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ENHANCING PHOTOSYNTHETIC CAPACITY AND ENERGY CONVERSION EFFICIENCY IN WHEAT

João Paulo Pennacchi

under supervision of Dr Ana Elizabete Carmo-Silva and

co-supervision of Prof Dr Martin A. J. Parry

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Declaration

Except were reference is made to other sources, I declare that the contents in this thesis are my work, in collaboration to the people mentioned in each of the chapters, and have not been submitted, in part or in full, for the award of a higher degree elsewhere.

João Paulo Pennacchi

Lancaster University

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Being grateful is to understand our limitations and recognize the beautiful capacity of the others to contribute to our good and personal growth.

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“I am but one member of a vast team made up of many organizations, officials, thousands of scientists, and millions of farmers - mostly small and humble - who for many years have been fighting a quiet, oftentimes losing war on the food production front”

Norman Borlaug

“Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim”

Francisco do Espírito Santo

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LIST OF ABREVIATIONS AND SYMBOLS

2-PG	2-phosphoglycolate
3-PGA	3-phosphoglycerate
A	Net CO_2 assimilation rate
A@Q	Net CO_2 assimilation at given light level
ADP	Adenosine-Bi-Phosphate
AHDB	Agriculture and Horticulture Development Board
Ald	Aldolase
Amax	Net CO_2 assimilation rate at high light and CO_2 levels
ATP	Adenosine-Tri-Phosphate
BBSRC	Biotechnology and Biological Sciences Research Council
BT	Booting
CA1P	Carboxy-D-arabinitol 1-phosphate
CAM	Crasulacean Accid Metabolism
CCM	Carbon concentrating mechanism
C_i	Intercellular CO_2 concentration
CIMMYT	Centro Internacional de Mejoramiento de Maiz y Trigo
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CTBP	2-Carboxytetritol-1,4-bisphosphate
DEFRA	Department for Environment, Food & Rural Affairs
DHL	Double-haploid lines
DM	Dry matter
DNA	Deoxyribonucleic acid
ERYCC	Earliness and Resilience for Yield in a Changing Climate
F-6-P	Fructose-1,6-biphosphate
FACE	Free-Air CO_2 Enrichment
FAPESP	Fundação de Apoio a Pesquisa do Estado de São Paulo
FBPase	Fructose-1,6-biphosphatase
G3P	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GenDist	Genetic distance
GGE	Genotype, genotype x environment

GM	Genetically modified
gs	Stomatal conductance to water vapour
GWAS	Genome-Wide Association Studies
H2	Broad-sense heritability
HA-GGE	Heritability-adjusted GGE biplot
ictB	Inorganic carbon transporter B
LAI	Leaf area index
LUE	Light use efficiency
MAS	Marker-assisted selection
NADPH	Dihydronicotinamide-adenine dinucleotide phosphate
NBT	New Breeding Technologies
NDVI	Normalized Difference Vegetation Index
NPP	Net Primary Production
NPQI	Normalized pheophytinization index
p	p value
PA	Post-anthesis
PAR	Photosynthetically Active Radiation
PCI	Productivity Capacity Index
PDBP	D-glycero-2,3-pentodiulose-1,5-bisphosphate
PEPC	Phosphoenolpiruvate carboxylase
PGK	3-phosphoglycerate kinase
PRI	Photochemical Reflectance Index
PRK	Phosporibolukinase
PSII	Photosystem II
Q	Light level
QTL	Quantative Trait Locus
r	Pearson product moment coefficient
R-5-P	Ribose-5-P
RAD	Solar radiation
RARSa	Ratio analysis of reflectance Chlorophyll a
RARSb	Ratio analysis of reflectance Chlorophyll b
RARSc	Ratio analysis of reflectance Carotenoid
RCA	Rubisco activase

RCI	Resilience Capacity Index
REML	Residual Maximum Likelihood
RIPE	Realizing Increased Photosynthetic Efficiency
RPE	Ribulose-5-P epimerase
RPI	Ribose-5-P isomerase
Ru-5-P	Ribulose-5-P
Rubisco	Ribulose-1,5-biphosphate carboxilase/oxigenase
RuBP	Ribulose-1,5-biphosphate
S-7-P	Sedoheptulose-1,7-biphosphate
SBPase	Sedoheptulose-1,7-biphosphatase
SDN	Site-Directed Nucleases
SE	Stem elongation
SEM	Standard error of the mean
SIPI	Structural Independent Pigment Index
SLA	Specific leaf area
SNPs	Single nucleotide polimorphirms
Sra	Simple Ratio
TALEN	Transcription Activator-Like Effector Nucleases
Tbase	Base temperature for degree day calculation in wheat
TILLING	Targeting Induced Local Lesions IN Genomes
TK	Transketolase
Tmed	Mean temperature between tha maximum and minimum
USDA	United States Department of Agriculture
WC	Water content
WI	Water Index
XuBP	D-xylulose-1,5-bisphosphate
Yp	Yield Potential
YSSI	Yield Stress Score Index
ZNT	Zink Finger Nucleases
εc	Light conversion efficiency
εi	Light interception efficiency
εp	Biomass partitioning efficiency
σ/μ	Variability

ABSTRACT

Wheat is one of the major grain crops worldwide and provides approximately 20% of the total caloric and protein input for the world's population. An increase in crop yields is required to meet the demands of the increasing world population in a challenging scenario of less predictable climatic conditions and sustainability requirements. There is an urgent need to develop crop plants that are more productive per land area and yield more stable outputs, without having to increase inputs of fertilizer or water. The canopy light-driven carbon uptake through photosynthesis is the primary determinant of plant biomass and yield. In this context, it is crucial to understand crop physiology and plant behaviour, with a focus on carbon balance, in contrasting environments, as well as to map the interactions between traits that drive wheat yields. Exploiting new methods of enhancing yield through successful strategies observed in other organisms can be used to achieve positive results for crop improvement.

The impact of contrasting environments in wheat development and yield was studied in a panel of elite wheat cultivars over 3 seasons in UK. Late sowing and lower rainfall decreased the duration of grain development, impacting on grain yields. Yield stability was related to the crop resilience to shorter grain development periods and was partially explained by genetic similarities between cultivars or by the presence of genes related to crop development.

The interaction between traits recognized as potential yield drivers and their stability and correlation to yield were studied in a double-haploid wheat mapping population. Traits correlated to light interception and use, and biomass allocation were strong yield drivers. Different lines adopt different strategies, based on these traits, to achieve high yields.

The possible effect of the insertion of the *ictB* gene, related to carbon concentration in the chloroplast in cyanobacteria and to improved yields in plants, was studied in genetically modified wheat plants. No improvement was observed for photosynthetic traits or yield in the transgenic plants when compared to control plants, although unforeseen experimental problems could have hidden the expected phenotype.

The achievements of this research contribute to improve the knowledge of wheat yield formation, its correlation to the carbon uptake process and stability in different climatic conditions. It may lead to a better understanding of new potential strategies to be applied in the breeding of genotypes for higher and more stable yields.

CHAPTER 1

GENERAL INTRODUCTION

1.1 FOOD SECURITY: PRODUCTION AND DEMAND

Food security can be defined as "a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (FAO, 2002). There are four main bases listed as requirements to ensure food security: a) physical availability of food, b) economic and physical access to food, c) appropriate and healthy use of food, d) stability of these factors over time (Schmidhuber and Tubiello, 2007; Friedrich, 2014). With the rise of global population, expected to reach over 9 billion by 2050 (Godfray *et al*., 2010), and the changes in food consumption patterns worldwide (Pingali, 2006), a broad increase of 70% in food production will be needed to meet the demands and ensure that the first, and most basic of the cited bases, is fulfilled (Schmidhuber and Tubiello, 2007, Tilman *et al*., 2011; Friedrich, 2014).

The increase in food production needs to be attained in a challenging scenario that, besides an increased demand for food, includes the effect of extreme climatic events on crop production and the need for sustainable use of resources such as energy, water and nutrients for agriculture (Evans, 2009; Berry *et al*., 2015; Tilman and Clark, 2015). There are three main approaches for increasing food production: the expansion of agricultural areas (limited by important natural ecosystems, geographical location and climatic conditions), the intensification of cropping systems (limited by the sustainable use of resources, gas emissions and climatic conditions) and an increase in crop yields. By 2050, increases in yield will be responsible for around 77% of the required increase in food production, with crop

intensification and land expansion accounting for a further 15% and 8%, respectively (Alexandratos and Bruinsma, 2012).

Rice, maize, wheat and soybean provide, directly or indirectly, two-thirds of the total calories and protein consumed worldwide every day and need to be important targets for research for crop improvement (Fischer *et al*., 2014). For these four main crops, a 2.4% yield increase per year is required to meet the expected demand by 2050, without expanding the current cultivated area (Ray *et al*., 2013). In opposition to the required yield increases, a global trend of yield plateau has been observed, specifically from 2002, with production increases being driven particularly by the expansion of land areas. This pattern was pushed mainly by the three main cereal crops (maize, rice and wheat) which represented 60% of the total expanded agricultural area (Grassini *et al*., 2013) (Fig. 1.1).

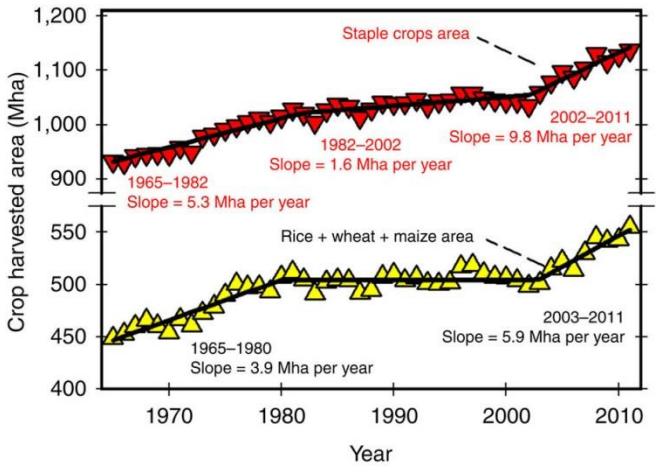

Figure 1.1 – Trends in total harvested area of staple crops and three major cereal crops. Staple crops include cereal, oil, sugar, pulses, fibre, tuber plus root crops. The three major cereal crops are rice, wheat and maize. Slopes of the fitted trilinear models are shown when significant (Student's t-test; $p < 0.01$, $n = 47$ years of yield data). Reproduced from Grassini *et al*. (2013).

Despite the general trend of yield plateau, different yield patterns can be observed around the globe. For the four most important crops (maize, rice, wheat and soybean), there are four main yield trends: yields never improved, yields stagnating, yield collapsed and yields still increasing (Ray *et al*., 2012) (Fig. 1.2). Monitoring these trends, and linking them to

environmental conditions, may provide information on plant responses and help in the development of new strategies to improve yields in the near future.

Wheat yield trends vary across the globe. There is a trend of increasing yields in US, Canada, South America and Russia and parts of Asia. Thirty seven percent of the wheat producing areas have stagnated yields, including important areas such as Australia, China, India, Turkey, Eastern and Western Europe and the central "Great Plain" area of the United States. In South-Eastern Australia, wheat yields have collapsed (Ray *et al*., 2012) (Fig. 1.3).

For wheat in the UK, the yield plateau trend started in around 1996 and continued until the present (Knight *et al*., 2012; DEFRA, 2016; FAOSTAT, 2016) with yields around 8 t ha^{-1}. Although the 2014 and 2015 harvests presented exceptional yields (8.7 and 9 t ha^{-1}, respectively), this is not enough to conclude that the yield plateau trend is over, since the yield in 2016 was back to the average 8 t ha^{-1}. The superior yields in 2014 and 2015 can be considered as a normal fluctuation around the mean, as are the low yields in 2012 (6.8 t ha^{-1}) (DEFRA, 2016).

Wheat is the biggest world crop in terms of land use, covering around 20% of the croplands worldwide, with an approximated total of 225 million hectares (FAOSTAT, 2016). The most intense cultivation areas are located in temperate latitudes in both hemispheres, but wheat is also cultivated in tropical areas, being present in all the continents: South and North America, Europe, Eastern Africa, Australia and New Zealand and Asia (Leff *et al*., 2004; FAOSTAT, 2016). Due to its better adaptation to drought and cold conditions wheat can be cultivated in areas where other cereals, such as maize and rice, are not well adapted (Leff *et al*., 2004).

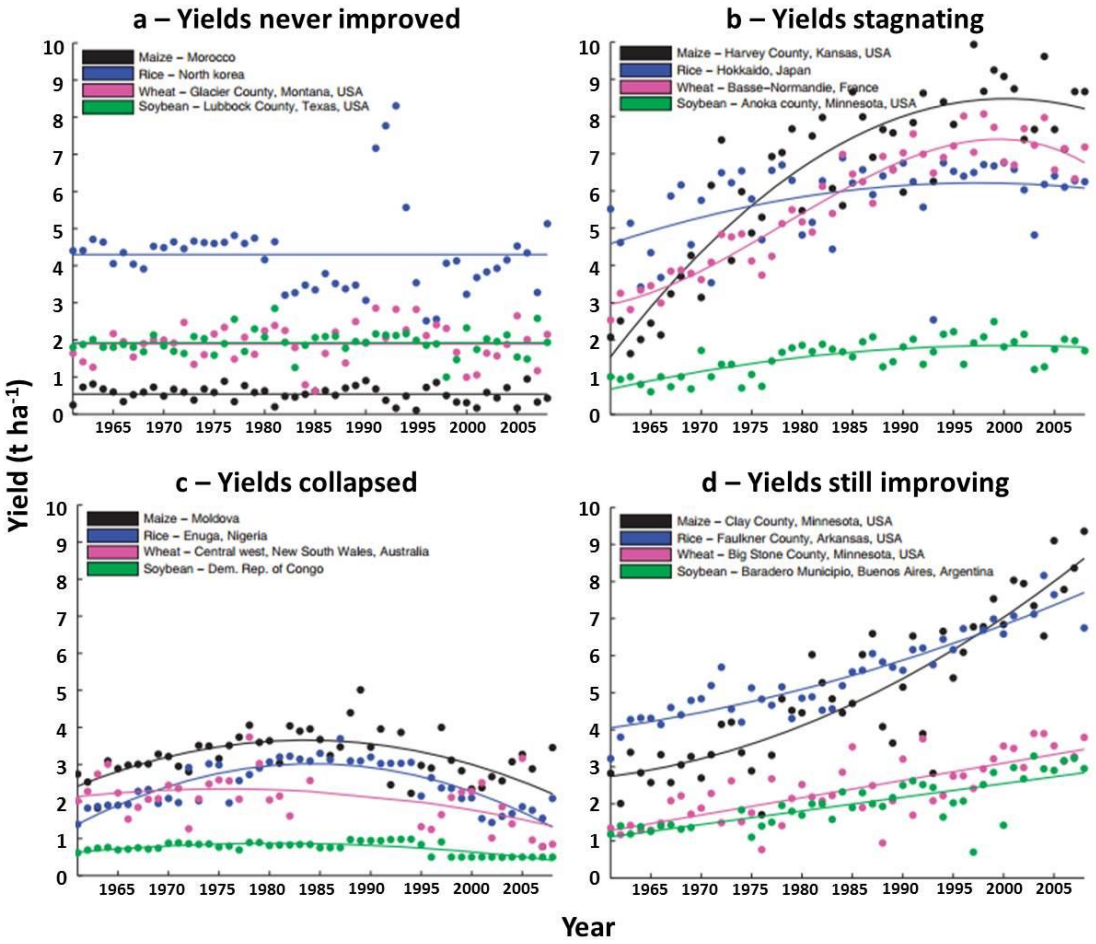

Figure 1.2 - Illustrative examples for each of the four types of global crop yield trends. The solid filled circles in each panel are the observed crop yields from various global locations to serve as illustrative examples. Colour codes indicate the crop. The solid curves are the statistical model fits to the data and similarly colour coded according to the crop type. (a) Yields never improved. (b) Yields stagnating. (c) Yields collapsed. (d) Yields still increasing. Reproduced from Ray *et al*. (2012).

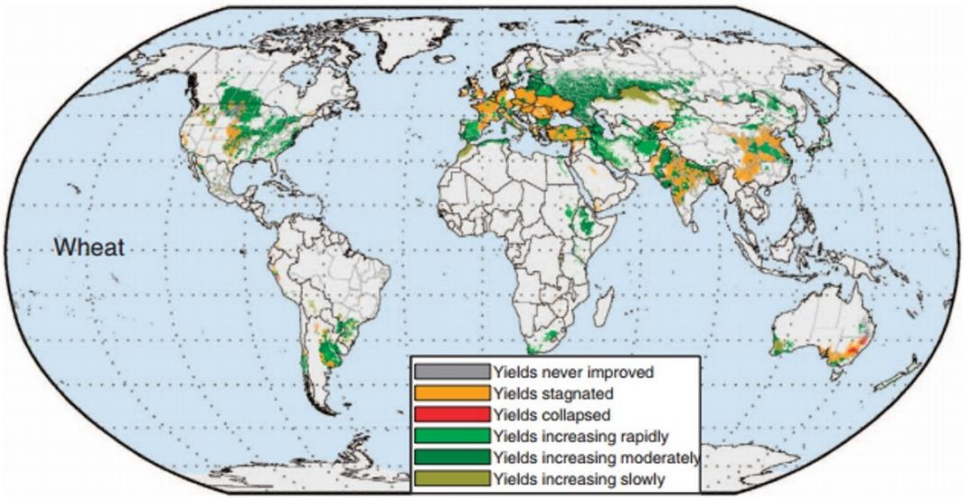

Figure 1.3 - Global map of current wheat yield trends. Yield trend status was determined at each political unit where wheat crop yields were tracked globally. The trends were divided into six categories and colour coded. Adapted from Ray *et al*., (2012).

1.2 WHEAT PRODUCTION AND IMPORTANCE

The wheat cultivars are usually separated according to their growth habits: winter and spring. Winter wheat cultivars are adapted to low temperatures and require a cold period that regulates flowering time according to day length (vernalization); they are sown in the autumn and harvested in the summer in a cycle of 10 months. Spring wheat cultivars do not require vernalization and are sown at the end of winter and harvested at the end of summer, in a cycle of 6 months (AHDB, 2015).

In the 2015/2016 season, global wheat production was 735.5 million tonnes, with a global average yield of 3.3 t/ha. China, India, the United States and Russia were the world's biggest wheat producers, followed by Canada, Ukraine, Pakistan and Australia (USDA, 2016). For the 2017 season an increase of 6.5 million tonnes is expected, including a 4.7 million tonne increase from Australia, correlated with exceptional weather conditions (USDA, 2016).

In terms of calorie intake, wheat represents 20% of all the calories consumed worldwide (FAOSTAT, 2016), highlighting its importance for food security. Although wheat production covers a considerable area of the globe, its consumption is not widespread. Globally, areas with high undernourishment rates are coincident with areas of low consumption and/or low production of wheat (Fig. 1.4) (FAOSTAT, 2016). Improving wheat tolerance to abiotic stress could allow the crop to be grown in areas where its cultivation is currently limited and help to address food security locally and globally.

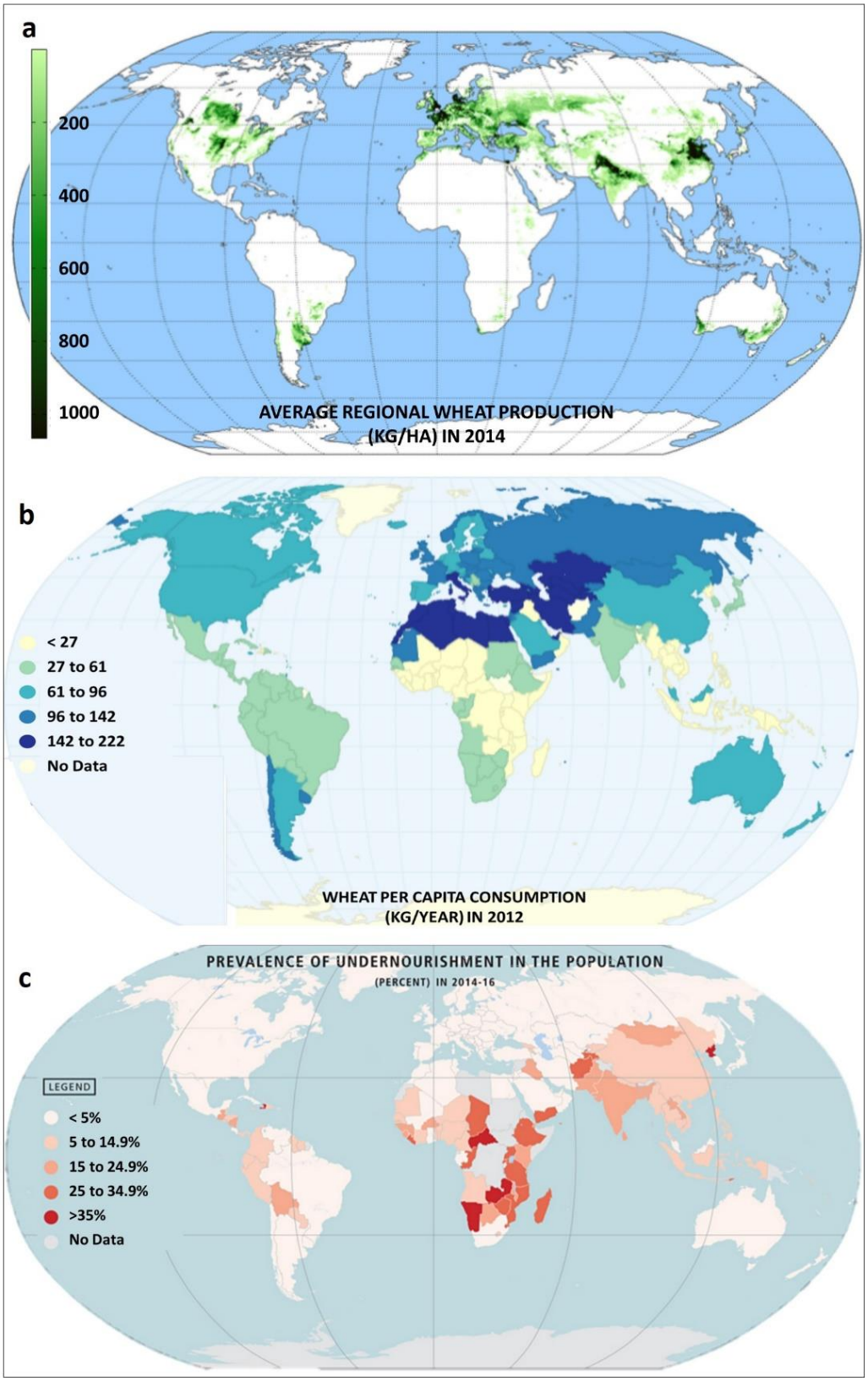

Figure 1.4 – Wheat production and consumption and its relation to undernourishment. (a) Average regional wheat production (kg/ha). (b) Wheat per capita consumption (kg/year). (c) Prevalence of undernourishment in the population (%). Adapted from FAOSTAT (2016).

