A physiological basis to crop improvement and agronomic development

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except as specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other university or similar institution. I further state that no substantial part of my dissertation has already been submitted for any such degree, diploma or other qualification at any university or similar institution. It does not exceed the prescribed word limit for the degree committee for Biology.

Celestin Ukozehasi

Dedication

This thesis is dedicated to the memory of my sister Furaha (1990 - 1994).

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"A marvelous view and breath of fresh air give hope as I am sitting on the top of a mountain. The satisfaction of having reached the top is great. The hike from the valley to the top was sometime tough. Climbing this mountain turned out to be quite an adventure. Just let me stay for a while, enjoying this view. At the other side I can see another mountain waiting for me to climb" (Bible: Psalms 121).

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Abbreviations and symbols

A	Rate of CO_2 assimilation
A_{max}	Maximum photosynthetic rate at saturating light and ambient CO_2
A_n	Net rate of CO_2 assimilation
ANOVA	Analysis of Variance
C_a	Atmospheric CO_2 concentration
CAM	Crassulacean Acid Metabolism
C_c	CO_2 concentration in the chloroplast
Chl	Chlorophyll
C_i	Intercellular CO_2 concentration
CIMMYT	International Maize and Wheat Improvement Centre
C:N	Carbon nitrogen ratio
CIP	Rwandan crop intensification programme
DI	Dual inlet
DM	Dry matter
DW	Dry weight
Eps	Earliness per se
E	Transpiration
ET	Evapo-transpiration
FAO	Food and Agricultural Organization of the United Nations
FW	Fresh weight
g	Diffusive conductance for CO_2 (g_c), and water vapour (g_w)
GA	Gibberellic Acid
GDP	Growth Domestic Product
g_s	Stomatal conductance
GS	Growth stage
G x E	Genotype by Environment interactions
HI	Harvest Index
IAEA	International Atomic Energy Agency
IFPRI	International Food Policy Research Institute
IRGA	Infrared gas analyzer
IVD	Inter-veins distance
JIC	John Innes Centre
kg	Kilogram
K_h	Leaf hydraulic conductance
K-S	Kolmogorrov-Smirnov
L	Length
LA	Leaf area
LAVD	Leaf-to-air vapor difference
LED	Light-emitting diodes
m	meter
mm	millimeter

MINAGRI	Ministry of Agriculture
MINECOFIN	Ministry of Finance and Economic Planning
MPa	MegaPascal
MRT	Mean Residence Time
MWL	Meteoric Water Line
N	Nitrogen
NIAB	National Institute of Agricultural Botany
NIH	Nitrogen Harvest Index
NILs	Near Isogenic Lines
NP	Nitrogen productivity
NISR	National Institute of Statistics of Rwanda
NUE	Nitrogen Use Efficiency
N_2	Atmospheric nitrogen
OM	Organic matter
PAR	Photosynthetic Active Radiation
PDB	A fossil Belemnite from the Pee Dee formation
Ped.	Peduncle
PGF	Plant growth facilities
pH	Negative logarithm of the hydrogen ion concentration in moles per liter; "p" refers to power of 10, "H" to hydrogen
ppm	parts per million
PRR	Pseudo response regulator
Rht	Reduced height
RWC	Relative Water Content
QTL	Quantitative trait loci
SD	Stomata Density
SLA	Specific leaf area
SMOW	Standard Mean Ocean Water
SPAD	Special Products Analysis Division (a division of Minolta)
TGW	Thousand grain weight
TW	Turgid weight
UCB	University of California at Berkeley
UK	United Kingdoms
UN	United Nations
USA	United States of America
WUE	Water Use Efficiency
WUE_i	Instantaneous Water Use Efficiency
Δ	Isotopic discrimination
Ψ_s	Soil water potential
Δ	Isotopic fractionation
~	About
$	US dollar
#	Number

Summary

Despite of the documented impacts of the so-called green revolution, food security in the world faces new challenges in terms of population growth, increases in no-agricultural land use (urbanization), and climate change. Trends in food security show that the world community is operating within two limits of food system: (i) the quantity of food that can be produced under a given climate; and (ii) the quantity of food needed by a growing and changing population. Therefore, taking food security successfully into the future requires novel approaches to boost agricultural productivity in order to balance food supply and demand without expanding the agricultural land.

To date, progress in wheat yield has been largely the result of the development of dwarf varieties through introgression of reduced height (*Rht)* genes. The height reductions arising from the presence of these genes increased yield by alteration of partitioning of dry matter and nitrogen in favour of the spike. However, increased partitioning through additional reductions in plant height is not likely; as comparative studies indicate that wheat yield is reduced when plants are shortened beyond a threshold, and most of the modern cultivars have reached the optimal height. Therefore, this dissertation aimed to identify the physiological attributes able to produce yield increases in the *Rht* genotypes with the optimal heights.

Approaches based on physiological understanding of yield are necessary for developing genotypes combining high yielding potential and agronomic traits of superior adaptation, and for understanding yield limiting factors. Yet, direct measurement of physiological variables is often difficult or expensive; as an example, measuring plant water status in the field is problematic, with techniques such as psychrometry generally only being suitable for laboratory studies. Therefore, proxy such as tissue RWC may be a good alternative measure of plant water status. We aimed to address these questions with three components of experimental research :(i) proxy-based screening to increased photosynthetic rate and water use efficiency in wheat; (ii) determinants of increased *HI* in lines with different *Rht* genes (*b*, *c*) when incorporated into contrasting background wheat genomes (*B, D*), and the relative effect on *N* partitioning during grain filling; (iii) analyses of stable isotopes (δ^2H, $\delta^{18}O$, $\delta^{15}N$ and $\delta^{13}C$) in an agronomic perspective in alley cropping systems associated with adjacent N_2 fixing trees, in terms of hydraulic redistribution, *N* availability and crop yields.

In this thesis, the proxy-based approach to crop selection was defined as a surrogate-based (proxy and surrogate used interchangeably) screening of cultivars for morphological, anatomical, and physiological traits of performance or crop environmental responses. The

research proposed steps for conducting a proxy-based crop selection programme. A comparative screening of 23 *Eps* cultivars and ranking for traits of photosynthetic and water use efficiency showed the correlative relationships of *SLA* to A_n, WUE_i, leaf *N*, $\Delta^{13}C$, K_h, leaf *RWC*, and *IVD*. Additionally, it was observed that *IVD* may influence *WUE* and A_{max}. It was suggested that these relationships of *SLA* to traits of photosynthesis possibly resulted from the association of *SLA* and the leaf biochemical characteristics.

Attention was also given to examining the mechanistic foundations that determine the relationship between plant height and yield. The results showed the straw-shortening significantly correlated both with A_{max} and K_h; and *SLA* decreased with the level of dwarfing; and the A_{max} related both K_h and *SLA*. Therefore, it was proposed that the straw-shortening may affects $A_{\max}$ by exerting a controlling influence over K_{h} through *SLA*. Moreover, both the partitioning of *N* to spike and the flag leaf *N* were related to plant height and growth stage. Additionally, the increased post-anthesis partitioning of *N* to grain associated with high *N* uptake rate and high *MRT* of *N* were probably the traits behind increased *NUE* and *NHI*. The data also indicated that increased grain number per spike, kernel weight and reduced peduncle length might be the driver of the increased *HI* in this experiment.

The test of the hypothesis that there might be practical application of the analyses of the natural abundance of stable isotopes (δ^2H, δ^{18}O, δ^{13}C, and δ^{15}N) and isotopic mixing model by *IsoSource* to understand plant interactions in terms of water redistribution and nitrogen transfer and uptake in agroforestry systems, indicated a consistent gradient in depletion of wheat xylem water δ^2H, δ^{18}O, and δ^{15}N in leaf as moving further away from the tree line. The data also reflected a consistent pattern of isotopic values (δ^2H, δ^{18}O, and δ^{15}N) in wheat in the proximity of the tree being similar to that of the tree, suggesting they were using the same source of water and *N*. Similarly, an isotopic mixing model data showed that the crops in the proximity of the trees accessed considerably amounts of the water and nitrogen redistributed by trees. The study also indicated the improvement in water use efficiency, chlorophyll content, grain number per spike, and grain yield for the crops nearest to the trees for a distance up to 5 m.

In conclusion, selection for increased *HI* should shift focus from reduced plant height to include increased grain number and kernel weight, increased partitioning of *N* to spike, reduced peduncle length, and low *SLA*. Finally, the hypothesis that efflux of water and *N* in agroforestry system from tree roots in topsoil and influences a number of physiological functions of neighbouring crops was confirmed by isotopic and physiological data.

Chapter 1 General introduction

"Things are complicated in this world and are determined by many different factors. We should look at a problem from different points of view and not from one point of view only" (Mao Tse-Tung, the Little Red Book).

1.1 Preamble

The United Nations (UN) forecasts the world human population will reach 9.4 billion by 2050 (Foulkes *et al.,* 2011). The world must therefore develop capacity to feed 10 billion within the next 40 years, and within the context of climate change (Cattivelli *et al.,* 2008; Hirel *et al.*, 2007). The climate change that is occurring over the years is partly responsible for modifying the crop production environment (Semenov *et al.,* 2012). Increased deviation in mean temperatures and precipitation are expected to dominate future changes in climate as they affect crop production (Hoffmann, 2011; Semenov & Halford, 2009; Jenkins *et al.,* 2009a; Solomon *et al.*, 2007). Consequently, it is expected that climate change could threaten food security in many areas of the World (Knox *et al.*, 2012).

Climate change could strongly affect the wheat crop that accounts for 21 % of food and 200 million hectares of farmland Worldwide (FAO, 2010), and constrain economic development in those countries that largely rely on agriculture. According to Hoffmann (2011), agriculture accounts for 20 to 60 % of GDP in most developing countries. Although wheat is traded internationally and developing countries are major importers (43 % of food imports), about 81 % of wheat consumed in the developing world is produced and utilized in the same countries (FAO, 2012; CIMMYT, 2005). This requires the breeding of new and high yielding cultivars of wheat that can resist various abiotic stresses or adapting existing cultivars to new production environments (Acquaah, 2008; Kurukulusuriya & Mendelsohn, 2008).

Global world demand for wheat is growing about 2 % per annum (Ortiz *et al.,* 2008). Meeting this demand will need to result from greater yield on existing croplands (Edgerton, 2009), because expanding agricultural production into remaining natural ecosystems is

environmentally unacceptable. Increases in wheat yield potential to date have resulted mostly from conventional breeding, and the contribution by physiology-driven breeding to date has been modest (Reynolds *et al.,* 2011; Braun *et al.,* 2010). However, there is now evidence that understanding traits at a physiological level could help to identify phenotypic interactions that could accelerate the progress of crop breeding (Shabala, 2013; Reynolds & Borlaug, 2006; Loss & Siddique, 1994).

1.2 Physiological approach to crop improvement: A conceptual background

While conventional plant breeding has relied heavily on empirical approaches to increase yield in the past, there is a broad consensus that strategies approaches based on sound physiological understanding of yield are also required if further yield gains are to be achieved (Fischer, 2011; Slafer & Araus, 2007; Jackson *et al.*,1996).

The knowledge of the physiological traits associated with gains in yield is essential to improve the understanding of yield limiting factors and to inform conventional breeding (Shearman *et al.,* 2005). There has been considerable discussion in the literature about the potential role of physiological research in crop breeding (Aisawi *et al.*, 2010; Slafer *et al.*, 2005). It is possible to argue that the physiological understanding of yield is of greater importance; According to Reynolds *et al.(*2011), yield progress from traditional breeding may be slowing and has become less efficient (in term of less progress per unit of breeding resources). It is also hard to see how functional genomics can deliver on yield or how genotype by environment interaction (G × E) can be elucidated without knowledge of physiological functions (Foulkes *et al.*, 2011).

1.2.1 The rationale for using physiological trait in crop breeding

Differences in developmental patterns among wheat are essential for improving adaptation and yield. Understanding the physiological basis of these traits is critical for their rational use in breeding. The word “trait” invokes several considerations; a trait may broadly be defined

to include developmental patterns, physiological processes, yield and its components, and plant environmental responses (Acquaah, 2008).

Crop growth and development depend on interaction of biochemical and physiological processes; the latter are under both genetic control and the influence of the environment, and the crop yield depends on the interaction between these processes (Acquaah, 2012). Therefore, improved knowledge of the factors involved in generating variation among cultivars would facilitate an efficient selection strategy.

Historically, the conventional approach to breeding, geared to cereal improvement, has been to start from yield and move towards underlying processes (Passioura, 1981; Acevedo & Ceccarelli, 1988; Jackson, 2001). Fischer (2011) named this strategy the "black box" and contrasted it to the "Ideotype" strategy which attempts to improve yield from understanding underlying processes. Of course, the challenge for conventional crop improvement has been to increase the precision of identifying the genotype of a complex trait; the degree of precision decreases with increased complexity of the character, and becomes very low for a highly complex trait such as yield (Reynolds *et al.*, 2011).

Therefore, the key question is whether selection for a given physiological trait as part of an integrated breeding approach could achieve results more quickly and efficiently than conventional breeding alone. It is already established that breeding for new cultivar with conventional breeding usually takes 10 to 12 years (Semenov & Halford, 2009; Acquaah, 2008). Many physiological traits appear to be of potential benefit to yield (Reynolds *et al.*, 2011). To assess which trait should be prioritized, alternate hypotheses may be tested empirically, based on conceptual understanding of physiological and biochemical constraints to performance. According to Jackson (2001), the following steps could be effective for incorporating physiological criteria into a breeding program;

i) Defining the yield limiting factors in the target growing environment

ii) Identification of physiological traits that may be used as indirect selection criteria

iii) Choosing the genotypes appropriate for evaluating trait expression

iv) Defining the protocol and measurement of trait expression and its association with performance

1.2.2 Defining the yield limiting factors in the target growing environment

Understanding of the factors limiting the performance of the genotype in the target environment is essential for improving breeding program through physiological research. It has been argued that the knowledge of limiting factors in the target environment would enable selection for cultivars that are most relevant to the target environment (Reynolds & Trethowan, 2007). Similarly, Cooper *et al. (*1995) suggested that an accurate defining feature of the target environment such as identifying constraints causing G x E interactions may lead to improved strategies of selection breeding.

Different approaches can be used to identify the factors limiting yield across the target environment (Jackson *et al.,* 1996): For instance qualitative local knowledge of the target environment may provide useful level of understanding. Similarly, agronomic trials could be used in which suspected limiting factors are manipulated to verify and quantify their effects (Nix, 1980). Climatic database of the region may add useful information such as rainfall variability, etc. Diseases screening trials could also be used (Jackson *et al.,* 1996).

1.2.3 Identification of physiological traits that may be used as indirect selection criteria

Identification of yield limiting factors may suggest the physiological traits that breeders could use as indirect selection traits. According to Jackson *et al.* (1996), to be of potential use in breeding, a trait must meet two broad criteria;

i) Evidence of genetic variability for a trait must exist

ii) Selection for a trait must be economically advantageous based on relative cost and benefits.

Additionally, Acquaah (2012) proposed that traits can be classified into two categories; (i) Simple traits associated with a particular morpho-physiological attribute, and (ii) integrative traits produced by the net effect of a number of simple traits. According to Reynolds *et al.(*2001) before the traits can be characterized, individual traits must be conceptualized and defined in terms of ; i) the stage of crop development they are pertinent, ii) the specific attributes of the target environment in which they are adaptive, and iii) their potential contribution to yield. Similarly, Jackson (2001) proposed two broad approaches to identify physiological traits to be used for crop improvement; i) Evaluating a set of genotypes for physiological performance in the presence of a known constraints of interest, and ii) Concurrently measuring putatively useful traits in the same genotype.

According to Reynolds *et al.* (2001), once identified, physiological traits affecting the response to a limiting factor may be used in two ways; i) as indirect selection criterion in core breeding programs, and ii) as selection criteria in introgression programs. Also, Fischer (2011) suggested that the use of physiological traits as indirect selection criteria should be based on their correlation both with the physiological performance of the crop and yield, their heritability, and their cost for measurement. The use of traits in association with yield as a combined selection index could also be considered.

1.2.4 Choosing the genotype appropriate for evaluating traits expression

The initial choice of germplasm is critical since conclusions will hinge on it being representative of breeding objectives (Ribaut *et al.,* 2001). According to Skovmand *et al.* (2001), the collection of germplasm may provide useful sources of genetic diversity, especially if the accessions originate from environments where yield constraints are similar to those in the target environment. Donor germplasm may also be identified outside locally adapted material being selected; for example, it may include improved material from other breeding programs which may have desirable characters, or material from related species.

Parent germplasm may also be either synthetically produced by artificially manipulating ploidy and backcrossing into elite lines or selected on the basis of a specific trait (Foulkes *et al.,* 2007). The number of lines to study must be sufficient to ensure a range of genetic diversity of the trait of interest.

1.2.5 Protocol and measurement of trait expression

The efficiency of selection for a physiological trait can be related to how accurate a trait is measured; thus, experimentation should take place to establish how and when measurement should be made to maximize the resolution for expression of the trait (Slafer *et al.,* 2007). The experiment should be managed optimally; because environmental factors such diseases, nutrient deficiency, and others, may affect the expression of physiological trait by genetic interaction by genotype (G × E). The experiment environment should also mimic the target environment factors. According to Hobbs & Sayre (2001), two groups of factors may interact with the expression of a trait;

i) Macro-environment (i.e., temperature, radiation, nutrient status, soil type, etc)

ii) Physiological factors (growth stages, small genetic diversity that may exist within fixed lines, phenology, etc).

Data should be collected to assess for consistent expression of traits of interest, and their association with physiological performance among genotypes. Multiple sampling would be necessary to reduce errors associated with measurement. A second phase of experiment may be needed to demonstrate a definitive genetic link between the trait and its performance in more closely related material such as homozygous sister lines. Finally, if the trait shows a strong association with the physiological performance, the nature of the association should also be examined. According to Jackson *et al.* (1996), the selection for specific trait both in the field and under controlled environments are likely to be more effective.

1.3 The reactions of photosynthesis in C_3 plant

The understanding of the process of photosynthesis is central to crop improvement for photosynthetic efficiency. The reactions that occur during photosynthesis involve two main processes: (i) light reactions, and (ii) carbon-fixation reactions.

1.3.1 The light reactions of photosynthesis

In the chloroplast, the pigment molecules (chlorophylls *a*, *b*, and carotenoids) are embedded in the thylakoids in discrete units of organization called "Photosystems". Two photosystems (PSI & PSII) are involved in the light reactions. The PSI and II, are spatially separated, and in work together simultaneously and continuously (Bruce *et al.*, 2010). The PSII is located primarily in the grana thylakoids; and the PSI is almost entirely in the stroma thylakoids and at the margins, or outer portions of either side of the grana thylakoids. The two photosystems are linked together by an electron transport chain.

Each photosystem consists of two closely linked components: an antenna complex, and a reaction centre. The reaction centre of each photosystem contains a special pair of chlorophyll *a* that is known as P_{700} and P_{680} in PSI and II respectively (The *"P"* stands for pigment, and the subscript *"700"* and *"680"* refers to the optimal absorption peak in nanometers). Each photosystem is also associated with a light-harvesting complex (but this does not contain a reaction centre).

In short, drawing from Taiz & Zeiger (2010), in PSII, light is absorbed by molecules of P_{680} in the reaction centre, either directly or indirectly by resonance energy transfer from antenna complex or light harvesting complex. When a P_{680} molecule is excited, its electron is transferred to a pheophytin, a modified chlorophyll *a* molecule. Pheophytin then passes the electron to PQ_A, a plastoquinone which is tightly bound to reaction centre. Next the PQ_A passes two electrons to PQ_B, another plastoquinone, which simultaneously picks up two protons from the stroma thereby becoming plastoquinol (PQ_BH_2). The PQ_BH_2 then joins a

pool of mobile plastoquinol molecules in the interior lipid portion of the thylakoid membrane, where it donates, one at a time, two electrons to the cytochrome b_6/f complex, and is oxidized back to PQ_B. The reduced cytochrome b_6/f donates the electrons to plastocyanin (a mobile electron carrier protein) in lumen. The protons released into thylakoid lumen via the cytochrome *b6/f* complex, and during the pumping of protons across the thylakoid membrane, generate an electrochemical proton gradient that drives the synthesis of *ATP* from *ADP* and P_i.

On the other hand, in *PSI*, light energy excites antenna molecules which pass the energy to the P_{700} molecules at the reaction centre. The excited P_{700} molecule passes electron to a special molecule called A_0. The electrons are then passed through a chain of carriers that includes phylloquinone (A_1), and Ferrodoxin. Electrons are transported from ferrodoxin to *NADP*, and this reduces *NADP* to *NADPH*. The electrons removed from the P_{700} molecule are replaced by electrons that are carried from *PSII* to *PSI* by plastocyanin.

1.3.2 The carbon fixation reaction

The *ATP* and *NADPH* generated by the light reactions are used to fix carbon to synthesize sugar. The reduction of carbon occurs in the stroma of chloroplast by means of reaction called "Calvin cycle". The starting and ending compound in the Calvin cycle is ribulose1,5 bisphosphate (*RuBP*). The cycle begins when three molecule of CO_2 enter the cycle and enzymatically (Rubisco catalyse this reaction) fixed to *RuBP*. The resultant 3 molecules of an unstable intermediate compound rapidly splits apart, and yield 6 molecules of phosphoglyceric acid (*PGA*), a three carbons compound. The 6 molecules of *PGA* are reduced to 6 molecules of phosphoglyceraldehyde (*PGAL*). Five of the 6 *PGAL* molecules are combined and rearranged to form three *RuBP*, the starting material. The extra molecule of *PGAL* is exported from chloroplast to the cytosol where through a series of reactions is

converted to sucrose, and which is exported from the leaf via vascular bundles to the other parts of the plant.

However, O_2 competes with CO_2 at the active site of *Rubisco* which also catalyzes the condensation of O_2 with *RuBP* to form one molecule of *PGA* and one of phosphoglycolate, in a process called photorespiration. Photorespiration is thought to be a wasteful process, yielding neither *ATP* nor *NADH*; and energy must be expended to salvage of the carbon in phosphoglycolate. The salvage involves the conversion of 2 molecules of phosphoglycolate into a molecule of amino acid serine and molecule of CO_2. The condensation of O_2 with *RuBP* occurs concurrently with CO_2. The conditions that alter the CO_2/O_2 ratio in favor of O_2 also induce condensation of O_2 (i.e. the close of stomata). It is argued that photorespiration acts to protect the photosynthetic apparatus from photoninhibition (i.e. when the leaf is exposed to more light than they can utilize) (Bruce *et al.*, 2010). Also photorespiration is the only way for the plant to remove phosphoglycolate, which is a toxic compound.

1.4 Fundamental for application of stable isotopes to physiological crop improvement

Over the past decades, there has been growing interest in the use of stable isotopes in plant physiological studies (Condon *et al.*, 2004; Unkovich *et al.*, 2001; Ehleringer *et al.*, 1993; Farquhar *et al.*, 1989; Richards & Caldwell, 1987). According to Griffiths *et al.* (1999), the natural abundance of stable isotopes can provide a quantitative framework for biological transformations and environmental influences on those processes.

1.4.1 Theoretical background for the use stable isotopes in plant physiology

The use of stable isotopes in plant physiological research requires knowledge of the fundamental principles of stable isotopes. According to Dowson & Brooks (2001), isotopes are nuclides of a single element that have different atomic weight. The word “nuclide” refers to any distinctive type of atom (Criss, 1999). An element can exist in several physically

distinguishable but chemically identical forms called isotopes, and each isotope having different number of neutrons but the same number of protons and electrons (i.e. ^{12}C, ^{13}C, ^{14}C). Nuclide may be classified into radioactive and stable (Michener & Lajtha, 2007); the radioactive nuclides are the ones that can decay into different forms of atoms. Stable nuclides do not decay, and may be subdivided into the radiogenic and the non-radiogenic stable nuclides, depending on their origins. According to Criss (1999), different processes can cause the stable non-radiogenic isotopes to vary in abundance;

i) Diffusion can produce abundance variations among the isotopes of any element simply because the various isotopes have different masses.

ii) Evaporation is another process that can cause isotopic fractionation.

iii) Through metabolic reactions, organisms also produce isotopic fractionations.

iv) Isotopic fractionations can also occur in a system that comprises two or more phases that contain a common element (i.e. ^{13}C is enriched during dissolution of CO_2 in water relative to air)

1.4.1.1 The carbon isotope effects

The isotopes are unevenly distributed among and within different compounds (i.e. most isotopes of carbon are 98.9 % of ^{12}C, with 1.1 % being ^{13}C), and this distribution can reveal information about the physical, chemical and metabolic processes involved in the isotope transformations. Variation in the $^{13}C/^{12}C$ ratio is the consequence of "isotope effects" (Farquhar *et al.,* 1989) which are expressed during the formation and destruction of bonds involving a carbon atom, or because of other processes that are affected by mass such as gas diffusion.

According to Farquhar *et al.* (1989) isotope effects are classified as being either kinetic or thermodynamic: The kinetic effect is the process that discriminates against the heavier isotope while the thermodynamic effects represent the balance of two kinetic effects at

chemical equilibrium in a system. Thermodynamic effects, like kinetic ones are temperature dependent. One example of kinetic effect is the difference between the binary diffusivity of $^{13}CO_2$ and that of $^{12}CO_2$ in air. One the other hand, an example of thermodynamic effect is the unequal distribution of isotope species among phases in a system (i.e. in CO_2 in air versus in CO_2 in solution).

The carbon isotope effects, denoted by α, are also called fractionation factors (O'Leary, 1993), and they are defined as the ratio of carbon isotope ratios in reactant and product:

$$\alpha = R_r/R_p \qquad (1.1)$$

where R_r is the $^{13}C/^{12}C$ molar ratio of reactant and R_p is that of the product.

1.4.1.2 Notation and standards

The analysis of stable isotopes is expressed in a differential notation, based on the comparison of mass spectrometric of the quotient of heavy to light isotopes for sample and a defined standard (Griffiths, 1998). Originally, Farquhar & Richards (1984) proposed that whole plant processes should be analyzed in the same terms as chemical processes (formula 1.1), however, because the absolute isotopic composition of a sample is not easy to measure directly (Farquhar *et al.*, 1989), rather, the mass spectrometer measures the deviation of the isotopic composition of the material from a standard.

According to Farquhar *et al.*(1989), the stable isotopes are reported as the measured difference in the isotopic composition of the sample χ and an accepted standard, and in term of dimensionless of δ values, termed "delta-values", defined by the formula;

$$\delta = \{R\ (\text{sample}) / R\ (\text{standard}) - 1\} \times 1000 \qquad (1.2)$$

Where the R values refer to the isotopes ratio; for instance $^{13}C/^{12}C$, and depending on the element of interest, this formula may define for example the $\delta^{13}C$, or $\delta^{18}O$. The factor of 1000 converts the δ value to per mil (‰).

The standards and their absolute values for the isotope used in this thesis are given (tab.1.1). The original standard for carbon and oxygen isotopes in CO_2 or carbonates was a fossil belemnite from the Pee Dee formation (PDB) which is no longer available but replaced by Vienna PDB (Ehleringer *et al.,* 1993). The standard for hydrogen and oxygen isotopes was the standard mean ocean water (SMOW) but has been replaced by Vienna-SMOW (Mook, 2001). The $^{15}N/^{14}N$ ratio of air is used to calibrate nitrogen isotope data (Criss, 1999).

Table 1.1 *Standards and their absolute abundance (Source: Dawson et al., 2002)*

Element	Isotope	Percent abundance	Ratio measured	Standard	Abundance ratio of standard
Hydrogen	^{1}H	99.984			
	^{2}H	0.0156	$^{2}H/^{1}H$	V-SMOW	1.5575×10^{-4}
Carbon	^{12}C	98.982			
	^{13}C	1.108	$^{13}C/^{12}C$	V-PDB	1.1237×10^{-2}
Nitrogen	^{14}N	99.63			
	^{15}N	0.3663	$^{15}N/^{14}N$	N_2	3.6764×10^{-3}
Oxygen	^{16}O	99.759			
	^{17}O	0.037		V-SMOW	2.0052×10^{-3}
	^{18}O	0.204	$^{18}O/^{16}O$	V-PDB	2.0672×10^{-3}

Farquhar & Richards (1984) proposed the use of Δ as the measure of the carbon isotope discrimination by the plant: they argued the Δ directly expresses the consequences of biological processes whereas composition δ_p is the result of both source isotopic composition and carbon discrimination. According to O'Leary (1993), the $\Delta^{13}C$ is computed as;

$$\Delta^{13}C = (\delta^{13}C_a - \delta^{13}C_p) / (1+\delta^{13}C_p / 1000) \quad (1.3)$$

Where $\delta^{13}C_a$ is the $\delta^{13}C$ value of air, and $\delta^{13}C_p$ is that of the sample. On the PDB scale, the carbon dioxide in air has a δ value of approximately - 8 ‰ (Ehleringer *et al.,* 1993). However, this value changes slowly becoming depleted in ^{13}C as consequence of anthropogenic emission. For example, from 1956 to 2007, the $\delta^{13}C$ in air has decreased from - 6.7 to - 8.1 ‰ (Keeling *et al.,* 1979; Michener & Lajtha, 2007).

1.4.2 Physiological basis of ^{13}C discrimination in C_3 plants

Carbon isotope composition of plants was first used to indicate photosynthetic pathway in plants (Bender, 1971): this is because phosphoenolpyruvate carboxylase, the primary carboxylating enzyme in species having a C_4 metabolism, exhibits a different intrinsic kinetic isotope effects and utilizes a different species of inorganic carbon that has isotopic composition at equilibrium different from that of Rubisco. The results of such survey have provided a broad base of the distribution of photosynthetic pathways among different phylogenetic groups and ecological zones (O'Leary, 1981).

According to Taiz & Zeiger (2010), plant leaves discriminate against ^{13}C though the chemical properties of ^{13}C are identical those of ^{12}C. Early workers recognized that diffusion and carboxylation were likely to be the principal causes of carbon isotope discrimination in plants (Farquhar *et al.,* 1982, 1989). A key to all these works has been the recognition that the carbon isotope fractionation in plant is related to C_i, the CO_2 in the intercellular air spaces of leaf.

According to O'Leary (1993), the carbon isotope fractionation in C_3 plants can be pictured by the relative limitation imposed during two steps of CO_2 uptake;

i) Diffusion of CO_2 into the leaf

ii) Fractionation at carboxylation site

In the first step, CO_2 diffuses from air into the leaf through the stomata to carboxylation site, whereby for a typical stomatal conductance, for every three CO_2 molecules entering a leaf, two retro-diffuse; because $^{12}CO_2$ is lighter than $^{13}CO_2$, it diffuses slightly faster toward the carboxylation site, creating a diffusion fractionation factor of - 4.4 ‰. In the second step, this CO_2 is taken up irreversibly by Rubisco, and which has an intrinsic discrimination value against ^{13}C of around - 30 ‰ (Taiz & Zeiger, 2010). However, two limiting cases can be considered (Ehleringer *et al.,* 1993); first, if the stomata are nearly closed, for instance in the

case of when leaves are exposed to water stress, the overall CO_2 uptake rate is limited by the initial diffusion process and the internal CO_2 concentration is low; in this circumstance, the carboxylation process takes up relatively more of the carbon species available, and the carboxylation isotope fractionation is not fully expressed. In this case, the isotope fractionation is reduced, theoretically approaching - 4.4 ‰ at very small apertures. Thus, for a C_3 plant, the $\delta^{13}C$ in this case should approach -12 ‰ (- 8 + - 4.4). On the other hand, if diffusion were infinite the stomata are relatively open, the internal CO_2 concentration approaches the external CO_2 concentration, and there is a facile transfer of CO_2 between the external and internal pool, allowing maximal retro-diffusion of ^{13}C. In this case, the diffusion approaches equilibrium and the observed fractionation approaches the carboxylation fractionation, and the leaf $\delta^{13}C$ of C_3 would approach - 38 ‰ (-8 + - 30). Real C_3 plants show behaviours intermediate between these two extremes.

According to Farquhar *et al.* (1989), the carbon isotope of a C_3 leaf plant can be predicted as;

$$\delta^{13}C_L = \delta^{13}C_a - a - (b - a)\,(C_i/C_a) \qquad (1.4)$$

where $\delta^{13}C_L$ and $\delta^{13}C_a$ are the carbon isotope of the leaf and atmosphere, respectively; a is the fractionation due to diffusion (- 4.4 ‰); b is the fractionation due to carboxylation (- 30 ‰); and C_i/C_a is the ratio of intercellular to ambient CO_2 concentrations.

The application of carbon isotope to plant physiology has become very productive, because equation (1.4) provides a link between the carbon isotope measurement and the intercellular CO_2 value in a leaf. Intercellular CO_2 levels are then directly linked with aspects of photosynthesis and stomatal constraints. As stomata close in C_3 plants or as water stress increases, the leaf carbon isotope is found to increase (Taiz & Zeiger, 2010). The carbon isotope measurement then becomes a direct proxy to estimate several aspects of water stress (Farquhar *et al.,* 1989). These applications include using isotopes to study plant performance

in both agricultural and ecological studies (Ehleringer *et al.,* 1993; Dawson *et al.,* 2002; Bowling *et al.,* 2008).

1.4.2.1 Source of variation of ^{13}C in C_3 plants

The major processes contributing to carbon isotope fractionation in plants are CO_2 diffusion and carboxylation (Farquhar *et al.,* 1989; Ehleringer *et al.,* 1993; Taiz & Zeiger, 2010). Additionally, the fractionation dues to the diffusion through the boundary layer of air above the leaf surface can also be considered as distinct from the effects of the diffusion through stomata and in the mesophyll cells (Vogel, 1993), although effects are small and largely ignored.

