hair length depends on species, genotype and status of the environment (Föhse et al., 1991 Gahoonia et al., 1997, Bates and Lynch, 2000). P and Fe deficiency trigger root hair formation of roots hair and is responsible for 90% P acquisition under limited availability.

In their work Gahoonia et al. (1997) identified barley and wheat genotypes with increased root hair density and length with associated enhanced P uptake under low P availability. In this study leaf pigmentation was more affected under N and P deficiency compared to control plants. A genotype dependent effect could not be established qualitatively but a low P caused increased chlorophyll damage than Low N (**Fig. 47**). It is well established that root system is correlated with agronomic productivity under limiting nutrient conditions. For example, deep rooting is associated with drought-tolerance in bean (Khan et al., 2010), wheat (Reynolds et al., 2007, Manschadi et al., 2008) and maize (Ribaut et al., 2009). We observed that the cv. HKBL 1385 displayed a strong and short intensive root sytem. In the previous studies in chapter 3, it displayed adaptability to low pH of 4.7 and recorded highest yield of 5.1 ton/ha compared to Nguzo (3.8) and HKBL 1512 (3.7) under field conditions. This cultivar recorded substantial yield in absence of exogenous N fertilizer suggesting that it has excellent N aquision mechanisms. The root system of Nguzo is elongated with intense lateral root network in response to low N. Nguzo was identified in various physiological studies in chapter 2 as highly tolerant cultivar displaying low ionic leakage and MDA as well as minimum and less chlorophyll damage when 14 barley cultivars were seedling were screened for salt stress tolerance. Reactions of roots system to other common stresses such has salt indicate that some tolerant plants such as halophytes have evolved Na^+ exlusion sequestration, therefore inhibiting Na^+ accumulation in the lateral roots (Vera-Estrella et al., 2005) and young leaves. Glycophytes however, can modulate root growth and root system architecture in response to salinity (Sun et al., 2008). Salt stress has an inhibitory effect on the elongation of primary roots but enhances lateral root development. It is therefore possible to use

variability in root phenotypes to screen plants phenotypes that can thrive under low N and P input environements.

5.0 General conclusion and the way forward

The current study in part attempted to identify key traits associated with physiological and molecular mechanisms that make some barley cultivars more resilient to abiotic stress than others. Measurements of changes in physiological parameters such as ionic membrane leakage, proline, chlorophyll, MDA sucrose and GB contents in response to short term exposure to increasing salt stress revealed genotypic responses that were dependent on duration of stress. Prolonged salt stress period (6 days) was better in discriminating against genotypes that were more susceptible versus tolerant to abiotic stress than short time (3 days). However, the sensitivity of one physiological assay to generate tolerant and susceptible clusters was not consistent across the genotypes. Transcript analysis of dehydrins genes revealed that SK_2 type *Dhn1* and a YSK_2 type *Dhn9* were dehydration responsive and could be used as potential markers for drought tolerance. In addition, downregulation of *Dhn3* and *Dhn4* transcript by salt stress was correlated with low accumulation of DHN proteins in Morex and Karne respectively. *Dhn3* and *Dhn4* genes are very close in the barley genome and displayed similar expression pattern in response to dehydration and salt stress. Future work on characterization of Kenyan barley may call for increase in the sample size of barley lines to be screened in order to increase the chances of identifying cultivars of both extremes.

Analysis of malting quality as function of fertilizer rate application showed that increased N application caused an increase in yield with concomitant rise in grain starch contant. Additionally, cultivars displayed variation in sensitivity to rates of N application and forms a basis for cultivar based fertilizer use and recommendation. Grain protein remained stable across the treatments since N application rate was based on soil analysis prior to sowing. Application rate of 30-40 kg/ha N did neither compromise yield nor present environmental risks. The potential use of the diversity of hordein polypetide fractions for cultivar identification was supported by the minimum effect caused by increase in N treatment. Hordein polypeptides genes were down regulated in the dry seeds. The need to conduct multi locational trials is necessary to test the perforamance of the same genotypes under different pH and precipitation conditions in order to ascertain the consistencies of the parameters. During germination, a upregulation of of *AMY1* transcript was correlated to high starch content in HKBL 1385 and Nguzo. In the root responses to N and P deprivation analyses, we conclude that improving the understanding of the root system response to abiotic stress can aid in identifying cultivars with enhanced

stress tolerance. However, much need to be unraveled to better understand molecular mechanisms in the nitrate and phosphate transporters in roots and leave in the putative tolerant and susceptible cultivars inorder to accelerate crops screening and development.

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