1.3 CLIMATE AND CROP PRODUCTION

Predicting the future impacts of climate on crop yields is not an easy task and depends on the precision of the climatic models as well as accounting for plant adaptation capacity (Parry *et al*., 2004; Challinor *et al*., 2014). In the past decades, heat and drought stresses have affected wheat, maize and rice crop yields more than flood and cold and this trend tends to be kept for the future (Lesk *et al*., 2016). Wheat and maize yields, for instance, decreased 2 to 3% in 2002 as a response to temperature and precipitation patterns in the two previous decades (Lobell and Field, 2007). For wheat, a 6% decrease in yield is expected for a 1°C increase in global air temperature (Asseng *et al*., 2014).

Heat stress influences wheat yield in multiple ways. In terms of the carbon fixation pathway, heat stress decreases photosynthesis, mainly, due to: a) reduced function of chloroplast and chlorophyll content (Xu *et al*., 1995); b) the slower reactivation of Rubisco by Rubisco activase (Salvucci and Crafts-Brandner, 2014); c) a decrease in abundance of small and large subunits of Rubisco (Demirevska-Kepova *et al*., 2005) and d) the increase in photorespiration due to changes in the relative solubility of O_2 and CO_2 (Long *et al*., 2004) and the decrease in Rubisco affinity to CO_2 (Jordan and Ogren, 1984), at higher temperatures. The carbon fixation process is also affected by the reduction in photosynthetically active tissue due to temperature-induced senescence in wheat leaves (Al-Khatib and Paulsen, 1984).

Yield components, such as grain growth and development, are directly influenced by heat stress. A reduction in grain number is correlated to the number of days of heat stress at the booting stage, when the spikelet starts to be formed from meristems in the spike (McMaster, 1997). Grain number is also reduced by heat stress (over 20°C) between spike initiation and anthesis, which can also cause flower sterility (over 30°C) (Saini and Aspinall, 1982). Grain weight is reduced by a temperature effect causing grain shrinkage (Dias *et al*., 2008), with less starch formation and accumulation in the grain (Spiert *et al*., 2006) and

shorter duration of the grain filling stage (Dias and Lidon, 2009). The translocation of assimilates from stem-storage or straight from leaf photosynthesis are influenced by temperature and impact on grain weight (Blum *et al*., 1994).

Like heat stress, drought stress has indirect and direct effects on productivity, being one of the major limitations to wheat yield in the UK (Dodd *et al*., 2011). Drought influences the carbon uptake by decreasing photosynthesis, mainly, due to: a) a direct decrease in stomatal conductance and intercellular carbon concentration (Flexas *et al*., 2004); b) a decrease in Rubisco activity and content (Parry *et al*., 2002); c) a decrease in the Ribulose-1,5-Biphosphate (RuBP) regeneration (Bota *et al*., 2004) and d) a decrease in the ATP synthesis (Farooq *et al*., 2009).

Grain growth and development is affected by drought at two different stages. Drought stress during the reproductive stage can cause pollen abortion, leading to inefficient fertilization and decreased number of grains (Saini and Westgate, 2010). Drought at grain filling can reduce the grain weight by reduced starch production and accumulation (Ahmadi and Baker, 2001) and by early senescence, which decreases the availability of assimilates from leaf photosynthesis (Madani *et al*., 2010) and shortens the grain filling stage (Plaut *et al*., 2004).

The future climatic predictions reinforce the likely reduction in rainfall and increase in temperature for Europe, and so heat stress may assume an important role in limiting condition to wheat yields in the future (Semenov and Shewry, 2011). The wheat crop growth cycle may be shortened to avoid the late dry and hot period of the season, shortening the grain filling and maturity stages, causing possible yield losses. Heat tolerance at the reproductive stage may be a crucial trait for wheat cultivars facing these conditions (Stratonovitch and Semenov, 2015).

1.4 BREEDING FOR YIELD POTENTIAL AND RESILIENCE

The breeding process for crop improvement in the second half of the last century was mainly focused on enhancing yield potential and contributed to maintaining production ahead of population growth (Araus *et al*., 2008). Yield potential can be defined as "the yield of a cultivar when grown in environments to which it is adapted, with nutrients and water non-limiting and with pests, diseases, weeds, lodging, and other stresses effectively controlled" (Evans and Fischer, 1999). Yield potential is distinct from the potential yield and the plant theoretical yield limit, which is based on the net primary production (NPP) (DeLucia *et al*., 2014) and predicted by simulation models with plausible physiological and agronomic assumptions (Evans and Fischer, 1999).

Between 1960 and 2000, crop yields in developing countries rose 208% for wheat, 109% for rice and 157% for maize (Pingali, 2012). Continued increases in yield potential could, once more, help to solve the current food demand problem. However, there are limiting points that are mostly related to replicating, at farm scale, the ideal growth conditions where yield potential is reached (Hengsdijk and Langeveld, 2009). The difference between the yield potential and the on-farm yield is defined as the yield gap (Lobell *et al*., 2009). One of the limiting factors is the nutrient use-efficiency, since availability of nutrients, such as nitrogen and phosphorus, can be reduced and cost increased, limiting their use and impacting on yield gains (Edgerton, 2009). Abiotic stresses, related to environmental pressures and climatic conditions, are the major factors that reduce actual farm yields and increase the yield gaps, and are responsible for 70% of yield decreases (Acquaah, 2007). Although increases in yield potential are possible (van Ittersum and Cassman, 2013) the research effort should be concentrated mutually on increasing yield potential and decreasing yield gap in future climatic conditions (Powell *et al*., 2012).

The combined use of biotechnology and breeding strategies can speed up and enhance the genetic gain of the breeding process (Tilman *et al*., 2002). The genetic gain of the breeding process is a function of: a) the degree of phenotypic variation present in the population; b) the probability that a trait phenotype will be transmitted from parent to offspring; c) the selection intensity and d) the length of time necessary to complete a cycle of selection (Moose and Mumm, 2008).

Yield, as a complex trait built by interactions of multiple traits and with a multi-gene influence, is not a simple target for breeding. The physiological breeding proposes the joint use of high-throughput methods of genotyping and phenotyping to evaluate the progeny of crosses between parents with contrasting, but complementary, characteristics related to yield (Reynolds and Langridge, 2016). Improving the understanding of the genetic and physiological background of yield-related traits could improve the efficiency of the selection intensity and cycle length in the breeding process, and increase the genetic gain (Reynolds *et al*., 2011).

The phenotypic variation in the population is greatly related to the selection of contrasting parents and the natural variation of the trait of interest (Reynolds *et al*., 2012). The rate at which a trait can be transmitted to next generations is related to the trait heritability (Falconer and Mackay, 1996). Heritability (also named repeatability or liability) is defined as the proportion of the phenotypic variance of a trait that is the result of genetic factors (Falconer and Mackay, 1996). Heritability is a characteristic of a specific population in a specific environment and its calculation in multiple target environments is crucial to predict the trait transmission accounting for the genotype x environment interaction (Mir *et al*., 2012).

In summary, the decision of which physiological traits to include in a breeding process to enhance crop yield needs to be made based on: their relative correlation to yield, the extent of genetic variation, heritability and genotype x environment interactions (Mir *et al*., 2012).

1.5 YIELD FORMATION

Plant dry matter is formed from the conversion of solar energy into compounds that are stored in form of biomass (Monteith and Moss, 1977). Mathematical models for explaining plant productivity are normally based on the Monteith and Moss (1977) model and define the production capacity or yield potential (Yp), in the absence of biotic and abiotic stresses, as a function of the available solar radiation (RAD) and the efficiency of plants to intercept radiation (εi), convert the solar radiation into biomass energy (εc) and the partitioning of the stored biomass in the plant organs (εp) (Formula 1.1) (Long *et al*., 2015).

$$Yp = RAD \cdot \varepsilon i \cdot \varepsilon c \cdot \varepsilon p \qquad \text{(Formula 1.1)}$$

Plant scientists have managed to improve greatly εi and εp since the Green Revolution and, even though there is still space for improvement, the energy conversion efficiency (εc) has been currently the main focus of research (Long *et al*., 2015). Traits, from the canopy to the anatomical level, related to the efficiency of light interception, energy conversion and biomass allocation are potential yield drivers (Fig. 1.5).

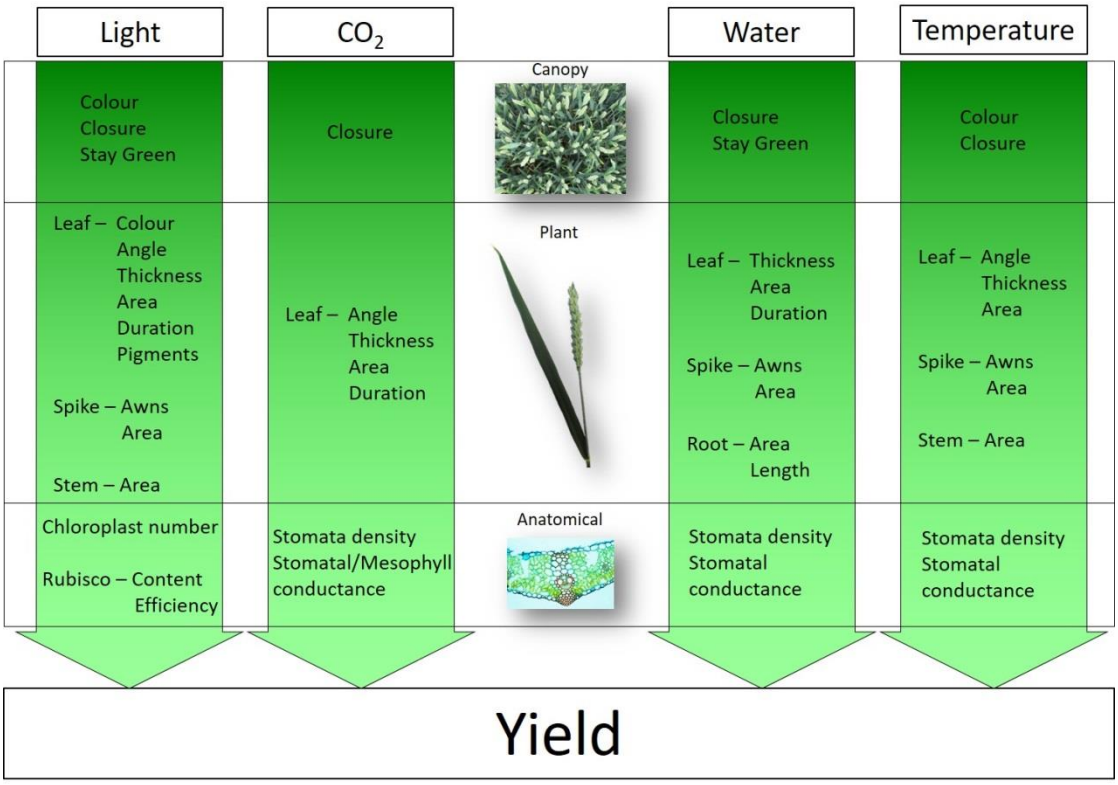

Figure 1.5 – Traits correlated to possible improvements in wheat yields at canopy, plant and anatomical levels. Adapted from Parry *et al*. (in press).

1.5.1 Traits that influence biomass production and yield

1.5.1.1 Light Interception

Leaf characteristics have a high influence on light interception efficiency. This is mainly related to how the leaves cover the soil area and for how long they are able to maintain photosynthetic activity. Keeping an active canopy for longer means intercepting more sunlight and increasing the energy input available for yield formation (Thomas and Howarth, 2000). For winter wheat, climatic conditions may constrain crop duration, with cold stress likely to happen early in the season, and heat and drought stresses at the end of the season (Semenov and Shewry, 2011). Even so, increasing early vigour (Ludwig and Asseng, 2010) and improving stay green (Farooq *et al*., 2011) can increase light interception efficiency without increasing the risks of facing stresses.

The genetic selection of cultivars with bigger seeds, made since the beginning of plant cultivation, generate taller plants, with bigger leaves and faster establishment. However, these traits were partially left behind by breeding during the Green Revolution which focused on the *Rht* dwarfing genes. Although the reduced height cultivars improved yield and mainly harvest index, which is related to the biomass partitioning to the grain, there is a need to balance the semi-dwarf phenotype with early vigour characteristics (Richards, 2000). Long coleoptiles, broad seedling leaves, large embryos, large coleoptile tiller, fast emergence and leaf expansion rate, large grains, low temperature tolerance and shallow crown depth are recognized as traits related to improved establishment and early canopy formation in wheat (Richards, 2000).

At the other end of the development cycle, there are different strategies to keep the canopy photosynthetically active for longer. Based on senescence initiation and rate, amount of pigments and the photosynthetic machinery, there are three main basic strategies of stay green: a) delaying the senescence initiation and maintaining the rate; b) starting senescence at normal time with a reduced rate and c) having an increased concentration of pigments and photosynthetic enzymes and keeping the initiation time and rate as normal (Thomas and Howarth, 2000). A combined strategy with a higher content of pigments, and delayed senesce initiation and slower rate could further improve stay green. However, a balance between keeping the canopy green and senescing is needed, as big part of the grain protein is formed from the remobilization of nitrogen from leaf protein during the grain filling stage (Hirel and Gallais, 2006; Foulkes *et al*., 2009).

Fast establishment and tillering are traits related to canopy development that improve area cover (Araus *et al*., 2002). In addition to increasing sunlight interception, a fast canopy closure helps weed control and decreases evaporation of soil water (Richards, 2000), this being particularly important in areas where the crop is susceptible to drought stresses (Araus

et al., 2002). Different strategies in terms of plant architecture and leaf characteristics influence light interception. Covering the area in multiple layers of smaller erect leaves is a more efficient strategy then having fewer layers of bigger horizontal leaves (Murchie *et al*., 2009). Bigger leaves at the top of the canopy tend to decrease light infiltration by increased reflection, as their capacity to absorb energy is limited by the quick photosynthesis saturation at elevated light levels (Burgess *et al*., 2015).

1.5.1.2 Light use efficiency

Light use efficiency can be defined as the ratio of net primary productivity (NPP) to intercepted sunlight radiation (Medlyn, 1998) or simply the crop photosynthetic efficiency (Araus, 2002). Photosynthesis is related to the energy conversion efficiency as it is the process underlying the conversion of sunlight (radiant energy) in plant biomass (chemical energy). It can be limited by the factors that influence photosynthesis such as CO_2 levels, water and nutrient availability, and temperature (Badger, 2013). For C3 photosynthesis, the maximum conversion efficiency of solar energy to biomass is 4.6% (Zhu *et al*., 2008).

Most traits related to light interception also influence light use. Canopy architecture influences light infiltration to leaves in the lower layers in the canopy, which do not reach optimal light conditions and decrease light use efficiency (Burgess *et al*., 2015). The activity of the central enzyme in the photosynthetic process, ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), is highly influenced by light levels. Rubisco activation state is controlled by the Rubisco activase enzyme, whose activity is regulated by the light mediated stroma ATP/ADP ratio. At low light intensities, Rubisco activase activity tends to be reduced, consequently reducing Rubisco activation and light use efficiency (Portis *et al*., 2008).

Specific leaf area (SLA) is the ratio of leaf area to leaf dry weight and is correlated to photosynthetic rate and yield (Badger, 2013). Higher values of SLA mean bigger and thinner

leaves and smaller values of SLA mean smaller and thicker leaves. At early stages higher SLA improves area cover and light interception, increasing canopy development and crop vigour (Richards, 2000; Badger, 2013). Although, as the area is covered and light interception reaches its maximum, high SLA increases shading and decreases light use efficiency by decreasing carboxylation rate per area (Richards, 2000; Badger, 2013; Walker *et al*., 2014). Bigger and thinner leaves at early stages and smaller and thicker leaves at the top layers of the canopy look to be traits to improve both light interception and light use efficiency in critical growth stages (Badger, 2013).

The pigment concentration in the leaves also influences the light use efficiency. Lower chlorophyll contents at the top of the canopy could improve light infiltration to the lower layers, without compromising photosynthetic levels, at saturating light conditions (Ort *et al*., 2011).

In terms of the CO_2 levels, changes in the external, intercellular and chloroplastic CO_2 concentrations affect the light use efficiency. The intercellular CO_2 concentration is influenced by leaf area and by the stomata density and stomatal conductance. The chloroplastic CO_2 concentration is influenced by the same factors as intercellular CO_2 concentration, plus mesophyll conductance (Flexas *et al*., 2008). The canopy closure, light infiltration and different assimilation rates down through the canopy can create a CO_2 gradient and influence light use efficiency (Parry *et al*., 2011). The rate of CO_2 fixation is dependent of multiple factors and is discussed in more detail further later.

The water availability is also a factor influencing light use efficiency and yield. The cell water content is correlated with assimilation rates and light use. Canopy closure and duration, leaf area and thickness and stomata density and stomatal conductance are variables that influence internal water content in the plant and change water use efficiency (Richards, 2002). Nutritional balance and a well-developed root system are also crucial to control water

intake and the balance between carbon absorption and transpiration (Hawkesford *et al*., 2013).

1.5.1.3 Biomass allocation

Great improvement has been achieved in harvest index since the Green Revolution mainly by the use of the semi-dwarf cultivars (Araus *et al*., 2002). Further increases in harvest index are possible but can be limited by plant architecture, nutrient remobilization, as well as by lodging resistance. A theoretical limit of 64% can be achieved for wheat harvest index without compromising lodging resistance (Foulkes *et al*., 2011). A number of possible strategies for improving harvest index are listed by Foulkes *et al*. (2011): a) increasing partitioning to spike growth and maximizing grain number; b) optimizing developmental pattern to maximize spike fertility; c) improving spike fertility through modifying its sensitivity to environmental cues; d) improving potential grain size and grain filling; e) identifying traits and developing genetic sources for lodging resistance and f) modelling optimal combinations of, and trade-offs between, traits.

1.5.2 Photosynthesis

Between the three general processes related to yield formation – light interception and use efficiency and biomass allocation – the one with the biggest possibility of improvement is light use efficiency, which is highly related to photosynthesis. A description of the photosynthetic process and its correlation to yield, its main limitations and potential improvements are presented below.

1.5.2.1 Photosynthesis reactions and their limitations

The use of sunlight radiation for the fixation of CO_2 from atmosphere through photosynthesis is the main driver of biomass accumulation in plants. Plants present different photosynthetic pathways and are usually classified according to the first products of carbon

fixation and the physical and temporal placement of the reactions (Taiz *et al*., 2014). C3, C4 and CAM are the three types of photosynthesis described in plants with intermediate pathways being reported as part of the evolutionary process (Lundgren and Christin, 2016). Photosynthesis happens in two complementary types of reactions inside the chloroplast: a) the light reactions that take place at the thylakoids and transform absorbed light energy into chemical energy in the form of ATP and reduced NADP (NADPH) and ferredoxin and b) the carbon reactions (dark reactions) that take place in the chloroplast stroma and use the chemical energy, generated by the light reactions, to operate the Calvin-Benson cycle to assimilate CO_2 and regenerate the acceptor substrate, ribulose-1,5-bisphosphate (RuBP) (Taiz *et al*., 2014).

Wheat is classified as a C3 plant, as the first product of CO_2 fixation is a 3-carbon molecule, the 3-phosphoglycerate (3-PGA). The process of carbon fixation in the Calvin-Benson cycle is split in three phases: a) the carboxylation phase where CO_2 is assimilated by Rubisco to ribulose-1,5-biphosphate (RuBP) generating the 3-PGA; b) the reduction phase where 3-PGA is reduced to glyceraldehyde-3-phosphate (G3P) using the ATP and NADPH generated by the light reactions and c) the regeneration phase where RuBP is regenerated from G3P in a series of reactions with ATP consumption (Raines, 2011; Taiz *et al*., 2014) (Fig. 1.6).

Rubisco is a dual-function enzyme that can carboxylate or oxygenate RuBP. When oxygenation happens (red arrow in Fig. 1.6), 3-PGA and toxic 2-PG (2-phosphoglycolate) are generated. Photorespiration is the process that recycles the toxic 2-PG and is related to decreases in the energy use efficiency, because of: a) the loss of pre-assimilated carbon as CO_2; b) the energy needed for regeneration of the RuBP used in the 2-PG formation to maintain steady state assimilation and c) the energy needed for re-assimilation of ammonia produced in recycling the carbon of the 2-PG (Taiz *et al*., 2014).