Cultivar variation in carbon isotopic composition is known to exist within crop species (Hubick & Gibson, 1993; Hall *et al.,* 1993; Condon *et al.,* 2004; Rebetzke *et al.,* 2006). The genetic control of $\Delta^{13}C$ appears to be strong in wheat; Condon *et al.* (2004) showed that genetic ranking was maintained at different sites and between plant grown in pots and in the field. Their work also indicated that broad sense heritability (proportion of total variance) of $\Delta^{13}C$ that can be ascribed to genotype, rather than to environment or to interactions between the two (G × E) ranged between 60 and 90 %, which suggests that $\Delta^{13}C$ is a trait with genetic control for which crop improvement strategies could readily exploit. Stable carbon isotope composition also varies among plant tissues (O' Leary, 1993); they argued that some of this variation is due to differences among the chemical components of plant tissue; for example, lipids can be as much as 10 ‰ lighter than the whole tissue (O' Leary, 1993; Badeck *et al.,* 2005); In contrast, cellulose and other carbohydrates are typically 1 to 2 ‰ heavier than whole tissue (Leavitt & Long, 1986), and lignin is typically 1 to 2 ‰ lighter; and the isotopic composition of tree rings is often enriched by 1.5 to 2 ‰ relative to foliage (Leavitt & Long, 1986). Other physiological and environmental factors can also affect the carbon isotope of C_3 leaf plant (Griffiths, 1999). One of emergent environmental pattern is that, isotopic

composition of the atmosphere affects the isotopic composition of the leaf plant (Farquhar & Lloyd, 1993); the $\delta^{13}C$ of atmospheric CO_2 is close to - 8 ‰. This value is slowly becoming more negative as the atmosphere becomes depleted in ^{13}C relative to ^{12}C. This depletion has arisen as a consequence of the anthropogenic emissions. The soil respiration (depleted in ^{13}C) can also affect the ^{13}C of the leaf produced near the soil surface as a result of abrupt decline in wind speed under closed canopy (Farquhar *et al.*, 1989; Jackson *et al.*, 1993). Latitudinal gradient in $\delta^{13}C$ of atmosphere has also been shown to exist, with $\delta^{13}C_a$ at 60^0 N being 0.2 ‰ more negative than that 60^0 S (Keeling *et al.*, 1989 cited in Ehleringer *et al.*, 1993). In global surveys of $\delta^{13}C$ over altitudinal gradients, Körner *et al.* (1988, 1991) found that plant $\delta^{13}C$ increased with altitude. Moorcroft & Woodward (1990) attributed the altitudinal gradient primarily to temperature effects on gas exchange, based on extrapolations from controlled environment studies. Similarly, consistent altitudinal gradients in plants ^{13}C were observed by Marshall & Zhang (1994); they observed that water use efficiency increased threefold over 2000 m altitude.

According to Lambers *et al.* (2008), the $\delta^{13}C$ was found to be less negative in the desert plants than in mesic plants, and in tissue produced during dry seasons. They also observed that annuals fractionate more strongly against ^{13}C than perennials; and, herbs fractionate more than grasses. Soil fertility, particularly the mineral nutrition of nitrogen, can affect the biochemical machinery for photosynthetic CO_2 assimilation (Farquhar, 1989); for example both Fu & Ehleringer (1992) and Fu *et al* (1993), observed that both in pot and field grown plants in high fertilizer treatment had significantly lower $\Delta^{13}C$ value than those grown at lower fertilizer level.

1.4.2.2 Implication of $\Delta^{13}C$ for C_3 crop improvement

The use $\Delta^{13}C$ as a proxy of long term water use efficiency (WUE) by C_3 plants has now been routine for more than a decade (Ehleringer, 1989, 1991; Ehleringer & Osmond, 1989;

Ehleringer *et al.*, 1993; Condon *et al.*, 2004; Rebetzke *et al.*, 2006). Recently, two new wheat varieties (Drysdale & Rees) have been brought to market in Australia using selection for drought tolerance and yield stability informed by $\Delta^{13}C$ as proxy for water use efficiency (Condon *et al.*, 2004; Passioura, 2006).

Traditionally, WUE has been defined as the ratio of net photosynthesis to transpiration (*A/E*). Farquhar *et al.* (1982) demonstrated that $\delta^{13}C$ in C_3 plant leaf provides a reliable index of water use efficiency because both WUE and $\delta^{13}C$ are controlled by intercellular CO_2 levels. This relationship can be described as with the formula (1.4) above. It was noted earlier that $\delta^{13}C$ is also related to WUE, and the relationship can be described as (Michener & Lajtha, 2007);

$$A = (C_a - C_i)\, g / 1.53 \qquad (1.5)$$

$$E = g\,(LAVD) \qquad (1.6)$$

$$WUE = (C_a - C_i) / [1.53(LAVD)] \qquad (1.7)$$

Where *A* is the net photosynthesis rate, *E* is the transpiration rate, *g* is stomatal conductance to water vapor, 1.53 is the ratio of diffusivities of water vapor and CO_2 in air (Campbell & Norman, 1998), and leaf-to-air vapor difference (*LAVD*) is the difference in water vapor concentration between the interior of the leaf and the surrounding atmosphere (Farquhar & Richard, 1984).

Because C_a is nearly constant in the atmosphere within a given year, WUE varies primarily with C_i and *LAVD*. If *LAVD* can be assumed constant among plant being considered at a given site, then plant $\delta^{13}C$ would be linearly be correlated with WUE. However, both environmental and anatomical factors (irradiance, soil moisture, salinity, air pollution, mesophyll conductance) have been recognized to influence the value carbon discrimination by plants (Griffiths, 1999; Farquhar *et al.*, 1989), therefore $\delta^{13}C$ can only relate to WUE in a comparative sense for crop under equivalent period, seasons, and environment conditions.

For instance Goldstein *et al.* (1989) proposed that when *LAVD* cannot be assumed constant or unknown, the intrinsic water use efficiency (*A/g*) can be inferred from isotope;

$$A/g = (C_a - C_i)/1.53 \quad (1.8)$$

1.4.3 Fundamentals of application of stable isotopes of ^{2}H and ^{18}O to plant hydraulic lift

Oxygen-18 and deuterium occur in water at abundances of 0.204 % of all oxygen atoms and 0.015 % of all hydrogen atoms, respectively (Clark & Fritz, 1997). These relative abundances change slightly as a result of the isotopic fractionations that accompany the evaporation from the ocean and other surface water and the reverse process of rain formation (*fig.1.1*). A correlation between $\delta^{2}H$ and $\delta^{18}O$ in fresh water was established, and the locus line of such sample in δ, and which is now known as the global meteoric water line and was defined by Gat *et al. (*2001) as;

$$\delta^{2}H = 8\ \delta^{18}O + 10\ ‰ \quad (1.9)$$

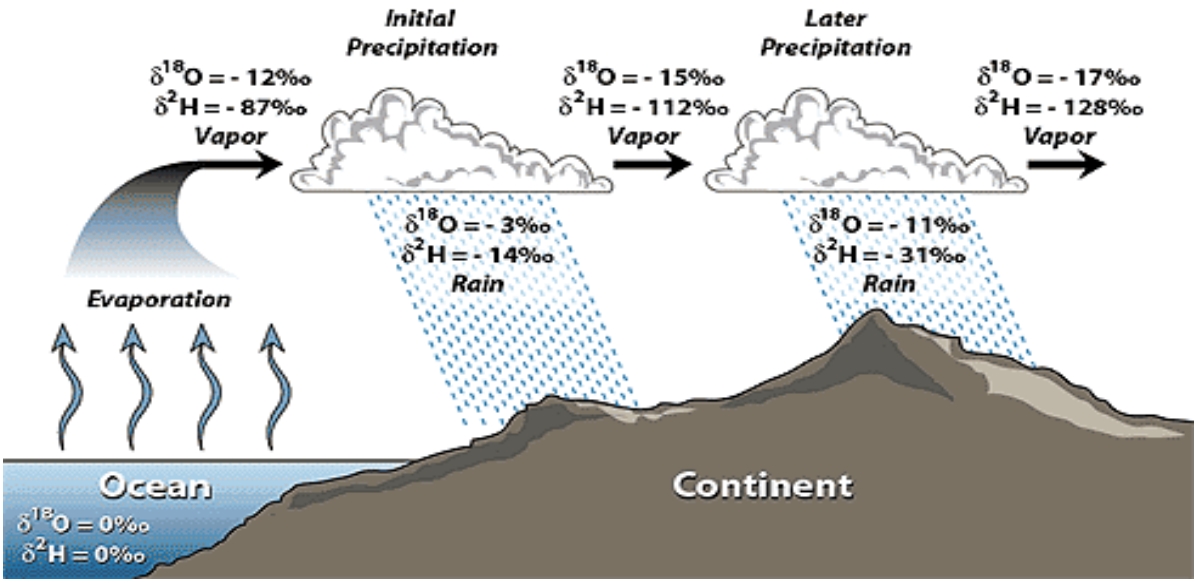


Figure 1.1 *Isotope fractionation that accompany evaporation of ocean & rain formation (Source:*Gat *et al.,* 2001*)*

The $\delta^{18}O$ and $\delta^{2}H$ isotope composition of plant are predominantly determined by the isotopic composition of water source (Dawson *et al.,* 2001), thus the processes that affect the isotopic composition of water source may influence the hydrogen and oxygen isotopic composition of

the plant. According to Marshall *et al.* (2007), the main sources of isotopic variation in plant water come from isotopic variation in precipitation, surface and soil water, and evaporation from the leaf surface during transpiration.

1.4.3.1 Isotopic fractionation associated with evaporation of surface water

Water –air interaction balances two opposing water fluxes: one upward from the surface and the other a downward one of atmospheric moisture (Gat *et al.,* 2001). At saturation (i.e. when the atmospheric humidity is 100 %), this interaction would bring the liquid water and air humidity into isotopic equilibrium with one another; for instance such a situation would occur at cloud base between the falling rain droplets and ascending air. When the air is unsaturated, a net evaporation flux results, in which the rate determining step is the diffusion of water vapor across the air boundary layer in response to the humidity gradient between the surface and the fully turbulent ambient air (Gat, 1996).

According to Mook (2001), three factors are involved in determining the overall isotope fractionation of surface water;

i) The equilibrium isotope fractionation of the liquid to vapor

ii) Fractionation resulting from the diffusion across the air boundary layer

iii) The back flux of the atmospheric moisture

1.4.3.2 Source of variation for isotopic composition of precipitation

An understanding of the process that controls the isotopic composition of precipitation is necessary for application of $\delta^{18}O$ and $\delta^{2}H$ to plant physiological research. According to McGuire & McDonnell (2007), the isotopic composition of precipitation is dependent upon several factors including isotopic composition of its vapor source (from ocean regions), fractionation that occurs as water evaporates into the air mass, rain formation processes, and air mass trajectory (*fig.1.1*).

The water cycle can be described by its marine part, which accounts for 90 % of the water flux (Mook, 2001). The isotope data of marine precipitation collected by the International Atomic Energy Agency (IAEA) show that isotope for marine precipitation for $\delta^{18}O$ is in the range of - 2.5 and - 3.0 ‰ , and with variable values for $\delta^{2}H$ of about - 14 ‰. As the marine air moves over the coast and across continental land masses, precipitation is initially closely aligned along the so-called Meteoric Water line (MWL)($\delta^{2}H = 8\ \delta^{18}O + 10$ ‰). Precipitation signals reflect isotopic effects (Michener & Lajtha, 2007), of which five have been recognized to determine the depletion in isotope value of continental precipitation, including altitude effect, distance from coast (continental effect), latitude effect, amount effect, and temperature as the overriding factor.

There are other factors to explain isotopic variation of precipitation (*fig.1.2*). Generally, more depleted isotopic values are found in winter rain, and enriched precipitation in the summer (*Gat et al.,* 2001). Precipitation also interact with plant covers as it fall, which can alter the isotopic signal of precipitation reaching the soil (Mook, 2001; *fig.1.2*); this could happens when part of the incoming precipitation is intercepted on the leaves of plant, and in part lost by evaporation; if further rain follow before the leaf is dried up, this can flush the enriched residual of the partially evaporated water to the ground.

1.4.3.2.1 The altitude effect

An altitudinal effect on the isotopic composition of precipitation has been reported in many studies conducted by IAEA around the World and has been found to vary from - 0.15 to - 0.6 ‰ per 100 m increase in elevation, and - 1 to - 4 ‰ per 100 m increase in elevation for $\delta^{18}O$ and $\delta^{2}H$, respectively (McGuire & McDonnell, 2007). For instance, detailed measurements in the western Oregon, USA, showed that $\delta^{18}O$ from individual rainfall event was strongly elevation dependent (- 0.22 to – 0.32 ‰ per 100 m increase in elevation) and that elevation explained between 63 and 89 % of the variance (McGuire *et al.,* 2005).

According to Mook (2001), the isotopic composition of precipitation changes with the altitude of the terrain and becomes more and more depleted in ^{18}O and ^{2}H at higher elevations. The altitude effect is temperature related, because the condensation is caused by the temperature drop due to the increasing altitude. Another factor associated with altitude effect is the evaporative enrichment of $\delta^{18}O$ and $\delta^{2}H$ in raindrops during their fall beneath the cloud base, which is large at low altitude where the cloud base is high above ground level (Gat, 1996).

1.4.3.2.2 Continental effect

The continental effect, also referred to as "the distance-from-coast effect" influences the isotope variation of precipitation; IAEA observed around the World, progressive $\delta^{18}O$ depletion in precipitation with increasing distance from the ocean; for example precipitation sample collected along west to East transect in Oregon , USA, showed an isotopic depletion in $\delta^{18}O$ of about - 1.5 ‰ per 100 km (Welker, 2000).

The continental effect has also been found to correlate with the temperature gradient and depends both on the topography and the climate regime (Gat *et al.,* 2001). According to Mook (2001), the extent to which a continental effect occurs depends also on the prevailing direction of the movement of air masses.

1.4.3.2.3 The latitude effect

The latitude effect is responsible for isotopic variations caused by progressive cooler temperatures that air masses encounter as they proceed from equatorial regions to higher latitudes with lower temperatures (McGuire & McDonnell, 2007). According to IAEA reports (Gat *et al.,* 2001), the latitude effect on $\delta^{18}O$ is ~ - 0.6 ‰ per degree of latitude for coastal and continental stations in Europe and USA, and up to - 2 ‰ per degree of latitude in the cooler Antarctic continent.

1.4.3.2.4 Amount effect

A relationship between the amount of precipitation and ^{18}O has been observed; small rains events are as a rule enriched in the heavy isotope than larger storms (Gat *et al.,* 1996). The amount effect has been attributed to evaporation and isotopic exchange of descending raindrops with atmospheric moisture, which affect more the rainfall of low intensity and low amount than a large storm. As the rainfall proceeds, humidity beneath the cloud base increases through time, reducing the evaporation loss of the raindrops; thus during long periods of rainfall, enrichment is less overall, and also because a greater proportion of the overall vapour is lost.

1.4.3.3 Soil water isotope composition

Water infiltrating from the surface drains through the void space in the soil. According to Mook (2001), the retention of water in the soil column and transport through it, does not, in itself affect the isotopic composition of the infiltrating water (*fig.1.2)*. However, when evaporation from within the topsoil occurs during dry intervals between rain events, this results in an enrichment of the heavy isotopes in the residual water. At some depth beneath the surface an evaporative front develops; above it water transport is predominantly in the gaseous phase; below it flow and diffusion in the water filled pore space dominate. An isotopic concentration profile develops, representing a balance between the upward convective flux and the downward diffusion of the evaporative signatures (Barnes & Allison, 1988). These enriched waters are then flushed down by subsequent rains, importing their evaporative isotope signature to the deeper soil water and groundwater (Clark & Fritz, 1997).

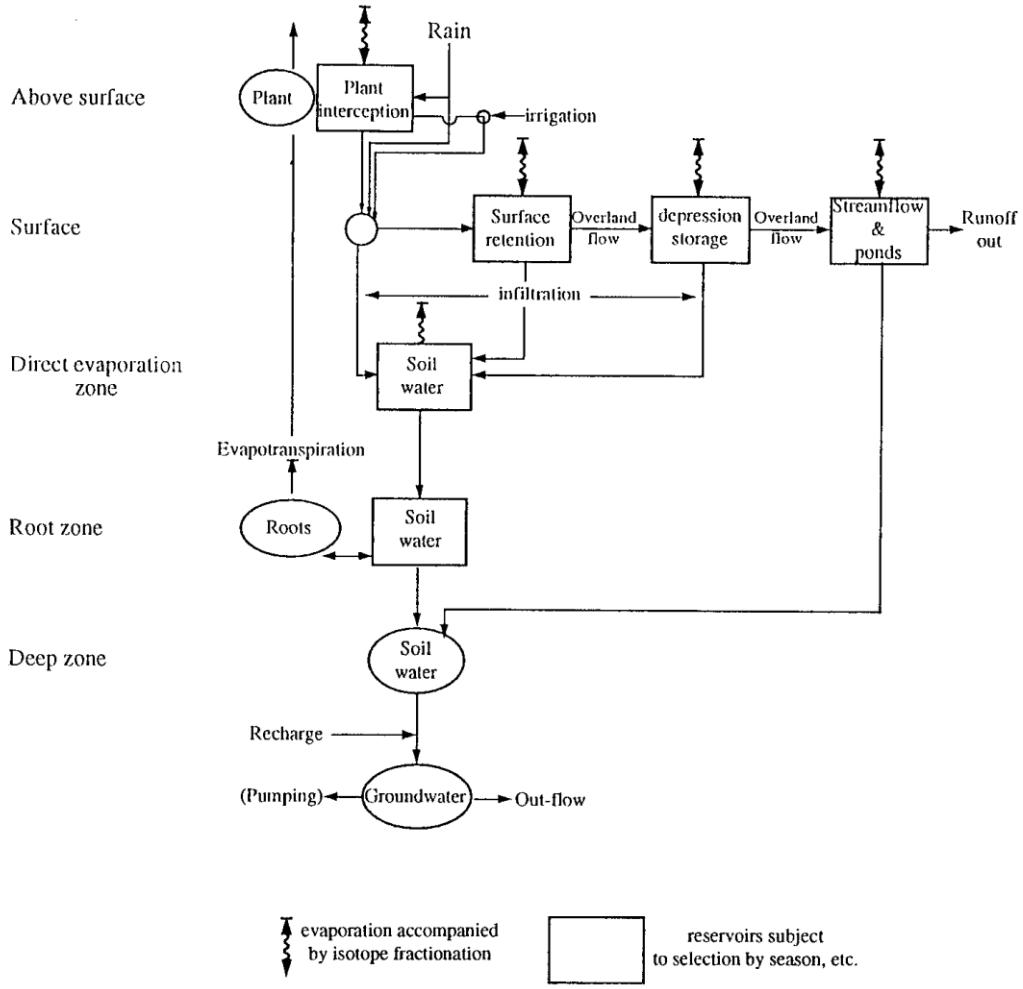


Figure 1.2 *Surface interception of precipitation and its movement into soil (Source:* Gat, 1996*)*

1.4.3.2 Isotope signatures of plant water

Water is not isotopically fractionated when taken up by plant (Dawson & Ehleringer, 1993). As a result, water in plant xylem carries the same isotopic signatures as the source water in the soil it is derived from, until it reaches the leaf (*fig.1.3*). Therefore, the isotope ratio of xylem water can be used as measure of the isotope of the main source of water being used by the plant.

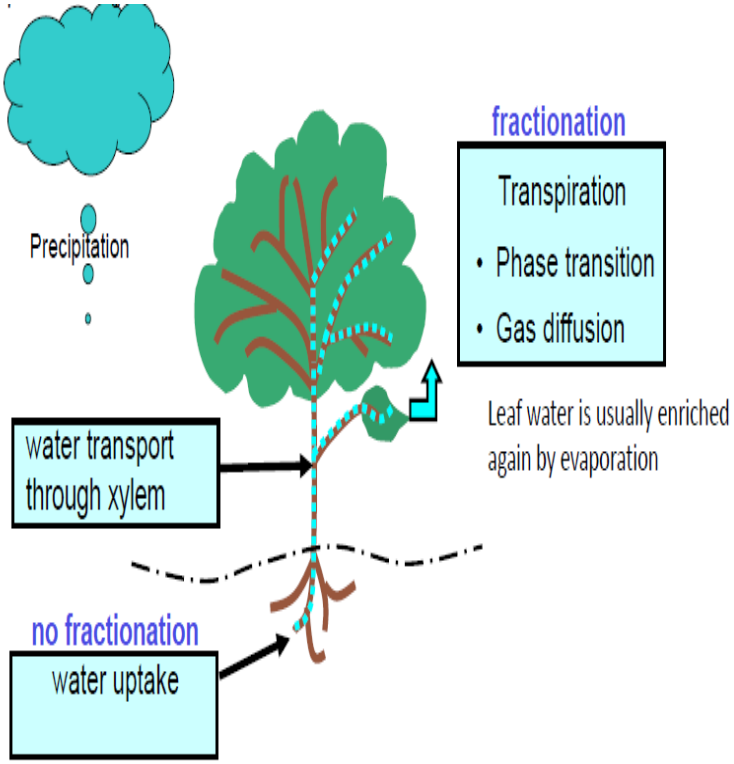


Figure 1.3 *Water movement in plant and isotopic fractionation*

1.4.4 Isotopic basis of plant nitrogen

There is a great deal to be learned about plants and their source of nitrogen, and among plant components. Understanding of the mechanistic relationship of *N* source and plant isotope signature would be an effective tool to elucidate the physiology of plant *N* nutrition. The physiological mechanisms that influence plant *N* isotopic signatures have been reviewed by Handley & Raven (1992), Högberg (1997), Evans (2001), and Robinson (2001).

The use of $\delta^{15}N$ in plant physiological research presents the potential for assessing contributions of various *N* sources to plant nitrogen nutrition in the field, including symbiotic nitrogen fixation and atmospheric deposition, and the interpretation of nitrogen in soil profiles. For instance Garten *et al.* (2007), used ^{15}N natural abundance measurements to assess the importance of N availability on the processes determining soil C dynamics in forest.

1.4.4.1 Sources of plant nitrogen and variation in $\delta^{15}N$

Many authors have used $\delta^{15}N$ data to draw inference regarding nitrogen source (Unkovich *et al.,* 2001; Evans & Ehleringer, 1993; Handley & Scrimgeour, 1997; Pate & Unkovich, 1999; Sanford *et al.,* 1995). The N_2 fixation by plant has been assessed using techniques first developed by Shearer and colleagues (Shearer & Kohl, 1986; Shearer *et al.,* 1983). Their technique relied on finding local reference species that would integrate the signal from available soil N that could then be compared with the signature of the presumed fixing species.

Recently, the potential of using $\delta^{15}N$ to understand plant N nutrition, and to trace the relative contribution of nitrogen fixation to plants and soil has been recognized (Evans, 2007). The assumption of this approach is that fractionation does not occur during N uptake and N fixation. The ability of plants to take up *N* directly via foliage has been recognized through experiment using ^{15}N in various gas or liquid sources as tracers (Boyce *et al.,*1996; Wilson & Tiley, 1998) or from leaf-chamber input-output budgets (Sparks *et al.,* 2001, 2003).

According to Evans (2007), the isotopic composition of nitrogen input and fractionation during transformation determine soil $\delta^{15}N$; a consistent pattern of soil $\delta^{15}N$ increases and N content decreases with soil depth has been observed across ecosystems. The mechanisms beyond this change were addressed by Nadelhoffer & Fry (1988), Brenner *et al.* (2001), and Baisden *et al.* (2002). Nadelhoffer & Fry (1988) hypothesized three possible mechanisms;

i) discrimination during decomposition

ii) differential preservation of components enriched in ^{15}N

iii) illuviation of ^{15}N enriched organic matter in deeper soil horizons

Results from field and laboratory experiments by Nadelhoffer & Fry (1988) indicated that the most likely mechanisms for the $\delta^{15}N$ increase with depth were input of litter at the soil surface that were isotopically lighter than organic matter (OM) and overall isotopic fractionation

during microbial processing of OM during decomposition. This was supported by Kramer *et al.* (2003) who examined soil $\delta^{15}N$ in relation to the degree of humification, and found that $\delta^{15}N$ increased with humification during microbial processing of OM.

1.5 The research context

The research of this thesis was undertaken in the context of the partnership between the government of Rwanda and the Cambridge Commonwealth Trust to support capacity building of Rwanda by enabling talented Rwandans to pursue postgraduate study at University of Cambridge in the area that contributes to the development of Rwanda. The genocide in nineteen ninety four in Rwanda, and ensuing wars and mass population displacement led to significant loss of human capacity, and strongly hit both academic and research institutions.

Rwanda is one of the poorest countries in the world (World Bank, 2007), with an average annual per capita income of only $245, more than half of all population (52 %) living in extreme poverty as measured by the international standard of $1 per day in income, and 67 % of rural poverty rate. Poverty in Rwanda is foremost a rural phenomenon. According to the national institute of statistics of Rwanda (NISR, www.statistic.gov.rw) 65 % of Rwandans rural resident are classified as poor. Therefore, empowering the agricultural sector is crucial for development. Agricultural research in Rwanda is carried out mainly in the public institutions; following the loss of considerable number of scientists during the Rwandan tragedies, Rwanda has gradually rebuilt its capacity (World Bank, 2007); however lack of trained scientist remains a problem. For instance, in its effort to intensify agricultural production system as an approach to food security, up to now, the Government of Rwanda is still relying on importing improved seeds from neighboring countries (Kathiresan, 2011).

1.5.1 The bio-physical environment of Rwanda

Rwanda is a small mountainous landlocked country of 26338 km^2 of area, of which 5.3 % occupied by water, and located at the centre of East Africa at 1.9403^0 S and 29.8739^0 E, and bordered by Uganda in north, Tanzania in the East, Burundi in the south and Democratic Republic of Congo (DRC) in west (*fig. 1.4*).

The landscape is dominated by hills with forested tops and cultivated hillsides ending in marshy valleys. With the altitude ranging between 970 and 4507 m, this equatorial country is characterized by a sub-equatorial climate (Vandoort & Van Ranst, 2003). According to the Rwanda Meteorology Agency (http://www.meteorwanda.gov.rw), temperature is relatively stable during the year (annual average of 20^0C), and ranges between 15 and 25^0C depending the altitude. However, diurnal fluctuations regularly exceed 12^0C. Enormous variability in space and time characterize the Rwandan rainfall regime; the highlands receive more rainfall (>2000 mm annually) than the lowlands, where the annual rainfall is about 1000 mm (*fig*.1.5*)*. Two rainy seasons alternating with two dry seasons can be distinguished; i) a short rainy season from mid September to mid December; ii) a short dry season from the second part of December to the beginning of February, iii) a long rainy season from February to the end of May, and iv) a long dry season from the beginning of June to the first half of September. Verdoodt & Van Ranst (2003) calculated the length of the dry and humid period in different zones of Rwanda, and found the water supply from rainfall during dry months is insufficient to meet the water demands and the crops have to rely on soil water reserves.

Figure 1.4 *Location of Rwanda in Africa (Source: World atlas maps)*

1.5.2 Agricultural context of Rwanda

Agriculture is the most important sector in Rwanda (MINECOFIN, 2012) in terms of contribution to GDP, employment, and foreign exchange. The agricultural sector accounts for 42 % of GDP, contributes significantly to national food self-sufficiency as over 90 % of all food consumed in the country is domestically produced. According to MINECOFIN (2006), 90 % of the economically active populations were employed in agriculture. About 87 % of rural households in Rwanda depend on agriculture as their main livelihood source (World Bank, 2007). Food crops dominate the Rwandan agriculture (potatoes, wheat, maize, cassava, beans, rice, and soybeans), reflecting the subsistence orientation. However, agricultural exports represent the main source of foreign exchange, and export earnings derive mainly from coffee and tea (MINECOFIN, 2012).

Agriculture in Rwanda is dominated by small-scale, subsistence-oriented family farming (MINAGRI, 2011). The use of improved inputs is still low, though since 2007, the Ministry of Agriculture has been distributing subsided seeds and fertilizer under the program of crop intensification (CIP). However, Rwanda remains in the group of countries with an overall low rate of fertilizer use (< 24 kg ha^{-1}). The scarcity of land in Rwanda is evident; with the

highest population density in sub-Saharan Africa (434 inhabitants per km², in 2014), with about 40 % land classified by FAO as having high erosion rate (MINAGRI, 2009), and the smallest average farm size (0.3 ha per rural resident).

According to the World Bank (2007), Rwanda clearly faces a major challenge with regard to land. Insights into the distribution of land in Rwanda can be gained from IFPRI grouping based on calculation from the National Institute of Statistics of Rwanda (NISR) data, three groups of land holding size can be recognized in rural area of Rwanda;

i) Landholding of less than 0.3 ha: approximately 40 % of rural household hold less than 0.3 ha. The average holding in this category is 0.11 ha. This group includes 11.5 % of all households holding no land (landless).

ii) Landholding between 0.3 ha and 1.0 ha: approximately 32 % of rural households hold between 0.3 ha and 1.0 ha. The average landholding per household is 0.58 ha in this group.

iii) Landholding of more than 1.0 ha approximately 26 % of rural households hold more than 1.0 ha. The average landholding per household is 1.94 ha in this category.

The scarcity of land in Rwanda becomes more evident when land endowments are compared to those of neighboring countries (*tab. 1.2*)

Table 1.2 *Land endowment in Rwanda compared to neighboring countries (Source: World Bank, 2007)*

	Rwanda	Burundi	Uganda	Kenya
Population density (people/km²)	335	274	136	58
Agricultural land/rural population	0.3	0.4	0.5	1.3
Arable land/rural population	0.2	0.2	0.2	0.3

Limited agricultural research and extension systems and low use of improved inputs contribute to the low productivity of Rwandan agriculture. Most of Rwandan farmers today have very limited contact with extension agents; in the past, agricultural extension was

considered the responsibility of the Government, and the responsibility for extension delivery assigned to MINAGRI. The extension was very top-down; technology recommendations were formulated centrally based on research results, and these recommendations were conveyed by extension agents to farmers, who were expected to adopt them exactly as formulated. The obvious ineffectiveness of the extension service led to the complete scrapping of the national extension service in 1998, and the devolution of responsibilities for technology transfer activities to the local level. In the absence of any follow up, public extension services basically disappeared, to be replaced by patchwork of project-funded initiatives, for which coverage is far complete and their technical competence is highly debatable.

Food security is a concern; fueled by high population growth averaging 2.9% per year, and modest income gains, demand for food has outstripped food production gains, contributing to a long term decline in national food self-sufficiency (World Bank, 2007). Rwanda remains a structurally food deficient country that imports annually at least 130000 tons of food, mainly (edible oil, wheat, sugar, rice, beans, maize, and cooking banana) (www.statistics.gov.rw).

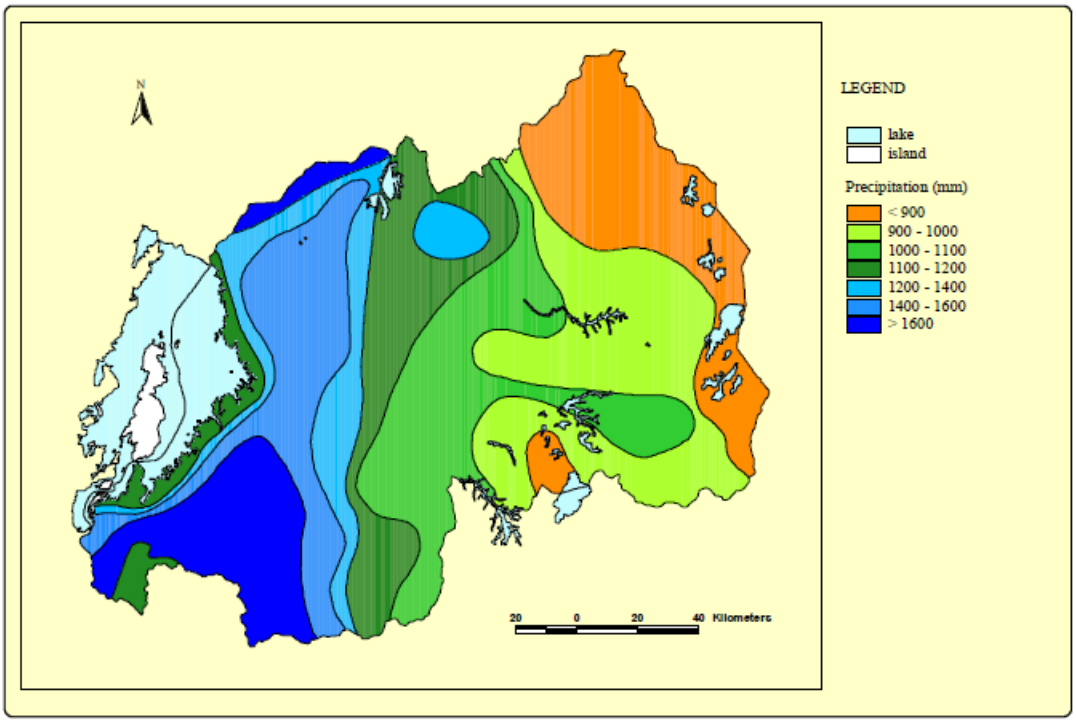


Figure 1.5 *Rainfall distribution in Rwanda (Source: Verdoodt & van Ranst, 2003)*

1.6 Research aim and questions

The aim of this research was to investigate the ways physiological research may be integrated to conventional breeding. The assumption is that selection for physiological traits could refine and enhance a breeding program. Central to this thesis was to address the key question of whether the physiological approach can be used to inform a crop improvement program.

To address the specifics of the main issue, research focused on the following specific questions;

i) Are there traits closely related to physiological processes that can be used as easy and cheap proxy of a particular component of performance for wheat?

ii) To what extent do variations in specific leaf area (SLA) account for variations in photosynthetic capacity and water use in wheat?

iii) Are there mechanisms linking leaf venation to photosynthetic rate and leaf water content in wheat?

iv) What is the mechanistic foundation that determines the relationship between plant height and grain yield?

v) Which physiological traits are related to grain yield and harvest index in semi-dwarf wheat?

vi) What factors underlie *N* partitioning to spike semi-dwarf wheat cultivars?

vii) What are the implications of hydraulic redistribution and nitrogen transfer for crop water and nitrogen use, wheat yield and its components, in agroforestry farming with the N_2 fixing *Alnus acuminata*?

1.7 Thesis structure

In this thesis the results of three years of research are presented. The thesis consists of the following five chapters, three of which are experimental;

Chapter 1 General introduction

Chapter 2 Proxy-based approach to physiological selection of wheat

Chapter 3 The Physiological consequences of *Rht* genes in winter wheat

Chapter 4 Hydraulic lift and N_2 fixing: Consequences of water efflux and *N* transfer for wheat production in agroforestry with *Alnus acuminata* on the terrace risers

Chapter 5 Major conclusions and outlook for further research

Chapter 1 General introduction

Before outlining the research aim and questions, structure, and content of the research in this chapter 1, the general introduction starts with the conceptual background of theoretical framework of physiological approach to crop breeding, and then continues with the underlying theories of fundamentals of application of stable isotopes to plant physiological research. Furthermore, it provides the research context for the field experiments in Rwanda, in which an overview of the bio-physical environment and agricultural context of Rwanda are given.