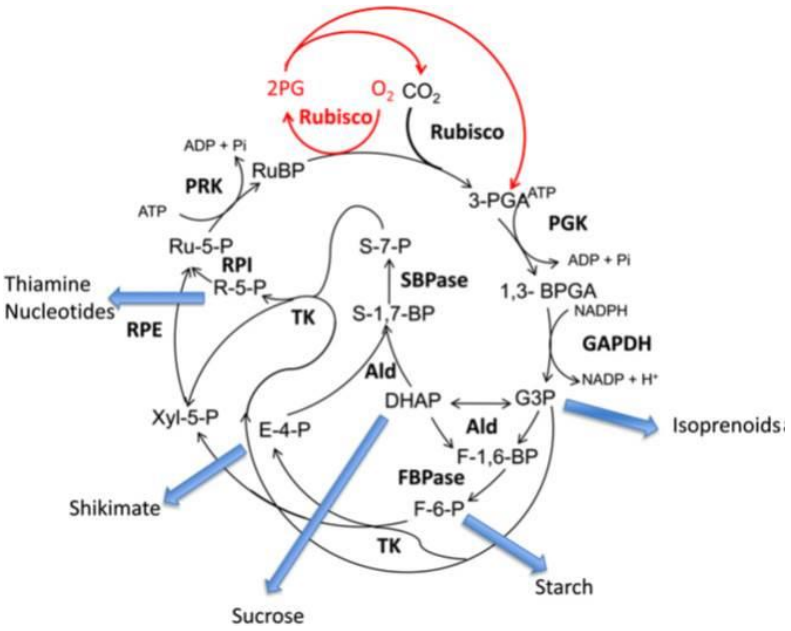

Figure 1.6 – The C3 cycle. The carboxylation reaction catalysed by Rubisco fixes CO_2 into the acceptor molecule RuBP, forming 3-PGA. The reductive phase of the cycle follows with two reactions catalysed by 3-PGA kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), producing G3P. The G3P enters the regenerative phase catalysed by aldolase (Ald) and either fructose-1,6-biphosphatase (FBPase) or and sedoheptulose-1,7-biphosphatase (SBPase), producing fructose-6-P (F-6-P) and sedoheptulose-7-P (S-7-P). F-6-P and S-7-P are then utilized in reactions catalysed by transketolase (TK), ribose-5-P (R-5P) isomerase (RPI), and ribulose-5-P (Ru-5-P) epimerase (RPE), producing Ru-5-P. The final step converts Ru-5-P to RuBP, catalysed by phosporibolukinase (PRK). The oxygenation reaction of Rubisco fixes O_2 into the acceptor molecule RuBP, forming 3-PGA and 2-phosphoglycolate (2PG), and the process of photorespiration (shown in red) releases CO_2 and 3-PGA. The five export points from the pathway are shown with blue arrows. Adapted from Raines (2011).

Photosynthesis can be limited by the CO_2 diffusion and by the biochemical pathways directly or indirectly involved in the carbon fixation (Sage *et al*., 2008). The CO_2 diffusion from the atmosphere to chloroplast is constrained by the resistance barriers of the boundary layer, stomata and mesophyll (Evans and Loreto, 2000; Flexas *et al*., 2008). The biochemical pathways that can influence photosynthetic rates are: a) the synthesis of ATP and NADPH in the thylakoid membrane during light reactions; b) the RuBP regeneration pathway in Calvin-Benson cycle; c) the carboxylation capacity of Rubisco and d) the feedback inhibition by accumulation of assimilates in the stroma and the shortage of inorganic phosphate availability (Lawlor and Keys, 1993).

The intercellular CO_2 concentration (Ci) affects the carbon fixation by the Calvin-Benson cycle by two different ways. At reduced C_i levels, the CO_2/O_2 ratio is decreased and Rubisco limits

photosynthesis by the lower carboxylation/oxygenation rate. In higher C_i levels, the carboxylation/oxygenation rate increases and Rubisco limitation is replaced by RuBP regeneration limitation and further by phosphate availability (Farquhar *et al*., 1980; von Caemmerer, 2000; Bernacchi *et al*., 2001; Bernacchi *et al*., 2003). The tipping point between Rubisco-limited and RuBP-limited photosynthesis happens around intercellular carbon concentrations of 300 µmol CO_2 µmol air^{-1}, for C3 species (von Caemmerer, 2000).

Photosynthetic rates are also influenced by light levels and by the efficiency of the photochemical reactions. At low light levels, in light-limited photosynthesis, the photosynthetic rate increases linearly with light intensity. In this phase, the carboxylation efficiency of Rubisco limits photosynthesis, as well as the chloroplastic CO_2 levels. At light-saturated conditions, the limitation to photosynthesis is related to the carboxylation capacity of Rubisco and RuBP regeneration (Badger, 2013).

1.5.2.2 Photosynthesis and yield

Intermediate steps of the Calvin-Benson cycle are involved in the synthesis of starch (in the chloroplast) and sucrose (in the cytoplasm and transported by the phloem). These molecules are involved in the production and storage of compounds that build plant biomass (Raines *et al*., 2011; Taiz *et al*., 2014) (blue arrows in Fig. 1.6). Although yield is determined by cumulative canopy photosynthesis, the correlation between biomass and instantaneous rate of photosynthesis per leaf area is commonly low, leading to the misconception that improving photosynthesis would not be useful to improve yields (Richards, 2000).

The free-air CO_2 enrichment (FACE) experiments showed that increases in photosynthetic rates lead to increased yields when other factors are not limiting. For grain crops, a 15% increase in grain yield was observed when doubling the CO_2 concentration (Ainsworth and Long, 2005). The yield increases under high CO_2 concentrations are linked to the decrease in photorespiration and the increase in photosynthetic efficiency (Walker *et al*., 2016).

Photorespiration, at the current CO_2 levels, can be responsible for reductions of 30% in net photosynthesis (Zhu *et al*., 2010) and 20% in plant yields (Walker *et al*., 2016).

Other evidence of increases in yield related to improvements in carbon fixation is reported in multiple studies. A study of a group of semi-dwarf wheat cultivars, released by CIMMYT between 1960's and 1980's, showed a trend of increasing yields to be correlated to their flag-leaf photosynthesis, stomatal conductance and photosynthetic capacity (Sayre *et al*., 1997). More recent studies in CIMMYT showed an increased yield for wheat cultivars selected by their flag-leaf photosynthetic rate and a correlation between the yield increases and net photosynthesis, stomatal conductance and intercellular carbon concentration (Fischer *et al*., 1998; Gutierrez-Rodriguez *et al*., 2000), as well as leaf porosity, canopy temperature and carbon isotope discrimination (Condon *et al*., 2008).

The grain filling process in wheat depends on two main sources of carbon: photosynthesis by leaves and by the ear during grain filling, and the mobilization of pre-stored carbohydrates from the stem (Blum *et al*., 1994). Photosynthesis can provide more than 60% of assimilates to the grain (Farooq *et al*., 2011). As it is the last leaf of the canopy to senesce and the most photosynthetically active leaf during grain filling, the flag-leaf can be responsible for 30 to 50% of the total assimilates supplied to the grain (Sylvester-Bradley *et al*., 1990). Wheat genotypes that sustain flag-leaf photosynthesis for longer periods produce better yields (Larbi and Mekliche, 2004).

1.6 POTENTIAL STRATEGIES FOR PHOTOSYNTHESIS IMPROVEMENT

The theoretical maximum efficiency of photosynthesis in wheat, at current atmospheric conditions, is 4.6%. The practical efficiency is not bigger than 2% and the average, in field conditions, is less than 1% (Zhu *et al*., 2008). Despite its key role and potential to improve plant yields, photosynthesis research has been under-explored (Lawson *et al*., 2012).

Improvements in photosynthesis could lead to an increase in yield potential by boosting the carbon fixation from the atmosphere and the availability of assimilates to be invested in plant biomass (Parry *et al*., 2011). It can also decrease the yield gap by alleviating the limitations of carbon fixation under stress conditions (Foyer and Shigeoka, 2011; Slattery *et al*., 2013) and improve nutrient, water and energy use efficiency (Carmo-Silva *et al*., 2015).

The main focus of research in photosynthesis improvement is related to overcoming the processes that limit photosynthetic efficiency, such as: CO_2 diffusion and concentration, RuBP regeneration, Rubisco efficiency and the efficiency of the light reactions. Possible improvements in each of these processes are discussed below.

1.6.1 CO_2 diffusion and concentration

The internal CO_2 level constrains C3 photosynthesis by influencing the ratio CO_2/O_2 at the Rubisco site and, consequently, the photosynthesis/photorespiration ratio. Increasing CO_2 to Rubisco can be achieved by facilitating its transport from the atmosphere, or, by concentrating it at the Rubisco site.

Facilitating CO_2 transport from the atmosphere is related to decreasing the boundary layer, stomatal and mesophyll resistances (i.e increasing conductance). The boundary layer conductance can be influenced by plant height with an increased boundary layer conductance to taller plants due to the wind and greater coupling between canopy and atmosphere (Jarvis and McNaughton, 1984). Leaf hairs can increase boundary layer humidity, decreasing transpiration, without significantly increasing the boundary layer resistance. Hairs can also reflect excessive radiation, resulting in lower canopy temperature (Ehleringer, 1983).

Stomata are responsible for 95% of all gas fluxes between the leaf and the environment and control water and CO_2 fluxes to and from the leaves. Stomatal characteristics including size, aperture/closure speed and response to different stimuli affect photosynthesis. The

increasing understanding of stomata dynamics and interaction between guard cells and mesophyll metabolism may suggest means to improve photosynthesis and water use efficiency (Lawson and Blatt, 2014). Mesophyll conductance improvement can increase carbon diffusion without significantly influencing water loss by the stomata (Flexas *et al*., 2008). Increased activity of aquaporins appears to be related to increased mesophyll conductance and can be exploited as a potential approach to facilitate diffusion (Uehlein *et al*., 2008). Increases in stomatal and mesophyll conductance could improve wheat net photosynthesis by 5% (Reynolds *et al*., 2009).

C4 and CAM plants have evolved different mechanisms to concentrate CO_2 to the Rubisco site. C4 photosynthesis is based on a physical separation of the CO_2 fixation reactions, where CO_2 is firstly fixed by phosphoenolpyruvate carboxylase (PEPC), which lacks the dual-functionality of Rubisco, in mesophyll cells. The fixed CO_2, in the form of organic C4 acids, is transported and made available at high concentrations via decarboxylation in the bundle sheath cells where Rubisco is exclusively located. In C4 plants Rubisco is therefore exposed to much higher CO_2/O_2 ratios, greatly decreasing photorespiration (Taiz *et al*., 2014). CAM photosynthesis uses a similar route but with a temporal, rather than spatial, separation between the primary and final CO_2 assimilation reactions. The first step catalysed by PEPC happens at night and the Calvin-Benson cycle during the day, when the products of the light reactions are available (Taiz *et al*., 2014).

Changing wheat C3 pathway to C4 pathway could improve highly the CO_2 concentration in the leaves, improving Rubisco carboxylation efficiency and photosynthetic rates. Based on the analysis of C3 and C4 photosynthetic efficiencies, a 50% increase in net photosynthesis can be reached by using this technology (Reynolds *et al*., 2009).

Current projects, the C4 rice (http://c4rice.irri.org/) and the CAM Biodesign (http://cambiodesign.org/) are investigating the effects of the insertion of C4 and CAM

pathways into C3 plants. The main limitations for these approaches to be practical are related to the multi-gene characterisation of C4 and CAM pathways, the need for accompanying anatomical adaptions and the better understanding of physical and temporal separations of carboxylation reactions (Covshoff and Hibberd, 2012; von Caemmerer *et al*., 2012; DePaoli *et al*., 2014). The complexity of the hexaploid wheat genome, as well as wheat evolutionary distance from C4 plants, can also be a complicating factor for the genetic manipulation involved in making the changes.

A different approach for increasing CO_2 concentration is to insert in plant cyanobacterial and algal chloroplastic carbon concentrating mechanisms (CCM), such as those involving carboxysomes (McGrath and Long, 2014) and pyrenoids (Raines, 2011). An improvement of 60% in net photosynthesis has been predicted by using this approach (McGrath and Long, 2014). The great advantage of this method, compared to the C4 adaptation, is that no anatomical transformations are required (McGrath and Long, 2014). Carboxysomes were successfully inserted into tobacco plants (Lin *et al*., 2014), but further studies to ensure the presence of other CCM components, such as bicarbonate transporters and evaluation of the photosynthetic efficiency of resulting plants, are still required.

The transformation of C3 plants with the insertion of single genes related to carbon concentration in cyanobacteria is also reported as a potential method to increase CO_2 concentration and photosynthesis in plants. However, a smaller effect in net photosynthesis is expected (9 to 16%) if compared with the insertion of carboxysomes (McGrath and Long, 2014). The *ictB* gene is recognized to be involved in carbon concentration in cyanobacteria and was firstly reported by Bonfil *et al*. (1998). Although its function in cyanobacteria is not well known (Price *et al*., 2012), it has been inserted in a range of higher plants due to its possible effects in net photosynthesis improvement. Improvements in the photosynthetic rate in *ictB* transgenic plants, compared to control plants, were reported in: *Arabidopsis* and

tobacco (Lieman-Hurwitz *et al*., 2003), soybean (Hay, 2012; Hay *et al*., 2017), tobacco (Simkin *et al*., 2015) and rice (Gong *et al*., 2015). The photosynthetic improvement was also correlated with increases in biomass/yield in the studied plants, except for tobacco (Lieman-Hurwitz *et al*., 2003) and rice (Gong *et al*., 2015).

1.6.2 Calvin-Benson cycle and Photorespiration

Approaches to improve photosynthesis through the Calvin-Benson cycle are related to increasing Rubisco carboxylation efficiency and/or the regeneration of RuBP. Rubisco is an inefficient and slow enzyme, and large amounts of the enzyme are required to keep adequate carboxylation rates, representing around 25% of leaf nitrogen (Parry *et al*., 2013). The main inefficiencies of Rubisco are: a) slow turnover rate; b) oxygenase reaction; c) low affinity to CO_2 and d) inhibition by sugar phosphates (Carmo-Silva *et al*., 2015). Changes in Rubisco amount and performance could lead to improvements in photosynthesis, but different approaches may be required for different crops and environments (Parry *et al*., 2013). At low to moderate light conditions, a 15 to 20% decrease in Rubisco amounts would decrease nitrogen needs by 10% without compromising photosynthetic rates. At high light conditions, higher amounts (or activity) of Rubisco would be needed in order not to limit photosynthetic rate (Parry *et al*., 2013).

Improving Rubisco performance could allow the reduction of Rubisco and, consequently, nitrogen amounts required to reach the same current photosynthetic rates (Hanson *et al*., 2016). Rubisco activity is regulated by the action of its activators and inhibitors. The Rubisco activation is controlled by the enzyme Rubisco activase (RCA) which is light and thermo sensitive (Salvucci and Crafts-Brandner, 2004; Carmo-Silva *et al*., 2015). RCA abundance has been linked to photosynthetic efficiency and yield in wheat (Ristic *et al*., 2009). Together with improving activation, decreasing inhibition can be a way to increase Rubisco efficiency. A number of sugar phosphates are known to bind to Rubisco causing its inhibition: 2-

Carboxy-D-arabinitol 1-phosphate (CA1P), D-xylulose-1,5-bisphosphate (XuBP), D-glycero-2,3-pentodiulose-1,5-bisphosphate (PDBP) and 2-Carboxytetritol-1,4-bisphosphate (CTBP) (Carmo-Silva *et al*., 2015). Improving Rubisco regulation could improve net photosynthesis by 10% (Reynolds *et al*., 2009).

Changes in Rubisco affinity to CO_2 and O_2 could also improve photosynthesis. There is large genetic variation in Rubisco specificity to CO_2 and O_2 within photosynthesizing species (Whitney *et al*., 2011). Exploiting this natural variation and deeply understanding Rubisco dynamics and the roles of small and large subunits in the carboxylation process is crucial to generate a better Rubisco (Whitney *et al*., 2011). Increasing Rubisco affinity to CO_2 and, consequently, its carboxylation rate, could result in a 60% increase in net photosynthesis (Reynolds *et al*., 2009).

Engineering a better Rubisco could enhance photosynthesis greatly at the current CO_2 levels. With the predicted increase in future atmospheric carbon concentration or with an improved Rubisco, photosynthesis tends to be limited by the levels of RuBP regeneration (Raines *et al*., 2011). Eight enzymes are directly involved in this process (Fig. 1.6) with different impacts in the regeneration rate (Raines *et al*., 2011). Analysis of antisense plants with reduced levels of each of the enzymes revealed sedoheptulose-7-biophosphatase (SBPase) as the biggest control point of the C3 cycle. Lower levels of SBPase are reported to be correlated to decreases in carbon fixation and plant growth (Raines *et al*., 2011) and overexpression of SBPase to improved photosynthetic rates and plant growth in tobacco (Lefebvre *et al*., 2005; Simkin *et al*., 2015). Improving RuBP regeneration could lead to a predicted 10% increase in net photosynthesis (Reynolds *et al*., 2009).

Successful application of the above mentioned approaches could improve photosynthesis and decrease photorespiration. Modifications in the photorespiratory cycle could result in decreased losses of carbon, nitrogen and energy and avoid accumulation of toxic compounds

(Peterhansel *et al*., 2013; Betti *et al*., 2016). Strategies of by-passing the photorespiration cycle are reported to improve plant growth in *Camelina sativa* (Dalal *et al*., 2015) and *Arabidopsis* (Kebeish *et al*, 2007). A transgenic approach of recycling glycolate in the chloroplast resulted in improved growth capacity in *Arabidopsis* (Maier *et al*., 2012).

1.6.3 Light reactions and Photoprotection

One of the biggest challenges in improving photosynthesis is to adapt the photosynthetic machinery to fluctuating light levels. Stomata and photosynthesis have different magnitudes of response to fluctuating lights leading to uncoupled patterns of CO_2 consumption/availability in these conditions (McAusland *et al*., 2016). Light availability can vary according to: time of day, season, geographic location, climatic conditions (as wind and cloud coverage) and canopy architecture (Murchie and Niyogi, 2011). At low light levels, photosynthesis is limited by light but, as light levels increase and the receptors reach their maximum capacity, light turns saturating to photosynthesis (Badger, 2013). Photoprotective mechanisms allow plants to dissipate the excess light and protect the photosynthetic machinery of photooxidative damage caused by photoinhibition (Long *et al*., 1994; Murchie and Niyogi, 2011). Photoprotection reactions cause a reduction in carbon fixation rates and maximum quantum yield of Photosystem II (Murchie and Niyogi, 2011). When light levels decrease, after a period of high light, plants will switch from the photoprotective to normal photosynthetic metabolism, but it can take from several minutes to hours to a complete adaptation. In conditions of fluctuating light, plants can last long periods with depressed photosynthesis causing possible yield losses of about 20% (Kromdijk *et al*., 2016). The use of a transgenic approach to speed up the recovery of photoprotection resulted in a 15% increase in plant biomass in tobacco growing under natural fluctuating light in the field. As the altered photoprotection pathway is similar in flowering plants, this approach has the potential to be expanded to food crops (Kromdijk *et al*., 2016).

1.7 THESIS OBJECTIVES

The main objective of this project was to improve the knowledge of wheat crop physiology and growth, with a special focus on energy conversion and carbon uptake through photosynthesis and its influence on productivity. It was based on two main approaches: exploiting the natural genetic variation of traits related to photosynthesis and yield (Chapter 2 and 3) or mimicking strategies of other species by transgene expression (Chapter 4). This included the studies of:

a) the influence of environmental pressures on wheat growth, production and resilience for 12 commercial cultivars over 3 consecutive seasons in the UK (Chapter 2);
b) the interaction between target traits for breeding for high yield and productivity in a wheat mapping population (Chapter 3);
c) the effect of the insertion of cyanobacterial *ictB* gene in wheat and its influence on carbon concentration, photosynthetic rates and yield (Chapter 4).

The achievements of this work are discussed in terms of the current and future possible contributions to the field of study and general research area (Chapter 5).

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CHAPTER 2

RESILIENCE TO SHORTENING OF GRAIN DEVELOPMENT STAGE LINKED TO WHEAT YIELD STABILITY IN THE UK

COLLABORATORS' CONTRIBUTIONS

The research presented in this chapter is an integrated analysis of three years of field experiments. The author was involved in the 2014 season experiment. The data from previous seasons was provided by the other researches, according to the references presented in the chapter. For the 2014 experiment, the author, Elizabete Carmo-Silva, John Andralojc and Luis Robledo-Arratia designed and performed the research. For the chapter, the author analysed the data and wrote the manuscript.

ABSTRACT

Ensuring food security in a changing climate is a major contemporary challenge that requires the development of climate resilient crops that perform well under variable environments, despite the occurrence of volatile climatic events and abiotic stress. Understanding the limitations imposed by different climatic conditions to crop development and yield could help the selection of genotypes with high yield resilience to non-ideal growth conditions. The phenotypic response - including developmental, photosynthetic and yield traits - to contrasting sowing date and rainfall, temperature and radiation patterns was studied for 12 commercial wheat cultivars, over three seasons, in the UK. Late sowing and lower rainfall reduced the grain development duration and impact in the yield response of the cultivars. Improving grain development rate and avoiding grain development shortening were the main strategies in achieving yield stability and were linked to the genetic similarities between the cultivars. These findings suggest that genetic variability for grain development duration and rate can be exploited to improve wheat yields.

2.1 INTRODUCTION

Arable crop research plays an important role in the context of sustainable and environmentally-friendly food production. It contributes to improve food security, not only by increasing crop yields but also by improving yield stability, despite sub-optimal growth conditions or geographic location (Maredia *et al*., 2000; Gregory and George, 2011). Extreme climatic events greatly influence crop development and yields (Schmidhuber and Tubiello, 2007). According to Ray *et al*. (2015), climatic variation can account for one third of crop yield variability. In wheat, high temperatures combined with limited water supply at critical growth stages are recognized as a major cause of yield loss (Ciais *et al*., 2005). Understanding the impact of climatic conditions to wheat is crucial for food security as the crop represents 20% of the calorific intake of the world's population (Braun *et al*., 2010).

As defined by Lobell *et al*. (2009), the yield gap is the difference between the yield potential and the actual on-farm yield for a given crop. Yield potential is the yield of a genotype when grown in environments to which it is adapted, without limitations of the recommended nutrients and water inputs and with biotic and abiotic stresses effectively controlled (Evans and Fischer, 1999). The actual yield is highly dependent on farm practice and environmental conditions, which together define how the genetic potential of a genotype is expressed. Components that influence the actual yield directly affect the yield gap (Lobell *et al*., 2009).

In wheat, breeders have significantly increased the yield potential over recent decades (Reynolds *et al*., 2009). The introduction of the dwarf genes technology, and its contribution to increase the harvest index, during the Green Revolution and the continued genetic selection for high yield genotypes in the post-Green Revolution period were the main drivers of wheat yield potential increases (Reynolds *et al*., 2009). Although further improvements in yield potential are still possible (van Ittersum and Cassman, 2013), research effort should be concentrated mutually in increasing yield potential and decreasing yield gap (Powell *et al*.,

2012). Understanding the response of different genotypes to environmental conditions and selecting for increased yield resilience under non-ideal growth conditions may help to improve the breeding for decreasing the yield gap (Fischer and Edmeades, 2010; Mueller and Binder, 2015).

Reynolds and Langridge (2016) highlight some important steps for the selection of genotypes for a specific phenotype in a breeding program: a) efficient phenotype evaluation in multiple environments; b) mapping of the influence of the genotype on the phenotype of interest; c) understanding the relative contributions of genetic and environmental factors to the phenotype of interest.

The evaluation of yield and yield resilience response of genotypes in multiple environments can be a complex process, mainly when the number of genotypes and environments increases. There are tools that can make this process more efficient. Productivity, resilience and stress indices can be reliable indicators of plant performance and plasticity (Thiry *et al*., 2016) and together with multivariate statistics and graphical analysis can facilitate the selection process (Yan and Tinker, 2005).

Understanding the link between genetic distances and phenotypic response of plants in different environments may help to predict their behaviour in specific climatic conditions (Letort *et al*., 2008; Brown *et al*., 2014). Genotypes that are genetically closer are expected to respond, phenotypically, more similarly when facing the same environmental conditions (Soriano *et al*., 2016). There are different methods for quantifying the genetic distances between genotypes and some of them may correlate better to specific traits (Crossa *et al*., 2010). A complex trait such as yield, for instance, is unlikely to be strongly correlated to the presence/absence of a single gene or the similarity of a specific section of a chromosome.

Quantitative genetics may address the challenge of understanding the relative contribution of genetics and environment in determining yield. In this context, heritability or repeatability

has been quantitatively defined to indicate how predictable a given phenotype is (Visscher *et al*., 2008) as it expresses how much of the observed trait variance is due to genetic factors, as opposed to environmental or stochastic variation (Minikel, 2013).

The influence of the environment on wheat development, physiological traits and yield components in the UK was investigated over three consecutive field seasons for 12 wheat commercial cultivars. The hypotheses tested were: a) that sowing date and rainfall, temperature and radiation patterns influence the crop development and yield patterns; b) that cultivars present different yield resilience to contrasting climatic conditions; c) that cultivars that are closer genetically tend to have a similar phenotype in each season; d) that broad-sense heritability can help to explain the grain yield variation and genotype x environment interaction and e) that the use of multivariate statistic methods and performance indices could help the cultivars' evaluation process.

2.2 MATERIAL AND METHODS

2.2.1 Plant material and field experiments

The ERYCC panel is composed of 64 wheat elite varieties, mainly from France and the UK, released between 1975 and 2008, selected according to their traits associated to adaptation to stress conditions, or, more specifically to their Earliness and Resilience for Yield in a Changing Climate (ERYCC) (Ober *et al*., 2013). This selection was part of a project involving the Agriculture and Horticulture Development Board (AHDB) and funded by a DEFRA (Department for Environment, Food & Rural Affairs) Sustainable Arable Link to characterize wheat cultivars for earliness and resilience traits and give breeders an indication of potential parents for further crosses (Clarke *et al*., 2012).

This wheat panel was grown at the Rothamsted Research farm, in Harpenden, UK, for three consecutive seasons, the first being harvested in 2012 and the last in 2014. Seeds for the first experiment were acquired from breeders involved in the ERYCC project and for the following

years were harvested from the previous experiment. Detailed information specific to each experiment is presented below (experiments identified by the year of harvest):

a) 2012: experiment planted at the Great Field 1&2, in a Typical Batcombe soil (Avery and Catt, 1995) after oilseed rape crop, in 2 x 1 m plots (2 m^2), with a sowing rate of 350 m^{-2}, organized in three randomized blocks; sown on 05/10/2011 and harvested on 17/08/2012 (Driever *et al*., 2014).

b) 2013: experiment planted at the Black Horse Field, in a Charity – Humble soil (Avery and Catt, 1995) after oat crop, in 3 x 1 m plots (3 m^2), with a sowing rate of 350 seeds m^{-2}, organized in three randomized blocks; sown on 12/12/2012 and harvested on 28/08/2013 (Carmo-Silva *et al*., submitted).

c) 2014: experiment planted at the Little Hoos Field, in a Typical Batcombe soil (Avery and Catt, 1995), after oilseed rape crop, in 9 x 1.8 m plots (16.2 m^2), with a sowing rate of 350 seeds m^{-2}, organized in three randomized blocks; sown on 15/11/2013 and harvested on 22/08/2014.

Application of fungicides, insecticides and herbicides, as well as fertilizers, was carried out according to Rothamsted farm practices in the three seasons.

For the analysis presented in this manuscript, a subset of 12 cultivars was chosen from the ERYCC panel, based on their contrasting behaviour for yield and gas-exchange traits (Table 2.1). Yield selection was based on cultivars' yield grouping according to the yield data for the 64 cultivars in 2008 (breeders test trials; Ober *et al*., 2013), in 2012 (Driever *et al*., 2014) and in 2013 (Carmo-Silva *et al*., submitted) seasons. Gas-exchange traits selection was based in photosynthesis at pre and post-anthesis in the 2013 season (Carmo-Silva *et al*., submitted). The cultivars presented in this analysis are: Avalon, Battalion, Brompton, Cadenza, Exsept, Gatsby, Gulliver, Humber, Oakley, Paragon, Savannah and Soissons. All the cultivars

presented in the subset were present in the wheat recommended list of the AHDB in the past, although, none of them is present on the 2017/2018 current list (AHDB, 2017).

Table 2.1 – Subset of cultivars of the ERYCC panel, their origin and growth habit and the selection criteria. Yield group is based on yield data for the ERYCC panel in three seasons (2008, 2012 and 2013) according to Carmo-Silva *et al*. (unpublished). Photosynthesis is based on pre and post-anthesis gas-exchange data for the ERYCC panel in 2013 season (Carmo-Silva *et al*., submitted).

Cultivar	Origin	Habit	Yield	Photosynthesis
Avalon	UK	Winter	Low	Low
Battalion	UK	Winter	High	Interm. (Similar pre and post-anthesis)
Brompton	UK	Winter	High	Interm. (Similar pre and post-anthesis)
Cadenza	UK	Facultative	Low	Intermediate
Exsept	Germany	Winter	Interm.	High
Gatsby	UK	Winter	High	High
Gulliver	UK	Winter	High	Low
Humber	UK	Winter	High	Interm. (Similar pre and post-anthesis)
Oakley	UK	Winter	High	Intermediate
Paragon	UK	Facultative	Low	High
Savannah	UK	Winter	High	High
Soissons	France	Winter	Interm.	High

2.2.2 Meteorological data

The meteorological data was acquired from the Rothamsted Meteorological Station at Rothamsted farm. The distance from the station to the experiments was, in a straight line: 100 meters for the 2012 experiment, 2.5 km for the 2013 experiment and 1.3 km for the 2014 experiment. The maximum and minimum daily temperature (°C), the daily rainfall (mm) and the radiation (MJ m^{-2}) were used. From this data, the accumulated rainfall and accumulated radiation for a specific period was calculated as the sum of the daily value from the first to the last day in the period considered. Average daily temperature (*Tmed*) was calculated as the mean maximum daily temperature and minimum daily temperature. Degrees day was calculated considering the base temperature (*Tbase*) for wheat crop as zero (McMaster and Smika, 1988) and by the equation system below:

$$Degreesday = \begin{cases} Tmed - Tbase, & \text{if } Tmed > Tbase \\ 0, & \text{if } Tmed \leq Tbase \end{cases} \quad \text{(Formula 2.1)}$$

The accumulated degrees day for a period of time was calculated as the sum of the degrees day from the first to the last day in the considered period.

2.2.3 Crop development monitoring and defining growth stages

The crop development was monitored across the season, using the Zadoks scale (Zadoks *et al*., 1974). The frequency of crop development monitoring depended on the crop stage and on the rate of change, being more frequent when crop development was faster and less frequent when crop development was slower. The crop cycle from sowing to harvest was divided into 5 stages: establishment (sowing to Z2.2), vegetative growth (Z2.2 to Z5.0), reproductive (Z5.0 to Z7.0), grain development (Z7.0 to Z9.0) and maturation (Z9.0 to harvest). Delay in sowing was calculated as the number of days between sowing and the limit date for early sowing of winter wheat in the UK; 15th of September (AHDB, 2011).

2.2.4 Gas-exchange measurements

For the three seasons, the response of net CO_2 assimilation (*A*) to the intercellular CO_2 concentration (*A* x C_i curve) was performed for flag leaves in plants at booting stage, Z4.3 to Z4.7 (Zadoks *et al*., 1974) using a portable infra-red gas analyser (IRGA) system (LI-COR 6400 and chamber 6400-40; LI-COR, Lincoln, USA). The methodology for harvesting plants and flag leaves is described by Driever *et al*. (2014). In the 2014 season, plants were harvested at post-dusk instead of pre-dawn. For the analysis presented in this manuscript two of the measured variables were considered: net photosynthesis at 400 µmol CO_2 µmol^{-1} air of ambient CO_2 (*A*(400)) and net photosynthesis at 1200 µmol CO_2 µmol^{-1} air of ambient CO_2 (*Amax*). Light intensities used for gas-exchange analysis were 1500 µmol of photons m^{-2} s^{-1} in 2012 (Driever *et al*., 2014) and 1800 µmol of photons m^{-2} s^{-1} in 2013 (Carmo-Silva *et al*., submitted) and 2014.

2.2.5 Yield measurement

The experiments were harvested using a Haldrup-C65 (Haldrup, Le Mans, France) plot combine. Grain and straw weights were measured by the combine and corrected to 100% of dry matter based on moisture of a subsample taken from the harvested plot, at harvest time. Harvest index was calculated by the ratio of grain to total above-ground biomass weight (grain + straw) at 100% dry matter. Linear mixed models were fitted to each year independently to evaluate a possible need to incorporate spatial heterogeneity into the covariance structure of the residuals.

Relative values of grain yield, biomass and harvest index were calculated for each cultivar by dividing the measured value for the cultivar by the average value of the whole ERYCC panel (64 cultivars) in the respective year. For instance, a relative grain yield value of 1 means that the cultivar had the same grain yield as the average of the ERYCC panel in that season.

2.2.6 Productivity, resilience and stress indices

The Productivity Capacity Index (PCI), Resilience Capacity Index (RCI) and Yield Stress Score Index (YSSI) were calculated based on the methodology proposed by Thiry *et al*. (2016) using the relative values for grain yield. The indices were calculated by comparing the 2013 and 2014 seasons relative to 2012. RCI (Resilience Capacity Index) was defined by the difference between grain yields in the two compared seasons (Formula 2.2). The yield difference was ranked from 1 to 10, with the biggest (most positive) value scored as 10 and the smallest (most negative) as 1. The intermediate values were allocated in the range between 1 and 10, according to their magnitude. PCI (Production Capacity Index) was defined by the average yield of each cultivar in the two compared seasons (Formula 2.3). The allocation of scores, from 1 to 10, was similar to RCI with the biggest average being scored as 10, the smallest as 1 and the others in between them. YSSI (Yield Stress Score Index) was calculated as the average of RCI and PCI, representing an index that integrates resilience and productivity in

multiple environments (Formula 2.4). Although the indices were designed to be used in a stress vs non-stress set of experiments, they usefully compared grain yields in different environments. The following examples explain the calculation of the indexes for the comparison between 2013 and 2012, where $Yield_{season}$ is the relative grain yield for each season:

$RCI_{2013\text{-}2012} = Yield_{2013} - Yield_{2012}$ (Formula 2.2)

$PCI_{2013\text{-}2012} = (Yield_{2013} + Yield_{2012})/2$ (Formula 2.3)

$YSSI_{2013\text{-}2012} = (RCI_{2013\text{-}2012} + PCI_{2013\text{-}2012})/2$ (Formula 2.4)

2.2.7 Genotyping and genetic similarities

Three genetic analyses were carried out for the ERYCC panel: one based on the cultivar's pedigree, one on genetic markers and one in the presence/absence of genes related to grain yield. For the pedigree analysis, the genealogic tree of the cultivars was built based on information from the breeders and from the wheat pedigree database website (wheatpedigree.net). The parental crosses that generated each cultivar were mapped as far as the available information permitted. From the pedigree data, the similarity between the cultivars was calculated using the package Kinship2 on R software version 2.6.1.

For the genetic markers analysis, the Axiom® Wheat Breeder's Array was used to genotype the cultivars using the Affymetrix GeneTitan® system, according to the procedure described by Affymetrix (Axiom® 2.0 Assay Manual Workflow User Guide Rev3). Allele calling was carried out using the Affymetrix proprietary software package Affymetrix Analysis Suite. The genetic distance (GenDist) for pairs of cultivars was calculated according to Gao *et al*. (2005). From the genetic distance matrix, a similarity matrix was calculated by:

$$Similarity_{ab} = 1 - GenDist_{ab} \quad \text{(Formula 2.5)}$$

where *a* and *b* are the two cultivars for which the similarity is being measured.

Clarke *et al*., (2012) have mapped the 64 cultivars of the ERYCC panel for the presence of a list of genes and the 1RS rye introgressions related to yield improvement in wheat (Table 2.2). Some cultivars from the ERYCC panel were also modified for introgressions from *Triticum turgidum spp. dicoccoides* related to drought resistance. Information for the emmer introgressions was obtained from RAGT Seeds, UK (Dr Peter Jack, RAGT Seeds, personal communication, August 2016). For the gene presence analysis, a similarity matrix was generated based on the presence/absence or gene copy number of genes and introgressions (Table 2.3).

Table 2.2 – Genes/introgressions and the linked phenotype for wheat plants

Gene/ Introgressions	Phenotype
Ppd1	Photoperiod insensitivity
Rht1	Reduced height
Rht2	Reduced height
Lr37	Resistance to Leaf Rust
Pch1	Resistance to Eyespot Disease
Sm1	Resistance to Orange Wheat Blossom Midge
Vrn-1	Vernalisation insensitivity
1RS (Secale cereale)	Disease resistant genes
T. turgidum spp. dicoccoides	Drought stress resistance

For all three types of genetic analyses, a hierarchical cluster analysis based on group average was carried out for the similarity matrixes, using GenStat 17th Edition (VSN International Ltd., Hemel Hampstead, UK). The similarity to Humber was used for the principal component analysis, given the higher values estimated for this cultivar for the YSSI index in 2013 and 2014 compared to 2012.

Table 2.3 – Gene presence/absence and introgressions for a subset of 12 genotypes of the ERYCC Panel. 1 and 0 mean the presence or absence of the gene/introgressions, respectively. For *Vrn-1*, numbers 1, 2 and 3 are the gene copy numbers (there is no information for Humber) and r and d means recessive and dominant, respectively. Data in this table was obtained from Clarke *et al*. (2012), the wheatpedrigree.net website and from Peter Jack, from RAGT Seeds UK.

Cultivar	Genes						Introgressions	
	Ppd1	*Rht1/Rht2*	*Lr37*	*Pch1*	*Sm1*	*Vrn-1*	Rye (1RS)	Emmer
Avalon	0	0	1	0	0	0	2 (r)	0	0
Battalion	0	0	1	1	1	0	2 (r)	0	0
Brompton	0	0	1	0	0	1	2 (r)	1	0
Cadenza	0	0	0	0	0	0	1 (d)	0	1
Exsept	0	0	1	0	0	1	3 (r)	0	0
Gatsby	0	1	0	0	0	1	3 (r)	1	0
Gulliver	0	0	1	1	0	0	3 (r)	0	1
Humber	0	0	1	1	0	0	-	1	0
Oakley	0	1	0	0	0	1	2 (r)	0	1
Paragon	0	0	0	0	0	1	1 (d)	0	0
Savannah	0	0	1	1	0	0	2 (r)	1	1
Soissons	1	1	0	0	0	0	2 (r)	0	0

2.2.8 Heritability

Broad-sense heritability (H^2) was calculated for grain yield in each season using the procedure described by Cullis *et al*. (2006), based on the ratio of the between cultivar variance component and the mean variance of the difference between two cultivar means, as estimated by best linear unbiased predictors (BLUPs). The GenStat 17th Edition software (VSN International Ltd., Hemel Hampstead, UK) was used.

2.2.9 GGE biplots

Genotype, genotype x environment biplots (GGE biplots) for grain yield were generated using GenStat 17th Edition (VSN International Ltd., Hemel Hampstead, UK). The GGE biplot of a trait consists of a graphical representation of the analysed genotypes in multiple environments, based on the use of multivariate principal component analysis. It allows an integrated analysis of the influence of different environments in the response of multiple

genotypes for a specific trait. Yan and Tinker (2005) described the methods to generate and interpret GGE biplots.

2.2.10 Statistical analysis

Principal component analyses were carried out for the integrated data for the three years. The data used for the analysis were: relative grain yield, biomass and harvest index; *A*(400) and *Amax*; accumulated degrees day, rainfall and radiation for each specific developmental stage and the full season; delay in sowing and the genetic similarity to Humber based on three different genetic analyses (pedigree, SNPs markers and presence/absence of a set of genes).

The Pearson Product Moment (PPM) coefficients were used to compare the correlations between traits. The analyses were performed using GenStat 17th Edition (VSN International Ltd., Hemel Hampstead, UK).

2.3 RESULTS

2.3.1 The 2013 and 2014 seasons had lower rainfall, mainly at reproductive and grain development stages

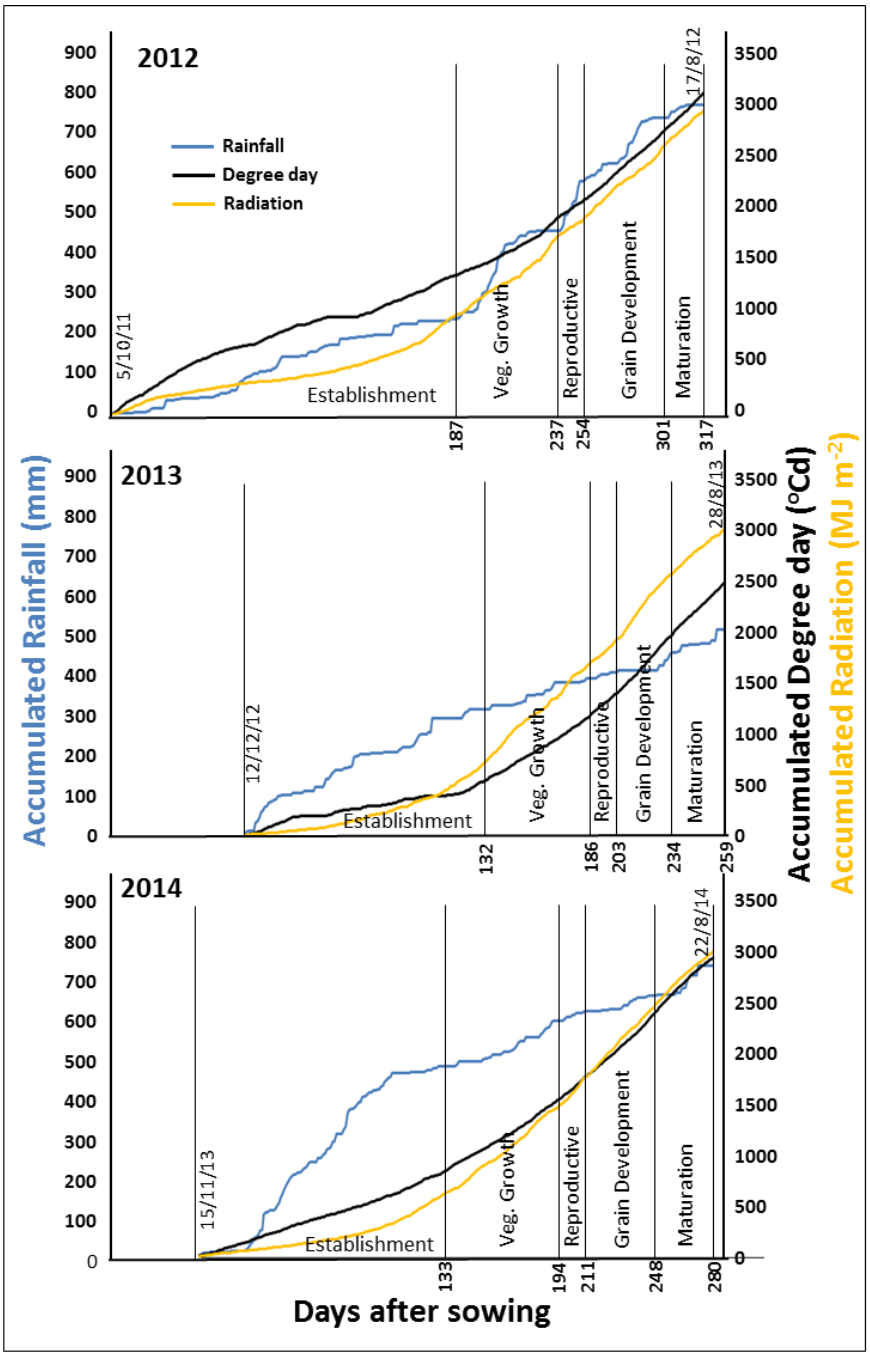

Figure 2.1 – Meteorological and developmental data for the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). The dates at the left and right hand side of the graphs correspond to the sowing and harvesting dates, respectively. The left *y* axis in each graph starts at the day of the earliest sowing date between the 3 years (5th of October) and the right *y* axis finishes at the day of the latest harvesting date between the 3 years (28th of August). Growth stages defined according to Zadoks scale: Establishment (sowing to Z2.2), Vegetative growth (Z2.2 to Z5.0), Reproductive (Z5.0 to Z7.0), Grain development (Z7.0 to Z9.0) and Maturation (Z9.0 to harvest)

The 3 seasons were characterised by different meteorological conditions that reflected in the accumulation of degrees day, rainfall and radiation (Fig. 2.1 and Table 2.4). Solar radiation, temperature and water availability are important factors that define plant growth and metabolism and can be limiting to wheat productivity if they are non-ideal in crucial stages of vegetative growth, reproductive and grain development. The 2012 season presented the highest rainfall accumulation over the season (768 mm) and in specific stages of vegetative growth (201 mm), reproductive (133 mm) and grain development (169 mm). The 2013 season presented the lowest rainfall accumulation over the season (506 mm) and in the specific stages of vegetative growth (75 mm), reproductive (19 mm) and grain development (48 mm). For the 2014 season the total rainfall accumulated over the season was closer to the 2012 season (739 mm) but unevenly distributed with a bigger concentration at the establishment stage. The rainfall accumulated at vegetative growth (115 mm), reproductive (26 mm) and grain development (40 mm) was much lower than the 2012 season and closer to the 2013 patterns (Fig. 2.1 and Table 2.4).