Chapter 2 Proxy-based approach to physiological selection of wheat

The second chapter deals with the proxies of physiological variables and traits. The efficiency of selection for physiological traits can be related to how well a trait is measured, and the nature of its association with performance. However, direct measurement of a particular physiological trait or plant response is often difficult or impossible. Taking the entire plant contextual approach, the study investigated the potential proxies in relation to photosynthesis and crop water relations, and how easier components are measured, and how these relate to

the physiological performance of the crop. Attention was given to proxies of leaf function; leaf thickness in relation to plant resource acquisition and use. Also leaf veins are at the core of the transport network for water, nutrients, and carbon for plant; yet the physiological connection between vein characteristics, leaf water supply and demand, and leaf performance are not easy to measure directly. This chapter provides the methods used to address the above issues, and discuss the results for specific leaf area (*SLA*), and inter-vein distance (*IVD*) as potential proxies of photosynthetic rate and water use efficiency.

Chapter 3 The physiological consequences of *Rht* genes in winter wheat

The third chapter focuses on mechanistic foundations that determine the relationship between plant height and grain yield in *Rht* lines. Considerable progress in wheat yield has been achieved through straw-shortening by introgression of *Rht* genes, and which has been associated with an increase in the *HI*, defined as the ratio of grain yield to total biomass. However, the relationship between grain yield and plant height has been proved to be parabolic; and the literature indicates that wheat yield is reduced when plant are shortened beyond a threshold optimum. The research of chapter 3 investigated for approaches to further increase both grain yield and HI within the reduced height of *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-D1c*, and compared to wild type in Mercia background. The research examined in the controlled environment, the partners of the determinants of *HI* in measurements of morphological, anatomical, physiological and yield components. Additionally, the partitioning of N at different growth stages was studied through the ^{15}N labeling experiment.

Chapter 4 Hydraulic lift and N_2 fixing: Consequences of water efflux and *N* transfer for wheat production in agroforestry with *Alnus acuminata* on the terrace risers

Below ground efflux of water from roots and nitrogen transfer among plants has been hypothesized, but it remains uncertain how important they are in facilitating physiological functions in intercropping farming. The fourth chapter presents results of a field experiment

conducted in north of Rwanda in the terraces field with agroforestry of *Alnus acuminata* and wheat. The study focused on the analyses of the natural abundance of stable isotopes $\delta^{2}H$, $\delta^{18}O$, $\delta^{15}N$, and $\delta^{13}C$, and an isotopic mixing model IsoSource to examine the patterns and consequences of the crop water and N acquisition from sources at different distance further away from the tree (1 m, 3 m, 5 m, and 7 m). Results indicated the crops in the proximity of trees exhibited isotopic values $\delta^{2}H$, $\delta^{18}O$, and $\delta^{15}N$ closer to that of the tree, and these improvements in water and nitrogen access resulted into increased grain yield for crops nearest the tree for a distance up to 5 m.

Chapter 5 Major conclusion and outlook for further research

In the concluding chapter, major conclusions and findings are discussed. Further research is also proposed.

Chapter 2 Proxy-based approach to physiological selection of wheat

"Even though it is often difficult to measure a particular physiological variable, it is certainly worthwhile determining its proxy" (This thesis).

Abstract

Approaches based on physiological understanding of yield are necessary for developing genotypes combining high yielding potential and agronomic traits of superior adaptation, and for understanding yield limiting factors. Physiological processes underlie the phenotype and yield observed in crops; plants respond to environmental and physiological stimuli through morphological, physiological, and metabolic modification occurring in all plant organs. Yet, direct measurement of a particular physiological variable is often difficult. This study was set to develop a proxy-based approach to wheat selection. After conceptualizing a theoretical framework of links between the traits of photosynthesis, water relations, leaf morphology and anatomy, and their likely proxies; a comparative screening of *23 Eps* wheat cultivars was conducted in field by means of photosynthetic gas exchange measurement, followed by isotopic measurements ($\Delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) in the leaf matter, and morphological and anatomical measurement (*SLA*, *IVD*, SD). Having ranked a number of traits according to their likely association with particular proxy, the results showed that photosynthetic rate and WUE were statistically significantly *($p<.01$)* associated with *SLA*, and *IVD*. Based on these results, the study concluded that *SLA* would be a potential proxy of both A_{max} and WUE in wheat, and surrogate measure of $\Delta^{13}C$, and that IVD could be proxy of leaf *RWC* and A_{max}.

Key words: Proxy-based selection, physiological traits, wheat, *SLA*, *IVD*

2.1 Introduction

2.1.1 Proxy-based crop selection: A conceptual approach to physiological research

To date, progress in yield *per se* has been achieved mainly through conventional breeding (Reynolds *et al.*, 2011). However, given the complexity of yield trait, it is apparent that a comprehensive approach to crop improvement has the greatest probability of achieving increased productivity. Given that any improvement in grain yield results from underlying physiological processes; approaches based on physiological understanding of yield are necessary for identification of traits putatively related to yield and adaptation, and selection criteria that could be exploited to complement the conventional breeding (Slafer *et al.*, 2005; Reynolds & Borlaug, 2006; Foulkes *et al.*, 2011). Yet, direct measurement of a particular physiological variable is often difficult.

In this chapter, we present a proxy-based approach to crop selection. Based on detailed knowledge of plant physiology, a definition was proposed; "*a proxy-based approach to crop selection is a surrogate-based screening of genotypes for morphological, anatomical, and physiological traits of performance or crop environmental responses".*

The proxy-based approach to crop selection is needed to accelerate the progress of breeding and at cheap cost. In this chapter, we propose six steps through which a physiological proxy is developed;

i) A particular physiological variable is identified

ii) Defining the trait through which a process can be measured

iii) Determining the growth stage at which the physiological variable is pertinent

iv) A proxy is proposed

v) A screening test is applied to a random set of cultivars

vi) The accuracy of the test is confirmed

Therefore, an idealized conceptual framework is proposed for making such a "proxy-based approach" operational in terms of physiological research in wheat (*fig.2.1*).

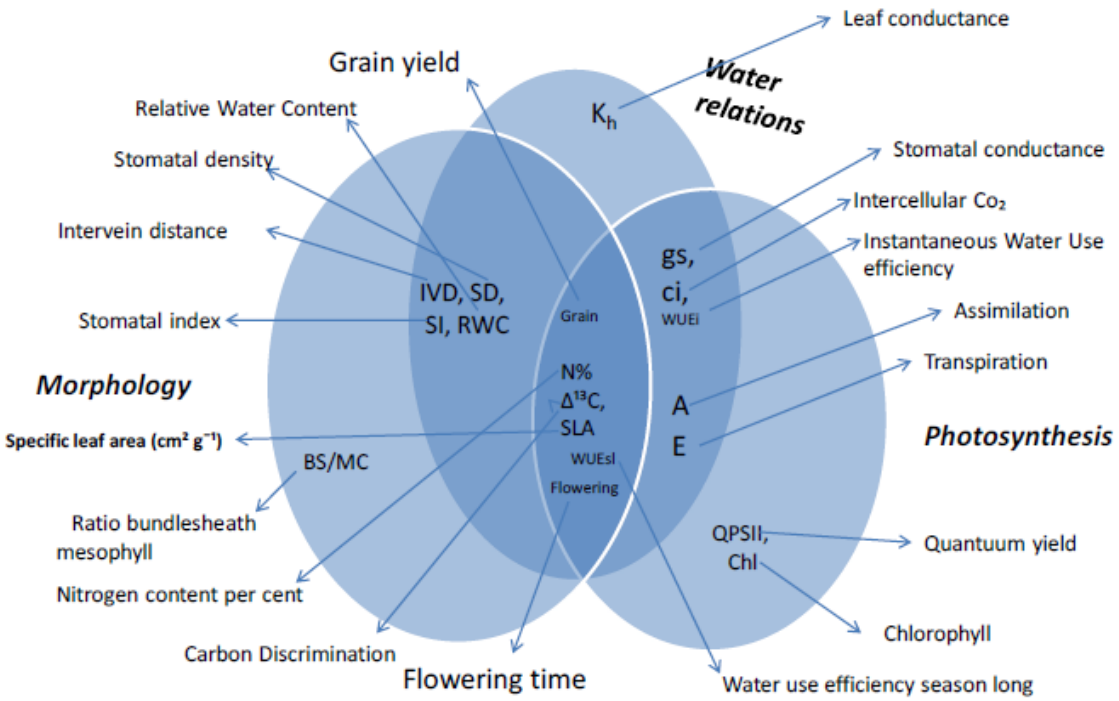


Figure 2.1 *Conceptual framework of interaction between physiological variables*

Taking the entire plant contextual approach, the research for this chapter aimed to answer the question of which physiological traits offer an easy and cheap proxy for a particular process in wheat. After theorizing the likely interaction between the physiological process of photosynthesis, water relations, leaf morphology and anatomy; attention was given to the proxies for photosynthetic and water use efficiency in wheat.

2.1.2. Background of earliness *per se* (*Eps*) of anthesis in wheat

Varietal differences in timing to anthesis independent of sensitivity to photoperiod and vernalization had been found (Ford *et al.*, 1981), and referred to as *"earliness per se (Eps)"* (Hoogendoorn, 1985*a*), or *"intrinsic earliness"* (Slafer, 1996). *Eps* genes have been identified on different wheat chromosomes, and some of them have been mapped as quantitative trait loci (QTL) for flowering time (Kuchel *et al.*, 2006). For example, Bullrich *et al.* (2002) mapped a *QTL* for *earliness per se* in the distal region of chromosome $1A^mL$ in a

cross between cultivated *(DV92)* and wild *(G3116) Triticum monococcum* L. accessions, which was designated *Eps-A^m1*.

On the other hand, Slafer (1996) reported that *Eps* genes not only regulate anthesis time but also affect the transition from vegetative to reproductive apices, early and late spike development, and stem elongation. As grain yield components are determined during these phases (Slafer & Whitechurch, 2001), it is therefore relevant to examine how *Eps* relate to the physiological processes of performance. The research of this chapter conducted a comparative screening of 23 *Eps* cultivars for photosynthetic and water use efficiency.

2.1.3 Photosynthetic gas exchange

Gas exchange implies the exchange of CO_2 and water vapor between the interior of the plant leaf and its surroundings (Larcher, 2003). The CO_2 diffusion to chloroplast is essential to photosynthesis (Lambers *et al.*, 2008). For photosynthesis to occur there must be a diffusion of CO_2 from the atmosphere into the leaf and into the carboxylation sites of Rubisco; first through the stomata, then through intercellular air space, and ultimately into chloroplast (Taiz & Zeiger, 2010). Of course, the main port of entry of CO_2 into the leaf is the stomatal pores, and the same port is traveled into the reverse direction by water vapor. The sharing of the of the stomata entry pathway by CO_2 and water vapor presents the plant with functional dilemma; the diffusion gradient that drives water loss is about *50* times larger than the gradient that drives CO_2 uptake (Taiz & Zeiger, 2010), with *1.6* molar ratio of diffusion of water vapor and CO_2 in the air. Therefore, it is obvious that the opening of stomata facilitates higher CO_2 uptake but unavoidably accompanied by substantial water loss. However, according to Sage & Sharkey (1987), this higher water loss rate also removes heat from leaves through evaporative cooling, keeping them relatively cool under full sunlight conditions.

The diffusion of CO_2 into leaf can be divided into two major components;

i) Gas phase

ii) Liquid phase

The gas phase of CO_2 diffusion includes; the boundary layer, the stomata, and the intercellular spaces to the leaf. And each of which imposes a resistance to CO_2 diffusion (Evans *et al.,* 2009). The boundary layer consists of relatively unstirred air at the leaf structure, and its resistance to diffusion is termed "boundary layer resistance". Taiz & Zeiger (2010) shows that the boundary layer resistance to diffusion decreases with leaf size; small leaves having a lower boundary layer resistance to CO_2 and water diffusion. After the diffusion through the boundary layer, CO_2 enters the leaf through the stomatal pores, and which impose resistance as well. There is also resistance to CO_2 diffusion in the intercellular air spaces that separate the sub-stomatal cavity from the wall of the mesophyll cells, causing a drop of ~ 5 ppm of CO_2 from the 400 ppm outside the leaf (Taiz & Zeiger, 2010).

The CO_2 diffusion of the liquid phase encompasses diffusion from the intercellular leaf spaces to the carboxylation sites in the chloroplasts, imposing the mesophyll resistance; and it is thought to be approximately one tenth of the combined boundary layer resistance and stomatal resistance when stomata are full open (Terashima, 1992). According to Larcher (2001), the stomata aperture (number, distribution, size, shape & mobility) is a cultivar-specific characteristic. By varying the width of the stomatal pores, a plant is able to control the entry of CO_2 into the leaf. In a steady state, the rate of CO_2 diffusion can be ascribed by Fick's first law (Lambers *et al.*, 2008). Hence:

$$A_n = g_c (C_a - C_c) = (C_a - C_c)/r_c \qquad (2.1)$$

where, g_c is the leaf conductance for CO_2, C_a and C_c are the mole fractions of CO_2 in the air and at the site of carboxylation, respectively; r_c is the inverse of g_c (leaf resistance to CO_2 diffusion). According to Lambers *et al.* (2008), the leaf conductance to CO_2, the g_c, can be

derived from measurements on leaf transpiration, which can also be described by Fick's first law in a similar way:

$$E = g_w (W_i - W_a) = (W_i - W_a) r_w \quad (2.2)$$

where g_w is the leaf conductance for water vapor; W_i and W_a are the mole of water vapor in the intercellular spaces and in air, respectively; r_w is the inverse of g_w, and E is the rate of leaf transpiration.

The ratio of CO_2 assimilation to transpiration which is termed instantaneous water use efficiency (WUE_i) of photosynthesis can be calculated as follow:

$$WUE_i = A_n/E = g_c (C_a - C_i)/g_w (W_i - W_a) \quad (2.3)$$

$$= C_a (1 - C_i/C_a)/1.6(W_i - W_a)$$

It has been suggested that the maximal rate of CO_2 assimilation (A_{max}), under natural conditions, at atmosphere CO_2 supply under optimal environmental conditions, is characteristic constitutional feature of specific crop cultivar (Larcher, 2001). The photosynthetic capacity, although characteristic of crop cultivar, is not a constant value; gas exchange patterns can change appreciably during plant growth and are also influenced by a number of external factors such as radiation, availability of CO_2, temperature, and supply of water and mineral nutrients (Griffiths, 1999; Geiger & Servaites, 1994). However, the literature shows that the variation in A_{max} among varieties and species are consistent enough so that the A_{max} is a useful parameter (Driever *et al.*, 2014).

The research for this chapter set out to investigate the potential for rapid, easier and low cost proxy for these photosynthetic gas exchanges.

2.1.4 Leaf morphology and anatomy

The influence of leaf morphology and anatomy on photosynthetic activity has long been recognized (Jellings & Leech, 1984; Garnier & Laurent, 1994). According to Nobel (1983), and Sharkey (1985), differences in photosynthetic capacity among plant cultivars may be

attributed to differences in biochemical, morphological and anatomical features of their leaves. Similarly, Taiz & Zeiger (2010) suggested that morphological aspects of the leaf such as leaf area can be a determinant in influencing the thickness of the boundary layer.

It appears that leaf thickness plays an important role in leaf functioning and relates plant strategy of resource acquisition and use (Vile *et al.*, 2005). Wide variation in leaf thickness among plant cultivars had been observed (Evans, 1999; Poorter *et al.*, 2009). Building both on Witkowski & Lamont (1991), and Roderick *et al.* (1999), it was shown that leaf thickness is closely related to *SLA* (the ratio of leaf area to leaf dry matter); as such *SLA* can be used as a proxy of leaf thickness. It was also suggested that *SLA* is a trait that may be up to *60* to *90 %* heritable (Rebetzke *et al.*, 2004; Songsri *et al.*, 2008). However, the measurement of leaf thickness is not straight forward.

Therefore, the research of this chapter was set to examine the mechanistic determining the possibilities of potential use of *SLA* for screening purposes in wheat. To guide the investigation, the study addressed the specific questions of;

i) To what extent do variation in *SLA* account for variations in photosynthetic capacity and water use in wheat?

ii) Are there mechanisms linking leaf venation to photosynthetic rate and leaf water content in wheat?

2.2 Material and Methods

The *23 Eps* cultivars (*Appendix A)* characterized in this study, form part of a broad physiological selection at *NIAB* from which selected cultivars are subsequently used for further selection for earliness of flowering. However, any other wheat cultivars could have been used for this experiment. The *Eps* lines were chosen as there was on-going agreement between the laboratory of physiological ecology and NIAB to phenotype these lines for photosynthetic performance on behalf of NIAB selection programme.

2.2.1 Photosynthetic gas exchange measurements

Photosynthetic performance can be assessed by gas exchange measurement (Lambers *et al.*, 2008). Snapshot measurements of photosynthetic gas exchange were performed on wheat leaves of 23 *Eps* cultivars in the field located at 52^0 *13´N,* 04^0 *59´E* at National Institute of Agricultural Botany (NIAB, Cambridge, the UK), using a portable LICOR LI-64000XT (LI-COR Inc., Lincoln, Nebraska, USA) (*fig. 2.2).* The plot size based on "hege" drill was 1.2 m² (1 m × 1.2 m). About 120 seeds (each cultivar) were sown in each entire plot. The field plots received a full schedule of agrochemical inputs according to the protocols used in the HGCA recommended list trials series (www.hgca.com). The plots were randomized designed with two replicates for each cultivar, and 46 plots were sampled.

Measurements were taken at anthesis (GS65), between 10h00 and 14h00, on fully expanded flag leaf of the main tiller (two leaves per plot, & *4* replications per cultivar) randomly chosen in the center of each plot. Parameters were set in the LI-COR as; Relative humidity to *60 - 80 %,* the block temperature at 20^0C, the CO_2 reference to *400 ppm*, flow rate at *400 µmol* s^{-1}, the photosynthetic active radiation (PAR) of *1000 µmol* quanta s^{-1} (the A/PAR curve was performed before the beginning of measurement, to determine the saturation point). After the setting of the parameters, a leaf was placed in the sensor head, and enclosed the chamber (and waited until the values got stable, ~ 2 minutes), and then measurement was

recorded on the LI-COR. The measurement was repeated on the second leaf in the same plot before moving to another plot, and the same leaves were marked for further sampling and analysis (*SPAD* measurements; *SLA*; $\Delta^{13}C$; leaf *N %*; & $\Delta^{18}O$).

The essence of gas exchange measurement is the direct measurement of photosynthesis from leaf gas exchange with an infrared gas analyzer (*IRGA*) which measures the carbon dioxide flux within sealed chamber containing a leaf sample. Air enters the chamber at a specified flow rate (*Fm)* measured and controlled by a flow–controller. The leaf changes the concentrations of CO_2 and H_2O inside the chamber. The magnitude of the difference in CO_2 and H_2O concentration between the air entering the chamber (C_e and W_e) and at the outlet (C_o and W_o) depends on the leaf gas exchange activity. The net photosynthetic rate (A_n) is then calculated following Von Caemmerer & Farquhar (1981):

$$A_n = Fm/L_a \{C_e - C_o (1-W_e) / (1 - W_O)\} \qquad (2.4)$$

The portable LI-COR 64000XT provides real time measurement of CO_2 uptake (A), transpiration (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i).

Figure 2.2 *Gas exchange measurement in the field with LI-COR*

2.2.2 Chlorophyll content measurement

From a physiological perspective, leaf chlorophyll content is a parameter of significant interest in its own: First, chlorophylls are the main pigments involved in the light capture for photosynthesis and other photochemical reactions (Marenco *et al.*, 2009); and the amount of solar radiation absorbed by a leaf was found to be largely a function of the foliar concentrations of photosynthetic pigments (Filella *et al.*,1995; Fritschi & Ray, 2007). Second, much of leaf nitrogen is incorporated in chlorophyll (Moran *et al.*, 2000). Third, pigments can be directly related to stress physiology and abiotic factors; for instance, it had been observed that chlorophyll content of the leaf generally decreases under stress and during senescence (Penuela & Filella, 1998; Larcher, 1995). Therefore, the measurement of leaf chlorophyll content may indicate photosynthetic variation among cultivars and provide other information about physiological performance of plants in their environment.

The chlorophyll measurement was taken non-destructively at anthesis (*GS65)* on a fully expanded flag leaf (two leaves per plot & four replicates per cultivar), using a hand held portable chlorophyll meter *SPAD-502* (Konica Minolta Sensing Inc., Osaka, Japan). The values measured by the chlorophyll meter *SPAD-502* correspond to the amount of chlorophyll present in the plant leaf. The values are calculated based on the amount of light transmitted by the leaf in two wavelength regions in which the absorbance of chlorophyll is different (Minolta, 1989).

The measuring head of the chlorophyll meter *SPAD-502* includes two *LEDs*, a red *LED* (peak wavelength: approx. *650 nm*) and an infrared *LED* (peak wavelength: approx.*940 nm*), that provide illumination. According to Minolta (1989), the *SPAD* unit values that appear in the display SPAD meter results from two processes: during calibration, the two *LED*s emit light sequentially without any leaf sample in the head, and the received lights are then converted into digital signals and the ratio of their intensity is calculated and stored in the unit's

memory. When a leaf is subsequently measured, the *SPAD-502* microprocessor outputs a processed value based on the ratio of the voltage produced by each wavelength relative to the value stored in the memory.

2.2.3 Specific leaf area ($cm^2\ g^{-1}$) measurement

The same flag leaf samples on which gas exchange and chlorophyll content measurements were taken in the field at *NIAB* were collected for specific leaf area (*SLA*) measurement. At anthesis (*GS65*), two flag leaves were sampled per plot (four replicates per cultivar): leaf was cut from plant, rapidly wrapped in moist paper, placed in plastic bag, put in cool box, and taken to the laboratory of physiological ecology at department of plant sciences (University of Cambridge) for further measurements.

In the laboratory, each leaf was recut under distilled water to remove the ligule, and placed immediately into a tube filled with distilled water, and stored in refrigerator at 4^0C for *6* hours to ensure fully rehydration of the leaves (Garnier *et al.*, 2001). After this period, the leaf blade was taken out of the tube, and blotted dry with tissue paper to remove any surface water, and immediately it was weighed to determine its saturated fresh mass.

The leaf area (*LA*), one side of the leaf, was measured with *ImageJ* (version *1.42q*, National Institute of Health, *USA*). Then, the sample was oven dried in a paper envelope at 75^0C for *24* hours. The leaf dry weight (*DW*) was obtained by reweighing the sample on micro-balance after oven drying. The *SLA* was calculated as the ratio of leaf area (*LA)* to dry weight (*DW*):

$$SLA\ (cm^2\ g^{-1}) = \frac{LA\ (cm^2)}{Dw\ (g)} \qquad (2.5)$$

2.2.4 Carbon discrimination ($\Delta^{13}C$) and leaf *N* measurement

The realization that $\Delta^{13}C$ in leaf matter could provide indirect measure of integrated variation in photosynthesis efficiency in C_3 plants over a growing season (Farquhar *et al.*, 1982; Farquhar & Richards, 1984) gave impetus to the prospects of its use in crop selection. The

utility of using $\Delta^{13}C$ in crop selection stems from the biochemical discrimination against ^{13}C during gas exchange: of course, in C_3, discrimination against ^{13}C by the carboxylating enzyme, Rubisco (~ *27 ‰*) is linked to photosynthesis via C_i/C_a, the ratio of intercellular to atmospheric CO_2 concentrations (Farquhar *et al., 1982*; Brugnoli *et al.,* 1988). This ratio reflects the relative magnitudes of net assimilation (A_n) and stomatal conductance (g_s).

Dawson *et al.* (2002) argued that $\delta^{13}C$ integrates photosynthetic activity through the period the leaf tissue was synthesized, and that leaf $\delta^{13}C$ values reflect the interplay among all aspects of plant carbon and water relations. Because of the integrative features of $\Delta^{13}C$ through time; Henderson *et al.* (1998) suggested that ^{13}C isotope can be used to assess traits that co-vary with gas exchange, *C* gain, and water relations.

Moreover, the photosynthetic capacity of leaf is related to its nitrogen content (Evans, 1989): Association between CO_2 assimilation rate per unit leaf area and the total leaf nitrogen per unit leaf area had been observed in many studies (Poorter & Evans, 1998; Reich *et al.,* 1994; Evans, 1983), and it had been argued that the reason for this relationship is the large amount of leaf organic nitrogen present in the chloroplasts, most of it in the photosynthetic machinery specifically Rubisco (Evans & Seemann, 1989).

At the Godwin laboratory (Cambridge University, UK), the dried ground leaf samples weighed (*1 mg*) into a tin capsule were analyzed for both $\delta^{13}C$ and percentage of nitrogen using Costech elemental analyzer attached to a Thermo Delta *V* mass spectrometer in continuous flow mode. The samples were introduced into the combustion reactor and flash combustion occurs. The sample and the tin capsule react in a temporarily enriched atmosphere of oxygen reaching temperatures of *1700 - 1800* ^0C and the sample is broken down into its elemental components. These combustion products are carried by a constant flow of Helium "carrier gas" through an oxidation catalyst of Chromium trioxide and then silver coated cobaltic oxide both kept at *1020* ^0C. The mass spectrometer software measures

the $^{12}C/^{13}C$ and the $^{14}N/^{15}N$ ratio, and *N* percentage in the sample. Reference standards from IAEA in Vienna are also run at intervals throughout the sequence and these values are used to calibrate to the international standards for $\delta^{13}C$ *PDB* and $\delta^{15}N$ in air.

The $\delta^{13}C$ value was used to compute the Δ^{13}C following Farquhar *et al.* (1982);

$$\Delta^{13}C = \left(\frac{\delta^{13}Ca - \delta^{13}Cp}{1+\delta^{13}Cp}\right)/1000 \qquad (2.6)$$

Where the $\delta^{13}C_a$ is the delta value of *C* in the air *(~ - 8 ‰)* and the $\delta^{13}C_p$ is the delta value of *C* in the sample.

2.2.5 Oxygen isotope analysis (δ^{18}O) in leaf matter

Oxygen isotope analysis of leaf samples was undertaken at Godwin laboratory (Cambridge University, the UK) using a Thermo Finnigan *TC/EA* attached to a Thermo Delta *V* mass spectrometer via a *conFlo 3*. Dried and ground leaf sample weighed (*0.1 mg*) and reference materials were placed in silver capsules, sealed and loaded into an auto-sampler for analysis. The samples and references are dropped automatically in sequence into a "high temperature conversion reactor" consisting of an outer ceramic mantle tube of aluminium oxide and an inner glassy carbon reactor containing a graphite crucible, glassy carbon granules and silver wool. The reaction temperature was 1450^0C. The gaseous products produced (H_2, *N2, CO*) are separated by a packed gas chromatographic molecular sieve column at a temperature of 90^0C, and then passed into the mass spectrometer via the *conFlo* for isotopic analysis.

According to Farquhar *et al.* (1998), the $\delta^{18}O$ of the leaf is largely determined by the integrated leaf to air vapor pressure gradient during photosynthetic gas exchange. This leaf air vapor pressure gradient changes with environment conditions (such as atmospheric humidity, soil moisture, air temperature) and plant responses to these environmental changes (Dawson *et al.*, 2002). Therefore, measurement of $\delta^{18}O$ of the leaf can aid with interpretations of differences in $\delta^{13}C$ among crop cultivars growing in the same conditions.

By considering concurrent variations of $\delta^{13}C$ and $\delta^{18}O$, one can distinguish between biochemical and stomatal limitations to photosynthesis.

2.2.6 Inter-vein distance (*IVD*) measurement

Leaf veins form the transport network for water, nutrients and carbon for plant (Brodribb & Holbrook, 2006). However, the underlying physical principles that connect veins pattern with photosynthesis remain unresolved. Here, we investigated the links between leaf venation to functional process of photosynthesis in wheat.

Leaf tissue of *5 mm* × *15 mm* to be used for vein measurement was cut in the middle area between the central vein and the leaf edge, on the same each leaf sample that was collected for stomatal measurement. Then, the tissues samples were fixed in a solution of *4%* para-formaldehyde overnight at 4^0C; and washed into *100 %* ethanol and dehydrated passing into ethanol concentration series of *30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %* and staying in each concentration for *45* minutes at 4^0C. Thereafter, samples were transferred into ethanol *95 %* with eosin *0.1 %*, and cooled overnight at 4^0C; and then moved into three series of *100 %* ethanol at room temperature for *45* minutes each.

The embedding followed through the sequences of steps; first under the mixture of *50 %* ethanol and *50 %* technovit, then under *100 %* technovit, and third, into mixture of *100 %* technovit and hardener *I* for *45* minutes per step. Thereafter, the samples were polymerized overnight in made up of technovit plus hardener *I* and *II* into mould covered with parafilm. Finally, samples were mounted with araldite on wooden block and kept cool and dry to harden fully over night at room temperature. Four μm thick cross sections were cut using a glass knife on microm *HM340E*; and stained with *0.1 %* toluidine blue. Sections were photographed on microscope at a magnification of ×40 using a digital camera (Nikon Coolpix *P5100*). The inter-veins distance was measured by means of *ImageJ* (version *1.42q*, National institute of health, *USA*), and measured following Dengler *et al.* (1993), as the distance

between the half of a major vein and half of minor vein (*fig.2.3*). The half of the major vein to the half of the minor vein was chosen due to the fact that water moves laterally from major to minor veins.

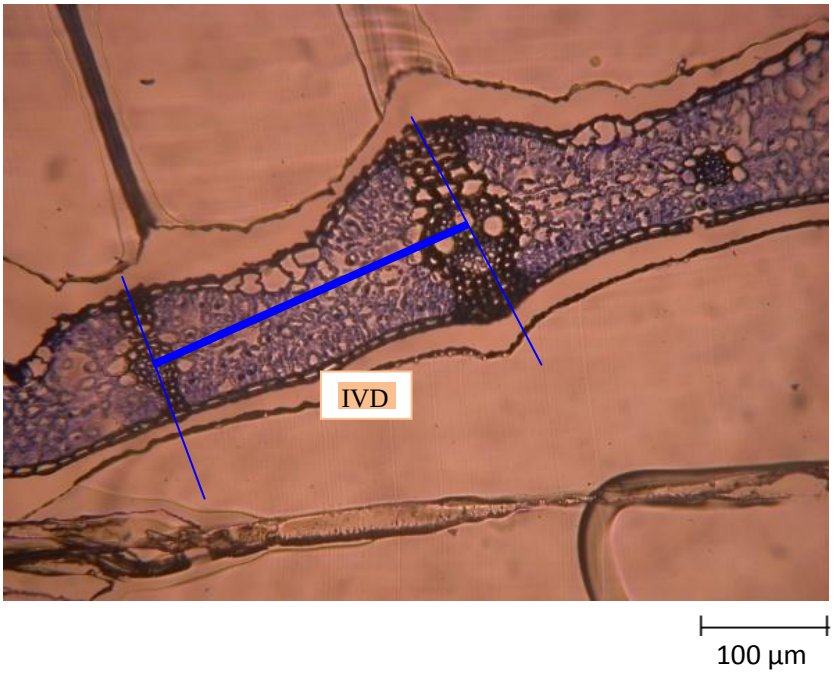


Figure 2.3 *The IVD measurement*

2.2.7 Leaf relative water content (*RWC*) measurement

The leaf relative water content (*RWC*) is a measure of its hydration status relative to its maximal water holding capacity at full turgidity (Mullan & Pietragalla, 2012). Measurement of leaf *RWC* may indicate the degree of water deficit and stress of plant (Bowman, 1989). The leaf water content status is intimately related to many physiological variables such as photosynthesis, stomatal conductance, transpiration, and so (Kramer & Boyer, 1995); therefore, a genotype with the ability to minimize stress by maintaining high leaf water content in stressed environment may have physiological advantage. The research of this chapter investigated the potential of using leaf *RWC* as a screening tool for physiological performance.

The two fully expanded intact flag leaf samples were collected at GS65 from two randomly chosen plants in each plot (4 replicates per cultivar) for RWC measurement. The samples

were immediately placed into pre-weighed plastic tubes and sealed the lid, placed in cooled container, and taken to the laboratory of physiological ecology (University of Cambridge, the UK) for measurement.

In the laboratory, the leaf fresh weight was measured as the weight of the tube containing the sample minus the weight of the tube. Thereafter, 1cl of distilled water was added to each tube containing the sample and was placed in refrigerator at 4^0C for *24* hours for leaf to reach full turgor; then, samples were taken out the tubes and blotted dry with paper towel, and the turgid leaf weight of the sample was measured; thereafter they were oven dried at 75^0C for *24* hours, and reweighed for dry weight afterwards.

The leaf *RWC* was computed following Barrs & Weatherley (1962);

Leaf $RWC\ (\%) = ((FW - DW) / (TW - DW))*100$ (2.7)

where; *FW*= fresh weight; *TW*= turgid weight; and *DW*= dry weight

2.2.8 Stomata density measurement

Leaf stomata density and distribution may affect remarquably the plant physiological process and its relations to environmental factors (Taiz & Zeiger, 2010; Lambers *et al.*, 2008; Raven *et al.*, 2005). In wheat , stomata occur on both sides 'adaxial and abaxial' of the leaf. The research of this chapter investigated how stomata density is related to physiological performance among 23 *Eps* wheat cultivars in term of photosynthetic and water use efficiency, and its potential use as a proxy for wheat.

The impression approach was used to determine the stomatal density. Both adaxial and abaxial side of the leaf were smeared with nail varnish in the middle area between the central vein and the leaf edge; after 20 minutes, the varnish print of 5 mm × 15 mm was peeled off from the leaf surface, mounted on glass slide, immediately covered with the cover slip, and lightly pressured with fine point twezers. Then a microscope image was taken on microscope under ×*40* magnification, using a digital camera (*Nikon Coolpox P5100*)(*fig. 2.4*).