The total radiation accumulated was similar for the 3 seasons, although, 2014 presented higher levels at reproductive and grain development stages. In terms of temperature, the accumulated degrees day over the season was much lower in 2013 (2411 °C day) than in 2012 (3114 °C day) and 2014 (2948 °C day), with the same pattern observed at grain development stage (Table 2.4).

In summary, the 2012 season had a higher accumulation of rainfall and degrees day in an even distribution over the season. It was likely that the 2013 season had lower soil water availability, mainly in the vegetative growth, reproductive and grain development stages. Moreover, at the grain development stage it was likely that the crop faced the combined effect of lower rainfall and high temperature. Although the accumulated degrees day was lower in 2013 for this stage (Table 2.4), it was due to the shortening of the grain

development stage duration. The average daily temperatures during grain development in 2013 were around 3°C higher than in the other two seasons (data not shown). It was also likely that the 2014 season had lower soil water availability, mainly at reproductive and grain development stages. The biggest amount of accumulated rainfall during the final days of vegetative growth could have kept soil moisture at reproductive and grain development stages higher than in 2013 (Fig. 2.1).

Table 2.4 - Accumulated rainfall, degrees day and radiation at each stage of development of UK field-grown wheat over three consecutive seasons (2012, 2013 and 2014) for the ERYCC Panel. Growth stages defined according to Zadoks scale: Establishment (sowing to Z2.2), Vegetative growth (Z2.2 to Z5.0), Reproductive (Z5.0 to Z7.0), Grain development (Z7.0 to Z9.0) and Maturation (Z9.0 to harvest).

	Days	Acc Rainfall (mm)	Acc Degrees day (°C day)	Acc Radiation (MJ m-2)
			2012	
Establishment	187	255	1379	986
Vegetative growth	50	201	502	717
Reproductive	17	133	212	223
Grain Development	47	169	756	780
Maturation	15	11	265	231
Season	317	768	3114	2938
			2013	
Establishment	132	311	546	733
Vegetative growth	54	75	589	903
Reproductive	17	19	254	258
Grain Development	31	48	596	659
Maturation	26	53	426	371
Season	259	506	2411	2924
			2014	
Establishment	133	483	822	600
Vegetative growth	61	115	695	849
Reproductive	17	26	257	321
Grain Development	37	40	622	692
Maturation	32	76	552	549
Season	280	739	2948	3011

2.3.2 Late sowing combined to reduced water availability have shortened the grain development stage in 2013 and 2014

In all the seasons, the crop was sown later than recommend for winter wheat crops (15th of September for winter wheat in the UK; AHDB, 2011). Late sowing can decrease the early growth of plants influencing their capacity to face low winter temperatures and delay plant

development. It can also influence the vernalization of winter wheat cultivars and flowering time. The latest sowing between the 3 seasons was recorded in 2013, with the sowing being 88 days late (on the 12th of December of 2012), followed by the 2014 season with 61 days (on the 15th of November of 2013) and 2012 with 20 days (5th of October 2011). The combination of late sowing and reduced rainfall in 2013 shortened the growing season (259 days), if compared to 2012 (317 days) and 2014 (280 days). It had a particular shortening effect at grain development stage, with 31 days in 2013, 37 in 2014 and 47 in 2012 (Fig. 2.1 and Table 2.4). Delay in sowing was negatively correlated to grain development duration ($r = - 0.92$, $p < 0.001$) in the integrated analysis for the 3 seasons.

2.3.3 Duration of developmental stages and meteorological conditions altered plant performance and grain yield patterns over the three seasons

The late sowing and meteorological conditions influenced the duration of growth stages and reflected in plant performance and grain yield patterns over the 3 seasons. The average yield of the ERYCC panel reached the highest value in 2012, the lowest in 2013 and intermediate in 2014 (data not shown). Multivariate analysis of principal components was used to integrate the data available for each season (Fig. 2.2). In terms of the cultivars grouping, 2013 presented a clear division between cultivars in two groups, suggesting a greater influence of the environment, possibly as a consequence of the previously mentioned reduced water availability and the reduced duration of some developmental stages (Table 2.4). Theoretically, if there was no environmental influence, the cultivars should group according to their genotypic similarities.

Specifically for the analysis of the 12 cultivars over the 3 seasons, grain yield and the duration of the grain development stage presented a positive correlation to PC1, inferring that longer grain development stages are positively linked to grain yield (Fig. 2.2). The duration of the grain development stage ($r = 0.44$, $p < 0.01$), as well as the accumulated radiation ($r = 0.56$, $p < 0.001$), rainfall ($r = 0.34$, $p < 0.05$) and degrees day ($r = 0.56$, $p <$

0.001), were correlated to yield. The shortening of the grain development stage in 2013 and 2014 influenced the way the cultivars yielded (Fig. 2.3).

The delay in sowing was in opposition to PC1 and to grain yield, meaning that early sowing favoured grain yields. This also explains the positive correlation of grain yield and duration of the establishment stage that was particularly longer in 2012 when the plants were sown earlier (Fig. 2.2).

2.3.4 The grain yield patterns of the 12 cultivars were more similar in 2013 and 2014 seasons in contrast to 2012

The GGE biplots were used as an integrative method of grain yield analysis for the 3 seasons (Fig. 2.5). In the biplots, each cultivar is represented by a score, an *x,y* coordinate in the Cartesian system, which represents the cultivar global relative grain yield. Seasons are represented by vectors connecting the graph origin to the season score. This vector defines an axis in which grain yield can be ranked to the specific season. The position of the cultivars in the axis represents its relative grain yield in the specific season. The dashed lines, orthogonal to the axis, can be used as a comparative scale, although it has no statistical meaning (Fig. 2.5). Cultivars that are placed near the origin tend to be more stable to yield variation as its relative position does not change drastically as the axis for each season is defined. However, they are unlikely to reach the highest yield values for a specific season.

A second approach was used to evaluate grain yield variation over the seasons, based on resilience (RCI), productivity (PCI) and resilience and productivity (YSSI) indices (Table 2.5).

In general terms, grain yield patterns in the 2013 and 2014 seasons were more similar and contrasting to 2012, which is evidenced by their distribution in the GGE biplot (Fig. 2.5). This can be a response to the shortening of the growth stages and reduced rainfall during 2013 and 2014. Lower soil water availability might also be the cause of the biggest variability of grain yield observed in 2013, that can be noted by the bigger range of values of relative grain yield and their error bars (2013 graph in Fig. 2.3) and also by the weak grouping of the

cultivars in the principal component analysis (red dots in Fig. 2.2) and the more extreme values for YSSI (Table 2.5). It is also stated by the lower value of grain yield broad-sense heritability for 2013 (H^2 = 0.74) in comparison to 2012 (H^2 = 0.86) and 2014 (H^2 = 0.82) which implies a bigger environment effect to grain yield. Lower heritability is related to a smaller effect of the genetic background and a lower predictability of the phenotype.

For the cultivars' photosynthetic behaviour at pre-anthesis, the 2012 and 2014 patterns were different from the 2013 pattern (Fig. 2.4). In 2013, Soissons and Savannah had higher photosynthetic rates than the majority of the other cultivars, which could be linked to their higher relative grain yield for this season (Fig 2.3 and Fig. 2.4).

2.3.5 Humber presented higher yield resilience to the conditions faced in 2013 and 2014 seasons

For the 12 cultivars analysis, when compared to the ERYCC panel grain yield variation, some cultivars maintained or improved their relative grain yield in the climatic conditions of 2013 and 2014, while others presented a decrease in their relative grain yield (Fig. 2.3).

Gatsby had the highest relative yield in 2012 but presented low yield stability at the 2013 and 2014 seasons' conditions (Fig. 2.3). The score for Gatsby in the GGE biplot, towards 2012 and far from 2013 and 2014 (Fig. 2.5) and its low values for PCI (Table 2.5 (1) and (2)) reinforced its low yield stability.

Avalon, Cadenza and Paragon, presented low resilience to the water limited conditions of the 2013 season. Although their grain yields were always lower than the ERYCC panel average, in the 2013 season they clearly presented a lower yield than the average and the others (Fig. 2.3). This behaviour is reinforced by their position in the GGE biplots, opposite the seasons' scores (Fig. 2.5) and the low values of RCI, PCI and YSSI for the 2013 season (Table 2.5 (1)).

Exsept showed stable yield over the seasons. It had relative grain yield always near the ERYCC panel average (Fig. 2.3), was positioned near the origin in the GGE biplot (Fig. 2.5) and presented intermediate values for RCI, PCI and YSSI (Table 2.5 (1) and (2)).

Humber presented the highest resilience to the conditions faced during 2013 and 2014. Its relative grain yield was near the average of the ERYCC panel in 2012, but was the highest for 2013 and 2014 (Fig. 2.3). It is also stated by its position in the GGE biplots, towards the 2013 and 2014 scores (Fig. 2.5) and the highest values for RCI, PCI and YSSI (Table 2.5 (1) and (2)). Soissons had a similar behaviour of yield resilience to Humber in the 2013 season but not in 2014.

Savannah, Brompton, Battalion and Gulliver were never the highest yielding cultivars between the 12, but were always in the intermediate range (Fig. 2.3) which is reinforced by their central positions in the GGE biplots (Fig. 2.5). They always presented high values for the PCI, but Battalion and Gulliver (in 2014) and Brompton (in 2013) presented a low RCI (Table 2.5 (1) and (2)). Savannah and Brompton were always above the ERYCC panel average in the 3 seasons.

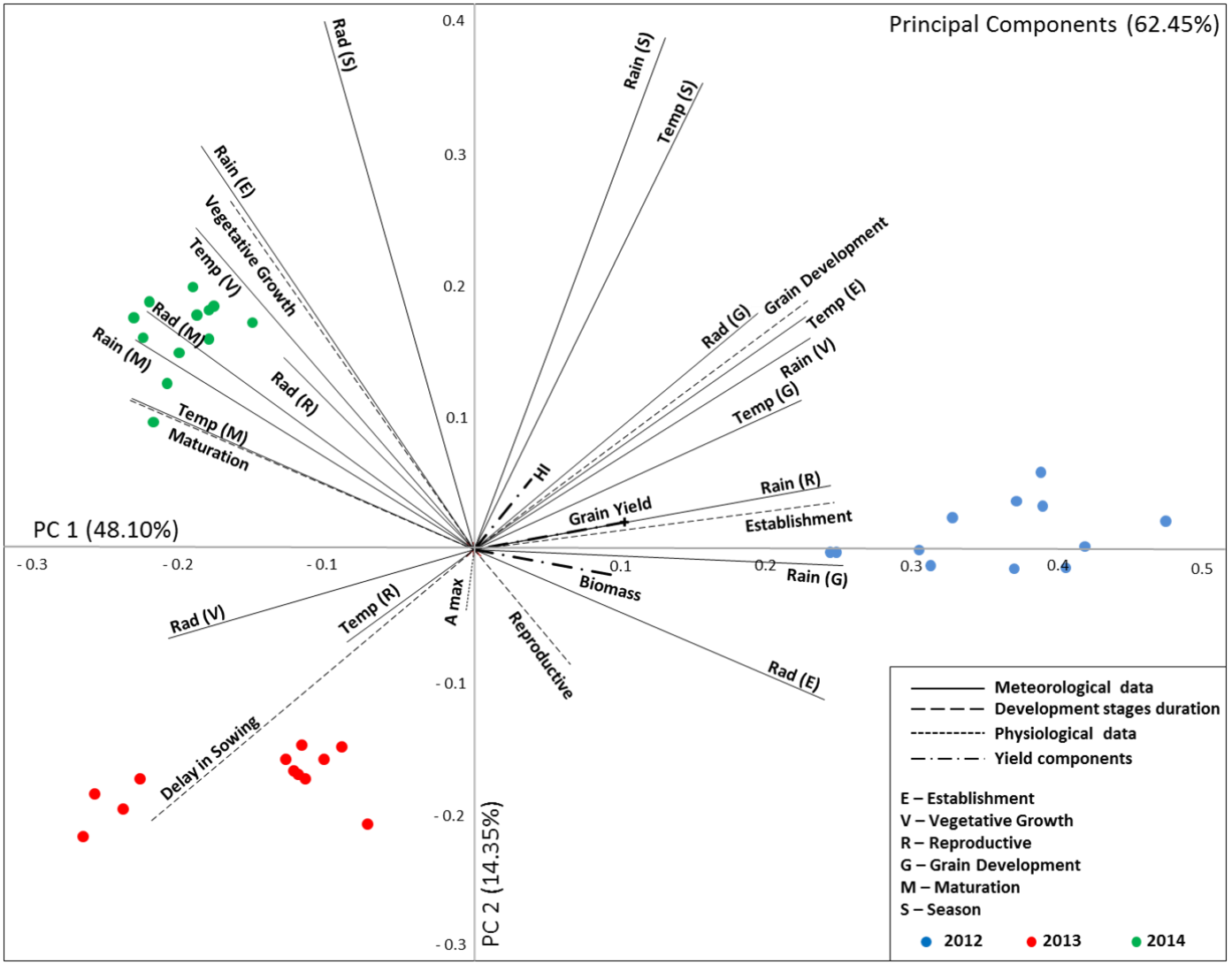

Figure 2.2 – Principal Component analysis for the integrated data for a subset of 12 cultivars of the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). PC1 and PC2 are the two first principal components. Temp, Rain and Rad (Stage) are respectively the degrees day, rainfall and radiation accumulated during a specific developmental stage or the full season. *A* (400) is the net photosynthesis at ambient CO_2 of 400 µmol CO_2 µmol^{-1} air and *Amax* is the net photosynthesis at ambient CO_2 of 1200 µmol CO_2 µmol^{-1} air and high light conditions. Delay in sowing is the number of days after the ideal sowing data for winter wheat in UK (15th of September) that the experiment was sown. Grain yield, Biomass and HI are, respectively, grain yield, biomass and harvest index relative based on the average values for the ERYCC panel in each year. Growth stages defined according to Zadoks scale: Establishment (sowing to Z2.2), Vegetative growth (Z2.2 to Z5.0), Reproductive (Z5.0 to Z7.0), Grain development (Z7.0 to Z9.0) and Maturation (Z9.0 to harvest).

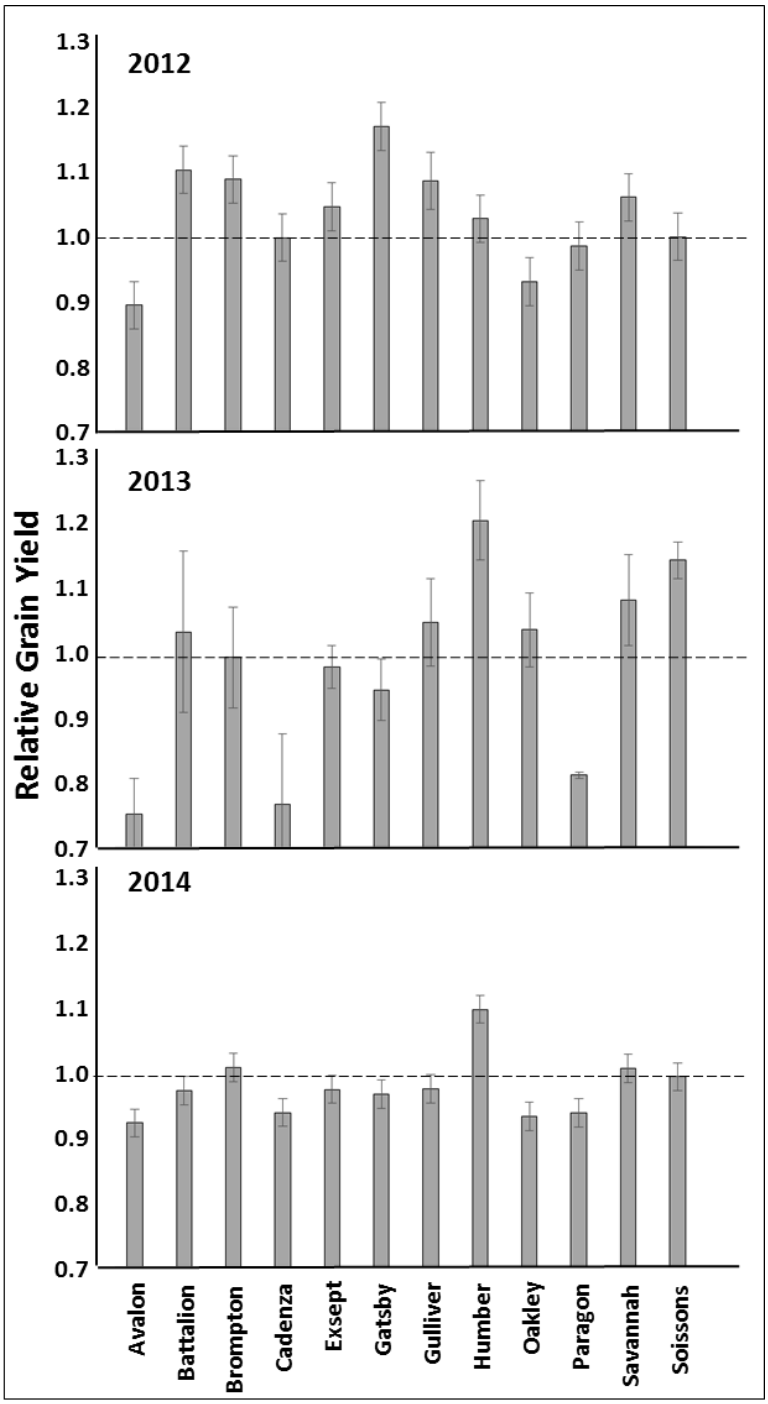

Figure 2.3 – Relative grain yield for a subset of 12 wheat cultivars of the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). Relative grain yield was calculated for each cultivar by dividing the grain yield of the cultivar (t ha^{-1}) by the average grain yield (t ha^{-1}) of the whole ERYCC panel (64 cultivars) in the respective year. ERYCC Panel average yield for each year was 12.3 t ha^{-1} for 2012, 7.9 t ha^{-1} for 2013 and 11.6 t ha^{-1} for 2012. The dashed line represents the average of the ERYCC panel for the respective year. Error bars are the standard error of the mean.

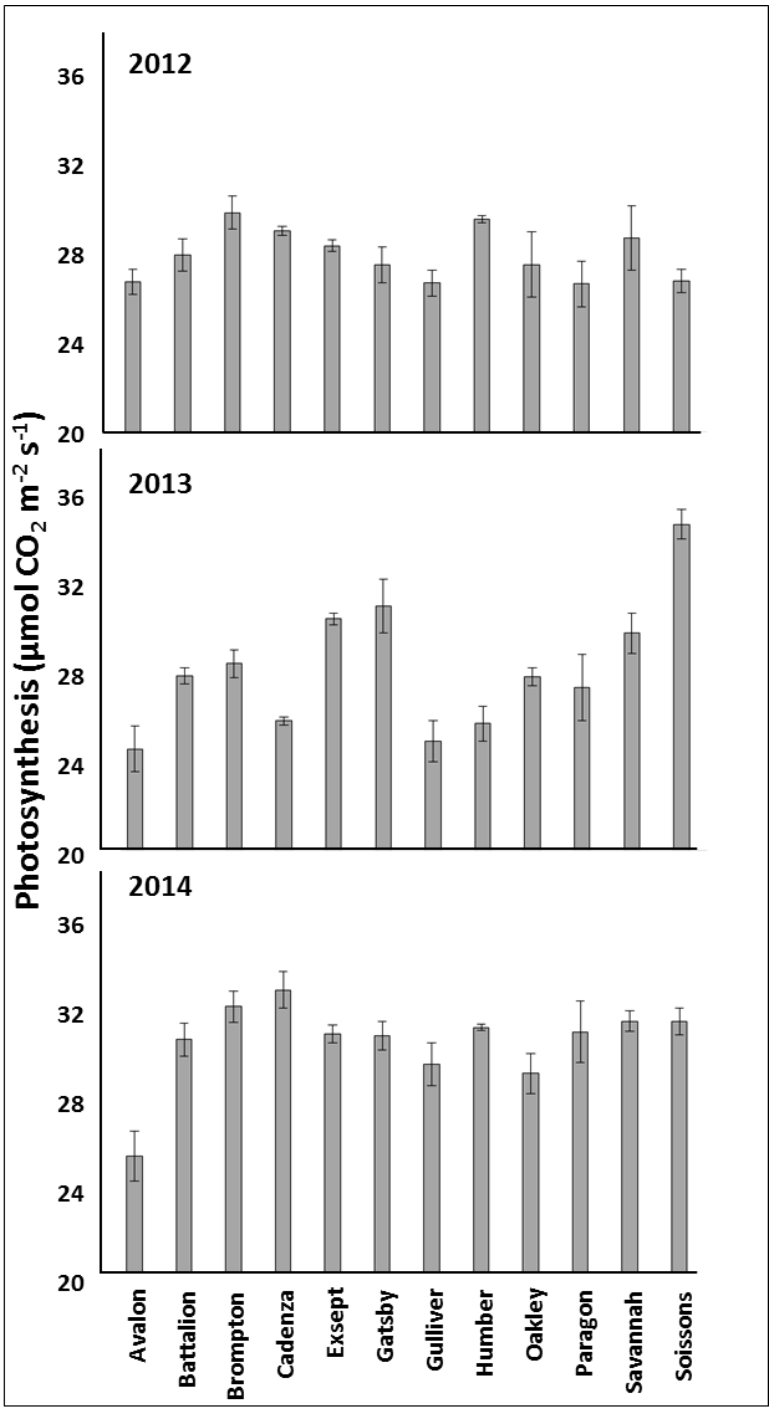

Figure 2.4 – Photosynthesis at pre-anthesis for a subset of 12 wheat cultivars of the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). Net photosynthesis (*A*400) at booting (Z4.5) measured at 400 µmol CO_2 µmol^{-1} air of ambient CO_2. Light intensities used for gas-exchange analysis were 1500 µmol of photons m^{-2} s^{-1} in 2012 (Driever *et al*., 2014) and 1800 µmol of photons m^{-2} s^{-1} in 2013 (Carmo-Silva *et al*., submitted) and 2014. Error bars are the standard error of the mean.

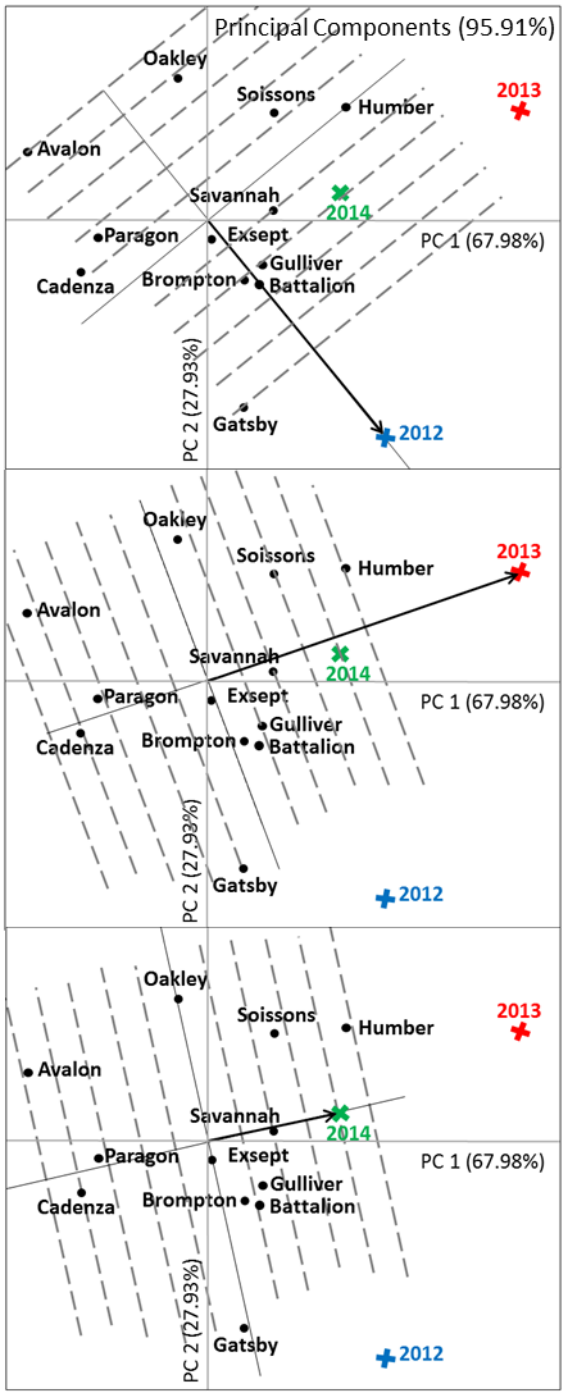

Figure 2.5 – Genotype, genotype x environment (GGE) biplot for grain yield for a subset of 12 cultivars of the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). PC1 and PC2 are the two first principal components. The arrow defines the main axis for each of the seasons. Dashed parallel lines are orthogonal to the axis and can be used to compare the grain yield scores for each cultivar in each season. As higher the cultivar is in the direction of the arrow, higher is its score.

Table 2.5 - Relative wheat grain yield comparison between the seasons of 2012 and 2013, or 2012 and 2014 for 12 wheat cultivars grown in UK. Relative grain yield was calculated for each cultivar by dividing the grain yield of the cultivar (t ha^{-1}) by the average grain yield (t ha^{-1}) of the whole ERYCC panel (64 cultivars) in the respective year. Red arrows down mean values in the fourth (lower) quartile; yellow arrows mean values in the second (up) and third (down) quartiles; green arrows up mean values in the first (higher) quartile. RCI, Resilience Capacity Index; PCI, Production Capacity Index; YSSI, Yield Stress Score Index. Cell colours vary from dark red (1) to dark green (10) with dark yellow as intermediate (5).

2012 vs 2013 (1)					
Cultivar	Relative Yield 2012	Relative Yield 2013	RCI	PCI	YSSI
Avalon	0.901	0.752	2	1	1.5
Battalion	1.117	1.045	5	9	7
Brompton	1.102	1.004	4	8	6
Cadenza	1.009	0.768	1	2	1.5
Exsept	1.058	0.989	5	7	6
Gatsby	1.186	0.952	1	8	4.5
Gulliver	1.100	1.060	5	9	7
Humber	1.039	1.224	10	10	10
Oakley	0.938	1.048	9	6	7.5
Paragon	0.996	0.815	2	3	2.5
Savannah	1.073	1.096	7	9	8
Soissons	1.010	1.160	10	9	9.5

2012 vs 2014 (2)					
Cultivar	Relative Yield 2012	Relative Yield 2014	RCI	PCI	YSSI
Avalon	0.901	0.930	9	1	5
Battalion	1.117	0.982	3	9	6
Brompton	1.102	1.019	5	9	7
Cadenza	1.009	0.947	5	4	4.5
Exsept	1.058	0.984	5	7	6
Gatsby	1.186	0.976	1	10	5.5
Gulliver	1.100	0.985	3	8	5.5
Humber	1.039	1.112	10	10	10
Oakley	0.938	0.941	8	2	5
Paragon	0.996	0.946	6	4	5
Savannah	1.073	1.017	6	8	7
Soissons	1.010	1.004	7	6	6.5

2.3.6 Yield resilience can be related to the cultivar's resilience to the shortening of the grain development stage

The yield resilience can be related to the different capacity of the cultivars to produce at shortened grain development stages. The reduction of the grain development stage was similar from 2012 to 2013 for Gatsby (16 days – 49 days of grain development in 2012 to 33 days in 2013) and Humber (17 days – 50 days of grain development in 2012 to 33 days in 2013). However, Gatsby produced around 18% more than the average in 2012 and below the average in 2013, while Humber produced near the average in 2012 and around 22% more in 2013 (Fig. 2.3 and Table 2.6). The same pattern is observed when comparing 2012 to 2014 with the decrease in the grain development being similar for the two, but with Gatsby (13 days shorter in grain development) yielding below the average and Humber (12 days shorter in grain development) yielding around 11% more than the average (Fig. 2.3 and Table 2.6).

Avalon, Cadenza and Paragon, the 3 cultivars with lowest yield in 2013, presented lower yield resilience between 2012 and 2013 when compared to cultivars that had the same shortening in the grain development stage, as Exsept, Savannah and Brompton, respectively (Fig. 2.3 and Table 2.6). The 3 cultivars (Avalon, Cadenza and Paragon) were classified as low yielding cultivars (Carmo Silva *et al.*, submitted) which reinforces the fact that low yield resilience can be linked to lower resilience to grain development shortening.

Soissons responded in contrasting ways to similar decreases in the grain development stage (10 days from 2012 to 2013 and 9 days from 2012 to 2014). In 2013 it yielded around 16% more than the average and in 2014 it was around the average (Fig. 2.3 and Table 2.6).

Table 2.6 – Shortening of grain development stage and the relative yield difference between the seasons of 2012 and 2013, or 2012 and 2014 for 12 wheat cultivars grown in UK. The relative yield difference is the percentage of change in relative yield from one season to the other for each cultivar when compared to the ERYCC panel average. For instance, Avalon had a relative yield of 0.90 in 2012 and 0.75 in 2013 which gives a reduction of 15%, shown in the table.

Cultivar	Grain development shortening (days)		Relative yield difference (%)	
	2012 to 2013	2012 to 2014	2012 to 2013	2012 to 2014
Avalon	14	3	-14.9	2.9
Battalion	19	7	-7.3	-13.5
Brompton	21	16	-9.8	-8.3
Cadenza	16	6	-24.0	-6.2
Exsept	13	9	-6.9	-7.4
Gatsby	16	13	-23.5	-21.0
Gulliver	17	14	-3.9	-11.4
Humber	17	12	18.4	7.2
Oakley	11	9	11.0	0.2
Paragon	22	13	-18.1	-4.9
Savannah	16	12	2.3	-5.6
Soissons	10	9	15.0	-0.7

2.3.7 Flowering time, grain yield and harvest index were influenced by the presence of specific genes

For the analysis of 64 cultivars of the ERYCC panel, the presence or absence of specific genes was correlated to plant behaviour. The presence of the *Ppd1* gene, related to photoperiod insensitivity and control of flowering time, was correlated to an extended reproductive stage in 2013 ($r = 0.74$, $p < 0.001$) and 2014 ($r = 0.67$, $p < 0.001$). Extended reproductive stage was achieved by early expansion of the spikes and not by a late start of grain filling, as the presence of the *Ppd1* gene was also negatively correlated to the duration of the vegetative growth stage in 2013 ($r = - 0.75$, $p < 0.001$) and 2014 ($r = - 0.74$, $p < 0.001$) but not to the grain development stage duration. In the conditions of reduced water availability in 2013 and 2014, plants with the *Ppd1* gene presence might have respond to other signals than the photoperiod and flowered early, as a strategy to shorten the crop cycle under non-ideal conditions.

The presence of the *Lr37* gene, related to leaf rust resistance, was correlated to grain yield in 2013 ($r = 0.32$, $p < 0.05$) and 2014 ($r = 0.26$, $p < 0.05$) and to harvest index in the 3 seasons

2012; r = 0.32, p < 0.05 / 2013; r = 0.45, p < 0.001 / 2014; r = 0.25, p = 0.051). Although signs of leaf rust were not seen at early stages in any of the seasons, the possible effects of the disease at late stages, combined with lower rainfall, could have increased senesce rate and limited grain yield by decreasing both the flag leaf photosynthesis and the biomass allocation to the grain mainly in 2013 and 2014.

No effects of the copy number of *Vrn-1* gene, related to the control of flowering time due to the vernalization process, were observed. A different behaviour of Cadenza and Paragon (facultative habit, with one copy of *Vrn-1* and dominant) to the other cultivars would be expected but was not observed. Clarke *et al*. (2012) showed that the vernalization requirement for the cultivars in the panel varied from 4 to 8 weeks of temperatures below 10°C. It suggests that, even on the years of late sowing, the length and low temperature levels that the crop faced during the winter were enough to the vernalization to be successful.

2.3.8 Genetic similarities to Humber are correlated to higher grain yield in 2013 and 2014

The different methods used to measure the genetic similarity between the cultivars agreed with each other. The pedigree and markers similarity were the most strongly correlated (r = 0.84, p < 0.001), followed by the genes and markers (r = 0.72, p < 0.01) and genes and pedigree (r = 0.57, p = 0.053). The similarities were calculated in reference to Humber due to its outstanding relative grain yield in 2013 and 2014. The similarity to Humber based on the SNPs markers was correlated to grain yield in 2013 (r = 0.63, p < 0.05) and 2014 (r = 0.84, p < 0.01). The similarities to Humber based on the pedigree (r = 0.81, p < 0.05) and on the gene presence (r = 0.64, p < 0.05) were also correlated to grain yield in 2014.

Savannah presented a high similarity to Humber for the SNPs and gene presence analyses (Fig. 2.6). This similar genetic background apparently influenced the behaviour of the cultivars over the three seasons as they presented a closer yield pattern (Fig. 2.3) and were

always placed in the same quadrant on the principal component analysis for each season (data not shown). In terms of the gene presence analysis, although some of the genes (*Pch1, Sm1, Vrn-1*) and introgressions (from rye and emmer) did not show a significant single effect in plant performance, an accumulated effect of their presence/absence could be leading these two cultivars to a closer behaviour. On the other hand, Soissons, which was always standing alone in the principal components analysis for each season (data not show), had low genetic similarity to the group (Fig. 2.6). This might be explained by its different origin (France) and breeding focus, as the wheat growth conditions in the UK and France can be contrasting.

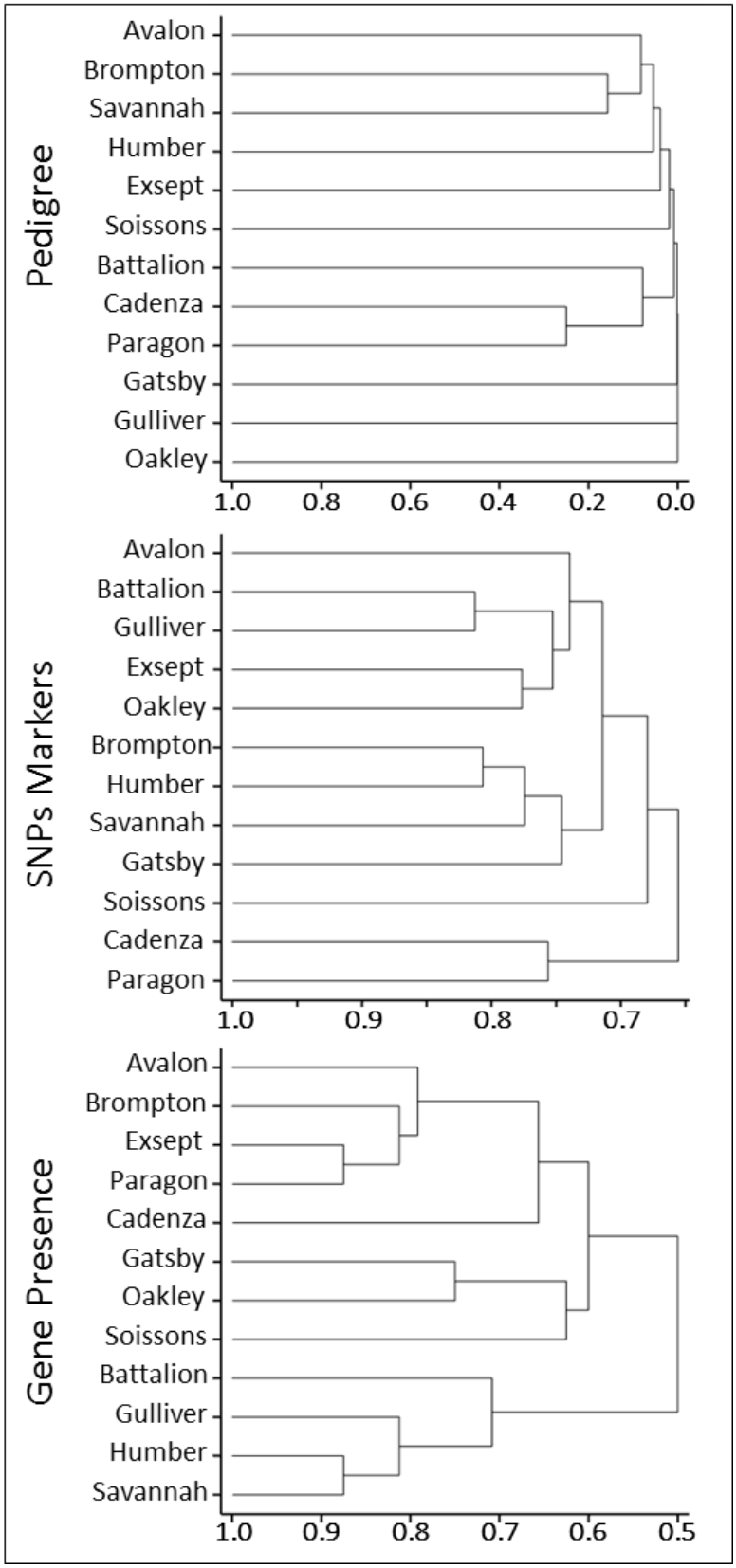

Figure 2.6 – Hierarchical Cluster analysis based on group average for similarity matrixes of pedigree, SNPs genetic markers and gene presence/absence for 12 cultivars of the ERYCC Panel grown in the UK over three consecutive seasons (2012, 2013 and 2014). The values in the *x* axis are the genetic similarities.

2.4 DISCUSSION

The ERYCC panel was grown at the Rothamsted farm in the UK for three consecutive seasons. Late sowing and reduced rainfall shortened the grain development stage and consequently influenced the yield patterns mainly in 2013. An analysis of 12 cultivars from the panel revealed that cultivars presented different yield resilience to the environmental constraints and that these differences could be partially explained by their genetic similarities. The results are discussed based on the different strategies presented by the cultivars to achieve higher yield resilience and the influence of their genetic background on this behaviour. The methodology of yield resilience evaluation is also discussed.

2.4.1 Grain development duration and rate and different strategies to yield resilience

The grain development stage is crucial for wheat grain yield definition (Evans and Fischer, 1999; Foulkes *et al*., 2002; Hunt *et al*., 1990). According to Semenov *et al*. (2014), in future climatic conditions, the wheat crop cycle might be shortened with yield being limited by the grain development duration and by the lower rainfall and high temperatures at this stage. Those conditions are similar to the conditions faced in the 2013 season: a shorter, drier and warmer grain development stage when compared mainly to 2012. For the current study, reduced water availability during the crop cycle was correlated to the reduced duration of the grain development stage, as also reported by Destro *et al*. (2001). Hunt *et al*. (1990) highlighted the importance of the grain development rate for wheat yield under shorter grain development periods. Grain development rate is the rate of accumulation of dry matter to the grain during grain development and filling. Higher grain yields can be achieved by having a longer grain development stage or by a higher grain development rate (Hunt *et al.*, 1990). The second strategy is particularly important under sub-optimal conditions when grain development duration is shortened, as observed in 2013 and 2014.

Humber, Soissons and Savannah were the only cultivars, among the 12 analysed, which were always at the same level or above the ERYCC panel average yield (Fig. 2.3) and that improved their relative grain yield from the 2012 to 2013 seasons (Table 2.6). It suggests a combination of productivity and stability in different climatic conditions (see YSSI index in Table 2.5), which would make them a likely selection for yield and yield resilience improvement. Gatsby, Cadenza and Paragon presented the lower yield resilience to the 2013 season's conditions. Ober *et al*. (2013) compared the relative yield for a subset of cultivars of the ERYCC panel in drought experiments. Humber presented an increase whilst Gatsby presented a decrease in relative grain yield under reduced water availability, which agrees with the current study.

Between Humber, Savannah and Soissons, higher yield resilience was achieved through different strategies: Humber and Savannah relied on an improved grain development rate in 2013, while Soissons relied on avoiding the grain development stage shortening.

The improvement in grain development rate for Humber and Soissons in 2013 is suggested by the fact that Gatsby and Cadenza, which presented a similar decrease in grain development duration to Humber and Savannah from 2012 to 2013, had a pattern of decreased yield resilience (Table 2.6). The grain development rate is related to the capacity of the plant to generate and transport dry matter to the grain. The two main sources of dry matter to the grain filling process are the assimilated carbon at post-anthesis and the reallocation of pre-stored carbon, mainly in stems. The first is related to photosynthesis at post-anthesis and the later to photosynthesis at pre-anthesis. Carmo Silva *et al*. (submitted) showed that for the ERYCC panel experiment in 2013, pre and post-anthesis flag leaf photosynthesis were positively correlated to grain yield and harvest index. Savannah presented one of the highest flag leaf photosynthetic rates in 2013 (Fig. 2.4) and Humber was selected by the capacity to maintain flag leaf photosynthesis from pre to post-anthesis in the same season (Table 2.1). These could be linked to different ways of achieving higher

relative grain yields and increasing yield resilience. In addition, Savannah and Humber presented the *Lr37* gene, which was positively correlated to harvest index and grain yield in 2013. The gene presence could have influenced the flag leaf photosynthetic active period by avoiding leaf rust pressure and early senescence.

In comparison to the average of the ERYCC panel, Soissons presented a shorter duration of the grain development stage in 2012 and a higher duration in 2013 which resulted in a smaller reduction of the grain development duration between 2012 and 2013 (Table 2.6). The 2012 reduced grain development stage was linked to an early maturation start and the 2013 extended grain development period to an early flowering and late start of maturation. Soissons presented the highest photosynthetic rates among the 12 cultivars in 2013 (Fig. 2.4), which could have also contributed to the higher relative yields. For the whole panel analysis, the presence of the photoperiod insensitivity gene (*Ppd1*) was correlated to the early flowering in 2013 ($r = 0.74$, $p < 0.001$). According to its agricultural characteristics (www.elsoms.com/agricultural-seed/soissons-winter-wheat), Soissons is not recommended for sowing before the second week of October, which could have also influenced its 2012 relative grain yield and the developmental stages duration.

In summary, under ideal growth conditions, the selection of cultivars for an increased grain development period could help to improve grain yields. In the predicted future climatic scenarios of a shorter, drier and warmer grain development stage, as proposed by Semenov *et al*., (2014), yield and yield resilience could be favoured by selecting cultivars based on their capacity to escape the grain development stage reduction, as reported to Soissons and/or by their improved grain development rate under limiting conditions. Although, Mashiringwani *et al*. (1994) reported the difficulties to define genetic inheritance of grain development rate, the fact that genetic similarities between the cultivars were linked to grain yield in the 2013 and 2014 seasons and the high similarity between Humber and

Savannah (for SNPs and genes analyses) (Fig. 2.6) can be a clue of the presence of genetic variation in grain development rate between the analysed cultivars.

2.4.2 Genotype evaluation and selection in multiple environments

The different methods used for evaluating the cultivars' performance in terms of grain yield and yield resilience over the 3 seasons were in accordance. The general positioning of the cultivars in the GGE biplot (Fig. 2.5) agreed with their rank for the RCI, PCI and YSSI indices (Table 2.5) when comparing grain yield for 2013 and 2014 seasons to 2012. RCI, PCI and YSSI index are recommended to the analysis of traits under control and specific imposed stresses (Thiry *et al*., 2016). The random climatic conditions faced during the 3 seasons and the contrasting behaviour of the cultivars in terms of relative grain yield could have limited the interpretation of the indices results. However, their agreement to the GGE biplot and principal component analysis suggests the possible use of them to compare traits in different seasons and/or environments.