The stomata density(SD) was calculated following Radoglou & Jarvis (1990b);

Stomata Density = Number of stomata / Leaf area (mm²) *(2.8)*

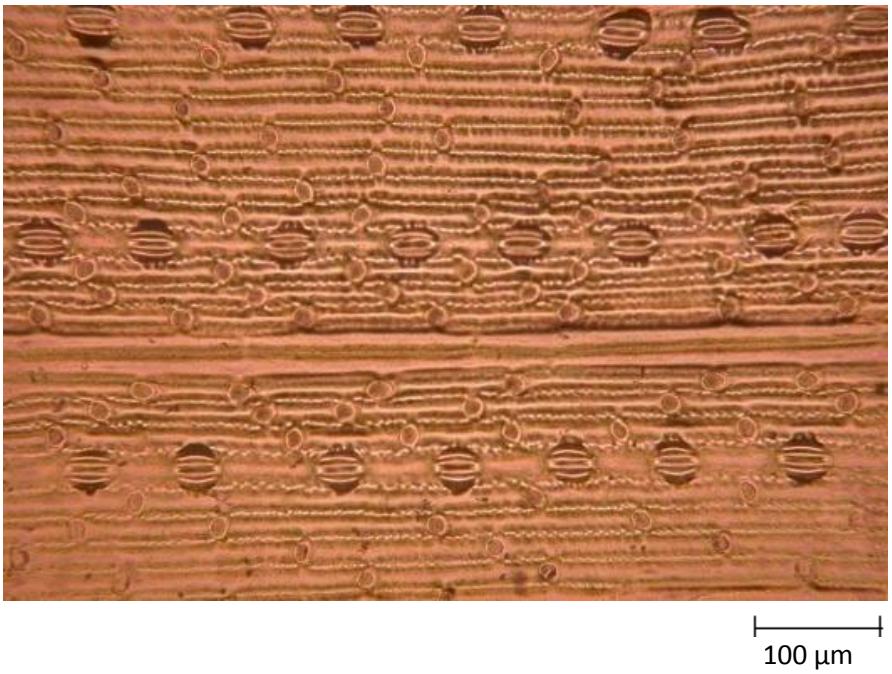


Figure 2.4 *Stomata density*

2.2.9 Statistical analysis

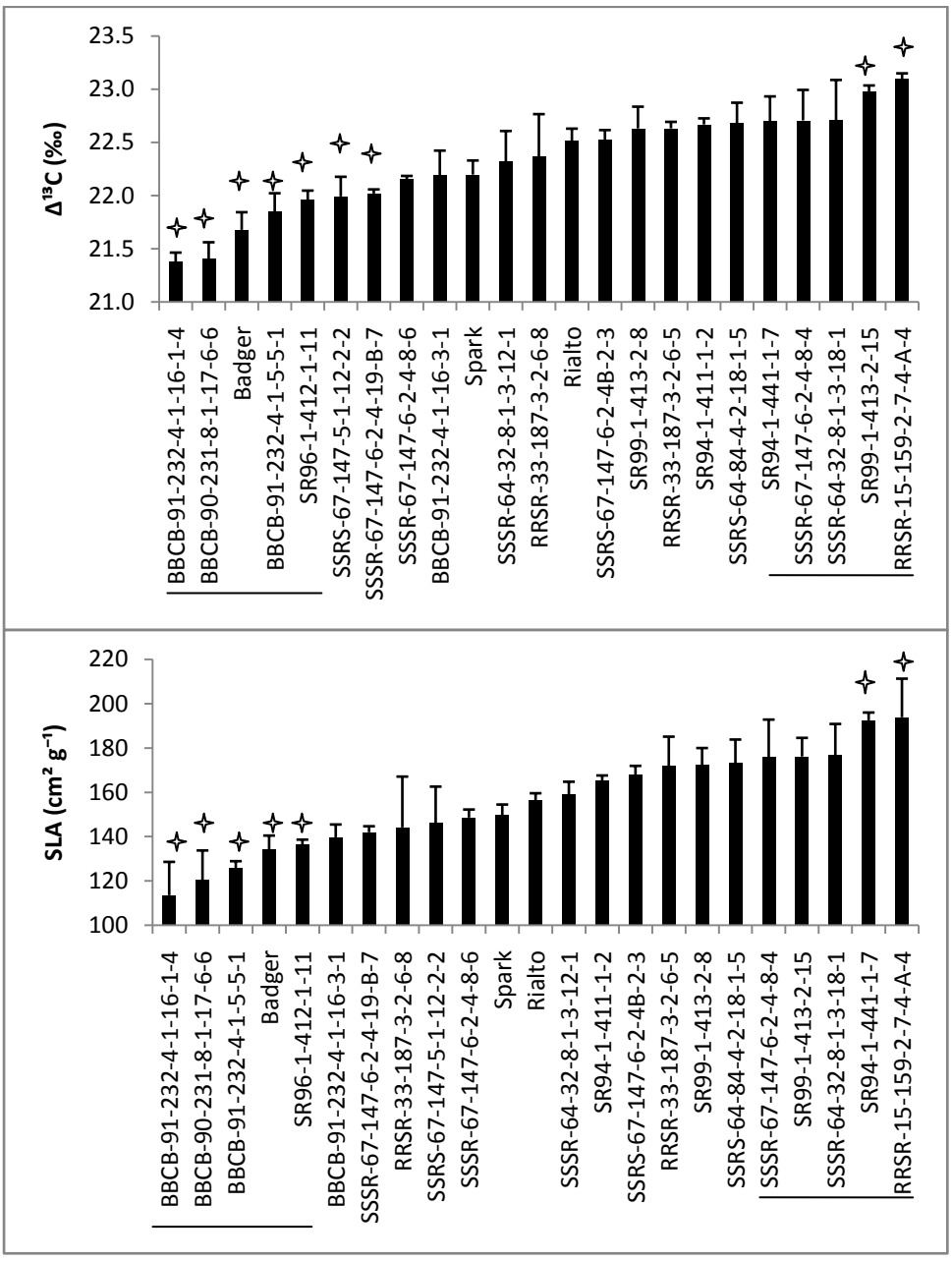
The statistical analysis of the data was performed using *SPSS 16.0* for windows (*SPSS Inc.*, Chicago, *IL*, *USA*). Data were explored for parametric assumptions of normal distribution, and homogeneity of variance: The test for normal distribution was performed using both histograms, and Kolmogorov-Smirnov test to produce *K-S* test and normal *Q-Q* plots. The Levene's test was used to test for homogeneity of variance. Then, graphing of means, ranking for variations in performance among cultivars, and the analysis for the linear relationships were performed by means of bar charts and scatter-plots respectively. Thereafter, the partial Pearson correlation analysis was conducted. Finally, data were subjected to the mixed analysis of variance (*Mixed ANOVA*) at $p<.01$; and Bonferroni test.

2.3 Results

The genotypic rankings for traits of physiological performance are reported, and followed by the relationships among variables of photosynthetic and water use efficiency.

2.3.1 Proxy-based ranking and magnitude of variability in performance of *Eps* cultivars

Ranking of the *23 Eps* cultivars with Rialto as control are presented for $\Delta^{13}C$ *(‰)* in leaf matter, *SLA* (*cm² g⁻¹*), and instantaneous water use efficiency (WUE_i, *µmol* CO_2 *mmol⁻¹* H_2O) (*fig.2.5*).



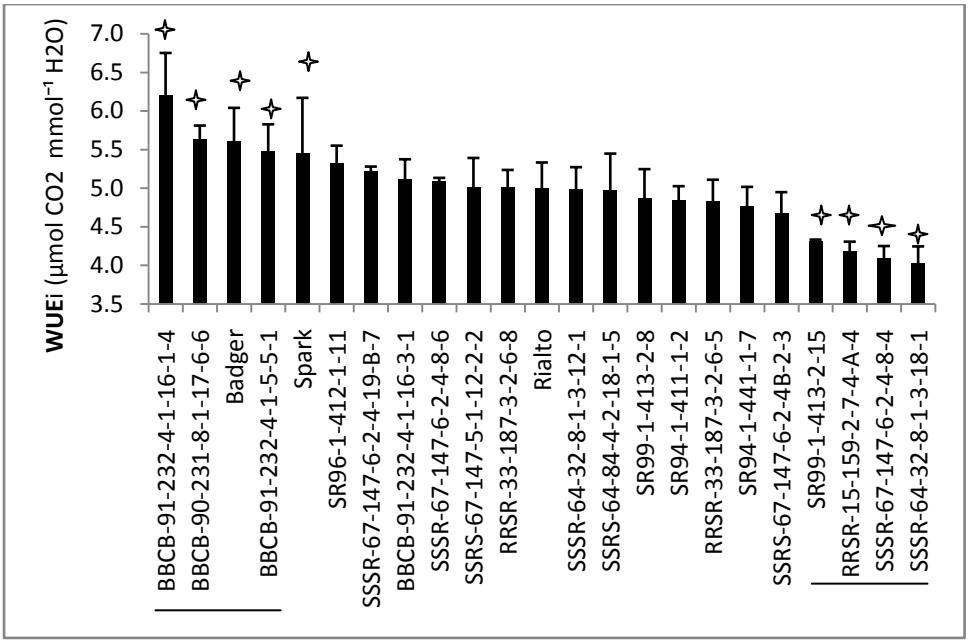


Figure 2.5 *Ranking for variation in $\Delta^{13}C$, SLA, & WUE_i*

The star (✧) on the top of the bar indicates statistical significance of difference depicted by the mixed ANOVA between the means of cultivars at $p < 0.01$. The bars represent the mean value ± SE. N=8

Genotypic ranking for $\Delta^{13}C$ values based upon leaf samples revealed consistency in rankings for both *SLA* and WUE_i. The $\Delta^{13}C$ in leaf organic matter was lowest in seven cultivars: *BBCB-91-232-4-16-1-4; BBCB-90-231-8-17-6-6; Badger; BBCB-91-232-4-1-5-5-1; SR96-1-412-1-11; SSRS-67-147-5-1-12-2-2; & SSSR-67-147-6-2-4-19-B-7*, with the mean values ranging between *21.4 ± 0.1 ‰* and *22.0 ± 0.0 ‰ (tab.2.1).* On the other range, the two cultivars: *RRSR-15-159-2-7-4-A-4; SR99-1-413-2-15*, showed the highest $\Delta^{13}C$ value with the mean in the range of *23.1 ± 0.1 ‰* and *23.0 ± 0.0 ‰.* The mixed *ANOVA* depicted statistically significant difference at *p<0.01* between cultivars (*fig. 2.5).*

Table 2.1 *The magnitude of variability in traits among Eps cultivars*

Variety	$\Delta^{13}C$	WUE_i	A_n	SLA	RWC	Chlor	SD	IVD	g_s	N	$\Delta^{18}O$
BBCB-91-232-4-1-16-1-4	21.4 ± .1**	6.2±.5**	31.9± 1.1**	114± 15**	83±2**	52.8±1.5**	60±3**	0.36±.01**	.47 ± .01**	4.4±.4*	24.4±.2**
BBCB-90-231-8-1-17-6-6	21.4 ± .2**	5.6±.2**	30.1± 1.8**	120± 13**	77±4*	50.4±.7*	57±3**	0.30±.02**	.41 ± .03**	4.3±.4*	24.7±.4**
Badger	21.7± .2**	5.6±.4**	31.8± 1.2**	134± 6**	83±2**	51.8±1.7**	62±3*	0.37±.00*	.41 ± .02**	4.1 ±.3	25 ±.2**
BBCB-91-232-4-1-5-5-1	21.8± .2**	5.5±.3**	28.1± .8**	126± 3**	77±1*	50.1±1.5*	61±2*	0.37±.01*	.40 ± .04**	4.2±.2*	25.3±.2**
SR96-1-412-1-11	22.0± .1**	5.3±.2**	27.6± 1.5**	137± 2**	78±2*	49.8±.2*	59±4**	0.32±.00**	.43±.02**	4.3±.2*	25.6±.1**
SSRS-67-147-5-1-12-2-2	22.0± .2**	5.0±.4*	26.5± 2.2*	146± 16*	76±1	50.2±.1*	62±6*	0.37±.00*	.40±.04**	4.2±.1*	25.6±.3**
SSSR-67-147-6-2-4-19-B-7	22.0± .0**	5.2±.1*	27.0± 2.5*	142± 3*	76±1	49.5±.3*	65±3	0.38±.01*	.38±.02**	4.2±.2*	25.7±.0**
SSSR-67-147-6-2-4-8-6	22.1± .0	5.1±.0	26.8± .7*	149± 4	75±3	48.4±.2	64±1	0.40±.01	.39±.01	4.0 ±.2	26 ±.1
BBCB-91-232-4-1-16-3-1	22.2± .2	5.1±.3	27.0± .9*	140± 6*	74±1	48.4±.3	65±4	0.42±.01	.40±.03	4.1 ±.3	26.2 ±.3
Spark	22.2± .1	5.4±.7**	29.8± 1.9*	150± 5	77±0*	48.2±.6	63±5	0.39±.00	.39±.01	4.0 ±.3	26 ±.0
SSSR-64-32-8-1-3-12-1	22.3± .3	5.0±.3	23.8± 1.3	159± 6	76±3	47.4±1.7	66±3	0.43±.00	.37±.05	4.1 ±.3	26.7 ±.1
RRSR-33-187-3-2-6-8	22.4± .4	5.0±.2	25.1± 1.0	144± 23*	76±1	48.0±.4	64±2	0.38±.00*	.33±.03	4.1 ±.1	27 ±.1
Rialto	22.5± .1	5.0±.3	24.1± 1.4	157± 3	74±1	47.6±.7	66±0	0.42±.00	.37±.02	4.0 ±.3	26.7 ±.2
SSRS-67-147-6-2-4B-2-3	22.5± .1	4.7±.3*	24.4± .1	168± 4*	71±2	47.9±.7	66±5	0.43±.01	.37±.04	4.0 ±.2	27 ±.0
SR99-1-413-2-8	22.6± .2	4.9±.4	23.2± .8	172± 8*	70±1*	45.8±.9*	69±0	0.43±.02	.37±.03	3.9 ±.1	27.7 ±.4
RRSR-33-187-3-2-6-5	22.6± .1	4.8±.3*	23.6± 2.1	172± 13	70±4*	47.0±.3	71±3**	0.44±.00	.32±.01**	4.0 ±.1	27.5 ±.3
SR94-1-411-1-2	22.7± .1	4.8±.2	24.6± 1.5	165± 2*	70±3*	47.9±1.0	69±1	0.42±.01	.33±.04	4.0 ±.2	27.7 ±.4
SSRS-64-84-4-2-18-1-5	22.7± .2	5.0±.5**	23.5± 1.3	173± 11**	68±5*	46.7±1.3	70±1	0.46±.00*	.35±.01	3.8 ±.2	27.5 ±.3
SR94-1-441-1-7	22.7± .2	4.8±.3*	23.5± 1.2	192± 4*	70±4*	46.9±.5	71±4**	0.43±.00	.32±.05*	3.8 ±.2	28 ±.0
SSSR-67-147-6-2-4-8-4	22.7± .3	4.1±.2**	23.4± 1.3	176± 17*	66±2*	46.4±.7	71±1**	0.44±.01	.31±.04**	3.6±.2*	28.2 ±.1
SSSR-64-32-8-1-3-18-1	22.7± .4	4.0±.2**	21.1± .8	177± 14*	67±1*	44.1±1.2*	73±1**	0.46±.00*	.30±.05**	3.9 ±.2	28.6±.3**
SR99-1-413-2-15	23.0± .1**	4.3±.0**	22.7± .6*	176± 8**	65±3**	45.7±.8*	75±2**	0.44±.01	.32±.04**	3.8±.3*	29.1±.1**
RRSR-15-159-2-7-4-A-4	23.1± .1**	4.2±.1**	22.4± 1*	194± 17**	65±4**	45.5±.6*	72±1**	0.47±.01**	.30±.05**	3.7±.1*	29.5±.3**

The values are means ± SE. Mean value that is statistically significant different compared to the control "Rialto" is marked. * designates a significant difference at $p< 0.05$, and the ** indicates the significance of difference at $p< 0.01$. The values for $\Delta^{13}C$ are expressed in ‰, for WUE_i in $\mu mol\ CO_2\ mmol^{-1}\ H_2O$, in $cm^2\ g^{-1}$ for *SLA*, in $\mu mol\ CO_2\ m^2\ s^{-1}$ for A_n, in % for RWC, *SPAD* index for chlorophyll content, in number per mm^2 for stomata density (SD on abaxial), and in *mm* for inter-vein distance (IVD). The g_s expressed in mmol $m^2\ s^{-1}$. The N content measured in %, and the $\Delta^{18}O$ expressed in ‰. N= 8.

Although the pattern of variation in leaf organic $\Delta^{13}C$ appeared as though it may has been driven by *SLA*, the trend was also observed in variation with *RWC* and *IVD*: cultivars with low *SLA* tended to have both higher leaf *RWC* and short *IVD*, while low leaf RWC and larger IVD were generally observed in cultivars with higher *SLA*. The photosynthetic net assimilation rate varied between *31.9 ± 1.1µmol* m^2 s^{-1} and *22.4 ± 1.0 µmol* m^2 s^{-1} *(tab. 2.1).* Plants tended to have higher stomata density on abaxial than adaxial side with stomata ratio in the range of *1.3* and *1.0.*

2.3.2 Relationships and Correlations between *SLA* and traits of photosynthetic rate

The extent to which a trait is useful as a selection criterion depends upon its relationships with others traits of performance. The regression analysis and partial Pearson correlation depicted consistently the relationships between *SLA* and the traits of photosynthetic efficiency (*fig.2.6).*

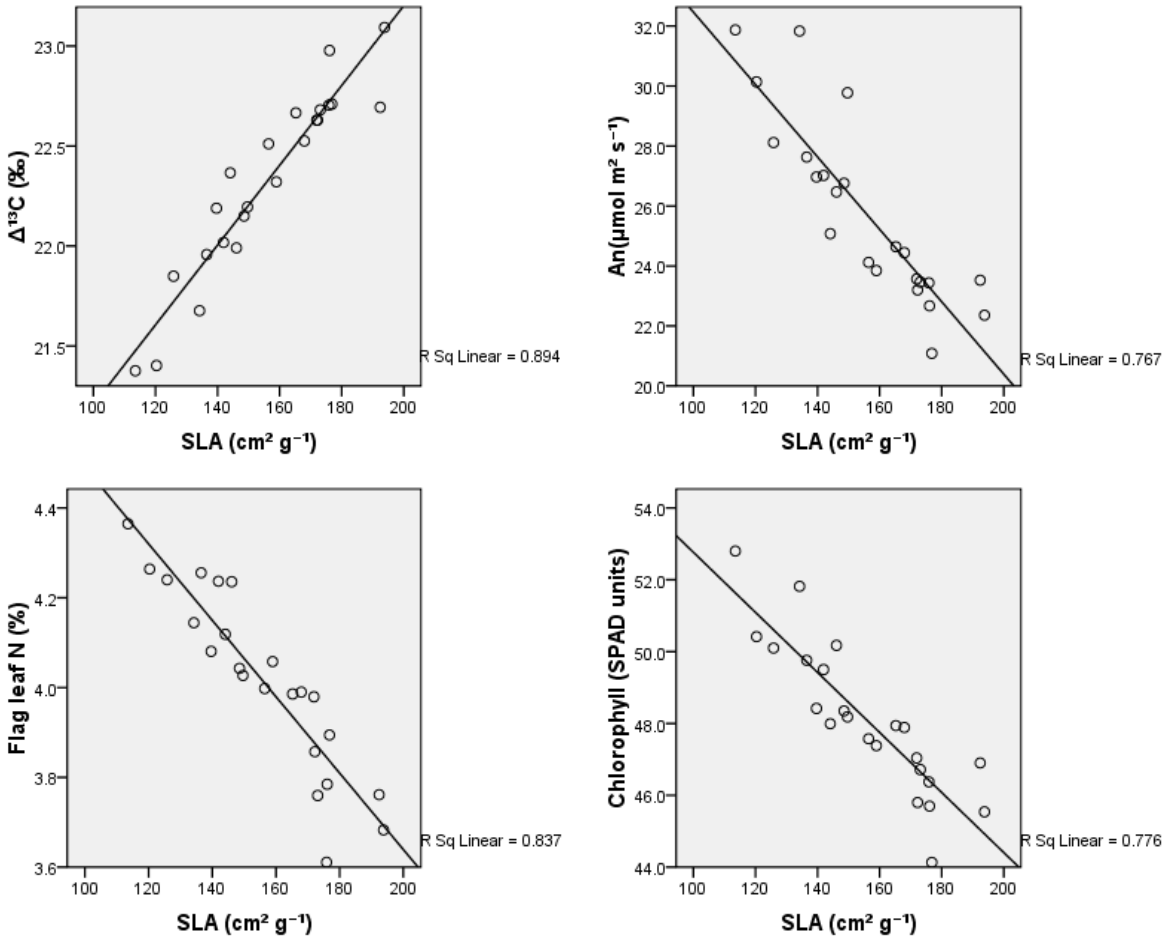
The experiment revealed an intimate positive association between *SLA* and $\Delta^{13}C$ (*fig.2.6).* The linear regression accurately described the significant correlation of $\Delta^{13}C$ to *SLA* (R^2*=0.89; p<0.01).* The cultivars with low *SL*A exhibited lower values of $\Delta^{13}C$ in their leaf matter than cultivars with higher *SLA* (*fig.2.6).* Because the value $\Delta^{13}C$ in leaf organic matter mainly results either from the effects of photosynthetic capacity or stomata conductance, or covariance of both (Dawson *et al.,* 2002); the regression analysis of the relationship between $\Delta^{18}O$ and $\Delta^{13}C$ was conducted and revealed a strong relationship (R^2= 0.77).

Table 2.2 *Pearson correlation between SLA, IVD, RWC, A_n, g_s, N, $\Delta^{13}C$, $\Delta^{18}O$, WUE_i, SD and chlorophyll Cont. (*df = 20*)*

Parameter	$\Delta^{13}C$	WUE_i	A_n	*SLA*	*RWC*	Chloro cont.	*SD*	*IVD*	g_s	leaf *N*
WUE_i	-.88**									
A_n	-.92**	.89**								
SLA	.95**	-.86**	-.88**							
RWC	-.92**	.90**	.89**	-.88**						
Chloro cont.	-.92**	.89**	.92**	-.88**	.90**					
SD	.92**	-.85**	-.86**	.91**	-.90**	-.87**				
IVD	.87**	-.76**	-.81**	.85**	-.79**	-.82**	.91**			
g_s	-.89**	.89**	.86**	-.87**	.87**	.87**	-.88**	-.75**		
leaf *N*	-.89**	.81**	.77**	-.91**	.89**	.84**	-.87**	-.83**	.82**	
$\Delta^{18}O$	.97**	-.91**	-.90**	.94**	-.94**	-.92**	.94**	.84**	-.92**	-.90**

**. Correlation is significant at the .01 level (1-tailed). The values for $\Delta^{13}C$ are expressed in *‰,* for WUE_i in *µmol CO_2 mmol^{-1} H_2O,* in *cm^2 g^{-1}* for *SLA,* in *µmol CO_2 m^2 s^{-1}* for A_n, in *%* for RWC, *SPAD* index for chlorophyll content, in number per *mm^2* for stomata density (SD on abaxial), and in *mm* for inter-vein distance (IVD). The g_s expressed in mmol m² s⁻¹. The N content measured in %, and the $\Delta^{18}O$ expressed in ‰.

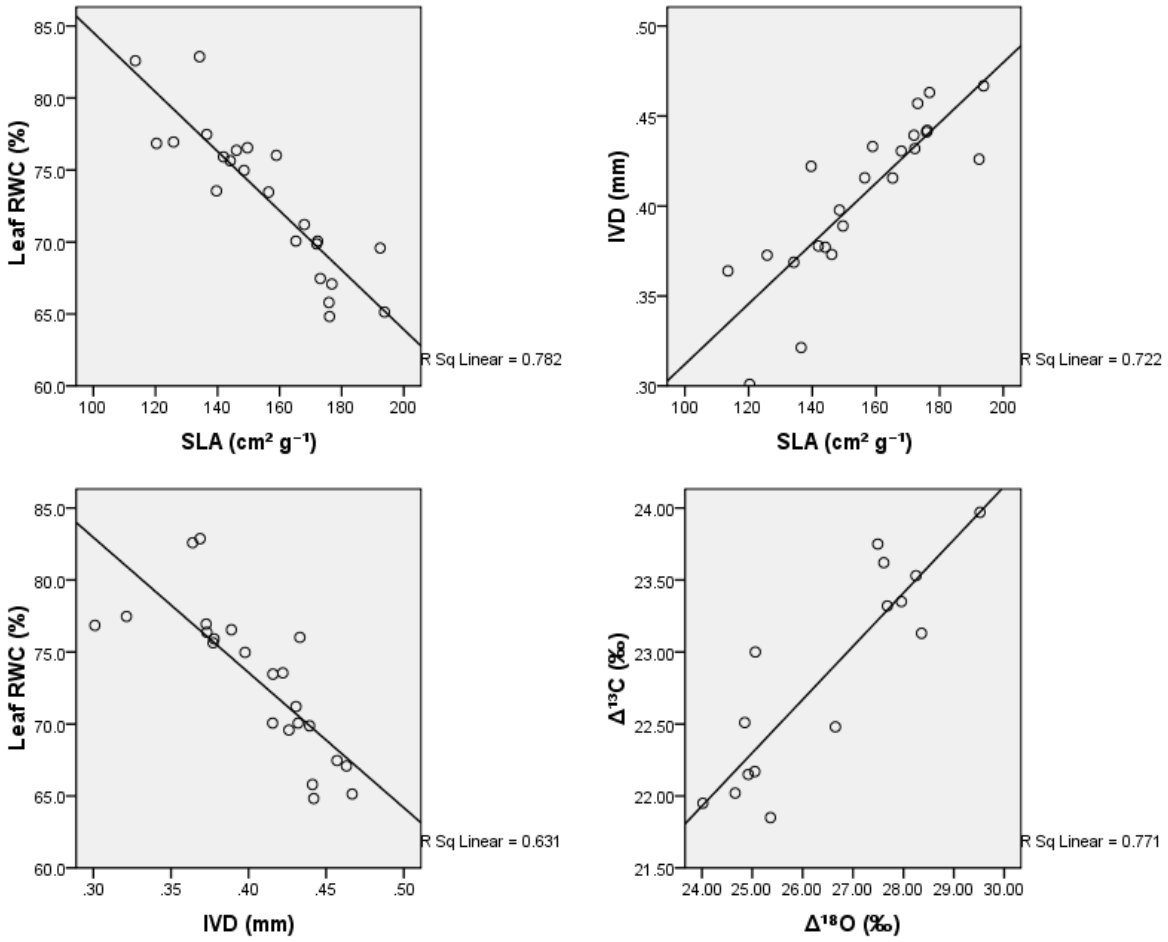
Significant statistical Pearson correlation (*$p<0.01$)* was observed between *SLA* and the flag leaf nitrogen content at anthesis (*$r= - 0.92$).* Similarly, the photosynthetic assimilation and chlorophyll content at anthesis were strongly related to *SLA* (*$R^2=0.77$; $R^2=0.78$* respectively). The stomata conductance was significantly related both to A_n, and WUE_i (R^2=0.73; R^2=0.80, respectively. All *ps* < .01).



The symbol represents individual measurement.
Figure 2.6 *Relationship between SLA and traits of photosynthetic efficiency*

2.3.3 Relationships and correlations between *SLA* and traits of water use efficiency

The link between *SLA*, leaf *RWC*, WUE_i, and *IVD* have been investigated, and the regression analysis indicated that *SLA* was significantly ($p<.01$) related both to the leaf *RWC* and *IVD* ($R^2 = 0.78$; $R^2 = 0.72$ respectively).



The symbol reprents individual measurement.
Figure 2.7 *Relationships between SLA, RWC, and IVD*

The variation of *SLA* among cultivars was associated with differences in leaf RWC, with dense leaves exhibiting relative higher water content than thin leaves. The values of SLA were statistically significant *($p<.01$)* related to the WUE_i *($R^2=0.75$).* Additionally, closer veins were associated and statistically significantly correlated with higher leaf relative water content, and lower *SLA* (*fig. 2.7).* Moreover, the Pearson correlation analysis indicated statistically significant correlation (*$p<.01$)* between *IVD* and leaf $\Delta^{13}C$ (*$r=-0.87$).*

2.4 Discussion

The research of this chapter dealt with the proxy-based approach to crop selection. The focus was particularly given to the proxies of photosynthesis and water use at the leaf level. The approach of *'proxy-based crop selection'* was introduced and defined. In contrast to the previous works (Ehleringer *et al.*, 1993; Rebetzke *et al.*, 2006; Bindumadhava *et al.*, 2006) that tended to focus on single tool such $\Delta^{13}C$ for selection, this study built on comprehensive examination of the interactions between physiological processes, to develop an integrated proxy of a particular physiological variable.

Emphasis in discussion will be led on the following three aspects;

i) Ranking for proxies and implications for selection of cultivars

ii) SLA as an integrated proxy of photosynthesis and water use efficiency

iii) The mechanism that links leaf venation to photosynthesis and water use

2.4.1 Ranking for proxies and implication for crop selection

Consistency of genotypic ranking is essential for breeding to be effective in selecting for a particular quantitative trait (Ehleringer *et al.*, 1993). Ranking of the 23 *Eps* cultivars for low $\Delta^{13}C$ based on leaf material, did show consistent pattern of variation, and were largely maintained in the ranking for low *SLA (fig.2.6).* In agreement with photosynthetic gas exchange results, the ranking for WUE_i were mostly similar to the ranks for both low *SLA* and $\Delta^{13}C$ in leaf organic matter; thus suggesting it is possible to relate WUE_i in wheat genotype to both *SLA* and carbon discrimination values of leaf matter.

The consistent pattern of ranking for *SLA* and $\Delta^{13}C$ among cultivars is of particular interest in the way SLA is cheap and easy to measure than $\Delta^{13}C$: it provides indication that *SLA* could be used in place of $\Delta^{13}C$ for selecting wheat cultivars. The results are in agreement with Farquhar *et al.* (1982) who first proposed that $\Delta^{13}C$ is a promising parameter for assessing and eventually selecting plant genotypes for water use efficiency in C_3 plants. The results are

also consistent with Vogel *(1993)* who suggested the $\delta^{13}C$ value in leaf matter of terrestrial C_3 plants growing under natural conditions range from - 22 to – 34 ‰; in close agreement, the $\delta^{13}C$ of leaf dry matter among the *23 Eps* cultivars tested in this study, varied between - 28 and - 31 ‰.

Many authors have exploited the ranking for selection of crop cultivars (Ehleringer *et al*., 1993; Acquaah, 2012), and their results showed ranking could be a powerful tool to make inference in crop selection: for example, Hall *et al*. (1993) used the consistency of ranking for $\Delta^{13}C$ and grain yield for selection of cowpeas, and they observed the ranking of accession for $\Delta^{13}C$ was remarkably consistent when the same genotypes were grown over different drought conditions, years, and date of sampling, but at the same conditions. Similarly, Garnier *et al.* (2001) based on ranking of species for functional traits, found the species ranking for a given trait remained mostly consistent in space and time. Further, Condon *et al.* (2004) used the genotypic ranking for selecting broad sense heritability of $\Delta^{13}C$ in wheat. Similary, Rajabi (2006) exploited ranking of leaf organic $\Delta^{13}C$ for selecting sugar beet cultivars for drought tolerance, and observed the ranking was closely maintained over time.

Overall, considering the consistent of the ranking for the proxies of photosynthetic rate and water use among the *23 Eps* cultivars screened over the research of this chapter, and the statistically significant difference between each particular cultivar and the control *'Rialto'*, five cultivars were proposed as selected for photosynthetic and water use efficiency, and to be submitted for further selection for earliness of flowering and yield. The selected cultivars were: *BBC-91-232-4-1-16-1-4; BBCB-90-231-8-1-17-6-6; Badger; BBCB-91-232-4-1-5-5-1; SR96-1-412-1-11*.

2.4.2 Specific leaf area *(SLA, $cm^2\ g^{-1}$)*: An integrated proxy of photosynthetic rate and water use in wheat

Identification of physiological traits contributing to superior performance of crop has been a long term goal of plant improvement (Reynolds *et al.*, 2011). The results in this chapter provided considerable evidence that *SLA* is a potential indicator of leaf photosynthetic rate and water use. In this section, the relationships between *SLA* and the traits of photosynthesis and water use are discussed and follow by the discussion of the patterns of trait of SLA and water use efficiency.

2.4.2.1 SLA and leaf biochemical characteristics of photosynthesis

The comparison of cultivars varying in *SLA* revealed a greater pattern of association between SLA and A_n (*fig*.2.6). Some sets of hypothesis could help explain the negative association between *SLA* and photosynthetic assimilation rate; the first explanation is by the mechanistic that links *SLA* to the leaf biochemical characteristics (Chlorophyll content, leaf *N*, Rubisco); According to Lambers *et al.* (2008) one mechanism leaves on a plant achieves a high A_{max} is by producing thicker (low SLA) leaves and which provide spaces for more chloroplasts per unit leaf area. Similarly, Evans & Poorter (2001) indicated that thicker leaves (thus low *SLA*) are associated with increases in number of chloroplasts and the amount of photosynthetic enzymes; thereby may enhance the photosynthetic capacity per unit leaf area. The *SLA* was investigated and used in this thesis as a proxy of leaf thickness; therefore leaf thickness per se was not measured in this study. However, the Rebetzke's group has studied some technique for the measurement of leaf width (Zhang *et al.*, 2014). In similar fashion, Farquhar & von Caemmerer (1980) argued that the capacity of the leaf tissue for photosynthetic CO_2 assimilation depends to a large extent on its Rubisco content.

Over the past years, a number of correlations have been uncovered relating photosynthetic capacity of the leaf to leaf *N* content (Evans, 1989; Schulze *et al.*, 1994; Reich *et al.*, 1994):

That is the higher leaf *N* content was found to be associated with higher rate of photosynthesis. The mechanistic causes of these relationships were attributed to the large amount organic *N* present in the chloroplasts, most of it in the photosynthetic machinery (Evans & Seemann, 1984). It had also been found that Rubisco and chlorophyll content both tend to increase with leaf *N* content (Evans & Terashima, 1988), they argued that with increased leaf *N*, the chlorophyll content and electron transport capacity increase. It was also observed that the amount of light absorbed by a leaf, and the diffusion of CO_2 through its tissue depend, at least partially, on its thickness (Agustin *et al.*, 1994; Syvertsen *et al.*, 1995). Therefore, the strong relationships obtained between *SLA* and both leaf chlorophyll and nitrogen contents *(fig.2.6);* provide indication that *SLA* would possibly be a good proxy to distinguish variation in photosynthetic capacity in wheat.

2.4.2.2 SLA and leaf water relations

The *SLA* appeared to be a potential indicator of plant water use efficiency and related to $\Delta^{13}C$ in this study (*tab*.2.3). In agreement with photosynthetic gas exchange results, the $\Delta^{13}C$ measured in the leaf dry matter were also negatively correlated with WUE_i as expected for C_3 plants (O' Leary, 1993; Farquhar *et al.*, 1982). The relationships between $\Delta^{13}C$ and WUE_i indicated it is possible to relate instantaneous photosynthetic gas exchange to estimate water use efficiency in wheat genotypes with $\Delta^{13}C$ values from leaf matter. Previous studies indicated there are several mechanisms by which $\Delta^{13}C$ in the leaf is regulated: According to Taiz & Zeiger (2010), in C_3 plants, genotypic variations in $\Delta^{13}C$ values in leaf results either from photosynthetic capacity or leaf conductance or the covariance of both.

The strong positive relationships obtained between $\Delta^{13}C$ and $\Delta^{18}O$ in leaf matter was further evidence supporting the data that the relationships between WUE_i and $\Delta^{13}C$ values in this study were mainly caused by variation in photosynthetic capacity. This view corroborate with Barbour *et al*.(2000) who used the measurements of $\Delta^{18}O$ in leaf matter to separate the

independent effect of photosynthetic capacity on carbon discrimination in C_3 plants. The results are also in accordance with Scheidegger *et al.* (2000) who proposed the potential of measuring both $\delta^{13}C$ and $\delta^{18}O$ in leaf organic matter to separate the independent effects of photosynthetic capacity and stomatal conductance on C_i/C_a. According to Dawson *et al.* (2002), whereas leaf $\delta^{13}C$ reflects C_i/C_a, $\delta^{18}O$ in leaf matter varies with ambient humidity, which in turn reflects changes in water use. Additional evidence in support that the variation in Δ^{13}C values among cultivars was due to difference in photosynthetic capacity came from the stomata conductance data: the cultivars with low values of Δ^{13}C exhibited both relatively higher stomata conductance, and higher photosynthetic assimilation rate compared to the cultivars with relatively higher value of Δ^{13}C (tab. 2.1).

Moreover, the finding of consistent positive relationships between *SLA* and $\Delta^{13}C$ is of particular interest *(fig. 2.6)*: it linked indirectly the WUE_i and *SLA*, and hence indicating the possibility of using *SLA* as surrogate measure of $\Delta^{13}C$ in selection for WUE_i in wheat. The proposition of using *SLA* as surrogate for $\Delta^{13}C$ and WUE_i is promising in the way it is easiest and cheap to measure while measurement of Δ^{13}C requires expensive analytical device makes it more expensive proxy to obtain.

Additionally, measurements based on *SLA* appears to be more repeatable than those based on biochemical: This can be deducted from the study of Hevia *et al.* (1999) who showed that *SLA* varied less than leaf phosphorus during the course of growing season. This conclusion can probably be extended to all foliar nutrients (Grimshaw & Allen, 1987). This finding is very important, because the discrepancies between reproducibility among experiment may well result in the unreliability of the results.

Closer association between *SLA* and leaf *RWC* (*fig. 2.7)* was observed in this research. Leaf water content is known to be related to several leaf physiological variables (Kramer & Boyer, 1995). For instance, Farquhar *et al.* (1989) argued that the leaf *RWC* closely reflects the

balance between water supply and transpiration rate. The argument was supported by Yamasaki & Dillenburg (1999) who suggested that leaf *RWC* is a useful indicator of plant water balance. Similarly, Joy (1985) conducted research to address the question of which parameter of water status should be used to measure water stress, and found the leaf *RWC* reflected recent loss of water by transpiration and the rate of flow of water into and through the plant. Genetic variation in leaf *RWC* was also observed by Lafitte (2007) who detected significant difference in leaf *RWC* among cultivars exposed to the same period of water exclusion.

Additional insights were provided by many authors that observed that decreasing of the leaf *RWC* of both C_3 and C_4 plants progressively decreased the photosynthetic CO_2 assimilation (Chaves, 1991; Cornic, 1994; Cornic & Massaci, 1996; Lawlor & Cornic, 2002; Lawlor, 2002). The data of this research are in agreement with those observations: the cultivars with low *RWC* exhibited both reduced photosynthetic assimilation rate and stomata conductance compared to the cultivars with relatively higher *RWC* (tab.2.1). The mechanistic causes of the relationship between leaf RWC and photosynthetic rate were provided by Lawlor (2002) who firstly suggested the decline in leaf *RWC* caused the decreases in stomatal conductance, slowing CO_2 assimilation; secondly, he suggested that the limitation of *RuBP* might be among the causes of decreased photosynthetic rate at low leaf *RWC*. Thirdly, he observed that at low leaf *RWC* the amount of many proteins was decreased: and he suggested that decreased *ATP* under low leaf *RWC* impaired proteins synthesis, through inadequate energy supply (Lawlor, 2002).

Taken together, the strong relationships between *SLA* and leaf *RWC*, and $\Delta^{13}C$ observed in this research, further confirmed the proposition that *SLA* constitutes a good estimate of plant water status.

2.4.3 The mechanism linking leaf venation to photosynthesis and water use

Leaf veins form the transport network for water, nutrients, and carbon for nearly all plants (Brodribb *et al.,* 2005). However, leaf venation is highly diverse within and across species (Cochard *et al.,* 2004b; Sack & Frole, 2006; Ellis *et al.,* 2009; Brodribb *et al.,* 2005). The aim of this research was to investigate the link of the structural properties of leaf venation to the functional processes of water use and photosynthetic efficiency. The priori hypothesis was that the distance water must flow, as determined by the position of leaf veins, should influence the leaf hydraulic properties of the plant.

Leaf venation in our study of *23 Eps* cultivars fell into distinct groups: the group of closer veins which demonstrated a strong association with leaves of low *SLA*, and another group of larger IVD which was associated with leaves of higher *SLA (fig.2.7).* Additionally, our data indicated the leaves with low *SLA (*also with closer veins*)* to be associated with higher leaf *RWC*, higher A_n, and higher WUE_i than leave with higher *SLA* and larger *IVD.*

These results corroborate with Scoffoni *et al.* (2012) who found that xylem cavitation which is often observed during dehydration, was better tolerated in their study by leaf with higher vein density. Another explanation was provided by Amiard *et al.* (2005) who argued that vein density would relate to photosynthetic functions because the vein surface area is thought to limit photosynthate transport away from the leaf.

In conclusion, our data suggested the link of *IVD* to the photosynthetic rate would probably be rooted in the effect of *IVD* on leaf RWC. Finally, our data provided basis for suggesting that *SLA* potentially constitutes the reliable proxy of photosynthetic rate and water use efficiency.

Chapter 3 The physiological consequences of *Rht genes* in winter wheat

"A wheat breeder who recently produced a high yielding variety of wheat, was asked what attributes gave it such capacity for yield; he replied, "I do not know…but I will list the traits of the variety, and it is for the physiologist to judge whether these may be the reasons for the high yield" (Donald, 1968).

Abstract

Considerable progress in wheat yield has been achieved during the so-called green revolution by the development of dwarf varieties through introgression of the *Rht* genes. The straw-shortening by these genes increased the harvest index (*HI)* by alteration of partitioning of dry matter and assimilates in favour of spike. However, the physiological basis of *HI* is not completely established yet. For example, comparative studies have shown that yield is reduced when plants are shortened beyond a threshold optimum. The aim of the investigation reported here was to identify the physiological attributes able to produce yield increases in *Rht* genotypes without further straw-shortening. Attention was given to examination in a controlled environment, the question of the mechanistic foundation that determine the relationship between plant height and yield in lines (*Rht-B1b*, *Rht-D1b*, *Rht-B1c*, *Rht-D1c)* with different *Rht* genes (*b*, *c*) when incorporated into contrasting background genomes (*B*, *D*); and the relative effects on *C:N* partitioning during grain filling. The results showed the straw-shortening was significantly associated with A_{max} and K_h *(p<.01)*. The *SLA* decreased with the level of dwarfing; and A_{max} significantly correlated with K_h and *SLA*: we therefore proposed that straw-shortening may affect both A_{max} and K_h by exerting a controlling influence over *SLA*. Similarly, both the partitioning of *N* to spike and the flag leaf *N* content were correlated with plant height and growth stage; the leaf *N* was highest at *GS65*. The data also indicated that, in short genotypes, increases in grain number, kernel weight, and reduced partitioning to the roots were the main driver of the increased *HI*. Moreover, the increased post-anthesis partitioning of *N* to grain associated with high *N* uptake rate and high *MRT* of *N* were the traits behind increased *NUE* and *NHI* in this experiment with wheat of reduced height. In conclusion, selection for increased *HI* of *Rht* wheat should shift focus from reduced plant height to include increased grain number and kernel weight, reduced partitioning to roots, increased partitioning of *N* to spike, reduced peduncle length, and low *SLA*.

Key words: *Rht*, wheat, *HI*, grain number, Partitioning to roots, *N* partitioning.

3.1 Introduction

To date, progress in wheat yield has been spurred in part by the widespread introduction of dwarfing genes (Reynolds *et al.,* 2011), and has been related to changes in plant morphology and function associated with the large increase in the *HI* (Fischer, 2011). From early 1960s, world-wide, wheat yield increased noticeably (at an average rate of 40 kg ha^{-1} $year^{-1}$) from 1 t ha^{-1} in 1960 to 2.6 t ha^{-1} in 2005 (Miralles & Slafer, 2007).

However, challenges to wheat production are still considerable as highlighted at a symposium involving scientists from wheat producing countries (Reynolds *et al.,* 2007). For example, a closer look at the average wheat yield during the last few years give cause of concern: the rate of yield increases between 1980 and 2005 was 36 kg ha^{-1} $year^{-1}$ (~14 % less that registered during the whole period from 1960), suggesting wheat yield might be leveling off (Miralles & Slafer, 2007). Moreover, global demand for wheat is predicted to increase at a faster rate (Rosegrant & Cline, 2003) than the annual gain in grain yield that are currently being realized (Shearman *et al.,* 2005). In addition, climate change impacts on agriculture indicate that there is a real risk of global wheat yield shortfall.

According to Calderini *et al*. (1995), one of the main attribute behind the increased wheat yield in the past, had been plant height, which has been systematically reduced as an immediate result of the introgression of *Rht* genes. Additionally, straw-shortening has also improved resistance to lodging (Paolillo & Niklas, 1996). However, a number of comparative studies have shown that wheat yield is reduced when plant are shortened too much, thus delimiting a range of plant height to optimum yield (Miralles & Slafer, 1995; Berry *et al.*, 2014). Therefore, the likelihood of further increasing wheat yield through additional reductions in plant height is rather low as several studies have indicated that most of modern wheat cultivars have reached the optimal heights (Reynolds *et al.,* 2011; Fischer, 2011; Foulkes *et al.,* 2007; Shearman *et al.,* 2005). Thus, it is clear that the aim of future wheat

improvement should be to increase grain yield within the optimal plant height. On that, Uppal & Gooding (2013) examined the effect of tillage systems on *Rht* genotypes; they found no evidence that the optimal ultimate crop height as modified by dwarfing genes, varies with tillage systems.

The investigation presented in this chapter aimed to identification of physiological traits able to produce wheat yield increases without further straw-shortening in lines (*Rht-B1b*, *Rht-D1b*, *Rht-B1c*, *Rht-D1c)* with *Rht* genes (*b*, *c*) of contrasting genomes background (*B*, *D*), and compared to the "Wild type" in Mercia background.

Specifically, the study addressed the following questions and hypotheses:

Research questions: (i) What is the mechanistic foundation that determines the relationship between plant height and grain yield?

(ii) Which physiological traits related to grain yield and harvest index in semi-dwarf wheat?

Hypotheses: (i) The N partitioning to spike is a trait related to plant height.

(ii) The distance that water must flow could influence the leaf hydraulic conductance and the photosynthetic rate of the crop.

The present introduction consists of five sections. Firstly, it provides the theoretical background of *Rht* genes involved in this study; secondly, it reviews the basis of the *HI*; thirdly, it deals with tillering of wheat; fourth, it discusses the regulation of flowering in wheat; and lastly, it gives background on *MRT* of *N* and ^{15}N labeling.

3.1.1 Theoretical background of *Rht* genes

The worldwide adoption of the reduced height (*Rht*) genes in wheat has proceeded since the early 1960_s (Flintham *et al.*, 1997; Bonnet *et al.*, 2001). These genes have been effective in reducing plant height by decreasing sensitivity of vegetative tissues to endogenous gibberellic acid (Keyes *et al.*, 1989; Rebetzke *et al.*, 2012). The mechanisms by which the *Rht* genes

effect a reduction in plant height are relatively well understood (Ellis *et al.,* 2004). According to Wilhelm *et al.* (2013), the *Rht* genes encode copies of the *DELLA* protein, a growth repressor, and each *Rht* gene contains a single nucleotide polymorphism that introduces a premature stop codon. The resulting *DELLA* proteins have a reduced sensitivity to *GA*: because the *Rht* genes affect *GA*-signalling, and *GAs* are involved in many development processes. The *Rht* genes have a range of effects on plant including the reduced coleoptiles length, decreased internode length, and shorter plant height (Gale *et al.,* 1985; Allen, 1989; Richards, 1992*b*; Peng *et al.,* 1999; Rebetzke *et al.,* 2004; Botwright *et al.,* 2005).

The height reductions arising from the presence of the *Rht* genes were associated with genotypic increases in *HI* and lodging resistance of wheat worldwide (Chapman *et al.,* 2007; Berry *et al.,* 2007), however, there has been no much precise physiological explanation of their effects on yield (Gale & Youssefian, 1985; Youssefian *et al.,* 1992*a, b*).

The genetics of final plant height is known to be complex, being determined by many genes. In wheat seventeen of the 21 chromosomes were found to determine genetical variation for height (Borner *et al.,* 1996). According to Worland (1996), it is possible to classify genes for height into those which increase or promote height, and those which reduce or suppress this character. Related to their response to exogenously applied gibberellins *(GAs)*, dwarf mutants can be divided into two categories (Reid *et al.,* 1996):

(i) *GA* sensitive mutants, where the absence or modified spectrum of endogenous gibberellins result in a dwarf plant, and in which normal growth can be restored by *GA* application.

(ii) *GA* insensitive mutants, that show a reduced response or complete insensitivity to applied *GA*. According to Gale & Gregory (1977), the *GA* insensitive mutants exhibit a reduced internodes length without reducing the length of the spike.

The *Rht-B1b, Rht-D1b, Rht-B1c* and *Rht-D1c* are among the twenty or so major genes affecting the plant height in wheat (Li et *al.,* 2012*a, b*). These four genes are genetically related, with *Rht-B1b* and *Rht-B1c* being alleles at a locus on chromosome *4B,* and *Rht-D1b* and *Rht-D1c* being alleles at a locus *4D* (Borner & Mettin, 1988; Pearce *et al.,* 2011; Wilhelm *et al.,* 2013). The greatest reductions in height are associated with *Rht-B1c* and *Rht-D1c* (Izumi *et al.,* 1981; Gale & Youssefian, 1985).

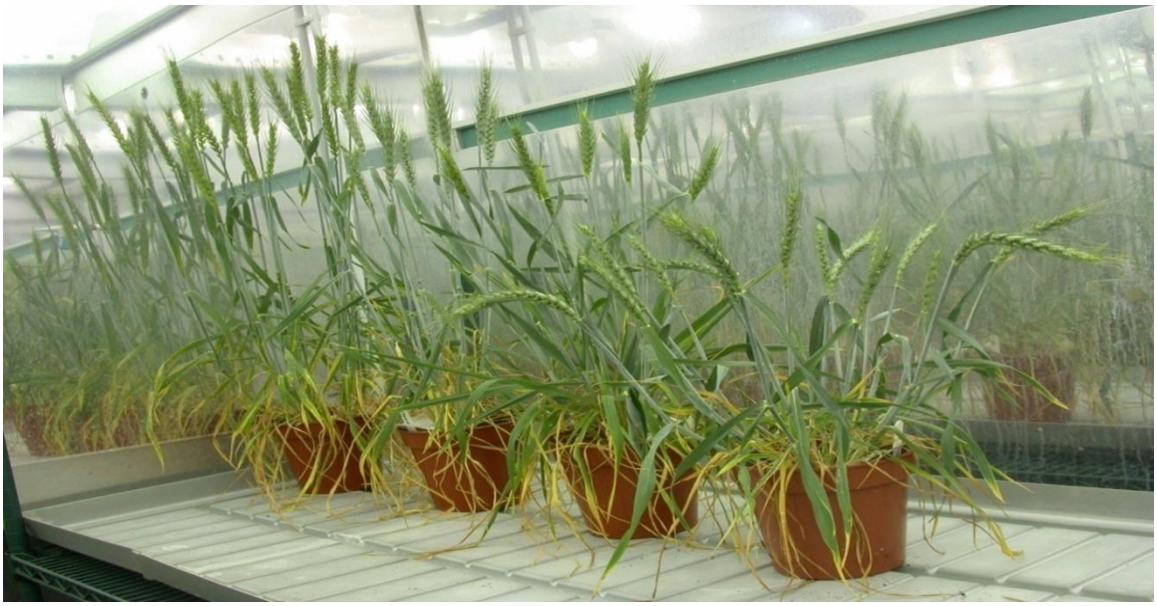


Figure 3.1 *The Rht lines tested in the experiment (Rht-B1b; Rht-D1b; Rht-B1c; Rht-D1c)*

3.1.2 The basis of the concept of *"Harvest Index"*

3.1.2.1 Historical background of *HI*

The increases in the fraction of above-ground biomass partitioned to useful parts of plant has been a feature of the selection and breeding of higher yield crops (Fitter & Hay, 1987). The *HI* appeared in the literature in the late 1950s (Hay, 1995), and it was linked with Donald's concept of the ideotype as a blueprint of wheat breading (Donald, 1968). Over the years since 1962, the use of term *HI* became more widespread because of the introduction of shorter-straw cereal varieties (wheat, rice, & barley), and the growing interest in the use of *HI* in interpreting the physiology of cereal crops (Hay & Walker, 1989).

Donald (1962) first defined the *HI* as the grain yield of a wheat crop expressed as a decimal fraction of total above-ground matter production. His definition has been criticized for ignoring the possibility of substantial variation in the partitioning of assimilates to below ground organs (Siddique *et al.,* 1990). For instance, Barraclough (1984) indicated that about 10 % of crop biomass is below ground at anthesis. To counteract this error associated with Donald's definition of *HI,* Akita (1989) used a correction factor of 5 % to allow for rice roots at harvest. In this context, some authors preferred to use two terms: *Actual harvest index* (following Donald's original concept), and *Apparent harvest index*, determined as a proportion of total dry matter including roots (Walker & Fioritto, 1984).

The concept of harvest index has been extended to the partitioning of nutrients, in particular, the nitrogen harvest index of seed crops, as ratio of nitrogen in grain to total nitrogen content of the plant biomass (Austin, 1980). It seems likely, therefore, that the *HI* concept is to stay in world literature in the future.

3.1.2.2 The mean residence time (*MRT*) and partitioning of *N*

Hirose (2011) defined the *MRT* of *N* as the expected length of time that a unit of *N* newly taken up from soil is retained in the plant before being lost. The *MRT* of *N* is estimated by adding ^{15}N labeled to soil, and its fate in plant is followed over time (Berendse & Aerts, 1987; Silla & Escudero, 2004).

The importance of grain nitrogen concentration to the baking quality and nutritional value of wheat is well established (Heitholt *et al.,* 1990; Foulkes *et al.,* 2009). Here, we are interested to examine the plant characteristics that display grain N concentration and exhibit genetic variability in Rht lines. We therefore hypothesized these characteristics in wheat are likely to be the *NUE*, *N* harvest index, and mean residence time of *N*, and they are likely related to plant height. Moreover, building on the concept of *MRT* of *N*, attention was also given to genetic variability in uptake rate of N among the semi-dwarf *NILs*. According to James &

Richards (2005), the ability to rapidly capture *N* from the soil, is expected to influence plant growth and competitive ability of plant in *N* limiting environment. Similarly, Golluscio (2007) argued that the *MRT* of *N* in plant would be an important indicator of plant adaptation to *N* stress.

3.1.3 The physiology and control of tillering in wheat

The importance of tillering capacity as a determinant of cereal yield has been recognized (Li *et al.,* 2003; Kuraparthy *et al.,* 2007). In wheat, tillering is one of the most important traits, because the tiller number per plant ultimately determines the number of productive spikes (bearing grains) per unit area (Dreccer *et al.,* 2013; Duggan *et al.,* 2005*a*), thus, the development of crop varieties with optimal tiller number could be a target for improving wheat yield. However, there is still a gap in the physiological knowledge of the control mechanisms governing tillering (Kebrom *et al.,* 2012; Dreccer *et al.,* 2013). Therefore, the research of this chapter dealt with the question of whether straw-shortening is associated with tillering in wheat.

In this section, we present a summary of the current knowledge of the factors controlling tillering. According to Assuero & Tognetti (2010), the process of outgrowth of axillary buds in grasses is known as *"Tillering"*. Tillering is a two-steps process (Kebrom *et al.,* 2012); initiation of a meristem in the axil of a developing leaf to form a bud, and its subsequent bud outgrowth. Tillering activity is quantified in term of "*Tiller number*" per plant; Bos & Neuteboon (1998) termed it "*Specific site usage*".

Tillering was found to be genetically regulated (Dreccer *et al.,* 2013). Rebetzke *et al.* (2008) suggested that the QTLs were involved in regulation of productive tillers. Classic genetic analysis has also led to the conclusion that tiller number is controlled by quantitative trait loci (Tang *et al.,* 2001). Moreover, a tiller inhibition gene *(tin)* was identified and mapped in wheat (Richards, 1988; Spielmeyer & Richards, 2004). Kebrom *et al.*(2012) reported that the

tin gene regulates tillering indirectly by controlling the timing of elongation of basal internodes of the main stem. Reduced tillering has also been related to earlier flowering (Gomez *et al.,* 1998). Hormonal control of tillering has also been revealed (Tomlinson & O'Connor, 2004; McSteen, 2009). Tillering and tiller growth have been shown to be stimulated by ethylene (Harrison & Kaufman, 1982). This observation was supported by Rajala & Palotone (2001); they proposed that tillering promotion by ethylene might be the consequence of ethylene mediated inhibition of auxin biosynthesis and movement.

The possible role of gibberellins in the tillering process has also been proposed; Raja & Peltone (2001), suggested that gibberellins tend to cause less development of axillary buds, and also to promote the elongation of already initiated tillers. Additionally, the role of photoperiod on tillering was proposed; Aamlid (1992) and Hay & Kirby (1991) suggested that photoperiod effect tillering mainly through the prolongation of tillering stage. The effect of the ratio of the red to far red light on tillering was observed (Ballarée *et al.,* 1990); they suggested that a low ratio of red to far red light signals for competition for light, and plant responds by inhibiting axillary bud outgrowth.

3.1.4 Basis of time to anthesis in wheat

Wheat varieties are divided into winter and spring based on the presence or absence of a requirement for a long exposure to cold temperature to induce flowering (Vernalization). In both spring and vernalized winter wheat varieties, photoperiod and growing temperature are the main factors affecting development rate and time to anthesis (Lewis *et al.,* 2008). According to Bentley *et al.* (2013), wheat is photoperiod sensitive, flowering only when daylength surpasses a critical length.

The physiological and genetic basis of the regulation of flowering time by photoperiod and vernalization has been documented (Turner *et al.,* 2005; Yan *et al.,* 2006; Beales *et al.,* 2007). On that, Hoogendoorn (1984) reported that sensitivity to photoperiod and vernalization are

controlled by major genes; he suggested that sensitivity to vernalization would be controlled by the presence or absence of insensitivity alleles on the major *Vrn* locus, while sensitivity to photoperiod would be regulated by insensitivity alleles at the *Ppd* locus. Additionally, Whilhem *et al.*(2013) showed that *Ppd-D1* was associated with both reduction in days to anthesis and height. According to Beales *et al.* (2007), the *Ppd-D1* is member of the Pseudo-response regulator *(PRR)* gene family; the *PRR* have been reported to be components of circardian clock (Matsushika *et al.*, 2007). Similarly, Ford *et al.*(1981) showed that sensitivity to photoperiod and vernalization was linked to the pattern of daylength and temperature in the region of the origin of the varieties. In accordance, Bentley *et al.* (2013) observed that photoperiod insensitive wheat flowered rapidly in both short and long days, whereas photoperiod sensitive wheat delayed in short days, and flowered rapidly in long days.

This chapter examined the extent to which *Rht* genes influences the time to anthesis in wheat.

3.2 Material and methods

This section focuses on proxies of yield components as consequences of straw-shortening of wheat. In addition to experimental setting in a growth chamber, and statistical analysis; attentions were given to the measurement of: *(i) HI* and partitioning of *N*; *(ii)* grain number and kernel weight; *(iii)* Tillering; and *(iv)* time to anthesis.

3.2.1 Plant material and conditions in growth cabinet

The measurements were collected on four set of near isogenic lines (NILs) in Mercia background: *Rht-B1b* (formerly *Rht1*) and *Rht-B1c* (formerly *Rht3*) being alleles on *B-Genome*; *Rht-D1b* (formerly *Rht2*) and *Rht-D1c* (formerly *Rht10*) being alleles on *D-Genome*; and wild type. The seeds were supplied to NIAB from the John Innes Centre (JIC) germplasm resources unit. The vernalization requirement of winter adapted germplasm was satisfied at NIAB with 8 weeks cold treatment in a vernalizing cabinet at 6^0C with an 8 hours photoperiod, applied to 2 weeks old seedlings for 6 weeks.

Thereafter, the seedlings were moved to the plant growth facility (PGF) of the University of Cambridge (Bateman St., Cambridge, the UK). There, the plants were placed into controlled environment growth cabinet (Conviron Ltd, Winnipeg, Canada) (*fig.3.2*). Individual seedlings were transplanted in pots of 14 cm height, 11 cm base width, and of 16 cm diameter at top, and filled with Levingtons *M3* compost (Scotts Professional, Bramford, UK). The pots were then arranged in randomized blocks with six replicates.

Figure 3.2 *The Rht plants in the growth cabinet at PGF*

During the entire growth period, the climate in the cabinet was set at: Temperature of 25^0C/23^0C (Day/night); *PAR* of 1200 µmol photons m^{-2} s^{-1} (measured at plant height); relative air humidity 40 %; 400 µmol CO_2 s^{-1}; and 16 hours day length. Two automatic watering regimes of 3 minutes duration were used during the experiment: until anthesis, the watering was achieved by thoroughly wetting capillary matting underneath the pots every 8 hours, and the second regime was set at *GS65*, where watering was only supplied at moisture limit of 50 % in pot, and the irrigation was suspended at *GS83 (Appendix B)*. A basal fertilizer, Osmocote *14-9-11*, was added at the rate of two tablets per pot at *GS20*.

3.2.2 The ^{15}N labeling and sampling

The ^{15}N label was applied to the plants in growth cabinet referred to in the section 3.2.1. In total 60 plants in pots were involved: 30 plants labeled with ^{15}N, and the other 30 plants for the control. At GS65, 50 ml of 2.5 mmol l^{-1} ^{15}N-labelled KNO_3 (99.97 %) was applied to each pot of the 30 pots (6 replicates per line), after BassiriRad & Caldwell (1992). After the labeling, the control and the labeled plants were treated equally.

For the recovery of ^{15}N in the plant parts, half of the labeled plants were harvested 24 hours after the application of ^{15}N label. Similarly, half of the control plants were harvested as well.

The other half of ^{15}N labeled plants were retained for *MRT* of *N* measurement, and harvested at *GS87*. Any unused ^{15}N after 24 hours, by the labeled plants, was removed by flushing four times with 2.5 liters (10 L) of tap water in each pot. The tillers with spike in each pot were harvested and dissected into roots, stem, leaves, and spikes. All root samples were triple rinsed with deionized water.

Thereafter, a composite sample for each plant component was collected per replicate, weighed for fresh mass and immediately frozen in liquid nitrogen. Then, the samples were freeze-dried for 48 hours in Modulyo 4K Freeze dryer (Edwards High Vacuum International, West Sussex, UK). After then, they were weighed for dry mass, and ground using ball mill (MM200 Mixer Mill, Glen Creston Ltd, UK). For each sample, a sub-sample of 1 mg was weighed into tin capsule and analyzed for total *N* and ^{15}N at the Godwin laboratory (University of Cambridge, the UK) as described in the section 2.2.4.

3.2.3 The ^{15}N-enrichment Calculations

With the ^{15}N data, we calculated the *MRT* of *N*, the *N* productivity, the nitrogen use efficiency (*NUE*), and the nitrogen harvest index (*NHI*). The ^{15}N values in plant parts (root, stem, leaf, & spike) were converted to the absolute isotope ratio (*R*) and the molar fractional abundance (*F*) following Teste *et al.* (2014);

$$R\ sample = \left[\left(\frac{\delta^{15}N}{1000}\right) + 1\right] \times R\ standard \qquad (3.1)$$

The absolute value (0.003678) of the natural abundance of ^{15}N in atmospheric N_2 was used as R standard.

$$F = \frac{R\ sample}{R\ sample\ +1} \qquad (3.2)$$

Then the mass –based fractional abundance (MF) was calculated as:

$$MF = \frac{F \times 15}{[(F \times 15) + (1 - F) \times 14]} \qquad (3.3)$$

The sample MF values resulting from the ^{15}N labeling were calculated by subtracting the MF of the control tissue from the MF of enriched sample, resulting in change in MF (ΔMF). Then, the excess sample tissue ^{15}N was calculated as:

$$Excess\ {}^{15}N\ (mg) = Tissue\ N\ concentration \times tissue\ mass \times \Delta MF \quad (3.4)$$

Thereafter, we expressed enrichment of plant as excess ^{14}N equivalent to highlight that N partitioning are shown in the common *N* form that the plant uses.

$$Excess\ {}^{14}N = Excess\ {}^{15}N \times \left(\frac{14}{15}\right) \quad (3.5)$$

The nitrogen productivity (NP) was calculated accordingly to Berendse & Aerts (1987), as the rate of dry matter production per unit of enriched ^{15}N in the plant (g dw mg^{-1} N).

The mean residence time (*MRT*) of ^{15}N was computed as per proposed by Hirose (2011) for both a steady and non steady system;

$$MRT\ of\ N\ (\#days) = N^{-}\Delta T/\Delta N \quad (3.6)$$

where N^{-} and ΔN are, respectively, the plant Excess^{14}N amount at second harvest, and the total Excess ^{14}N amount recovery after 24 hours, and ΔT is the number of days between the first and second harvest.

The nitrogen use efficiency (*NUE*) of ^{15}N was calculated as the product of the nitrogen productivity of ^{15}N and the mean residence time of ^{15}N in the plant (Berendse & Aerts, 1987):

$$NUE\ (g\ dw\ mg^{-1}N) = NP \times MRT \quad (3.7)$$

The ^{15}N harvest index (^{15}*NHI*) was computed following Andersson & Johansson (2006);

$$^{15}NHI = Excess\ {}^{14}N amount\ in\ the\ grain / Excess\ {}^{14}N amount\ in\ the\ whole\ plant \quad (3.8)$$

3.2.4 The time to anthesis and plant height measurement

The time to flowering was scored using the Zadoks scale (Zadoks *et al.*, 1974), and recorded at *GS65* as the number of the days by which 50 % of spikes of a line have extruded 50 % of their anthers. The plant height was measured at *GS87*, as the length (in cm) of individual

culms from the soil surface to the top of the spike. Similarly, the peduncle length (uppermost internode of the stem) was scored at *GS87*, as the length (cm) from the last node on the stem to spike collar.

3.2.5 The measurement of the leaf hydraulic conductance (K_h)

The K_h is a measure of how efficiently water is transported through the leaf, determined as the ratio of water flow rate through the leaf (mmol m^2 s^{-1}) to the driving force, the water potential difference across the leaf ($\Delta\psi$, MPa^{-1}) (Sack & Holbrook, 2006).

The K_h was measured using *PMS* pressure chamber by over-pressurizing method. The leaf was cut to fit the chamber, and weighed for fresh weigh on analytical balance. The chamber was gradually pressurized with compressed air until the bubble of sap appeared at the cut; at this point the balance pressure was note as ψ_1. Subsequently, the leaf was over-pressurized at 5 bars for five minutes in excess of initial balance pressure. The extruded sap was removed from the cut end of the leaf. Then the pressure was released from the chamber and waited for five minutes, after which the pressure was increased again till the sap extruded to the cut end of the leaf (noted as ψ_2). Thereafter, the pressure was slowly released, and the leaf was removed from the chamber and weighed for saturated weight. Leaf was scanned and the leaf area was measured by means of imageJ. The dry weight was collected after oven drying of the leaf for 24 hours at 75^0C.

The K_h was computed as:

$$K_h \text{ (mmol m}^2 \text{ s}^{-1} \text{ MPa}^{-1}) = \Delta \text{ fresh weight} \times \text{time (second)}^{-1} \times \text{leaf area}^{-1} \times \Delta\psi^{-1} \quad (3.9)$$

3.2.6 The A/C_i responses curve measurement and A_{max} determination

The A/C_i response curve was measured on flag leaf (2 leaves per plant, and three replicates per line) at *GS65* using a LI-COR -6400XT set in auto-programme mode. The parameters were set in the Li-COR as: The relative humidity to 60 %, the block temperature at 25^0C, the

CO_2 reference to 400 ppm, the PAR to 1200 µmol quanta m^2 s^{-1}, and the ambient CO_2 value was set as (all in ppm): 50, 100, 150, 250, 350, 500,700, 900, 1200,700, 400. Once the measurement was completed, the data was downloaded from the LI-COR 6400XT to the computer. The data collected from the A/C_i response curve was entered into a programme called "*Photosyn assistant windows software for analysis of photosynthesis, version 1.1*" (Dundee Scientific, Dundee, UK) to derive A_{max}. The programme uses an iterative procedure to make an estimation of A_{max} from the A/C_i curve obtained through gas analysis (Harley *et al.,* 1992; Wullschleger, 1993).