For the GGE biplots, the length of the vector for each season represents the variation of the trait (grain yield in this specific case) between the cultivars (Yan and Tinker, 2005). For instance, the different sizes of 2013 and 2014 vectors mean that the variation of grain yields between the 12 cultivars was much bigger in 2013 than in 2014 (Fig. 2.5). Yan and Holand (2010) proposed a heritability-adjusted GGE biplot (HA-GGE), where the length of the vectors would be proportional to the square root of the broad-sense heritability for the specific environment. The vectors would then represent the proportion of all the variance for the trait which was explained by the genotypic variance in this specific environment. For the current study, 2013 and 2014 would present a change in their vector lengths, with the 2014 vector being bigger than the 2013 vector, as the heritability for grain yield was 0.82 and 0.74 for the 2 years, respectively. The HA-GGE looks a promising strategy to include even more information into the GGE biplots and help on the decision of selecting genotypes in breeding

process. The software required for HA-GGE biplots was not available at the time of writing this work. In summary, the GGE biplots presented a friendly integrated representation of relative grain yield for the 12 cultivars over the 3 seasons. The method improved the evaluation of cultivars' performance to yield and yield resilience in different environments. These benefits could be enhanced when analysing a bigger number of cultivars, environments and their interaction as required in breeding programs evaluation.

2.5 CONCLUSIONS AND FINAL COMMENTS

Late sowing and lower rainfall shortened the grain development stage in 2013 and 2014. This has changed the yield and yield resilience patterns of the 12 cultivars when comparing these seasons to 2012. Humber, Savannah and Soissons presented higher yield and yield resilience when compared to the ERYCC panel average over the 3 seasons, but relied on different strategies to achieve higher yield resilience in shorter grain development stages. Humber and Savannah presented a bigger grain development rate while Soissons presented a smaller reduction in the grain development stage. The similar behaviour of Humber and Savannah may be linked to their genetic similarity which suggests that genetic variation to grain development rate can be exploited to improve yield and yield resilience. Lower broad-sense heritability for relative grain yield was observed in the 2013 season, suggesting a link between heritability and the environmental constraints to plant growth and development. The use of GGE biplots and performance indices improved the process of the cultivars' evaluation in terms of yield and yield stability over the 3 seasons.

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CHAPTER 3

DISSECTING THE MAIN YIELD DRIVERS IN A DOUBLE-HAPLOID WHEAT MAPPING POPULATION IN THE UK

COLLABORATORS' CONTRIBUTIONS

The author, Elizabete Carmo-Silva and John Andralojc designed and performed the research. The author and Stephen Powers analysed the data. The author wrote the manuscript.

ABSTRACT

Improving crop yields may act as a solution to ensure food security under future scenarios of the growing world population, changes in food consumption patterns, climate change and limited use of resources to agriculture. Defining traits that can be reliable cornerstones to yield improvement and understanding their interaction and influence to yield formation is an important part of the success of breeding programs for high yield. Traits that can drive yield increases, such as light interception and use efficiency, and carbon assimilation and allocation, were intensively phenotyped in a double-haploid wheat mapping population for two consecutive seasons in the UK. Traits were analysed for their correlation to yield, variability and broad-sense heritability. Canopy cover and reflectance, biomass production and allocation to stems and leaves and flag leaf photosynthesis at pre and post-anthesis were correlated to plant productivity and contributed in explaining different strategies of wheat lines to reach higher grain yields. The contrasting strategies and the correlations between grain yield and multiple traits over the season reinforced the complexity of this trait and the need of an integrated analysis to understand and improve wheat grain yield.

3.1 INTRODUCTION

Global food security is defined as a situation where all people at all times have access to safe, nutritious and affordable food that provides the foundation for active and healthy lives (FAO, 2002). Ensuring food security in the near future is challenging, mainly considering the predicted scenarios of a growing world population (Godfray *et al*., 2010), changes in food consumption patterns (Pingali, 2006), extreme climatic events (Tilman and Clark, 2015) and the need for sustainable use of resources in agricultural activities (Berry *et al*., 2015).

Increasing food production is one of the alternatives in ensuring that food availability is kept ahead of food demand. Food production can be increased by: expansion of croplands, intensification of land use and increase in yields. Increasing yields might be responsible for around 77% of food production increases by 2050 (Alexandratos and Bruinsma, 2012). In opposition to the required yield increases, a trend of stagnating yields has been observed for the main crops around the world (Grassini *et al*., 2013). For rice, maize, wheat and soybean, which represent two-thirds of the total caloric intake worldwide (FAOSTAT, 2016), yields stagnated in around 2002, with increases in production being pushed by expanding the cultivated land area (Grassini *et al*., 2013). For wheat, in the UK, yields have stagnated around 8 t ha^{-1} since 1996 (Knight *et al*., 2012).

The Green Revolution was successful in increasing wheat yields by the use of the dwarf genes approach (Araus *et al*., 2008). It was related to a higher efficiency in allocating plant biomass to the grain during grain filling, which led to an increase in harvest index (Richards, 2000). This approach cannot be further exploited for the current and future situation as the wheat harvest index has reached values close to its theoretical limit (Foulkes *et al*., 2011). Understanding yield formation and dry matter accumulation is therefore crucial to reveal new target traits to be exploited in the process of breeding for high yield (Reynolds and Langridge, 2016).

Yield formation is a process of energy conversion and accumulation. The radiative energy available in sunlight is intercepted by plants and converted during photosynthesis to molecules that can be stored in the form of dry matter and allocated to plant organs (Monteith and Moss, 1977). Therefore, crop yield is a function of the incident sunlight and the efficiency of the plant to intercept and convert light into biomass, as well as the efficiency of partitioning of the biomass to the organ of economic interest (Long *et al*., 2015). Traits, from canopy to molecular levels, correlated to any of the above mentioned steps of yield formation, are potential targets for the breeding of high yielding genotypes.

In wheat, light interception is mainly related to canopy cover and architecture. By increasing canopy longevity, plants can intercept more energy from sunlight (Thomas and Howarth, 2000). Early vigour, fast leaf expansion and development of area cover, and late senescence (stay green) are related to an extended photosynthetic active period of the canopy and are related to yield in wheat (Richards, 2000). At the leaf level, smaller and more erect leaves at the top of the canopy, and bigger and more horizontal leaves at the lower levels, are related to a better light interception (Murchie *et al*., 2009), as well as lower concentrations of chlorophyll in the top leaves compared to lower leaves to enable better light distribution (Ort *et al*., 2011).

Light use efficiency is the ratio of net primary productivity (NPP) to intercepted photosynthetically active radiation (PAR) (Medlyn, 1998) or simply the crop photosynthetic efficiency (Araus, 2002). At current atmospheric conditions, C3 photosynthesis has a theoretical maximum efficiency of 4.6%. However, practical efficiency is around 2% and the average, in field conditions, less than 1%, showing potential for improvement (Zhu *et al*., 2008). Possible strategies to improve photosynthesis, and consequently light use efficiency, include overcoming the limitations imposed to the photosynthetic process, such as: CO_2

diffusion and concentration, the regeneration of Calvin-Benson cycle intermediates, Rubisco efficiency and light reactions (Raines, 2011).

Free-air CO_2 enrichment (FACE) experiments showed that increased photosynthetic rates are related to yield increases, when other conditions are not limiting (Ainsworth and Long, 2005). Previous studies highlighted the positive influence of photosynthesis to wheat yields (Sayre *et al*., 1997; Gutierrez-Rodriguez *et al*., 2000; Condon *et al*., 2008) and specifically the importance of flag leaf photosynthesis as an important source of photoassimilates during grain filling (Sylvester-Bradley, 1990; Larbi and Mekliche, 2004).

Together with flag leaf photosynthesis, the reallocation of carbohydrates stored in plant organs is an important driver of grain yield formation in wheat. Biomass stored, mainly in stems and leaves, can play an important role in yield definition, since a significant portion will be reallocated to the grain during grain filling (Blum *et al*., 1994).

There is a multitude of plant traits, in different structural levels, that may contribute to yield improvement. Selecting possible targets can be a challenging and confusing process, although there are some directions that can make the process more efficient. Selection of target traits for breeding for high yield need to be based on: a) the correlation between the trait and yield; b) the variability of the trait for the species or inside a population; c) the trait stability (heritability) and d) the influence of genetic x environment interaction to the trait (Mir *et al*., 2012). Although the selection of traits is a crucial step for the breeding of high yield genotypes, understanding traits interaction and trade-off, as well as their behaviour along the crop cycle have an important role in defining yield improvement strategies.

The influence of multiple traits to wheat productivity and their interaction to define different strategies to reach higher yields were studied for a double-haploid wheat mapping population, during two seasons, in the UK. The hypotheses tested were: a) that traits influencing light interception and conversion and biomass allocation are linked to wheat

yields differently over the season; b) that high yields can be reached by multiple strategies of combining the above mentioned traits; c) that wheat grain yield can be predicted through mathematical modelling based on multiple phenotypic traits.

3.2 MATERIAL AND METHODS

3.2.1 Plant material and field experiments

A mapping population composed of double-haploid lines (DHL) generated by Syngenta was used. For the selection of the population, a total of 12 wheat cultivars (for which mapping populations were previously generated) were grown in randomized block design experiments (4 blocks) and were screened for their photosynthetic traits, leaf characteristics and biomass production at early stages by Dr Elizabete Carmo-Silva at Rothamsted Research. Five-week old plants of the 12 cultivars were analysed with a portable infra-red gas analyser (IRGA) system (LI-COR 6400 and chamber 6400-40; LI-COR, Lincoln, USA) for the response of net CO_2 assimilation to the intercellular CO_2 concentration (A x C_i curves) at light levels of 1500 µmol photons m^{-2} s^{-1}. The youngest fully expanded leaf was used to the gas-exchange analysis and was also measured for their leaf area and thickness. Total above ground biomass was determined after gas-exchange measurements. The two parents were chosen based on their contrasting behaviour for photosynthesis at ambient CO_2 (400 µmol CO_2 µmol air^{-1}) and high light, specific leaf area and biomass production (Fig. 3.1). None of the parents was included in the ERYCC Panel cultivars presented in Chapter 2. The selection was recommended by David Feuerhelm from Syngenta.

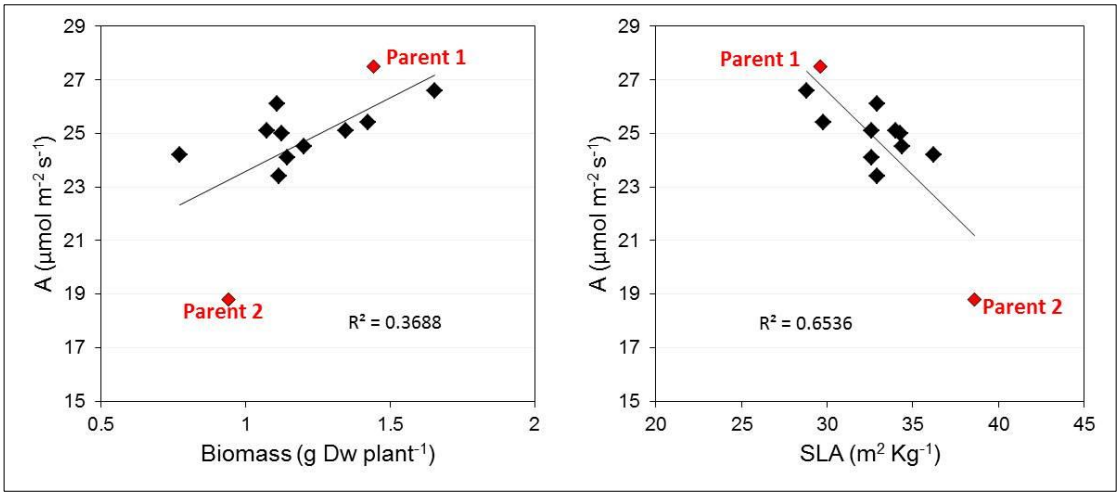

Figure 3.1 – Net photosynthesis (*A*) vs biomass and net photosynthesis (*A*) vs specific leaf area (SLA) for a set of 12 wheat cultivars for the selection of mapping population parents. Data and graphs kindly provided by Dr Elizabete Carmo-Silva.

The mapping population, composed by the two parents and 119 lines, was grown at the Rothamsted Research farm, in Harpenden, UK, for two consecutive seasons, with the first sown in October 2014 and harvested in August 2015, and the second sown in October 2015 and harvested in August 2016. Detailed information of each experiment is presented below (experiments identified by the year of harvest):

a) 2015: experiment planted at the Pastures field, in a Typical Batcombe soil (Avery and Catt, 1995) after oilseed rape crop, in 2 x 1 m (2 m^2) plots of 6 rows, with a sowing rate of 350 seeds m^{-2}, organized in three randomized blocks; sown on 20/10/2014 and harvested on 23/08/2015.

b) 2016: experiment planted at the Delafield field, in a Batcombe soil (Avery and Catt, 1995) after oilseed rape crop, in 4 x 1 m (4 m^2) plots of 6 rows, with a sowing rate of 350 seeds m^{-2}, in a completely randomized design; sown on 12/10/2015 and harvested on 24/08/2016.

Application of fungicides, insecticides and herbicides, as well as fertilizers was done accordingly to Rothamsted farm practices in the three seasons.

3.2.2 Meteorological data

The meteorological data was acquired from the Rothamsted Meteorological Station at the Rothamsted Farm. The distance from the station to the experiments was, in a straight line: 1 km for the 2015 experiment and 1.6 km for the 2016 experiment. The maximum and minimum daily temperature (°C), the daily rainfall (mm) and the daily radiation (MJ m^{-2}) were used. From this data, the accumulated rainfall and accumulated radiation for a specific period was calculated as the sum of the daily value from the first to the last day in the period considered. Average daily temperature (*Tmed*) was calculated as the mean of the maximum daily temperature and the minimum daily temperature. Degrees day was calculated considering the base temperature (*Tbase*) for wheat crop as zero (McMaster and Smika, 1988) and by the equation system below:

$$Degreesday = \begin{cases} Tmed - Tbase, & \text{if } Tmed > Tbase \\ 0, & \text{if } Tmed \leq Tbase \end{cases} \quad \text{(Formula 3.1)}$$

The accumulated degrees day for a period of time was calculated as the sum of the degrees day from the first to the last day in the considered period.

3.2.3 Crop development

Crop development was monitored across the season, using the Zadoks scale (Zadoks *et al*., 1974). The frequency of crop development monitoring depended on the crop stage and on the rate of change, being more frequent when crop development was faster or less frequent when crop development was slower. Senescence was measured from anthesis to the end of the season using the wheat senescence scale (Pask and Pietragalla, 2012).

3.2.4 Phenotyping

For 2015, a full set of measurements was taken at three specific development stages: stem elongation (SE – Z3.2), booting (BT – Z4.5) and 7 days post-anthesis (PA – Z6.5). Other

measurements were carried out over the season and the frequency of measurement is detailed in the information presented below:

- Leaf cover: horizontal pictures, parallel to the plots, were taken weekly from March (Z2.4) to August (Senescence score 10) using a digital camera. The pictures were analysed using the BreedPix software that outputs the area covered by green tissue as a percentage of the total area (Casadesus *et al*., 2007). From this data, the following indices were calculated: early vigour, accumulated green area, highest leaf cover and stay green (Fig. 3.2).

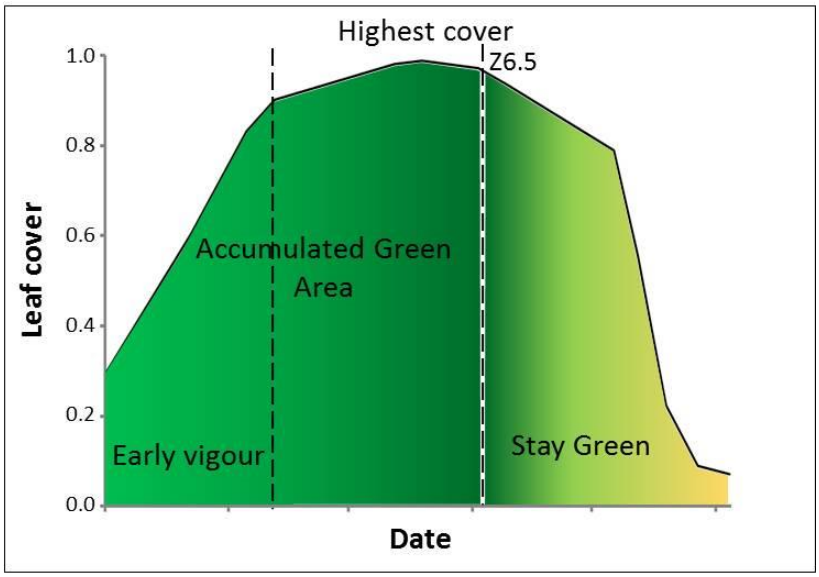

Figure 3.2 – Graphic representation of indices calculation from the outputs of the BreedPix software for the 2015 season. The indices are: early vigour (accumulated green area from the 4/3/15 until 15/4/15, when plots reached an average of 90% leaf cover), accumulated green area (accumulated green area from 4/3/2015 to Z6.5), maximum leaf cover and stay green (accumulated green area from Z6.5 to harvest).

- Leaf Area Index (LAI): measured at soil level at the 3 crop development stages analysed (SE, BT and PA) using the LI-COR LAI-2200 plant canopy analyser (LI-COR, Lincoln, US). Three measurements were taken between the 2nd and 3rd row of each plot at 25, 50 and 75% of plot length and averaged to a single value per plot.

- Height: average crop height at the 3 crop development stages (SE, BT and PA).

- Peduncle length: the distance between the flag leaf insertion and the bottom of the spike was measured for three plants per plot at PA and averaged to a single value per plot.

- Reflectance: measured 75 cm above the canopy at the 3 crop development stages (SE, BT and PA) using the HandySpec System (TEC5, Oberursel, Germany) reflectance meter. From the reflectance measurements a set of traits was calculated, using the provided software. The indices' definitions and calculation are described by Pietragalla et al. (2012), where R_{number} is the reflectance value at the specific wavelength: Normalized Difference Vegetation Index (NDVI = $[R_{900} - R_{680}]/[R_{900} + R_{680}]$), Simple Ratio (Sra = R_{900}/R_{680}), Ratio Analysis of Reflectance Chlorophyll a (RARSa = R_{675}/R_{700}), Ratio Analysis of Reflectance Chlorophyll b ((RARSb = $R_{675}/[R_{650} \times R_{700}]$), Ratio Analysis of Reflectance Carotenoid (RARSc = R_{760}/R_{500}), Normalized Pheophytinization Index (NPQI = $[R_{415} - R_{435}]/[R_{415} + R_{435}]$), Structural Independent Pigment Index (SIPI = $[R_{800} - R_{435}]/[R_{415} + R_{435}]$), Photochemical Reflectance Index (PRI = $[R_{531} - R_{570}]/[R_{531} + R_{570}]$) and Water Index (WI = R_{970}/R_{900}).

- Gas-exchange Analysis: plants were harvested from the field at pre-dawn and stems placed in water-filled tubes in cold and dark cabinets until the time of measurement, as detailed described by Driever *et al*., (2014). Gas-exchange traits were measured at BT and PA for the flag leaf in one plant per plot with a portable infra-red gas analyser (IRGA) system (LI-COR 6400 and chamber 6400-40; LI-COR, Lincoln, USA) using the multiphase flash method (Loriaux *et al*., 2013). Plants were analysed at an ambient CO_2 concentration of 400 µmol CO_2 µmol air^{-1} and light levels (Photosynthetically Active Radiation) of 1800, 1000, 500, 250, 100 µmol photons m^{-2} s^{-1}. Maximum net photosynthesis (*Amax*) was measured at 1200 µmol CO_2 µmol air^{-1} and 1800 µmol photons m^{-2} s^{-1}.

- Flag leaf and plant characteristics: the flag leaves analysed for gas-exchange measurements were used for the following measurements: leaf thickness, length and width, chlorophyll content, flag leaf area and weight. Plant shoot weight was also measured. The measurements were done at BT and PA. At PA, for the plant shoot, stem thickness and number of green leaves were also determined. The following spike characteristics were measured at PA: spike dry weight, length, width, presence of awns and number of spikelets.

- In-season biomass analysis: at PA, a 50 cm row (in the first quarter of the third row in the plot) was manually harvested and the following measurements were taken: tiller count and leaves, stems and spike dry weight, according to methodology described by Pask and Pietragalla (2012).
- End-of-season biomass analysis: at physiological maturity (Z9.9), a 50 cm row (in the third quarter of the third row in the plot) was manually harvested and tiller count, straw, spike and grain dry weight, grain moisture and grain number were measured. The following traits were calculated: harvest index, grain weight per ear, grain number per ear, thousand-grain weight, estimated yield, and estimated biomass production (Pask and Pietragalla 2012).
- Combine harvest: the experiments were harvested using a Haldrup-C65 (Haldrup, Le Mans, France) plot combine. Grain weight for each plot was measured. Grain moisture was measured using a sub-sample of grains from each plot, at harvest time, and grain weight was normalized to 15% moisture content. Grain weight per plot was corrected for the two rows harvested by hand and grain yield estimated in tons per hectare at 85% dry matter.

Phenotypic traits were grouped into four categories: leaf and canopy (leaf cover, leaf area index, height, peduncle length and flag leaf and plant characteristics), biomass (in-season and end-of-season biomass traits and grain yield from the combine), reflectance (reflectance indices) and gas-exchange (traits measured and calculated from the IRGA analysis).

For the 2016 season, the following measurements were taken using the same methodology described for 2015: leaf cover (weekly from March (Z2.4) to August (Senescence score of 10)), leaf area index (at Z4.5 (BT), Z6.5 (BT), Z7.5, Z7.9 and Z9.4) and reflectance (at Z3.7, Z5.7, Z6.5 (PA), Z7.1, Z7.5, Z7.8, Z7.9, Z9.4 and Z9.7). End of season biomass analysis was performed as in 2015 season for a 25 cm row. Yield was calculated as in 2015, based on the Haldrup harvest data.