3.2.7 The yield components and Harvest index measurements

The pathways to yield are collectively called "yield components" (Acquaah, 2012). The grain crop producing tillers such as wheat, the yield components are: tillers with seeds bearing spikes, grain number and kernel (grain) weight. Breaking down a complex trait into components might facilitate the finding of selection criteria to improve this characteristic.

The tiller number per plant was obtained as a count of tiller with seeds bearing spikes, and the average of the number of tillers per plant for each line was computed. The whole plant biomass (including the roots) was placed into an envelope and transported to the laboratory of physiological ecology (University of Cambridge, UK), there the samples were freeze dried for 48 hours in Modulyo 4K Freeze dryer (Edwards High Vacuum International, West Essex, UK). Thereafter, the sample was weighed before and after threshing.

The harvest index (*HI*) was calculated as the ratio of grain yield to the whole plant dry weight (including roots) (Walker & Fioritto, 1984). The grain number per spike was determined by the total number of kernels of the spikes divided by total number of spikes (Fischer, 2011). The grain weight was calculated as the thousand grain weight (TGW) divided by 1000 (Reynolds *et al.,* 2001).

3.2.8 Statistical analysis

All statistical analyses were performed using *SPSS 16.0* for window (SPSS Inc., Chicago, IL, USA). Firstly, the data was explored for parametric assumptions of normal distribution and homogeneity of variance using Kolmogorrov-Smirnov (*K-S*) and Levene's tests respectively. Then, graphing of means was performed using bar charts, and the analysis for linear relationship was conducted by mean of scatter-plots. Thereafter, the data was subjected to the partial Pearson correlation analysis. The one-way-independent *ANOVA* was performed at $p<.01$, followed by the post hoc test using Bonferroni test at significance level $p<.01$.

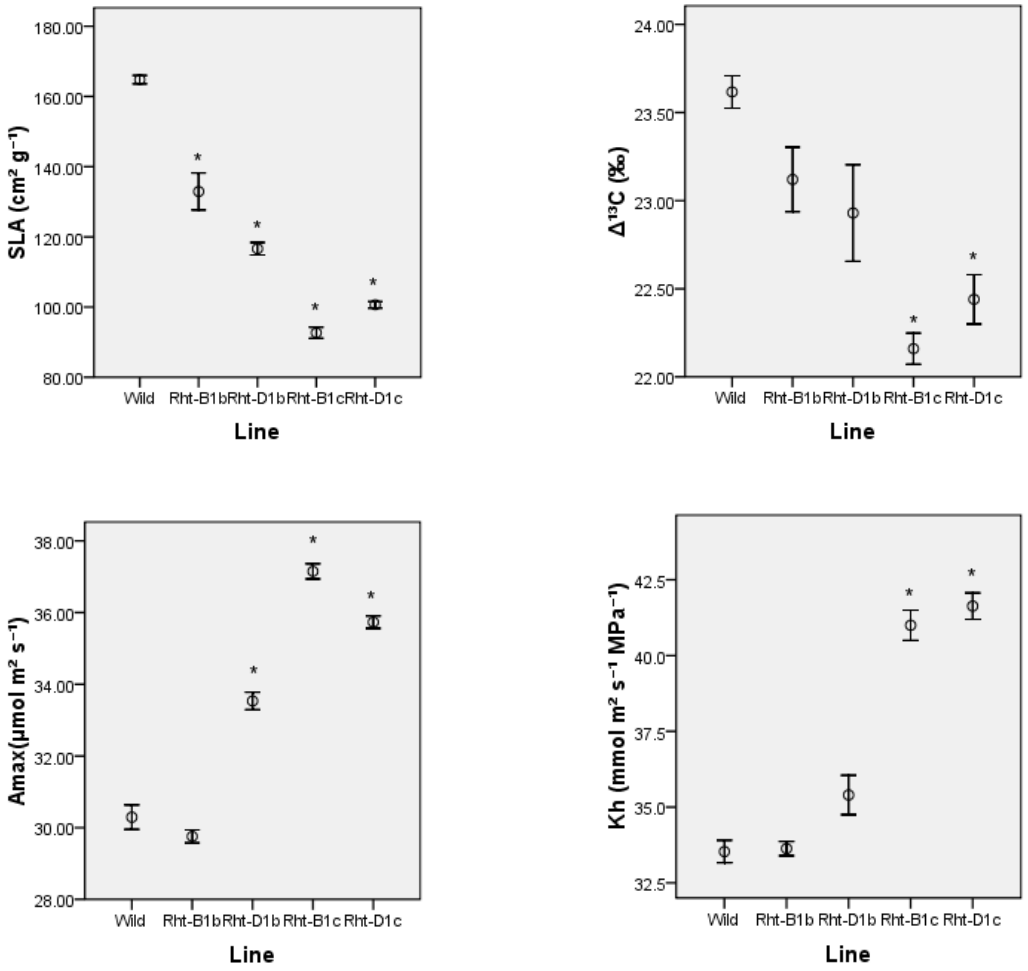
3.3 Results

Here, the effect of straw-shortening on photosynthetic rate and leaf hydraulic conductance, yield components, and partitioning of *N* to grain are shown. Firstly, the variation among the lines in A_{max}, K_h, *SLA*, and $\Delta^{13}C$ are presented as compared to Wild type. Similarly, the consequences of those *Rht* genes on yield components (*HI*, grain number per spike, *TGW*, tillering, days to anthesis, and plant height) are shown. Lastly, the effects of straw-shortening on nitrogen use efficiency and partitioning (*NP*, *MRT*, *NUE*, flag leaf *N* at different *GS*, & *NHI*) are provided.

3.3.1 The effects of straw-shortening on photosynthesis and leaf hydraulic conductance

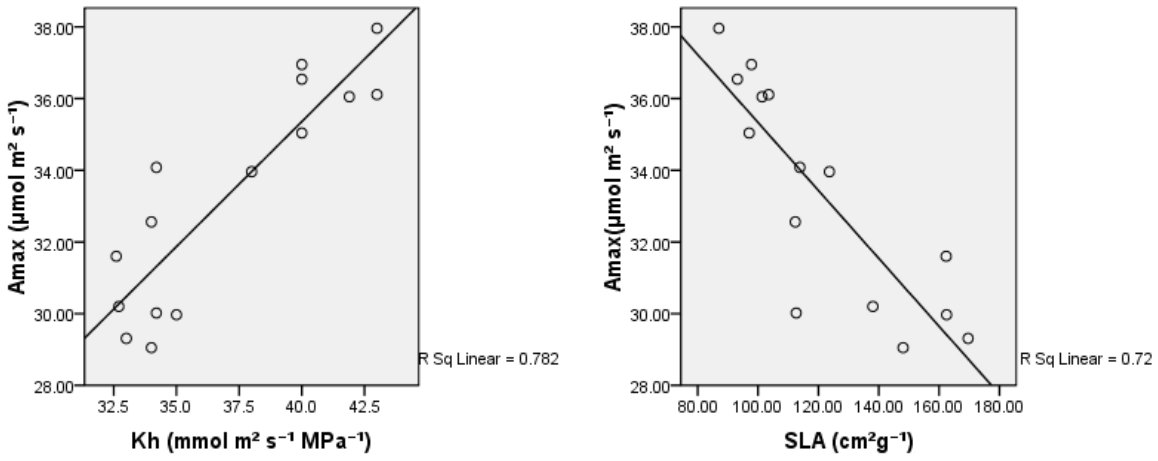
The *Rht* genes may have improved significantly ($p<.01$) both the photosynthetic capacity (33.5 ± 0.5 µmol; 37.2 ± 0.4 µmol; 35.7 ± 0.3 µmol, all per $m^2\ s^{-1}$; for *Rht-D1b*; *Rht-B1c*; and *Rht-D1c* respectively) and the leaf hydraulic conductance with the level of straw-shortening (41 ± 1.0 mmol; 41.6 ± 0.9mmol, all per $m^2\ s^{-1}\ MPa^{-1}$; for *Rht-B1c*; and *Rht-D1c* respectively) compared to the control (30.3 ± 0.7 µmol $m^2\ s^{-1}$; 33.5 ± 0.7 mmol $m^2\ s^{-1}\ MPa^{-1}$; for A_{max}; and K_h respectively). The *K-S* test for assumption of normal distribution showed

that the data did not deviate significantly from normal at *p<.01*. Similarly, the Levene's test indicated that the assumption of homogeneity of variance was tenable at p<.01.



* The means difference is significant at p<.01. The bar represent the genotypic mean ± SE. N= 6

Figure 3.3 *Variation in traits of photosynthesis and water use among Rht lines*



*: The mean is significantly different to *WT* at *p<.01* (Bonferroni test). The symbol represents individual measurement.

Figure 3.4 *Relationships of A_{max}, K_h and SLA*

The $\Delta^{13}C$ values in leaf organic matter (22.16 ± 0.18 ‰; 22.44 ± 0.28 ‰; for *Rht-B1c*; and *Rht-D1c* respectively) and the *SLA* (92.7 ± 3.1 $cm^2\ g^{-1}$; 100.6 ± 1.9 $cm^2\ g^{-1}$; for *Rht-B1c*; and *Rht-D1c* respectively) were significantly (p<.01) lower in shorter lines (*Rht-B1c*, *Rht-D1c*) than in taller ones (23.12 ± 0.37 ‰; 132.9 ± 10.5 $cm^2\ g^{-1}$ for *Rht-B1b*; and 22.93 ± 0.55 ‰; 116.6 ± 3.5 $cm^2\ g^{-1}$ for *Rht-D1b*) and the wild type (23.62 ± 0.18 ‰; 164.8 ± 2.4 $cm^2\ g^{-1}$; for $\Delta^{13}C$ and *SLA* respectively) (*Appendix D*). The plant height varied cultivars; the tall wild type with 60.3 ± 1.5 cm and the shortest *Rht-D1c* with 32 ± 0.6 cm tall. The *Rht-B1c* was 37 ± 1.5 cm, *Rht-B1b* was 58.7 ± 0.7 cm, and the *Rht-D1b* was 51.3 ± 0.9 cm. The height of *Rht-B1b* and the wild type did not differ significantly at $p< 0.01$.

The one-way independent *ANOVA* revealed there was a significant (p<.01) effect of straw-shortening on the level of A_{max}, K_h, $\Delta^{13}c$ and *SLA*. The Bonferroni post hoc test (all *ps<.01*) revealed (*fig*.3.3) that the A_{max} for *Rht-B1b* did not significantly differ to the wild type, and that *Rht-D1b* genes significantly increased the A_{max} compared both to Wild line and *Rht-B1b*. The *Rht-B1c* significantly increased the A_{max} compared to the wild type, *Rht-B1b*, and *Rht-D1b*, but did not significantly differ to *Rht-D1c*. Similarly, the same test (all *ps<.01*) showed that K_h for wild type, *Rht-B1b*, and *Rht-D1b* did not differ significantly (*fig.3.3)*, but the K_h was significantly higher for *Rht-B1c* compared to Wild line, *Rht-B1b*, and *Rht-D1b*, but did not differ significantly to the *Rht-D1c*.

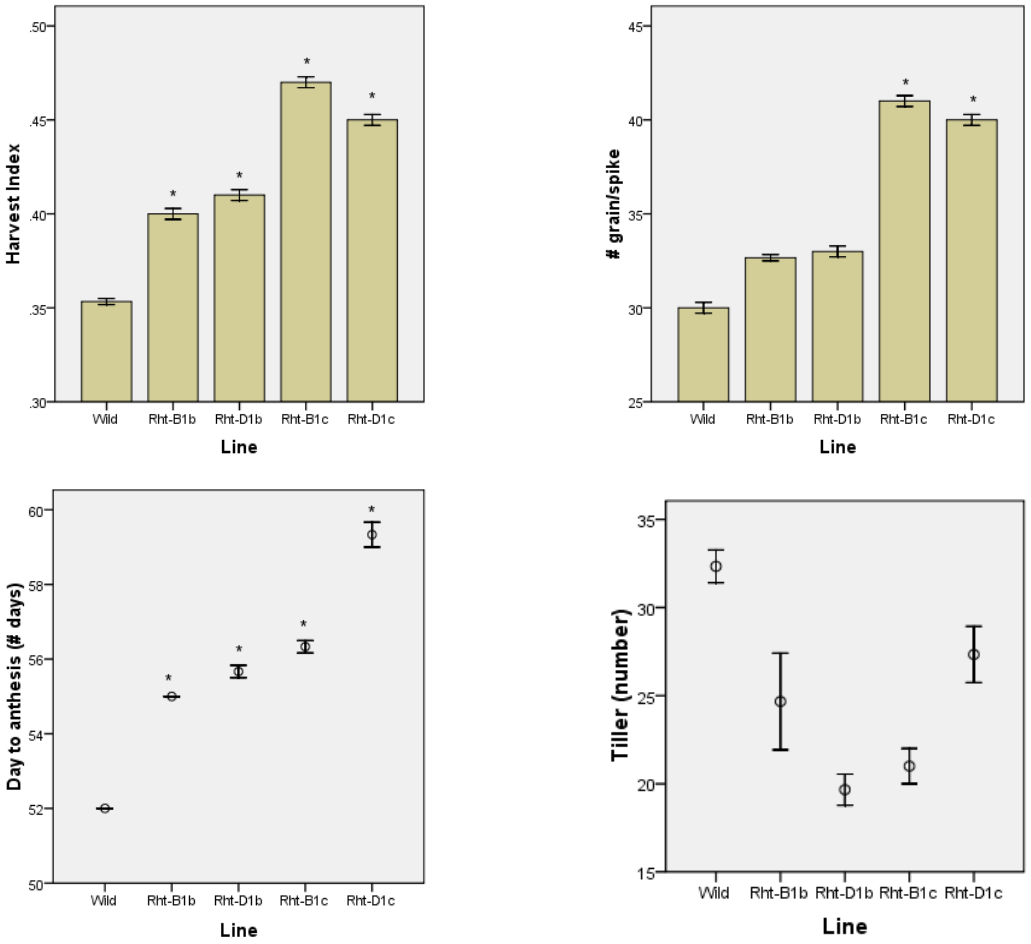
The data also showed that A_{max} was related both to *SLA* (R^2=0.72) and leaf hydraulic conductance (R^2=0.78).

3.3.2 The effects of straw shortening on yield components

The straw-shortening may have significantly (*All ps<.01*) increased the *HI*, grain number, *TGW*, but did not influence the level of tillering (*fig.3.5*). The Bonferroni post hoc test (all *ps<.01*) revealed that the *Rht-B1b* displayed a significant increased *HI* (0.40 ± 0.01) compared to the wild type (0.35 ± 0.0) but did not differ to the *Rht-D1b* (0.41 ± 0.01), and

was significantly less than both *Rht-B1c* (0.47 ± 0.01) and *Rht-D1c* (0.45 ± 0.01). However, the grain numbers were not significant different from Wild type, and both *Rht-B1b* and *Rht-D1b* (*fig.3.5).* The *Rht-B1c* yielded more grain number (41 ± 1) than *Rht-D1c (40 ± 3),* but did not significantly differ.

The straw-shortening significantly (p<.01) lengthened the duration to anthesis (*fig.3.5).* The *Rht-B1b* days to anthesis (55 days) were significantly different (p<.01) to *Rht-D1c* (59 days) and the wild type (52 days), but did not differ to the time to anthesis of both *Rht-D1b* (56 days) and *Rht-B1c* (56 days). The straw-shortening by *Rht-D1c* lengthened significantly the duration to anthesis compared to all other lines (*fig*.3.5).



* The mean difference is significant at p<.01. Error bar represent SE of mean for HI and grain number. The bar for tiller represent Mean ± SE. N=6

Figure 3.5 *The impact of Rht genes on yield components*

The Pearson correlation analysis showed that the *HI* was significantly related to plant height, grain number, TGW, and the peduncle length (*tab.3.1*). However, when the effect of grain number on *HI* was controlled (in the analysis), the *HI* did not significantly correlate to both plant height and the peduncle length, but significantly correlated with grain weight ($r=-.73$, $p<.01$). But, when the effect of plant height was controlled, the grain number on spike still did significantly correlate to the *HI* ($r=.70$, $p<.01$; one-tailed).

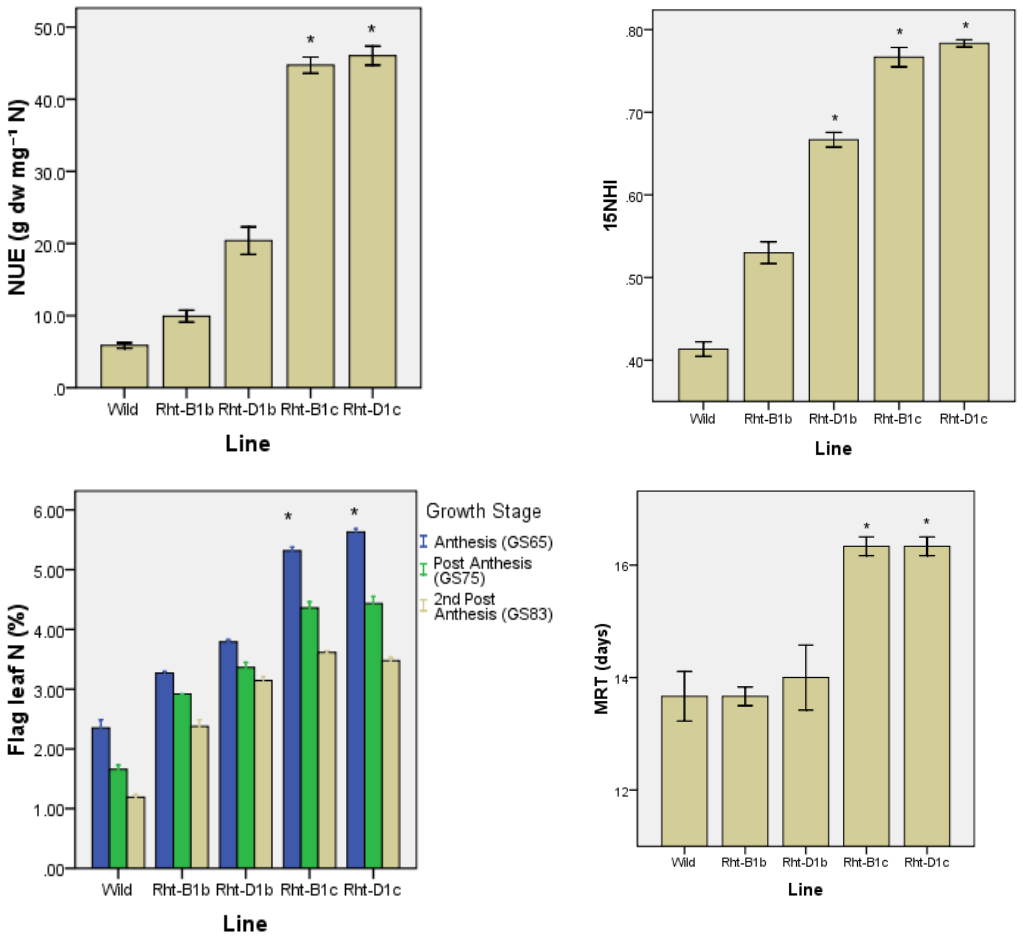
Table 3.1 *The Pearson correlations for traits of performance*

Parameter	*SLA*	*IVD*	A_{max}	$\Delta^{13}C$	$\Delta^{18}O$	K_h	*# tiller*	*# grain*	*TGW*	*DANT*	*Ped L.*	*HI*	*RWC*	biomass	*NP*	*NUE*	*MRT*	*NHI*	g_s	*Height*
IVD	.87**																			
A_{max}	-.85**	-.86**																		
$\Delta^{13}C$	.80**	.65**	-.71**																	
$\Delta^{18}O$	.89**	.80**	-.88**	.88**																
K_h	-.77**	-.88**	.88**	-.56*	-.81**															
# tiller	.36	.12	-.34	.23	.32	-.19														
# grain	-.85**	-.89**	.86**	-.63**	-.84**	.88**	-.27													
TGW	.94**	.88**	-.83**	.73**	.87**	-.83**	.36	-.92**												
DANT	-.80**	-.81**	.70**	-.51*	-.72**	.78**	-.29	.79**	-.81**											
Ped L	.63**	.79**	-.69**	.45*	.67**	-.85**	.06	-.90**	.79**	-.73**										
HI	-.94**	-.91**	.88**	-.69**	-.88**	.87**	-.36	.92**	-.96**	.82**	-.76**									
RWC	-.87**	-.90**	.92**	-.74**	-.86**	.84**	-.38	.84**	-.90**	.70**	-.71**	.91**								
biomass	-.92**	-.92**	.91**	-.67**	-.87**	.88**	-.37	.90**	-.91**	.89**	-.74**	.91**	.90**							
NP	-.88**	-.93**	.92**	-.66**	-.87**	.93**	-.24	.95**	-.90**	.83**	-.85**	.91**	.88**	.96**						
NUE	-.86**	-.91**	.91**	-.70**	-.90**	.93**	-.21	.97**	-.90**	.79**	-.88**	.90**	.86**	.93**	.98**					
MRT	-.79**	-.81**	.81**	-.63**	-.83**	.85**	-.21	.85**	-.83**	.74**	-.84**	.85**	.81**	.82**	.91**	.89**				
NHI	-.91**	-.90**	.87**	-.62**	-.85**	.87**	-.42	.88**	-.92**	.88**	-.74**	.93**	.90**	.98**	.94**	.90**	.84**			
g_s	-.85**	-.83**	.89**	-.69**	-.84**	.81**	-.29	.94**	-.90**	.69**	-.79**	.89**	.85**	.85**	.86**	.90**	.73**	.82**		
Height	.84**	.92**	-.90**	.64**	.84**	-.93**	.17	-.94**	.88**	-.85**	.87**	-.88**	-.84**	-.95**	-.98**	-.98**	-.86**	-.90**	-.87**	
Grain mass	-.92**	-.93**	.92**	-.66**	-.88**	.90**	-.33	.93**	-.92**	.89**	-.78**	.94**	.90**	.99**	.97**	.94**	.83**	.98**	.88**	-.96**

**. Correlation is significant at the .01 level (1-tailed). *. Correlation is significant at the .05 level (1-tailed). The values for $\Delta^{13}C$ are expressed in *‰,* for WUE_i in $\mu mol\ CO_2\ mmol^{-1}\ H_2O$, in $cm^2\ g^{-1}$ for *SLA*, in $\mu mol\ CO_2\ m^2\ s^{-1}$ for A_{max}, in *%* for RWC, *SPAD* index for chlorophyll content, in number per mm^2 for stomata density (SD on abaxial), and in *mm* for inter-vein distance (*IVD*). The g_s expressed in mmol $m^2\ s^{-1}$. The K_h measured in mmol $m^2\ s^{-1}\ MPa^{-1}$. The NUE is expressed in g dw mg^{-1} N. The tiller in number, and grain in grain number per spike , and the $\Delta^{18}O$ expressed in ‰. The *TGW* is measured in g. The peduncle length is expressed in cm. The biomass is measured in kg, dw. The *NP* is expressed in g dw mg^{-1} N. The height is measured in cm. Both the *MRT* and day to anthesis are measured in days. The grain mass is measured in kg dw.

3.3.3 The consequences of *Rht* genes on nitrogen partitioning

The one-way independent *ANOVA* showed that the straw-shortening increased significantly ($p<.01$) both the *^{15}NHI*, and *^{15}NUE*, and that it influenced significantly the *MRT* of *N*. Additionally, the Bonferroni post hoc test ($p<.01$) indicated that *Rht-B1c* and *Rht-D1c* significantly increased the *^{15}NUE* (44.7 ± 2.2 g dw mg^{-1} N; 46.0 ± 2.6 g dw mg^{-1} N; for *Rht-B1c* and *Rht-D1c* respectively) compared to the wild type (5.9 ± 0.8 g dw mg^{-1} N). It also showed that the *^{15}NHI* for *Rht-B1b* did not significantly differ to the wild line (*tab.3.2*). The *^{15}NHI* and the *^{15}NUE* were linearly significantly related (*fig.3.6*). The experiment showed that the flag leaf nitrogen content was at its highest level at *GS65* and exhibited genetic variability at anthesis, but did not differ significantly at *GS87* (*fig.3.6, & Appendix C*).



* The mean difference is statistically significant at p<.01. Error bar represent *SE* of the mean. N= 6. NUE is on total plant dry weight.

Figure 3.6 *The effects of straw-shortening on nitrogen partitioning*

Table 3.2 *The Bonferroni post hoc test for ^{15}NHI*

Dependent Variable:15NHI (N= 6)

	(I) Line	(J) Line	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	Wild	Rht-B1b	-.11667	.02789	.019	-.2446	.0113
		Rht-D1b	-.25333*	.02789	.000	-.3813	-.1254
		Rht-B1c	-.35333*	.02789	.000	-.4813	-.2254
		Rht-D1c	-.37000*	.02789	.000	-.4979	-.2421
	Rht-B1b	Rht-D1b	-.13667*	.02789	.006	-.2646	.-.0087
		Rht-B1c	-.23667*	.02789	.000	-.3646	-.1087
		Rht-D1c	-.25333*	.02789	.000	-.3813	-.1254
	Rht-D1b	Rht-B1c	-.10000	.02789	.050	-.2279	.0279
		Rht-D1c	-.11667	.02789	.019	-.2446	.0113
	Rht-B1c	Rht-D1c	-.1667	.02789	1.000	-.1446	.1113

*. The mean difference is significant p<.01

3.4. Discussion

The research of this chapter dealt with how the straw-shortening affects the traits of photosynthesis and leaf hydraulic conductance, yield components and partitioning. The aim was to identify the physiological traits able to produce yield increases without further straw-shortening. Attention was also given to test the hypothesis that distance water must flow should influence the leaf hydraulic conductance and the photosynthetic rate of the crop. Moreover, considerations were given to develop proxies of these traits. Particularly, the research experimented the ^{15}N labeling to the study of *N* partitioning.

This section discusses the physiological traits that determine the relationship between plant height, and yield and how these traits can be applied to crop selection. Firstly, the implication of straw-shortening on photosynthetic capacity and leaf hydraulic conductance are discussed; then there is examination of the determinants of *HI* and how they are related to plant height; and finally, the consequences of straw-shortening for the partitioning of *N* are assessed.

3.4.1 Implication of straw-shortening on photosynthesis and leaf hydraulic conductance

Uncovering the mechanistic foundations that underlie the relationship between plant height and leaf hydraulic conductance would broaden the physiological understanding of straw-shortening for crop improvement. The K_h is a key physiological variable for water relations (Sack & Scoffoni, 2012). We evaluated the new hypothesis that link plant height to leaf hydraulic conductance and photosynthetic capacity, in lines of different level of dwarfing. We hypothesized that the distance water must flow should influence the leaf hydraulic conductance and the photosynthetic rate of plant. This was based on Brodribb *et al.* (2005) who observed that the maximum net assimilation rate (A_{max}) was coupled to the capacity of the leaf vascular system to supply water to photosynthesing cells.

We found an intimate association between level of dwarfing, leaf hydraulic conductance (K_h), the photosynthetic capacity (A_{max}) and *SLA* (*fig*.3.3 & 3.4). Combining these observed

relationships enabled us to uncover the mechanistic that links straw-shortening to photosynthetic rate. Both leaf K_h and A_{max} increased with the level of dwarfing, *Rht-B1c* and *Rht-D1c* exhibiting the highest rate. A single regression accurately showed that A_{max} related both to leaf K_h and *SLA*, increasing with increases in leaf K_h and decreases in *SLA* (*fig.3.4*). A similar analysis illustrated the effect of straw-shortening to both *SLA* and flag leaf $\Delta^{13}C$ (*fig.3.3*).

The results lead to the suggestion that the straw-shortening effects A_{max} by exerting a controlling influence over K_h and *SLA*. Primarily, there may be the physical control of leaf K_h by the distance to traverse by water; this may link secondarily the leaf K_h to A_{max} because of the coordination between A_{max} and leaf K_h (Brodribb *et al.*, 2005).

The second mechanistic that explains the link between straw-shortening and A_{max}, is by *SLA*: The *Rht* genes might affect the size of *SLA*, as a result, A_{max} would be affected as the research of the second chapter of this thesis established the connection between *SLA* and A_{max} (also, *fig.3.4*). This finding corroborates with both Lecain *et al.* (1989) and Keyes *et al.* (1989) who observed that the flag leaf of dwarf wheat had thicker leaf than the tall one. In agreement, later, Morgan *et al.* (1990) found more chlorophyll, protein, and Rubisco content per unit leaf area in the dwarf than the tall isolines, and ascribed the effect of dwarfing genes on photosynthesis to a greater density of cells capable of photosynthesis.

3.4.2 The determinants of Harvest Index of reduced height plant

Historically, the increase in *HI* of *Rht* lines was seen as the effect of *Rht* genes to limit stem extension growth, decreasing assimilate demand for this organ and diverting it to the developing ear which is not itself dwarfed (Flintham *et al.*, 1997). We evaluated the various physiological variables that may have effects over the *HI*, and the possible relationships among them.

Grain number and kernel weight

We found that both grain numbers per spike and kernel weight significantly ($p<.01$) correlated to *HI* (*tab.3.1*). The increases in grain number on spike were consistently associated with the level of dwarfing (*fig.3.5*), with *Rht-B1c* and *Rht-D1c* exhibiting highest grain number compared to the other lines. However, the number of grain on spike was partially offset by reduction in kernel weight, indicating a trade-off between grain number and kernel weight on spike. Whether the reduced grain size is a competitive response to the increase in the number of kernels on spike or the primary effect of straw-shortening, was not clear. Based on the similar results of chapter four of this thesis, we suggest the former might be the cause.

The height reduction arising from straw-shortening by the *Rht* genes has been proposed as the driver of the increases in *HI* (Rebetzke & Richards, 2000). The *HI* is an integrative trait including the net effect of many physiological processes (Li *et al.*, 2012). Our data indicated that the *HI* significantly correlated with all these traits of plant height, peduncle length, grain number, kernel weight, and partitioning of dry matter to roots. But when the effect of grain number was held constant (partial correlation), the *HI* significantly correlated only with kernel weight and could not correlate with plant height (*tab.3.1*). However, when the effect of plant height was controlled, still the *HI* significantly correlated with both grain number and kernel weight, indicating they might be the key determinants of *HI*.

Some of the possible explanation of the increased grain number in *Rht* lines was proposed by Youssefian *et al*. (1992*a, b*), they argued that it derives from increased partitioning of assimilates to the developing ear as a consequence of reduced demand for stem elongation therefore resulting into improved florets fertility.

Plant height

One of the main attributes modified to increase the *HI* has been plant height, which has been systematically reduced (Slafer *et al.*, 2005). Our results indicated the increased adding effect of *Rht-B1b*, *Rht-D1b*, *Rht-B1c* on A_{max}, *HI*, and grain number on spike, but with relative reduction for *Rht-D1c* compared to *Rht-B1c* (*fig*.3.3, & 3.5), thus, showing there might penalty for reducing height beyond *Rht-B1c*. This finding is in accordance with several other studies (Berry et al., 2014; Miralles & Slafer, 1995) that showed wheat yield is reduced when plant is shortened excessively.

Nitrogen partitioning and use efficiency

The concept of *HI* was extended to the partitioning of *N*, in particular, the nitrogen harvest index (NHI), as the ratio of nitrogen in grain to total nitrogen content of the plant biomass including roots (Austin, 1980). The grain *NHI* is important trait in relation to the baking quality and nutritional value of wheat. We therefore examined the extent the straw-shortening influences the *NHI*, using the ^{15}N labeling as the experimental technique. The study indicated straw-shortening increased the ^{15}*NHI* linearly with the level of dwarfing (*fig*.3.6). The inclusion of roots *N* in *NHI* calculation showed that the root *N* content varied from 10 - 20 % of the total *N* amount in the plant, indicating the partitioning of *N* to roots affect the *NHI*. The root *N* was influenced by straw-shortening, with the *Rht-B1c* and *Rht-D1c* showing the lowest partitioning of *N* to the roots compared to other lines.

The study also examined the effect of straw-shortening on *NUE* and *MRT* of *N*. Our data indicated that *NUE* increased linearly with the level of dwarfing, and the shortest lines (*Rht-B1c*; *Rht-D1c*) exhibited the highest MRT of *N*. According to Golluscio (2007), a high *NUE* and a high *MRT* of *N* in plant would be an important adaptation indicator of plant to *N* stress. The increased *NUE* is also important for plant breeding and crop production to meet the challenge of low input cost and low pollution to the environment (Delgado, 2002).

This study also confirmed that the post-anthesis partitioning is the driver of *HI*; at anthesis, the flag leaf nitrogen content exhibited genetic variability while at physiological maturity, there was no indication of such variability. This finding was consistent with the ^{15}N labeling experiment results that indicated similar trend.

The time to anthesis

The current research also evaluated the effects of straw-shortening on earliness of flowering. The data indicated the *Rht* genes may lengthen the duration to flowering (*fig. 3.5*). We speculated that the effect of straw-shortening on lengthening the time to anthesis would be the consequences of *Rht* genes on lengthening the stem elongation phase. This is in agreement with Reynolds *et al*. (2009) who suggested that the variation in the lengthening of stem elongation phase means the cultivars may differ in their earliness of flowering.

In conclusion, the study indicated that the partitioning of *N* to spike and roots are the characteristics related to plant height and growth stage. Moreover, the selection of wheat cultivars for increased *HI* should shift focus from reduced plant height to include increased grain number and kernel weight, reduced partitioning to roots and reduced peduncle length.

Chapter 4 Hydraulic lift and N_2-fixing: Consequences of water and nitrogen effluxes for wheat production in agroforestry with *Alnus acuminata*

"All the rivers run into the sea; yet the sea is not full; unto the place from which the rivers come, thither they return again" (Bible: Ecclesiastes 1:7)

Abstract

Whilst substantial information is available concerning both the process of hydraulic lift and N_2 fixation, there is a gap in the knowledge of the extent and the magnitude of utilization by neighboring plants of the water and nitrogen redistributed in the top soil under the field conditions. We hypothesized that effluxes of water and nitrogen from plant roots systems in the topsoil profile may facilitate a number of physiological functions of neighboring plants. Therefore, a field experiment was conducted in an andic soil of high lands in northern Rwanda to address the questions of the extent and consequences of water and nitrogen efflux from tree roots for an intercropping system of wheat and *Alnus acuminata*. The study involved analyses of natural abundance of stable isotopes δ^2H, $\delta^{18}O$, $\delta^{15}N$ and an isotopic mixing model *"IsoSource"* to quantitatively determine the proportional contribution of water and nitrogen sources respectively to the crop isotope signatures at different distances from the trees (*1 m*, *3 m*, *5 m*, *& 7 m*). Similarly, other physiological proxies such as $\Delta^{13}C$, *SLA*, and grain number and yield were used to evaluate the consequences of water and nitrogen efflux from roots for crop water status and yield, respectively. We noted that *Alnus acuminata* exhibited the hydraulic lift and *N* redistribution. The data indicate significant ($p<.01$) gradient in depletion of wheat xylem water δ^2H and $\delta^{15}N$ signatures moving further away from the tree line. The results have also shown the improvement in both water status and chlorophyll content (as determined via proxies of $\Delta^{13}C$, *SLA*, and *SPAD*, respectively) for the crops nearest to the trees for a distance of up to *5 m*. The study provided quantitative evidence that the improvement in water and nitrogen status may have been brought about by hydraulic lift and redistribution, likely to be associated with N_2 fixation and transfer, and resulted in increased wheat grain number and yield nearest to the trees.

Key words: Hydraulic lift & redistribution, water effluxes, N_2 fixing & transfer, isotopic proportional sources, *Alnus acuminata*, Rwanda.

4.1 Introduction

4.1.1 Hydraulic lift and redistribution

Water transfer by roots between spatially separated soil compartments of differing water status has been shown by many authors (Dawson *et al.,* 2002). The term *"hydraulic lift"* was coined to describe this process (Richards & Caldwell, 1987). The hydraulic lift was defined by Caldwell *et al.* (1998) as the passive movement of water from roots into soil layers with lower water potentials, while other parts of the root system in moister soil layers are absorbing water. Burgess *et al.* (1998) named it "*hydraulic redistribution*" arguing it occurs throughout the root systems whenever a water potential gradient exists across soil layers spanned by roots.

According to the hydraulic lift hypothesis (Caldwell, 1988), at night, when transpiration is reduced, root water potential rises above Ψ_s in drier soil layers, and water movement occurs passively down a water potential gradient (Jensen *et al.,* 1961; Schippers *et al.,* 1967; Dickson *et al.,* 1979; Shone & Flood, 1980; Corak *et al.,* 1987). Evidence in support of hydraulic lift is the result of stomata opening and closing comes from the study of plants having Crassulacean Acid Metabolism (CAM); Caldwell *et al.* (1998) observed CAM exhibited hydraulic lift during the day.

The origins of hydraulic lift in plants remain an open question. Optimality theory (Givnish, 1986) would suggest that if a plant is to pay a cost in terms of giving up water to the surrounding soils, then there should be some benefits for this behavior. A number of authors (Richards & Caldwell, 1987; Caldwell & Richards, 1989; Dawson, 1993) have pondered the possible benefits of hydraulic lift for the plant exhibiting it; they suggested the hydraulic lift can facilitate the day time transpiration by supplying water overnight to the upper soil layer where it can be re-utilized the following day.

Results from several experiments suggested that a considerable amount of water is lifted; Wan *et al.*(1993) estimated that hydraulically lifted water ranged from 14 % of daily *ET* to roughly 1/3 *ET* for the shrub *Artemisia tridentata*. For example, Emerman & Dawson (1996) estimated that a mature (~ 20 m tall) maple tree lifted 102 ± 54 *l* of water per night over the course of a *5* day period. These field estimates are in agreement with laboratory measurements of the amount of water that can be moved by hydraulic lift; Bovel & Baker (1985) and Baker & Bovel (1988) reported that an average of 42 % and 31 % of daily transpiration were supplied by water efflux by roots overnight into dry soil compartments of Bermudagrass and Cotton, respectively.

In most plant communities, roots length density decreases exponentially with depth (Jackson *et al.,* 1996). This root distribution combined with some direct evaporation from the soil surface results in drying of the soil profile from the surface downward (Caldwell *et al.,* 1998). It has been argued that because soil tends to dry from the surface downward and nutrients are usually most plentiful in the upper soil layers, lifted water may provide moisture that facilitates favorable biogeochemical conditions for enhancing mineral nutrient availability, and the acquisition of nutrients by roots (Caldwell *et al.,* 1998). Hydraulic lift may also prolong or enhance fine root activity by keeping them hydrated. Also, release of water into upper soil layers provides a source of water for neighboring, shallow-rooted plants, to utilize as a source of water.

Studies of localization of water efflux from roots indicated that much of water loss may occur in young roots (Watt *et al.,* 1998). Work with graminoids (Watt *et al.,* 1996), and trees (Dawson, 1993, 1996) and Eucalyptus (Phillips & Riha, 1994) showed that efflux of water into the soil is localized in the young portions of the root system where the casparian band and suberin lamellae of the hypodermis are not fully formed. According to Dawson (1998),

water efflux can also occur at junctions within the highly branched fine roots system where roots are less than 2.5 mm in diameter.

Measurement of the isotope composition of water in the various components of the water has enabled the identification of water masses and the tracing of their interrelationships (Gat, 1996). One of the applications is the use of the distinct isotopic signature of various sources to quantitatively determine their proportional contribution to the mixture signature in an end product (Phillips & Gregg, 2003). This application can be used to characterize the proportional source of water the plant is using. For instance, Yakir & Yechieli (1995) have exploited this feature to distinguish between saline groundwater and flash floods at the Dead Sea shore. According to Dawson *et al.* (2002), and Gat (1996), the stable isotope analyses of both hydrogen and Oxygen of different water sources provide a powerful tool towards quantifying the contribution of different sources to the plant water uptake.

However, the magnitude of water redistribution by hydraulic lift under the field conditions is not fully understood yet. The research of this chapter aimed to elucidate the extent of water effluxes and its consequences on wheat intercrops in agroforestry system with *Alnus acuminata*.

4.1.2 The N_2 fixing and transfer

The practice of agroforestry on agricultural land has been the subject of considerable research (Akinnifesi *et al.*, 1998). There is a sense that resource use is increased under agroforestry systems (Vandermeer *et al*., 1998); for example, belowground transfer of nitrogen among plants has been hypothesized, from a N_2-fixing source plant to a non-fixing plant sink (Haystead *et al.*, 1998; Arnebrant *et al.*, 1993; He *et al.*, 2009). The nitrogen transfer from N_2-fixing trees can be a major source for the associated crops in low-inputs farming system. According to Lal (2004) the *N* is the most important nutrient limiting crop production in the tropical small-scale farming; therefore integrating the N_2-fixing trees into farmland may

improve the *N* supply of the cropping system. However, it remains uncertain of what mechanism drives *N* transfer.

Several mechanisms involving *N* transfer from N_2 fixation by plants have been hypothesized by different authors, via: roots grafts or root-to- root contacts (Caldwell & Richards, 1986); roots exudations (Teste *et al.,* 2014); and mycorrhizal networks (He *et al.,* 2003). But it is not clear how important it is quantitatively. It is also argued that after root exudation, two basic mechanisms may be transporting ions and simple amino acids to neighboring plants; the first is mass flow that moves ions along with the flow of water and the second is the diffusion of ions along concentration gradient without the flow of water (Teste *et al.,* 2014).

According to Evans (2001), whole plant and leaf *N* isotope composition are determined by the isotope ratio of the external *N* sources. The physiological mechanisms that influence plant *N* isotopic signature have been reviewed by Evans (2001), and earlier by Högberg (1997) and Handley & Raven (1992).

The study of *N* transfer among plants could have potential in agro-farming under low external inputs (Jensen, 2005; Wichern *et al.,* 2008). Scientists initially hoped that quantifying $\delta^{15}N$ could be used to trace the relative contribution of N_2 fixation to plants and soils (Michener & Lajtha, 2007). Many authors have used $\delta^{15}N$ data to draw inferences regarding *N* sources (Garten *et al.,* 2007; Evans *et al.,* 2007; Phillips & Greggs, 2003). For example, Schulze *et al.* (1991) employed the natural abundance of $\delta^{15}N$ values in Acacia savannas to estimate the nitrogen fixation by the *Acacia melifera* trees on aridity gradient in Namibia, and found that about 71 % of nitrogen was fixed. Similarly, Shear *et al.* (1983) used the natural abundance of $\delta^{15}N$ in tissues of *Prosopis grandulosa* to estimate the N_2-fixation by these trees, and concluded it is feasible to use variation in natural abundance of $\delta^{15}N$ as an index of N_2-fixation. Additionally, studies in the California Sonaran desert indicated Prosopis woodland fixed a significant amount of N_2 based on soil *N* accumulation beneath (Virginia & Jarrell,

1983); since desert soils are often deficient in nitrogen, they argued that N_2-fixing by prosopis tree might be the basis of that soil *N* accumulation.

There would be great deal to be learned from $\delta^{15}N$ of plant tissues and their sources of *N*. The aim of this research was to experiment if the analyses of the natural abundance of $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$ coupled to an isotopic mixing model "IsoSource" can be applied to determine the relative transfer of *N* from a N_2 fixing tree to wheat and hydraulic redistribution in an agroforestry system in the field.

4.2 Material and Methods

4.2.1. Description of research area

The research described in this chapter took place between October *2013* and March *2014* at three sites located in Northern Rwanda (*fig. 4.1, & 4.2*). The site at Rurembo was located at *01° 53'S; 29° 57'E* and elevation 2245 m. The second site at Kirezi was at elevation of 2269 m and at *01° 54' S*; *29° 57' E*. The third site at Cyansure was located at *01° 55' S*; *29° 57' E*, and elevation of 2248 m. The area was selected for this research because it is the main wheat growing region of Rwanda, and the sites were selected based on the availability of bench terraces field with trees old enough to exhibit hydraulic lift and nitrogen fixing. The terraces were chosen in the middle of watershed in each site (avoiding the crest and valley floor).

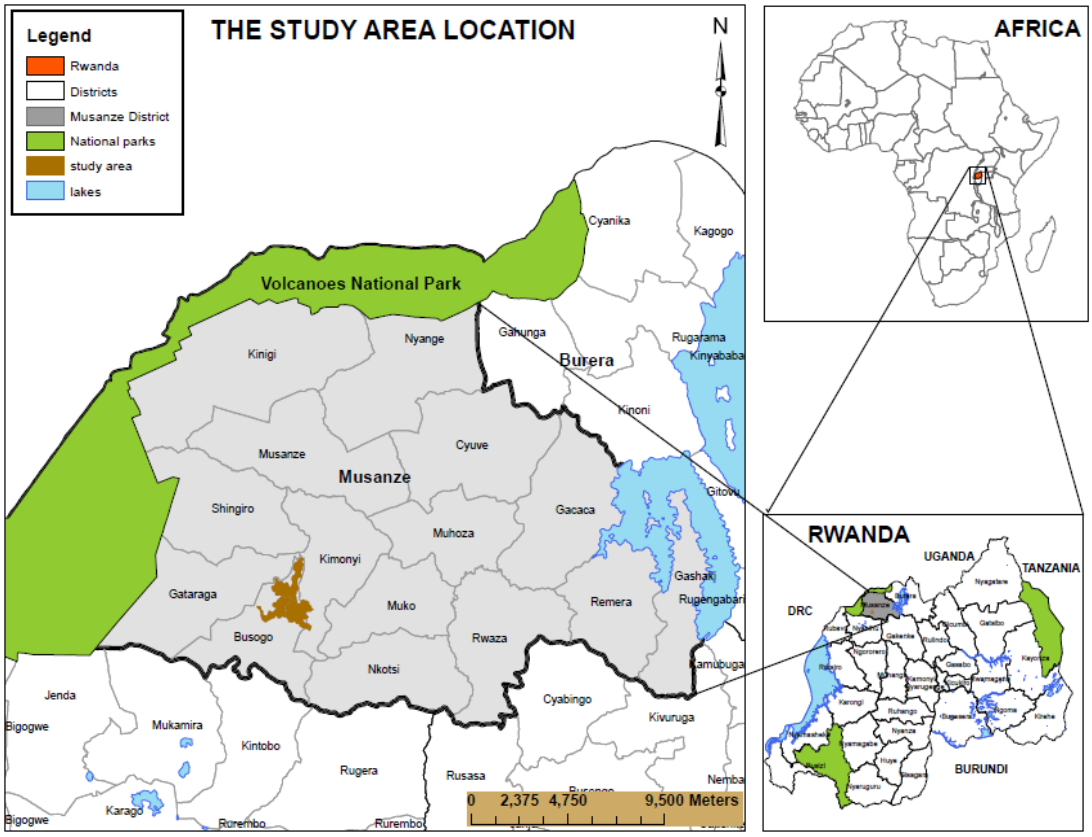


Figure 4.1 *Location of the study area (field experiment)*

The rain distribution across the whole region is bimodal, characterized by long and short rain seasons that allow two cropping seasons a year (*fig. 4.3*). Based on climatic data of the local

weather station in Busogo, the average annual rainfall over the period *2011-2013* was 1640 mm, with average annual temperature of *15°C* (*fig.4.3*). The soils of the area are Alfisols (*Ultic tropudalf*), Inceptisols (*Andic eutropept*; *Typic dystrandept*; *Entic eutrandept*), and Mollisols (*Cumulic hapludoll*) (*fig.4.2*). However, the soil type in the actual all fields experiment was *Ultic tropudalf*. The *pH* (in water) of the soil was *6.7*, and the dominant crops are wheat, potatoes, maize and peas. Potatoes and wheat are the main cash crops in the area.

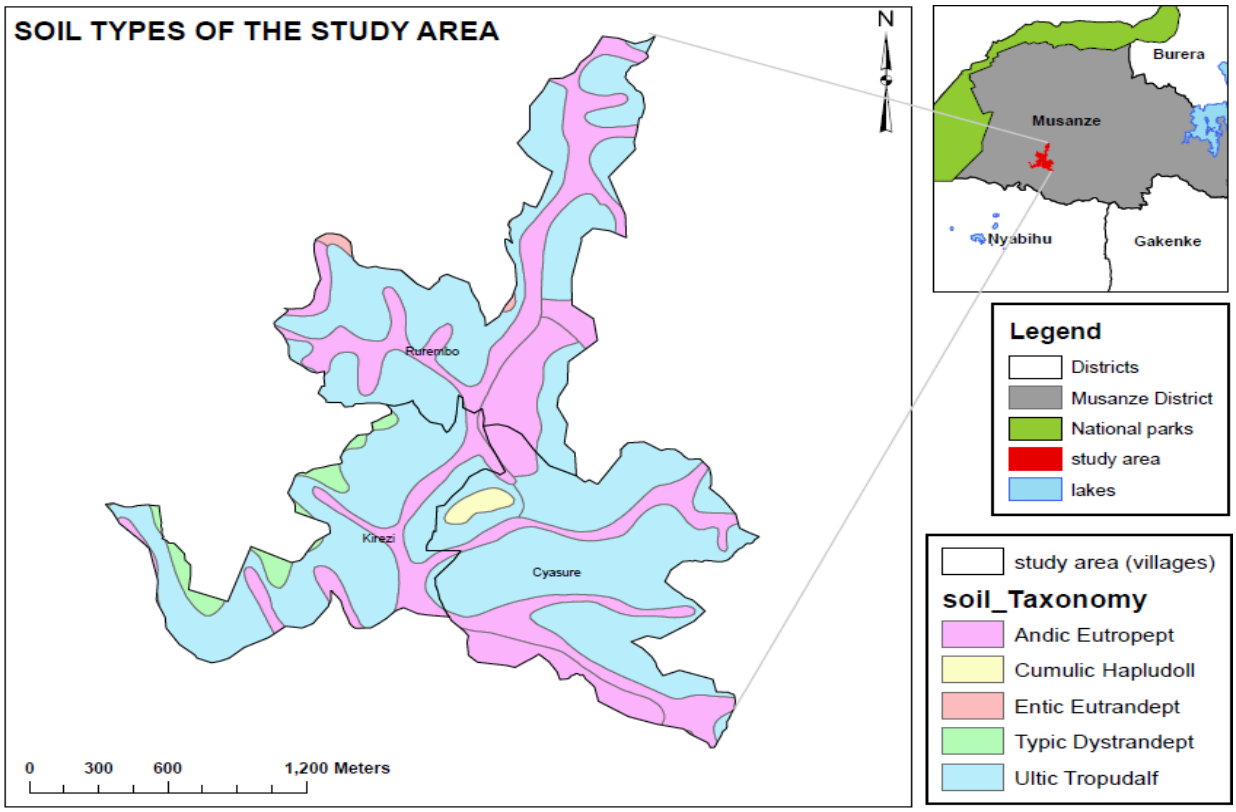


Figure 4.2 *The soil taxonomy of the sites (Kirezi,Cyansure, Rurembo) (Data source: GIS unit MINAGRI, Rwanda)*

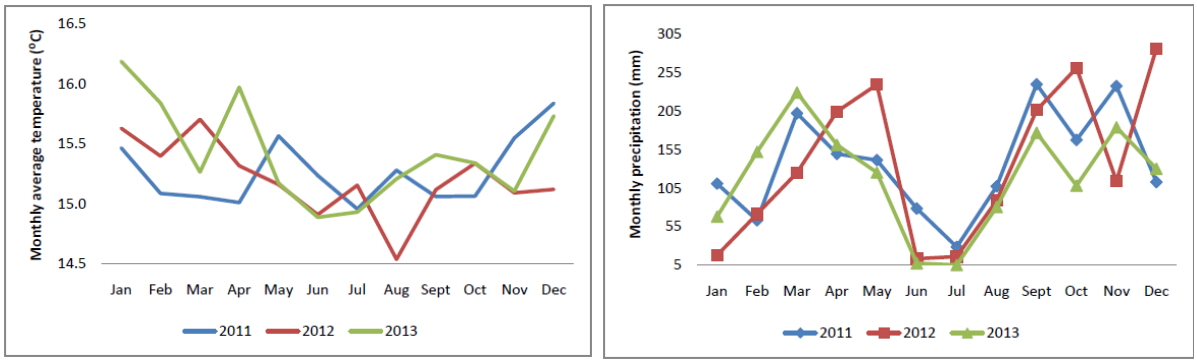


Figure 4.3 *Distribution of temperature and rainfall over the period 2011-2013 on the study area*

4.2.2 Experimental setting and data collection

The experiment was conducted on the farmers' fields' terraces that were established in *2010* with transplants of *Alnus acuminata* trees on the terrace risers. Two consecutive benches of the terraces are separated by a terrace riser (*fig. 4.4*) which may prevent the crop on the two benches to interact. The spacing between trees was 5 m, and the terrace width was 8 m with wheat (Variety Bisagi in all the fields of the study) grown on the bench (*fig.4.4*). The wheat planting density was 150 plants m^{-2}. DAP (Di-ammonium phosphate) was applied as basal fertilizer at the rate of 130 kg ha^{-1}, and the urea (46%) was applied as topdress fertilizer at the rate of 100 kg ha^{-1}. The trees on the terraces were regularly pruned (three time a year).

Figure 4.4 *Experimental field*

Initially, samples for each water sources (rain, soil, & tree) were collected in exetainer for isotopic measurements of δ^2H and $\delta^{18}O$. A total of 15 samples of rain water, 40 of soil water, and 15 twigs for tree water were collected.

Soil was sampled from each plot at four points along the slope in the terrace at 1 m, 3 m, 5 m, and 7 m from tree, and three replicates. Soil samples were taken using a soil auger at depth of 0-20 cm and a composite of three samples. A total number of 40 composite soil samples were taken. These soils were air dried and sieved to less than 2 mm for $\delta^{15}N$ measurement. Additionally, three twigs were sampled for each plot, and stem of wheat at 1 m, 3 m, 5 m and 7 m from the tree. Each twig and wheat stem sample was placed in an exetainer for water

extraction later in the laboratory. The flag wheat leaf samples for measurements of *SLA*, and $\Delta^{13}C$ and $\delta^{15}N$ were collected at four points in the bench of the terrace further away from tree (1 m, 3 m, 5 m, 7 m) and three replicates at *GS65* as per described in section *2.2.3* of this thesis. The chlorophyll content of flag leaf was estimated using the chlorophyll meter *SPAD-502* as per section *2.2.2*. The yield components (grain number, kernel weight, *HI*, grain yield) measurements were taken at *GS87* as described in *3.2.4*. The grain yield (kg ha^{-1}) was computed following Acquaah (2012):

*Grain yield (kg ha^{-1}) = number of spikes per m^2 *grain number/spike*(TGW/1000)*10* (4.1)

4.2.3 Extraction and vacuum distillation of water

The water was extracted by distillation from the twig, wheat stem, and soil samples using a vacuum manifold with five units (*fig.4.5*), by Mr. Glyn Jones, Technician, Physiological Ecology Group (Department of Plant Sciences, University of Cambridge, UK). Samples had been stored in exetainers, which were placed carefully onto needles, and initially frozen in polystyrene cups of liquid nitrogen. The residual air was removed from each exetainer one sample at a time by the vacuum system, each unit was isolated with a stopcock and the liquid nitrogen replaced with a beaker of hot water (~70°C). Dewars were placed on condensers and regularly refilled with liquid nitrogen. When the distillation was complete, the water in the condenser was transferred to central collection tube with liquid nitrogen, which could then be removed, warmed and transferred via Pasteur pipette to pre-labeled container.

Figure 4.5 *Extraction and distillation of water by vacuum manifold with five units*

4.2.4 Isotopic analysis

The water samples in vials with inserts were exported to the Center for stable isotopes biogeochemistry (University of California at Berkeley, *USA*) for analysis of δ^2H and $\delta^{18}O$. The δ^2H in water was analyzed by IRMS. According to the Centre for isotopes (at UCB) protocol (http://nature.berkeley.edu/stableisotopelab/analyses/water-analysis/), each sample of 1 mL was analyzed in dual inlet (*DI*) using a hot chromium reactor unit (H/Device ^{TM}) interfaced with a thermo Delta Plus *XL* mass spectrometer. Standards were added to every run and corrected for differential drift of standards with different isotope ratios. The $\delta^{18}O$ in water was analyzed by continuous flow (*CF*) using a Thermo Gas Bench *II* interfaced to a thermo Delta Plus *XL* mass spectrometer. In brief, 200µL of water for both standards and samples were pipette into 10 mL glass vials (*Exetainer*, *Labco Ltd*., *UK*) and quickly sealed. The vials were then purged with 0.2 % CO_2 in helium and allowed to equilibrate at room temperature for at least 48 hours. The $\delta^{18}O$ in CO_2 was then analyzed.

The $\delta^{13}C$ and $\delta^{15}N$ in leaf samples of wheat and *Alnus acuminata*, as well as the $\delta^{15}N$ in soil samples were analyzed at Godwin laboratory (University of Cambridge, *UK*) as described in the section *2.2.4* of this thesis.

4.2.5 Data analysis

One of the aims of this research was the application of the distinct isotopic signatures of various sources (water sources, or nitrogen sources) to quantitatively determine their proportional contribution to the mixed signature in crop tissue. According to Phillips (2001), when *n* isotope systems are used to determine the proportional contributions of *n+ 1* sources to a mixture, standard linear mixing models can be used to mathematically solve for the unique combination of source proportions that conserves mass balance for all *n* isotopes. For example, with one isotope system and three sources, following Phillips & Gregg (2003), such a system of mass balance equation can be solved to determine the proportions ($\int_A, \int_B, \int_C$) of source isotopic signatures (δ_A, δ_B, δ_C) which coincide with the observed signature for the mixture (δ_M):

$$\delta_M = \int_A \delta_A + \int_B \delta_B + \int_C \delta_C \qquad (4.2)$$

$$1 = \int_A + \int_B + \int_C$$

In this method, a programme called IsoSource performes this computation; the user inputs the isotopic signatures of the sources and crop, along with the source increment and the mass balance tolerance. Then, the programme firstly iteratively creates each possible combination of source proportions (that sum to 100 %) by some small increment (referred to as "source increment") such as 1 %. Secondly, the predicted isotopic signature for the mixture is computed as each combination is created. Thirdly, these predicted mixtures are compared with the observed crop signature; if they are equal or within small tolerance (referred to as "mass balance tolerance") such as ± 0.1 ‰, then this combination of source proportions represents a feasible solution and is stored in a data set. We inputted the isotopic signatures of

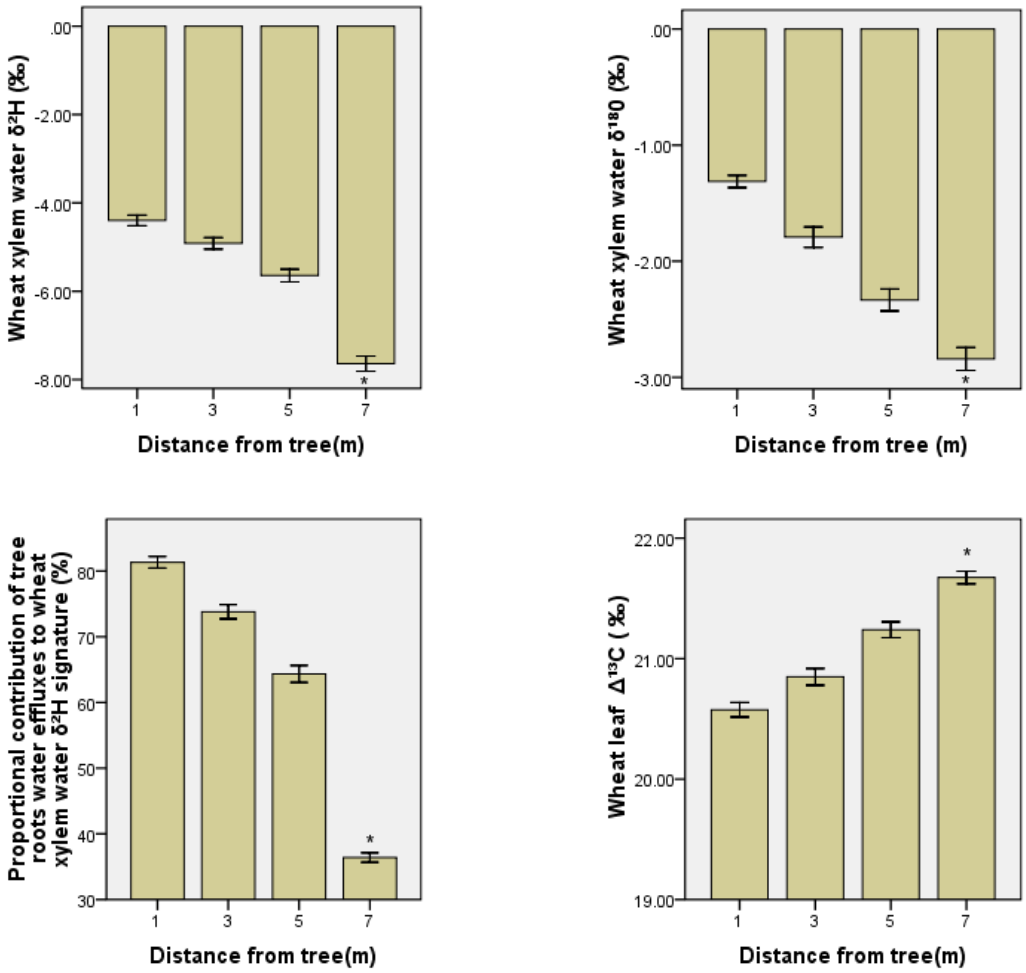
the sources and of the crops, obtained from the Berkeley analysis, into the IsoSource (which can handle up to ten sources). Thereafter, statistical analysis was conducted as described in the section *3.2.5* of this thesis.

However, caution should be taken into account about the limitations of this method in that it assumes that source signatures that fall closest to that of the plant (mixture) provide greatest contribution; therefore, the reliability of the isotopic determined contribution depends on the similarity of sources and plant isotopic signatures (Phillips & Gregg, 2003). Nevertheless, these limitations do not undermine the validity of *IsoSource*, nor the analysis of natural abundance of stables isotope as it has been shown by Phillips & Gregg (2003), and Sierra *et al*. (2007).

4.3 Results

4.3.1 The hydraulic redistribution and uptake

The crops in the proximity of trees up to 5 m exhibited significantly ($p<.01$) isotopic values of stem water δ^2H and $\delta^{18}O$ (*tab*. 4.1) which were closer to those of tree twigs (- 1.60 ± 0.02 ‰; and - 4.88 ± 0.07 ‰ for $\delta^{18}O$ and δ^2H respectively), indicating they were using the same source of water. For the crop furthest from the trees at 7 m, the stem water isotopic signature was closer to that of precipitation. The wheat xylem water signature of δ^2H and $\delta^{18}O$ consistently became more negative moving further from the trees (*fig 4.6)*. Additionally, the wheat in the proximity of trees consistently showed relatively low values of leaf matter $\Delta^{13}C$ (20.58 ± 0.12 ‰; 21.67 ± 0.11 ‰ at 1m and 7 m respectively) and *SLA* which increased moving out into the terrace (*fig. 4.6, & Appendix E).*



* Statistically significant different to 1m, 3m, 5m at (all *ps<.01*); Error bars represent *SE* of the mean. N=15

Figure 4.6 *Water effluxes from the tree roots and redistribution to wheat intercrops*

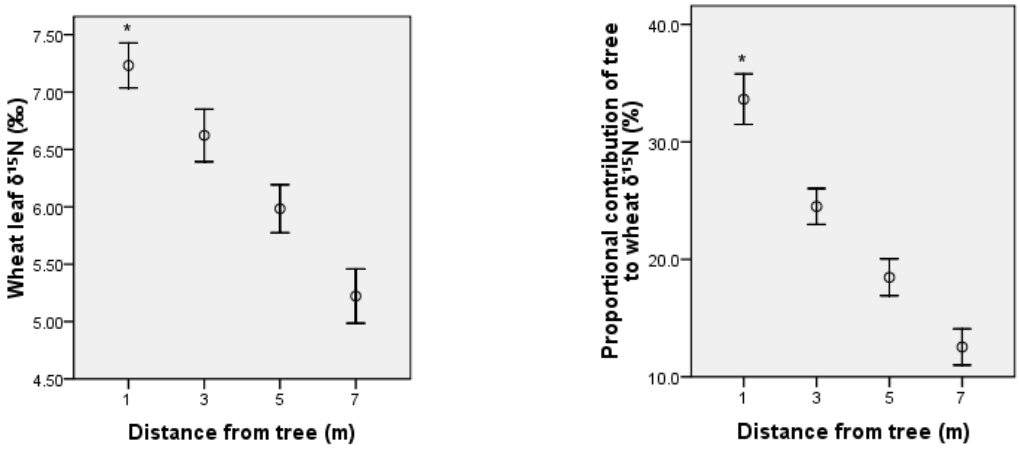
The isotopic mixing model indicated that water accessed by the tree accounted for 81 ± 2 % of the wheat stem water at 1 m, and that the contribution of the tree water to wheat water consistently declined as moving further from trees (*fig.4.6).*

Table 4.1 *The isotopic values (Mean across sites) for crop at different distance from the tree, and for sources of water and nitrogen (N=15)*

Isotope	Distance from tree(m)	δ value of crop (Mean ± SE)	δ Source (Mean ± SE)
$\delta^{18}O$	1	-1.41 ± 0.11	Rain:-2.94 ± 0.01
	3	-1.79 ± 0.18	Twig water:-1.60 ± 0.02
	5	-2.33 ± 0.19	Soil water (0-20cm)= -2.18 ± 0.18
	7	-2.84 ± 0.20	
$\delta^{2}H$	1	-4.40 ± 0.24	Rain:21.76 ± 0.25
	3	-4.92 ± 0.31	Twig water:-4.88 ± 0.07
	5	-5.64 ± 1.11	Soil water (0-20cm)= -10.02 ± 0.86
	7	-7.64 ± 1.34	
$\delta^{15}N$	1	7.23 ± 0.52	Tree:7.50 ± 0.13
	3	6.62 ± 0.77	Soil (0-20cm) :1.38 ± 0.02
	5	5.98 ± 0.62	
	7	3.22 ± 0.83	

4.3.2 The redistribution of nitrogen and capture

The $\delta^{15}N$ of the crop indicated consistent gradient declining with the distance from the N_2 fixing trees (*fig.4.7*). The wheat nearest to trees showed the $\delta^{15}N$ signature values closer to that of the tree; at 1m from the tree the wheat signature was 7.23 ± 0.52 ‰ relative to the tree (7.50 ± 0.13 ‰), while further at 7 m, the crop ^{15}N signature was 3.22 ± 0.83 ‰. The isotopic mixing model indicated that tree *N* may have provided 33.6 ± 4.3 % of the crop *N* at 1 m. Additionally, the chlorophyll content of wheat leaf was significantly higher at 1 m and 3 m from the tree, which declined moving further out into the bench (*fig.4.8).*

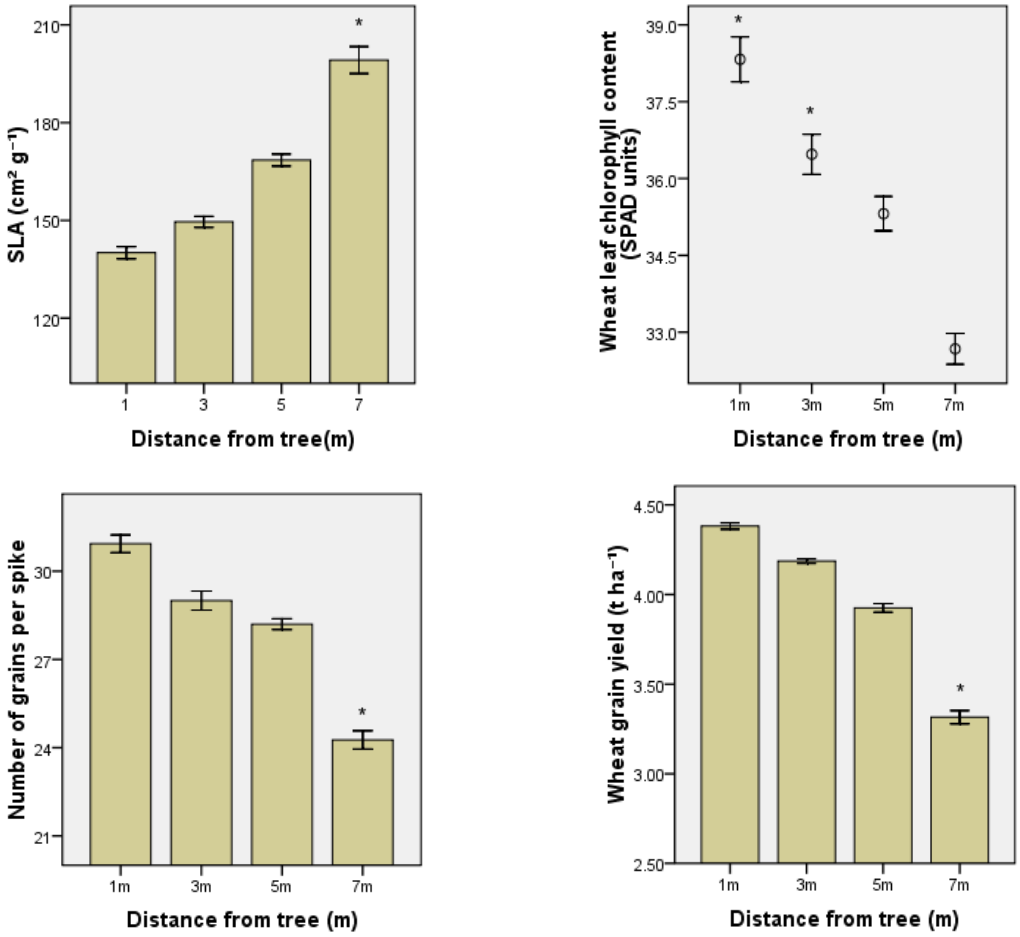


*Statistically significant different (*ps<.01*); Bar represent Mean ± SE (across sites). N=15

Figure 4.7 *Nitrogen transfer from the N_2 fixing trees to wheat*

4.3.3 The effects of water and nitrogen efflux for wheat production

Both the grain number per spike, and yield were consistently ($p<.01$) higher (4.4 ± 0.04 t ha^{-1} grain yield; 31 ± 1 grain number) at 1 m from the tree and declined as moving further in the terrace. The lowest grain yield (3.3 ± 0.07 t ha^{-1}) and least grain number (24 ± 3 grain number) on spike were always registered at 7 m from the tree. Both grain number per spike and grain yield at 1 m, 3 m and 5 m did not significantly differ at $p<0.01$ (*fig.4.8*), but significantly differed to that at 7 m.



*Statistically significant different (all *ps<.01*); Error bar (for *SLA*, grain number, and grain yield) represents *SE* of mean; Bar for chlorophyll content represent Mean ± SE (across sites). N=15.

Figure 4.8 *Variation in water and nitrogen efflux for wheat at different distances from the trees*

4.4 Discussion

The use of stable isotopes to elucidate the physiological processes in plants has increased in the past three decades (Marshall *et al.*, 2007; Teste *et al.*, 2014). The understanding of the mechanistic underlying the physiological processes behind stable isotopic composition signatures gives new tools to encapsulate plant resources acquisition and use to inform agronomic development. For example, there is a sense that isotopic mixing model coupled to the physiological measurements could be a way to link water and nitrogen sources used by plant to other aspects of their water and nitrogen relation respectively. We therefore tested the hypothesis that there might be practical application of the natural abundance of stable isotopes of $\delta^{2}H$, $\delta^{18}O$, $\delta^{15}N$, and isotopic mixing modeling "*IsoSource"* to understanding of resources (in term of water and nitrogen) redistribution in agroforestry systems.

The study dealt with the dynamics of water and nitrogen in intercrop of *Alnus acuminata* and wheat in the field. We focused on water and nitrogen because they are the most important resources influencing plant functions. The discussion of the main results raised out of this research will be led on: (i) Hydraulic lift and redistribution; (ii) the transfer of fixed *N*; and (iii) the implications of water redistribution and nitrogen transfer in the intercropping of wheat and *Alnus acuminata*.

4.4.1 Hydraulic lift and redistribution

The results of this research supported the hypothesis of hydraulic lift (Richards & Caldwell, 1987) and indicated pattern of hydraulic redistribution within the agroforestry system of *Alnus acuminata* and wheat. A closer examination of wheat xylem water $\delta^{2}H$ and $\delta^{18}O$ signatures showed such sequence: the xylem water of wheat nearest trees was enriched in both $\delta^{2}H$ and $\delta^{18}O$ and became depleted linearly moving further from the trees (*fig.4.6*). This feature of the data also reflected a consistent pattern of isotopic values in wheat xylem water

in proximity to trees being similar to that of the tree twig water (*tab.4.1*), suggesting they were using the same source of water.

These observations were strongly supported by the data of both $\Delta^{13}C$ in wheat leaf organic matter, and SLA (*fig.4.6*): there was consistent observation of low carbon discrimination in the leaf matter of wheat and low *SLA* of wheat in the proximity of the trees for a distance up to 5 m; and values of these two parameters increased when moving further from the trees. Additional evidence of redistribution of water in this agroforestry system came from the isotopic mixing model data; it revealed that the crop in the proximity of the trees may have accessed considerably the water redistributed by trees (*fig.4.6*). Therefore we propose that the trend in isotopic signature of wheat xylem water at different distances from the trees was most likely the result of hydraulic efflux from the tree roots dispersed in the topsoil.

This study showed that our understanding of plant interactions within a community could be enhanced through isotopic work. It also indicates that *Alnus acuminata* exhibits hydraulic lift. This idea is in accordance with the work of Caldira *et al.* (2001) on positive biodiversity-production relationship: they used the $\Delta^{13}C$ data to establish a relationship between species richness and productivity, and concluded that water use efficiency and productivity were higher when plants were grown in mixtures. In a related fashion, Dawson (1998) used isotopes of hydrogen and oxygen isotopes to determine the reliance of a species on winter precipitation versus fog. The application of stable isotopes of $\delta^{2}H$ and $\delta^{18}O$ has also helped to distinguish time of day that plants use their water in relation to a transition between conditions of low soil moisture availability and short episode of high soil moisture availability (Plamboeck *et al.*, 1999; Williams & Ehleringer, 2000b).

4.4.2 Nitrogen transfer

The transfer of nitrogen has been hypothesized to exist where plants with contrasting nutrients-acquisition strategies (N_2-fixing and non-fixing) co-occur (Dawson *et al.,* 2002; *He et al.,* 2009). However, there remains controversy about whether belowground *N* transfer occurs (Ikram *et al.,* 1994; Johansen & Jensen, 1996); and so far research on *N* transfer in agroforestry has conventionally assumed that *N* transfer occur via the decomposition of legume litter and pruning residues in soil (Jalonen *et al.,* 2009). We addressed these questions with assessment of natural abundance of $\delta^{15}N$ and an isotopic mixing model to determine the proportional contribution of *N* sources to the crop *N*. The literature has more recently been highlighting the need to study of *N* transfer among plants towards agro-farming under low external inputs (Hauggaard & Jensen, 2005; Wichern *et al.,* 2008); therefore we aimed to inform options for optimizing the interactions of plants for improved production and efficient use of *N* resources.

The data from this study provided an indication that N transfer from N_2-fixing trees can be a considerable N source for the associated crop in agroforestry farming (*fig.4.7*). Similarly, a comparison of the $\delta^{15}N$ signatures of the tree and the wheat revealed that the crops in the proximity of trees exhibited value closer to the tree $\delta^{15}N$ and declined as moving further in the terrace (*tab.4.1*). Our results are consistent with works of many authors (Handley & Scrimgeour, 1997; Robinson, 2001; Evans, 2001; Stewart, 2001) who suggested that the $\delta^{15}N$ of leaf tissues reflect the net effect of $\delta^{15}N$ of the sources used by that plant. Our findings are also in accordance with the works of both Moyer *et al.*(2006) and Lu *et al.*(2013) who showed that *N* transfer among plants can occurs through release of *N* compounds from the N_2-fixing plant leading to uptake by a non N_2-fixing plant. These findings are also in agreement with Hadley & Raven (1992) who suggested that there is no evidence of

fractionation either of $\delta^{14}N$ or $\delta^{15}N$ during its physical movement (passive and active uptake) across living membranes of plants.

The relative N transfer of 33.6 ± 4.3 % at 1 m from the tree also agrees with Sierra & Daudin (2010) who assessed in situ the ^{15}N transfer from stem-labeled trees to associated grass and found that the transfer of the added ^{15}N was limited in space (up to 1m from trees) and was on average 33 %. Similarly, Snoeck *et al*. (2000) noted 13 to 42 % of ^{15}N transfer from the legume trees to coffee. Nevertheless, such data should be interpreted with caution; for example, Daudin & Sierra (2010) observed that grass presented a preferential uptake of *N* released by the tree; if that is the case, then this preferential *N* uptake may cause discrepancy in isotopic mixing model results. Similarly, Sierra *et al* (2007) argued that *N* transfer from N_2 fixing trees may involves direct and indirect pathways; i.e. *N* transfer could be indirect if *N* exudates from the roots of tree were taken by soil microorganisms and passed through microbial turnover (Høgh-Jensen, 2006); in that case the isotopic mixing model could not resolve such system because it takes into account only *N* sources. The pruning regime (frequency and intensity) was also argued to be another factor that may affect *N* transfer by limiting the rate of N_2 fixation (Nygren *et al.*, 2000).

Moreover, Sanchez *et al.* (1997) argued that the roots of trees are often able to capture nutrients at the depths beyond the reach of most crop and redistribute them into topsoil, and this can be an additional nutrients input in an agroforestry system; in accordance with our data on hydraulic redistribution, this provides additional evidence to substantiate these connections between a direct transfer of *N* belowground.

Finally, the question of which mechanisms drive the *N* transfer between plants remains unsolved up to date; several mechanisms have been hypothesized; release of *N* in exudates (Høgh-Jensen, 2006), roots-grafts (Caldwell & Richards, 1986), and mycorrhizal netwoks

(He *et al.*, 2003). We therefore recommend further research into the mechanisms by which plants transfer *N* to their neighbors.

4.4.3 Implications for wheat production

This research has shown that hydraulic redistribution and nitrogen transfer by plant roots systems may facilitate a number of important physiological functions. We observed an improvement both in wheat grain number and yield nearest the tree, and a consistent decline moving further away from the trees (*fig.4.8*). A closer examination of the whole data lead us to suggest this pattern of improved number of grain and yield may has been brought about by hydraulic redistribution and nitrogen transfer in this agroforestry system of *Alnus acuminata* and wheat.

The data of this work are supported by Lott *et al.* (2003) who observed the *Grevillea robusta* trees improved maize productivity by increasing the proportion of annual rainfall captured or accessing deep water reserves with the soil profile; and their observation was in accordance with Ong *et al*. (1992) that indicated improvement in annual rainfall utilization of 40 to 80 % in *Cajanus cajana* and groundnut agroforestry systems. In the same fashion, Rockstrom (1997) argued that there is considerable scope to improve agricultural productivity by agroforestry practice in dry region like Niger where only 6-16 % of rainfall was reported to be used by pearl millet, and most the reminder was lost by evaporation or deep drainage.

Moreover, our results are in agreement with number of other authors who noted that improvement in water and nutrients in the top soil enhanced production factors: Long ago, Newbould *et al.* (1971) observed that uptake of nitrogen, phosphorous, and calcium from the topsoil by ryegrass was strongly influenced by the topsoil water content. Similarly, Richards & Caldwell (1987) noted that the persistence of hydraulic lift over appreciable periods in the topsoil layer had several implications for rhizosphere processes and plant nutrients acquisition (i.e. improving ion mobility, prolonging life span of fine roots).

Also, Rundel *et al.* (1982) noted that soil water and nitrogen content in the upper 60 cm beneath *Prosopis glandulosa* was increased and attributed that improvement to be the result of hydraulic lift and symbiotic nitrogen fixation. The findings of this study are also in agreement with the work of Muthuri *et al*. (2005) that observed some complementarities of resource use between *Alnus acuminata* and Maize at Thika, in semi-arid Kenya. Similarly, Peden *et al.* (1993) found that the yield of bean grown with *Alnus acuminata* was 50 % higher than in a field without trees: they attributed this effect to the ability of *A. acuminata* to fix N_2 and which may have been transferred to associated crop via the soil.

Chapter 5 Major conclusions and Outlook for further research

"The two methods of crop improvement: Physiological and conventional selections should not be considered as antipoles. In the breeding programme, they actually complement each other" (This thesis).

The thesis presents findings of research that is all about physiology-driven crop improvement and wheat breeding. The physiological understanding of crop improvement is necessary for developing genotypes combining high yielding potential and agronomic traits of superior adaptation, and for understanding yield limiting factors.

The research described in this dissertation aimed to address these challenges with three experimental research: Chapter 2 dealt with the proxy-based approach to physiological selection of traits; Chapter 3 addressed the physiological attributes determining increased *HI* in *Rht* genotypes and the consequences of *Rht* genes on yield components in winter wheat; and Chapter 4 focused on analyses of the natural abundance of stable isotopes ($\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$) in an agronomic perspective in terms of hydraulic lift and redistribution, *N* availability and crop yield, in agroforestry systems associated with N_2 fixing trees.

Further research is proposed at the end of the chapter. The major conclusions that are discussed are:

(i) The proxy-based approach to physiology-driven breeding may have potential to identify optimal ideotypes for the combined trait package.

(ii) Low *SLA* is a mechanism that may improve photosynthetic efficiency in wheat.

(iii) The *Rht* genes benefit wheat yield beyond mere reduction of plant height.

(iv) The understanding of plant interactions in terms of hydraulic and *N* redistribution and uptake in agronomic perspective may be enhanced by analyses of natural abundance of stable isotopes ($\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$) coupled to an isotopic mixing model "*IsoSource*".

The proxy-based approach to physiology-driven breeding may have potential to identify optimal ideotypes for the combined trait package.

In contrast to previous works (Blum, 1990; Ehleringer *et al.*, 1993; Condon *et al.*, 2004; Rajabi, 2006) that tended to rely on a single tool (i.e., $\Delta^{13}C$) as an indirect selection criterion, this study stressed the importance of comprehensive examination of the interactions between physiological processes and crop environmental responses, to develop an integrated proxy of a particular physiological variable. The research also focused on the steps for incorporating physiological criteria into a breeding programme.

It is possible to argue that the physiological approach to crop selection is of greater importance in terms of identification of traits that could be used either as selection criteria in core breeding or in introgression programme. The approach likely has clear benefit to be cost efficient and may achieve results quickly than genetic breeding. The cost of screening for a trait is of prime importance for breeder in term of gain from higher selection intensities. The research of this thesis aimed to contribute to advance of physiology-driven breeding; as the literature survey of crop physiology by Jackson *et al.* (1996) revealed that little effort is devoted to develop outputs and ideas from physiological research in the context of crop breeding.

The proxy-based approach advocated in this thesis involves surrogate-based screening for morphological, anatomical, and physiological traits or crop environmental responses. The central attribute to a proxy is the quality to be easy to measure, affordable, and reliable. The research of chapter 2 introduced and defined the concept of *"Proxy-based approach to physiological selection of trait"*, and proposed steps for conducting a proxy-based crop selection programme.

The approach described in this thesis should guide breeders working in physiology-driven breeding; this has also potential application in pre-breeding, and could be useful in fund-

limited research programme. There is a sense that physiological approach to crop selection is needed as indicated by the survey of breeders by Jackson *et al.* (1996).

Low *SLA* is a mechanism that may improve photosynthetic efficiency in wheat.

The influence of leaf morphology and anatomy on photosynthetic and water relations has long been recognized (Wilson & Cooper, 1967; Sharkey, 1985). The research of chapter 2 and 3 of this thesis have clearly shown how *SLA* significantly correlated with both photosynthetic (A_{max}, $\Delta^{13}C$, leaf *N* content, Chlorophyll content) and water use variables (leaf *RWC*, K_h, *WUE*, *IVD*). As indicated by Witkowski & Lamont (1991), *SLA* reflects the combined effects of both density (dry mass per unit volume) and thickness. Lambers *et al.* (2008) argued that thicker leaves (thus low *SLA*) have more chloroplast, proteins, photosynthesizing cells, and *Rubisco* content per unit area. Similarly, Lambers & Poorter (1992) indicated that thicker leaves have a high proportion of vascular tissue.

As revealed by the research of this thesis, there is a sense that low *SLA* may be an important tool for screening for crop resources acquisition and use efficiency. First, it is likely that the boundary resistance to diffusion of CO_2 decreases with low *SLA* thus permitting high photosynthetic uptake of CO_2. Drawing from Murchie *et al.* (2008), low *SLA* may improve canopy structure and thus permit a higher leaf area index (*LAI*: leaf area per unit ground); this may increase light interception in the canopy, which may result into improved radiation capture and use efficiency.

Selection for low *SLA* may be one route for improving photosynthesis through increasing the Rubisco content of leaves. In principle, the rate of photosynthesis can be increased further by increasing the total amount of photosynthetic machinery per unit leaf area. In practice, there is an optimal concentration of leaf *N* which is determined partly by leaf thickness (Murchie *et al.*, 2008). Furthermore, light absorbance depends strongly on chlorophyll content per unit

leaf area (Evans, 1998), and chlorophyll concentration index (*SPAD*) varied systematically with *SLA* in this study.

The findings of the research of this thesis raised the possibility to propose that low *SLA* may potentially be used for screening purposes in crop selection for photosynthetic efficiency in wheat.

The *Rht* genes improve wheat beyond mere reduction of height.

Uncovering the mechanism that underlies the relationship between plant height and harvest index would potentially inform the strategies for improvement of wheat with optimal height. The height reduction arising from straw-shortening by the *Rht* genes has long been proposed as the driver of the increases in *HI* of wheat (Youssefian *et al.*, 1992*a, b*). The research of chapter 3 in this thesis has revealed three other mechanisms by which the *Rht* genes may also improve the *HI* of wheat: (i) Low *SLA*, (ii) increased *MRT* of *N*, (iii) and increased grain number on spike.

The result of chapter 3 clearly indicated that the *Rht* genes may decrease the *SLA* and that there may be a threshold of level of dwarfing beyond which this effect could not happen (the *SLA* decreased with the level of dwarfing up to *Rht-B1c*, and the Rht-D1c exhibited the *SLA* a bit higher than the *Rht-B1c*). The lines with low *SLA* consistently exhibited both higher rate of A_{max} and K_h compared to that of higher *SLA*. Therefore, the thesis proposes that straw-shortening may improve A_{max} through the effects of lowering the *SLA*: Three explanations support this proposition; (i) both the research of Chapter 2 and 3 have consistently shown strong correlation between low *SLA* and high A_{max}; the literature also indicates that low *SLA* is associated with higher photosynthetic machinery; (ii) the other explanation is the effect of low *SLA* on canopy structure, *LAI*, reduced boundary resistance to CO_2 diffusion, and radiation capture (as explained in above section); (iii) the third mechanism is the association of low *SLA* and short *IVD* as shown in this thesis; this association is linked to increased

photosynthetic rate through the connection of short *IVD* and higher K_h as indicated by Brodribb *et al.*(2005), and it has been shown that increased K_h improves the efficiency of water delivery to photosynthesizing cells and allowing stomata to remain open (Scoffoni *et al.*, 2012).

Increasing the *MRT* of *N* is likely the other mechanism by which straw-shortening improves the *HI* of wheat: the research of chapter 3 has consistently shown that the shortest lines (Rht-*B1c, and Rht-D1c*) exhibited increased the *MRT* of *N* than the relatively tall lines. Selection for increased *MRT* of *N* may also be important in enhancing the stay-green of the leaf, and therefore could provide an additional route to increase photosynthetic efficiency. The increased *MRT* of *N* also is a characteristic possibly related to *NUE* as indicated by the results of this research. The high *MRT* of *N* has been proposed to be an important indicator of plant to N stress (Golluscio, 2007). There is a sense that selection for high *MRT* of *N* may be one of the strategies to meet the challenge of low input cost and low pollution to the environment (Delgado, 2002).

As shown in chapter 3, it is likely that the *Rht* genes benefit the *HI* by effect on grain number and kernel weight on spike. The grain number and kernel weight advantage of straw-shortening apparently derives from increased partitioning of assimilates to spike as a consequences of reduced demand for the stem elongation; and Youssefian *et al.* (1992b) has shown this may increase the total number of florets viable at anthesis. However, there may be competition effects on kernel weight associated with increases in grain number on spike. This assertion may explain the notion of reduced grain yield associated with excessive dwarfing: It is possible that extreme straw-shortening increases grain number too far at the expense of kernel weight (resulting in lighter grains that may be caused by greater competition for assimilates due to greater number on spike), which may nullifies the yield benefit. Therefore, realizing the yield advantage of *Rht* dwarfs depends upon achieving a balance between the

increases in grain number and kernel weight. For this to be realized, selection for grain length in addition to grain number would be one of the strategies; an evidence in support come from Gegas *et al*.(2010) that shows grain length and grain volume are controlled independently.

The understanding of plant interactions in terms of hydraulic and *N* redistribution and uptake in an agronomic perspective may be enhanced by analyses of natural abundance of stable isotopes ($\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$) coupled to an isotopic mixing model "*IsoSource*".

The understanding of physiological basis of stable isotope signatures of the critical plant resources water, carbon, and nitrogen may be an approach to encapsulate plant interactions, and resources acquisition in agroforestry systems. The research of chapter 4 employed the analyses of natural abundance of stable isotopes ($\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$) supplemented with an isotopic mixing model "*IsoSource*" to investigate for the redistribution of water and nitrogen, and their effects on wheat production in intercropping of *Alnus acuminata*. The key to this research was that the distinct isotopic signatures of various sources of plant water and nitrogen can be identified, and their relative contribution to plant isotopic signatures could be determined by *IsoSource*.

The results indicated that *Alnus acuminata*, a N_2-fixing tree, may have exhibited hydraulic lift and redistribution of nitrogen. The evidence came from the realization that the crop in the proximity of tree has shown isotopic value of water and nitrogen closer to that of tree; indicating they may have been using the same source of water and nitrogen. This observation was strongly supported with the *IsoSource* data that indicated that the crop near the tree may have accessed considerably water and nitrogen from the tree. Based on these results, it is possible to argue that the analyses of natural abundance of stable isotopes coupled to *IsoSource* may be a potential approach (compared to enriched isotope approach which is difficult to apply in the field conditions) to plant physiological studies in the field.

The research has also shown that the wheat grain yield has increased in the proximity of tree compared to the distance further away in the bench: Three explanations may be possible; the improvement in grain yield near the tree may be either the result of the direct uptake by the crop of the water and nitrogen redistributed by the tree, or the effect of enhanced nutrients availability and acquisition facilitated by the moisture from hydraulic redistribution by tree. There is also indication that the roots of tree may able to capture nutrients at the depth beyond the reach of crop and redistribute them into the topsoil (Sanchez *et al.*, 1997), and this can be an additional nutrients inputs that may result into improved crop yield in the proximity of tree.

The findings of this research may have practical implications in agroforestry systems in terms of dimensioning of the width of alley or the terrace bench.

Suggestions for further research

Further research is needed to understand the mechanism by which extreme straw-shortening reduces yield. This could be supported by work for identification of *QTL* for reducing height without reducing grain yield or seeking independent control of kernel weight. Further work should also be carried out to better understand the genetic control of *SLA* and investigate whether reliable genetic marker can be identified. Additional further studies are needed to determine (i) the root properties that may regulate efflux of water, and (ii) mechanism by which plants transfer *N* into the soil. The investigation on how the proxy-based selection approach could be integrated with markers development is needed: this may accelerate the deployment of trait in breeding programme.

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Appendices

Appendix A: List of EPS varieties

BBCB-91-232-4-1-16-1-4

BBCB-90-231-8-1-17-6-6

Badger

BBCB-91-232-4-1-5-5-1

SR96-1-412-1-11

SSRS-67-147-5-1-12-2-2

SSSR-67-147-6-2-4-19-B-7

SSSR-67-147-6-2-4-8-6

BBCB-91-232-4-1-16-3-1

Spark

SSSR-64-32-8-1-3-12-1

RRSR-33-187-3-2-6-8

Rialto

SSRS-67-147-6-2-4B-2-3

SR99-1-413-2-8

RRSR-33-187-3-2-6-5

SR94-1-411-1-2

SSRS-64-84-4-2-18-1-5

SR94-1-441-1-7

SSSR-67-147-6-2-4-8-4

SSSR-64-32-8-1-3-18-1

SR99-1-413-2-15

RRSR-15-159-2-7-4-A-4

***Appendix B**: The Zadoks scale decimal code for growth stage (GS) of cereal*

	Germination
00	Dry seed
01	Water uptake (imbibition) started
03	Imbibition complete
05	Radicle emerged from seed
07	Coleoptile emerged from seed
09	Leaf just at coleoptile tip
	Seedling growth
10	First leaf emerged
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
14	4 leaves unfolded
15	5 leaves unfolded
16	6 leaves unfolded
17	7 leaves unfolded
18	8 leaves unfolded
19	9 or more leaves unfolded
	Tillering
20	Main shoot only
21	Main shoot and 1 tiller
22	Main shoot and 2 tillers
23	Main shoot and 3 tillers
24	Main shoot and 4 tillers
25	Main shoot and 5 tillers
26	Main shoot and 6 tillers
27	Main shoot and 7 tillers
28	Main shoot and 8 tillers
29	Main shoot and 9 or more tillers
	Stem elongation
30	Pseudo stem erection
31	1st node detectable
32	2nd node detectable
33	3rd node detectable
34	4th node detectable
35	5th node detectable
36	6th node detectable
37	Flag leaf just visible
39	Flag leaf ligule/collar just visible
	Booting
40	- - - - - - -
41	Flag leaf sheath extending
45	Boot just swollen
47	Flag leaf sheath opening
49	First awns visible
	Heading (Inflorescence emergence)
50	First spikelet of head visible
53	1/4 of head emerged
55	1/2 of head emerged
57	3/4 of head emerged
59	Emergence of head complete
	Pollination (Anthesis)
60	Beginning of pollination
65	Pollination half complete
69	Pollination complete
	Milk Development
70	- - - - - - -
71	Kernel watery
73	Early milk
75	Medium milk
77	Late milk
	Dough Development
80	- - - - - - -
83	Early dough
85	Soft dough
87	Hard dough
	Ripening
90	- - - - - - -
91	Kernel hard (difficult to separate by fingernail)
92	Kernel hard
93	Kernel loosening in daytime
94	Overripe, straw dead and collapsing
95	Seed dormant
96	50% of viable seed germinates
97	Seed not dormant
98	Secondary dormancy
99	Secondary dormancy lost

***Appendix C*:** *The flag leaf nitrogen concentration at different growth stages in Rht lines (Rht-B1b; Rht-D1b; Rht-B1c; Rht-D1c) and wild type.*

Line	N (%) at GS65	N (%) at GS75	N (%) at GS83
Wild Type	2.12	1.5	1
Wild Type	2.89	1.54	1.21
Wild Type	2.04	1.93	1.36
Rht-B1b	3.19	2.93	1.96
Rht-B1b	3.38	2.91	2.44
Rht-B1b	3.24	2.91	2.73
Rht-D1b	3.81	3.07	2.94
Rht-D1b	3.65	3.37	3.13
Rht-D1b	3.91	3.65	3.36
Rht-B1c	5.57	4.04	3.5
Rht-B1c	5.21	4.73	3.61
Rht-B1c	5.17	4.31	3.73
Rht-D1c	5.6	4.78	3.64
Rht-D1c	5.83	3.98	3.28
Rht-D1c	5.46	4.54	3.51

***Appendix D**: Genetic variability of Rht lines (Rht-B1b; Rht-D1b; Rht-B1c; Rht-D1c) and wild type in the traits of photosynthesis and partitioning.*

Line	SLA ($cm^2 g^{-1}$)	IVD (mm)	A_{max} ($\mu mol\ m^2\ s^{-1}$)	$\Delta^{13}C$ (‰)	$\Delta^{18}O$ (‰)	K_h ($mmol\ m^2 s^{-1}\ MPa^{-1}$)	Tiller (#)	Grain (#)	TGW (g)	DAT (days)	Ped. L. (cm)	*HI*	Biomass (dw, kg)	NP (g dw $mg^{-1}N$)	^{15}NUE (g dw $mg^{-1}N$)	MRT (days)	^{15}NHI	gs ($mmol\ m^2\ s^{-1}$)	Height (cm)	Grain mass (dw, kg)
WT	162.3	.38	31.6	23.35	27.96	32.6	30	30	41	52	18.3	.35	1.08	.47	6.9	13	.38	.26	60	.38
WT	162.5	.37	29.97	23.97	29.52	35	31	31	38	52	14.8	.35	1.09	.46	6.3	12	.42	.28	58	.38
WT	169.6	.35	29.31	23.53	28.25	33	36	29	39	52	16.9	.36	1.08	.36	4.4	12	.44	.24	63	.39
Rht-B1b	138.1	.35	30.2	23.13	28.36	32.7	25	33	35	55	13.7	.4	1.30	.50	6.6	13	.48	.30	58	.52
Rht-B1b	148.1	.38	29.05	23.75	27.49	34	15	33	36	55	14.2	.39	1.36	.79	11.5	14	.57	.26	60	.53
Rht-B1b	112.7	.34	30.02	22.48	26.65	34.2	34	32	35	55	16.8	.41	1.40	.83	11.7	14	.54	.28	58	.57
Rht-D1b	112.4	.33	32.56	23.32	27.68	34	19	34	35	56	18.6	.41	2.02	1.36	16	12	.66	.30	51	.83
Rht-D1b	113.9	.35	34.08	21.85	25.36	34.2	17	32	34	55	17.8	.4	1.92	1.20	17.3	14	64	.31	53	.77
Rht-D1b	123.7	.32	33.96	23.62	27.61	38	23	33	35	56	14.3	.42	1.91	1.77	18	16	.7	.27	50	.80
Rht-B1c	87	.30	37.96	21.95	24.02	43	19	40	30	56	11.2	.48	2.35	2.63	43.4	16	.78	.38	38	1.1
Rht-B1c	97.8	.29	36.95	22.51	24.85	40	19	42	31	57	8.8	.47	2.36	2.55	41.6	16	.79	.41	39	1.1
Rht-B1c	93.2	.30	36.54	22.02	24.66	40	25	41	30	56	9.2	.46	2.25	2.82	49.1	17	.72	.37	34	1.0
Rht-D1c	103.5	.29	36.11	22.15	24.92	43	21	39	32	60	9.1	.45	2.49	2.75	43.5	16	.77	.34	33	1.1
Rht-D1c	97.1	.29	35.04	22.17	25.05	40	30	41	31	58	6.6	.44	2.48	3.04	48.3	17	.78	.35	32	1.1
Rht-D1c	101.3	.30	36.05	23	25.06	41.9	31	40	31	60	8.6	.46	2.50	2.72	43.3	16	.8	.37	31	1.2

***Appendix E**: The crop SLA, $\Delta^{13}C$, grain number and yield and the proportion of crop $\delta^{2}H$ and $\delta^{15}N$ accounted from trees at different distance.*

Location	Replicate	Distance (m)	SLA ($cm^2\ g^{-1}$)	Grain (#)	Yield ($t\ ha^{-1}$)	$\Delta^{13}C$ (‰)	$\delta^{2}H$ (%)	$\delta^{15}N$ (%)
Rurembo I	I	1	141	31	4.36	21.15	80	9.5
	I	3	138	30	4.16	21.22	77	8.4
	I	5	148	29	4.04	21.58	71	7
	I	7	163	22	3.39	21.89	43	3.9
	II	1	145	32	4.32	21.39	85	32.6
	II	3	149	30	4.12	21.44	83	26.8
	II	5	152	29	4.01	21.44	71	23.6
	II	7	165	24	3.29	21.62	47	20.5
	III	1	139	30	4.34	21.37	80	49.6
	III	3	141	29	4.20	21.37	73	40.5
	III	5	189	28	4.08	21.63	58	38.7
	III	7	189	22	3.35	21.74	42	35.2
Rurembo II	I	1	122	31	4.33	21.19	86	41
	I	3	155	29	4.27	22.16	67	27.1
	I	5	182	28	4.04	22.62	63	19.1
	I	7	289	23	3.26	22.69	34	4.9
	II	1	163	32	4.35	20.53	84	54.7
	II	3	167	30	4.19	21.17	77	42.7
	II	5	177	28	4.02	21.32	74	42
	II	7	227	25	3.36	21.95	35	34.6
	III	1	151	33	4.34	20.45	83	36.6
	III	3	163	31	4.06	20.53	75	17.4
	III	5	177	29	3.95	20.54	64	11.6
	III	7	227	24	3.40	22.25	31	0.2
Kirezi I	I	1	119	32	4.51	20.27	82	51.1
	I	3	127	31	4.29	20.73	86	27.3
	I	5	154	29	4.04	20.9	76	4.5
	I	7	164	23	3.47	21.39	38	0.9
	II	1	163	32	4.52	20.52	92	32.5
	II	3	166	31	4.30	21.08	84	32
	II	5	167	28	4.04	21.07	78	13
	II	7	205	30	3.46	21.35	32	11
	III	1	135	34	4.51	20.03	91	58.2
	III	3	138	28	4.16	20.38	57	30.6
	III	5	155	29	3.87	20.83	36	18
	III	7	167	29	3.46	21.59	27	3.3
Kirezi II	I	1	143	33	4.52	20.57	68	15.5
	I	3	147	32	4.20	20.65	66	12.5
	I	5	162	31	3.98	21.39	62	11.4
	I	7	203	22	3.47	21.41	34	9.4

	II	1	159	33	4.53	20.41	73	25
	II	3	163	27	4.33	20.57	69	23
	II	5	169	28	3.84	21.23	65	23.6
	II	7	199	24	3.52	21.71	30	22.2
	III	1	138	30	4.54	20.24	88	13.8
	III	3	159	32	4.32	20.42	86	6.3
	III	5	195	29	3.87	21	58	4.8
	III	7	201	25	3.47	21.13	33	0.9
Cyansure	I	1	135	28	4.15	20.02	75	21.7
	I	3	142	25	4.05	20.29	70	21.1
	I	5	179	26	3.87	21.09	65	19.3
	I	7	199	24	3.26	21.63	42	16.6
	II	1	125	27	4.21	20.17	78	49.7
	II	3	159	26	4.07	20.37	68	41.1
	II	5	169	27	3.93	21.39	61	34.6
	II	7	196	25	2.33	21.59	39	21.6
	III	1	123	26	4.17	20.32	75	13.1
	III	3	129	24	4.07	20.35	69	10.8
	III	5	154	25	3.31	20.56	63	6.1
	III	7	193	22	3.23	21.16	39	2.9