3.2.5 Statistical analysis

The method of residual maximum likelihood (REML) was used to fit a linear mixed model to each measured trait to test for any statistically significant ($p < 0.05$, Chi-squared test) variation due to spatial trends over the rows and columns in the field design. Predicted means by the model fitted to each trait were used in subsequent analyses. Pearson Product Moment (PPM) coefficients were calculated for all the pairs of traits measured in order to evaluate pairwise correlations.

For 2015 data, the lines were ranked according to yield. For the lines in the higher quartile, an analysis of traits influencing yield was performed. For each trait, values were ranked as high (upper quartile), intermediate (two intermediate quartiles) and low (lower quartile). The traits included in this analysis were chosen based on their correlation to yield, their heritability and variability in the population, and collinearity to other traits.

The analyses were performed using the GenStat 17th Edition software (VSN International Ltd., Hemel Hampstead, UK).

3.2.6 Grain yield modelling

Linear mixed models were used to predict yield as a combination of the measured traits for the 2015 season. Two models were fitted using Forward and Backward Stepwise methods. The traits that were coincident for the two models were used as forced terms (terms always present in the model) and traits that just appear in one of the methods were used as free terms (that can be present or not in the model).

For the 2015 and 2016 seasons, a second modelling approach used the reflectance indices and the definition of yield as a function of the available sunlight radiation (RAD), the efficiency of the vegetation to intercept (εi) and convert energy from sunlight to dry matter (εc) and the partitioning factor (εp) (Long *et al.*, 2015). From the definition of the reflectance

traits, NDVI is related to leaf area, PRI to light use efficiency and WI to relative water content. A model to predict biomass accumulation and grain yield for each line was proposed as:

$$Biomass = (\sum_{i}^{f}(RAD.NDVInorm\,.\;LUE\,.\;(WI^{-1})) \qquad \text{(Formula 3.2)}$$

$$Yield = Biomass\,.HI \qquad \text{(Formula 3.3)}$$

where *Biomass* is the predicted accumulated biomass, *Yield* is the predicted yield, *RAD* is the sunlight radiation in the period, *NDVInorm* is the normalized NDVI, *LUE* is the light use efficiency calculated from PRI, *WI* is the water index and *HI* is the harvest index; *i* and *f* are the measurement points.

NDVInorm was calculated from NDVI multiplied by a factor related to leaf cover indices. The correction factor for each line was calculated by the chosen leaf cover index for the line divided by the average of the index for the mapping population. The indices used for each of the periods in the season were: early vigour from the initial stages to SE; accumulated green area from SE to PA; stay green from PA to the end of the cycle. *LUE* was calculated from PRI using the conversion factor defined by Wu *et al*. (2015).

As the first measurements in each season were made around SE, values for NDVI, PRI and WI were estimated for the early stages (around Z2.4). NDVI was estimated as 25% of the NDVI at SE, PRI and WI were estimated as the same values as SE. Biomass accumulated was very low and considered zero. Pearson coefficients were calculated between the modelled and measured grain yield for the two seasons.

3.2.7 Heritability

Broad-sense heritability (H^2) was calculated for all the traits measured in each season using the procedure described by Cullis *et al*. (2006), based on the ratio of the between cultivar variance component and the mean variance of the difference between two cultivar means,

as estimated by best linear unbiased predictors (BLUPs), using the results of the linear mixed model analyses. The GenStat 17th Edition software (VSN International Ltd., Hemel Hempstead, UK) was used.

3.2.8 Variability

The variability of each measured trait was calculated by the ratio between the standard deviation of the trait (σ) and the trait average (μ). This represents a normalized variation of each trait for the mapping population in the season.

3.3 RESULTS

3.3.1 Traits within the same group of measurement presented high collinearity

The total number of measured/calculated traits was over 150 for the 2015 season and multiple correlation trends between them were observed. The Pearson coefficient (r) indicates the level of correlation between a pair of traits. It varies from a strong negative correlation (-1) to a strong positive correlation (+1). Positive correlation between a pair of traits means that increases in one trait are related to increases in the other. Negative correlations have an opposite meaning, with increases in one trait being related to decreases in the other or *vice versa*. As some traits were measured using the same instrument, at the same time, or were calculated from a single measurement, a common trend of collinearity was observed inside particular groups of traits (black bordered triangles in Fig. 3.3). Strong positive and negative correlations were observed between pairs of traits mainly inside the groups of reflectance indices and gas-exchange measurements (Fig. 3.3). Considering the high number of traits measured in the current study, when collinearity was high inside a group, the selection of a few or a single trait that was correlated to the trait of interest (grain yield) and to the other traits inside the group was adopted to simplify the interpretation of the results.

3.3.2 Correlation to grain yield varied at different developmental stages

The correlation between grain yield and specific measured traits changed throughout the season according to different developmental stages. This suggests that some traits might be more or less important to yield definition in different stages of crop development. Correlation to yield is one of the important characteristics of a trait to be chosen as a potential target for yield improvement, as well as heritability and genetic variability. Several traits were significantly correlated to yield, but also correlated to other traits in the same group (Fig. 3.3). For reflectance indices at SE stage, for instance, NDVI, SRA, RASRc and SIPI were positively correlated to yield but also strongly correlated to each other. The set of traits presented in Table 3.1 was selected according to their correlation to yield, variability and heritability. Traits that correlated to the selected trait and had a weaker correlation to yield

Canopy cover and longevity traits correlated to grain yield, such as: accumulated green area until Z6.5 ($r = 0.43$, $p < 0.001$), highest cover ($r = 0.42$, $p < 0.001$), early vigour ($r = 0.27$, $p < 0.01$) and stay green ($r = 0.26$, $p < 0.01$) (Table 3.1). Although accumulated green area and highest cover have similar correlation to yield and heritability ($H^2 = 0.77$ for both), accumulated green area presented bigger variability ($\sigma/\mu = 0.03$) than highest cover ($\sigma/\mu = 0.01$) in the population (Table 3.1). It can be explained by the fact that every line covered nearly 100% of the soil area at their highest cover, but the slope of the curve and the accumulated area previous to the highest cover presented different patterns.

Grain yield was also correlated to the plant biomass accumulated at PA. Biomass accumulation in stems ($r = 0.23$, $p < 0.05$) and leaves ($r = 0.25$, $p <0.01$), as well as total plant biomass ($r = 0.24$, $p < 0.05$), correlated to grain yields (Table 3.1). The positive correlation between biomass accumulated in stems at PA and grain yield may be explained by the biggest availability of dry matter to be re-allocated from the stem to the grain during grain filling. Leaf biomass at PA was also correlated to specific leaf area ($r = 0.39$, $p < 0.001$) and

leaf area index ($r = 0.42$, $p < 0.001$) at PA and to stay green ($r = 0.54$, $p < 0.001$). These multiple correlations are related to a bigger capacity to intercept and convert energy at PA, generating photoassimilates that sustain grain filling and thus contribute to grain yield.

NDVI, as a measurement of green biomass, was correlated to the leaf cover traits as well as to grain yield at SE ($r = 0.49$, $p < 0.001$) and BT ($r = 0.28$, $p < 0.01$) (Table 3.1). NDVI correlation to yield decreased during the season (at PA, $r = 0.17$, $p = 0.06$). PRI, as an indirect measurement of canopy light use efficiency, was correlated to photosynthesis and other gas-exchange traits, as well as to grain yield at SE ($r = 0.48$, $p < 0.001$), BT ($r = 0.44$, $p < 0.001$) and PA ($r = 0.35$, $p < 0.001$) (Table 3.1). WI is a measurement of canopy water stress, inverse to the canopy water content. This explains its negative correlation to yield, as higher values of WI are related to lower canopy water content. WI was negatively correlated to yield at SE ($r = -0.36$, $p < 0.001$), BT ($r = -0.21$, $p < 0.05$) and PA ($r = -0.42$, $p < 0.001$) (Table 3.1), suggesting that water could be limiting to grain yield, especially at PA.

Flag leaf photosynthesis was correlated to grain yield at all light levels as well as high CO_2 concentration (*Amax*) at BT (Table 3.1). At PA, the correlations were less strong and only significant at light levels higher than 1000 µmol photons m^{-2} s^{-1}. Other gas-exchange traits were also correlated to yield for some of the light levels at BT, including stomatal conductance and electron transport rate. At BT, both A and *gs* were correlated to yield at all the light levels. At PA, A correlated to yield at the two higher light levels and *gs* was not correlated to yield (Table 3.2). For both, BT and PA, the correlation between A and *gs* was strong at the high light levels, showing a diffusion limitation to photosynthesis. This correlation decreased at lower light intensity, mainly at 100 µmol photons m^{-2} s^{-1}, suggesting that photosynthesis was limited not just by diffusion, but also by the biochemical pathways involved in carbon fixation and light reactions (Table 3.2).are not shown.

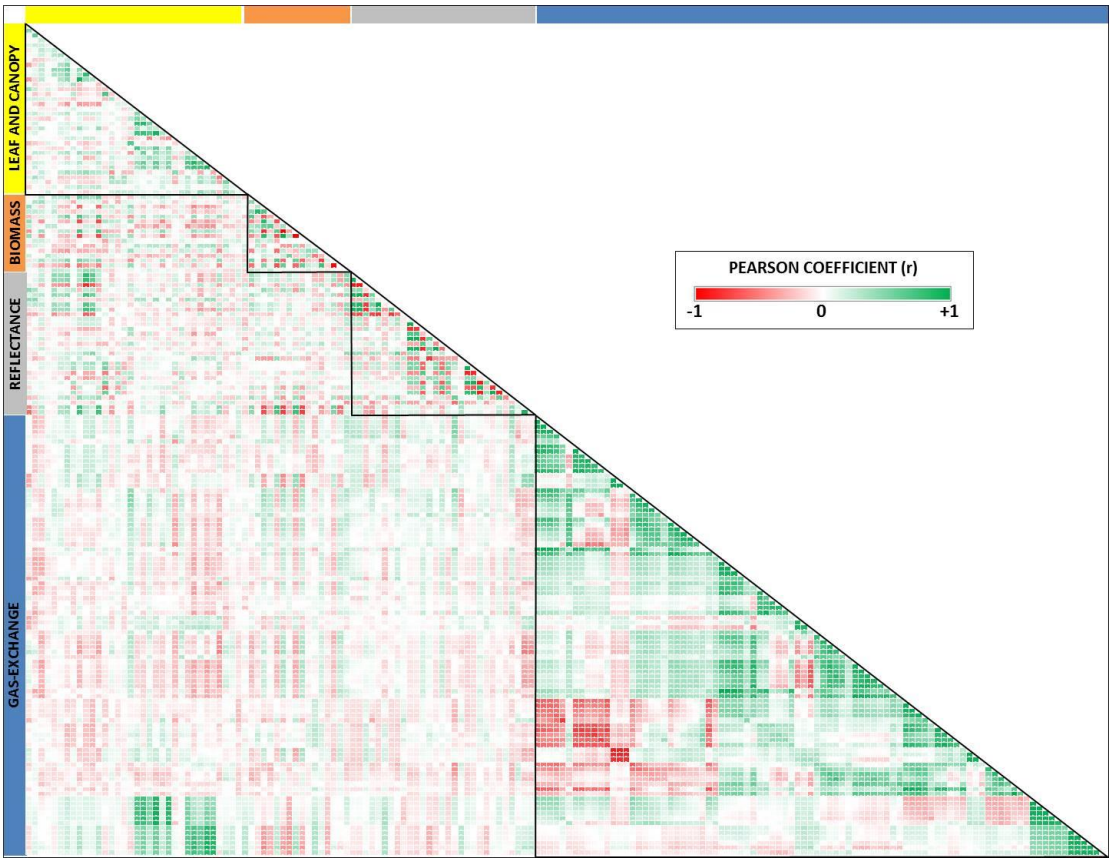

Figure 3.3 – Pearson coefficients' matrix for traits measured for a double-haploid wheat mapping population grown in UK in 2015. Values for the Pearson coefficient varies from -1 (dark red, meaning strong negative correlation between the pair of traits) to +1 (dark green, meaning strong positive correlation between the pair of traits), through 0 (white, meaning no correlation between the pair of traits). Black bordered triangles limit the correlations within a specific group of traits related to Leaf and Canopy, Biomass, Reflectance and Gas-exchange.

Table 3.1 – Physiological traits' Pearson coefficients to grain yield, variability and heritability in a double-haploid wheat mapping population, grown in the UK in 2015. Traits grouped according to Leaf and Canopy, Biomass, Reflectance and Gas-exchange. Significance levels for correlations according to a double-tailed F test: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. Variability is calculated by the ratio of the standard deviation and the average of each trait and means a normalized dispersion index. Heritability is calculated according to Cullis *et al*. (2006) and represents the ratio between the genetic variance per total variance for each trait. SE, stem elongation (Z3.2); BT, booting (Z4.5); PA, 7 days post-anthesis (7 days after Z6.5). NDVI, Normalized Difference Vegetation Index; PRI, Photochemical Reflectance Index; WI, Water Index. A@Q, flag leaf net photosynthesis (*A*) at ambient CO_2 and a specific light level (Q); *Amax*, net photosynthesis at 1200 µmol CO_2 µmol air^{-1} and at light level of 1800 µmol photons m^{-2} s^{-1}.

	Leaf and canopy						Biomass		
	Early vigour	Acc. green area	Highest cover	Stay green			Leaves weight (PA)	Stem weight (PA)	Total biomass (PA)
Correlation (r)	0.27**	0.43***	0.42***	0.26**			0.25**	0.23*	0.24*
Variability (σ/µ)	0.09	0.03	0.01	0.09			0.10	0.09	0.08
Heritability (H^2)	0.50	0.77	0.77	0.78			0.42	0.42	0.24
	Reflectance indices								
	NDVI (SE)	PRI (SE)	WI (SE)	NDVI (BT)	PRI (BT)	WI (BT)	PRI (PA)	WI (PA)	
Correlation (r)	0.49***	0.48***	-0.36***	0.28**	0.44***	-0.21*	0.35***	-0.42***	
Variability (σ/µ)	0.02	0.70	0.01	0.01	1.98	0.02	0.33	0.01	
Heritability (H^2)	0.71	0.89	0.57	0.79	0.87	0.70	0.93	0.79	
	Gas-exchange								
	A@Q1800 (BT)	*A*@Q1000 (BT)	*A*@Q500 (BT)	*A*@Q250 (BT)	*A*@Q100 (BT)	*Amax* (BT)	*A*@Q1800 (PA)	*A*@Q1000 (PA)	
Correlation (r)	0.28**	0.29**	0.28**	0.28**	0.23*	0.21*	0.18*	0.19*	
Variability (σ/µ)	0.07	0.06	0.05	0.04	0.05	0.05	0.07	0.06	
Heritability (H^2)	0.59	0.61	0.61	0.47	0.28	0.54	0.59	0.61	

Table 3.2 – Pearson coefficients between net photosynthesis (*A*), stomatal conductance (*gs*) and grain yield at booting (BT; Z4.5) and 7 days post-anthesis (PA; 7 days after Z6.5) in a double-haploid wheat mapping population, grown in the UK in 2015. Significance levels for correlations according to a double-tailed F test are: ^{NS} $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

	Light levels (µmol photons m^{-2} s^{-1})				
	1800	1000	500	250	100
	BT				
A - *gs*	0.92**	0.88**	0.82**	0.76**	0.55**
A - Yield	0.28**	0.29**	0.28**	0.28**	0.23*
gs - Yield	0.22*	0.21*	0.21*	0.21*	0.21*
	PA				
A - *gs*	0.80**	0.85**	0.77**	0.73**	0.49**
A - Yield	0.18*	0.19*	0.17^{NS}	0.12^{NS}	0.08^{NS}
gs - Yield	0.16^{NS}	0.17^{NS}	0.18^{NS}	0.16^{NS}	0.16^{NS}

3.3.3 High yielding lines presented different strategies to build grain yield

Lines in the high yielding group (higher quartile for grain yield within the population) presented different strategies to achieve higher productivity, based on the traits with stronger correlation to yield (Table 3.1). Some lines relied on increased early vigour and green mass accumulation with lower photosynthetic rates. Others relied on the re-allocation of pre-stored biomass to the grain or in the maintenance of carbon uptake levels in the flag leaf during grain filling, for instance.

The highest yielding line (223) in the population in 2015 presented a higher canopy cover and longevity, and higher biomass accumulation at PA, despite lower photosynthesis per leaf area at BT and PA (Fig. 3.4). Line 246 had lower canopy cover and longevity and intermediate values of accumulated biomass at PA, but higher values for photosynthesis per leaf area at BT and PA. Line 399 presented intermediate values for canopy cover and longevity, biomass accumulated at PA and photosynthesis at BT and PA. Although there is no significant correlation between BT and PA photosynthesis difference to yield, line 140, which kept the same levels of photosynthesis from BT to PA, reached the second highest yield in the

population, suggesting the importance of flag leaf photosynthesis maintenance to sustain grain filling.

The two parents of the mapping population presented intermediate yields and different patterns for the analysed traits (Fig. 3.4). The photosynthesis patterns were coincident to the observed during the preliminary experiments that led to selection of the mapping population with Parent 1 presenting higher photosynthesis values than Parent 2 (Fig. 3.1 and Fig. 3.4). The correlations between yield and multiple traits and their variation over the season, as well as the diverse strategies of achieving high yields, reinforce the complexity of grain yield and the need for an integrated approach for its understanding and prediction.

3.3.4 Different modelling methods presented different correlations between predicted and measured yield

Two modelling strategies were used to predict grain yield using the measured traits. The first approach, for the 2015 season, used a linear mixed model with 13 terms: harvest index, height (SE), height (PA), NDVI (SE), stay green, stem thickness (PA), WI (PA), AQ100 (PA), ETR (PA) Flag leaf SPAD (BT), height (BT), highest cover and PRI (BT). The model was able to explain 57% of yield variability (R^2 = 0.57; r = 0.76, $p < 0.001$), which is relatively low considering the amount of traits measured. The high collinearity between traits (Fig. 3.3) and the small variability of some of the traits within the population can explain the relatively poor fitting of this model.

The second approach was based on the theoretical definition of yield and used: accumulated radiation, reflectance and leaf cover indices, and harvest index to predict biomass accumulation and yield (Fig. 3.5). Modelled grain yield and measured grain yield had a correlation of 0.59 (R^2 = 0.34, $p < 0.001$) and 0.64 (R^2 = 0.40, $p < 0.001$) for 2015 and 2016 (Fig. 3.6), respectively. For 2016, biomass accumulation was responsive to high NDVI and LUE, until spike emergence (Z5.7). Maintenance of NDVI and an increase in WC, as well as higher radiation levels, kept the biomass accumulation rate until Z7.5, even with a decreased

LUE. From Z7.5, biomass accumulation rate decreased with the decrease of NDVI, WC and LUE (Fig. 3.5).

The modelled yield for 2015 presented, in general, lower values than in 2016, even though the measured yield was relatively higher for 2015 (Fig. 3.6). It may be related to the lower number of measurements made over the season in 2015 (3) in comparison to 2016 (10) that could have resulted in a bigger uncertainty for the model. It may also explain the reduced fitness of the model for 2015.

	Leaf and Canopy			Biomass			Reflectance								Gas-exchange			Yield
Lines	Early vigour	Acc. green area	Stay green	Leaves weight (PA)	Stems weight (PA)	Total biomass (PA)	NDVI (SE)	NDVI (BT)	PRI x 100 (SE)	PRI x 100 (BT)	PRI x 100 (PA)	WI (SE)	WI (BT)	WI (PA)	Flag Leaf A (BT)	Flag Leaf A (PA)	Flag Leaf A (PA-BT)	Yield (85% DM)
Correlation to Yield	0.27	0.43	0.26	0.25	0.23	0.24	0.49	0.28	0.48	0.44	0.35	-0.36	-0.21	-0.42	0.30	0.19	NS	-
Line 223	3.2	11.2	3.2	29	79	139	0.92	0.95	-1.5	0.2	-2.1	0.93	0.90	0.79	17.0	15.2	-1.8	10.7
Line 140	3.3	11.1	3.4	24	56	110	0.92	0.94	-0.4	0.5	-1.8	0.94	0.92	0.79	17.5	17.5	0.0	10.7
Line 315	3.1	11.2	3.0	29	68	129	0.92	0.95	0.0	0.2	-1.5	0.93	0.91	0.78	17.5	14.7	-2.9	10.6
Line 022	2.6	10.6	2.5	22	77	144	0.91	0.95	-0.8	0.7	-2.1	0.94	0.90	0.79	17.5	16.2	-1.4	10.5
Line 331	2.9	10.9	2.9	24	63	124	0.91	0.96	-1.2	0.9	-2.6	0.95	0.91	0.79	18.1	16.6	-1.5	10.4
Line 405	3.1	10.9	3.0	26	72	133	0.91	0.95	-1.3	0.5	-0.9	0.95	0.92	0.79	18.2	15.7	-2.6	10.3
Line 367	2.8	11.3	2.9	27	67	132	0.93	0.96	0.1	1.0	-1.7	0.93	0.89	0.78	17.7	15.6	-2.2	10.2
Line 348	3.5	11.2	3.4	23	68	122	0.92	0.96	-0.3	0.6	-1.5	0.94	0.87	0.79	18.6	16.6	-2.1	10.2
Line 126	3.2	10.9	3.1	25	67	125	0.92	0.94	-0.4	0.6	-1.3	0.94	0.93	0.78	18.4	16.3	-2.2	10.2
Line 395	2.6	11.1	2.7	23	68	130	0.93	0.95	-0.2	0.9	-1.1	0.93	0.89	0.78	19.2	16.7	-2.5	10.2
Line 143	3.0	10.6	2.9	26	73	134	0.91	0.96	-1.7	0.9	-1.2	0.95	0.90	0.79	18.0	17.0	-1.0	10.2
Line 210	3.2	11.1	3.2	28	74	138	0.91	0.96	-1.3	0.6	-1.9	0.94	0.90	0.78	17.6	14.3	-3.3	10.2
Line 394	2.3	10.6	2.4	23	74	147	0.93	0.96	-0.4	1.1	-2.3	0.93	0.87	0.79	18.4	14.8	-3.6	10.2
Line 364	2.8	11.0	2.9	26	76	143	0.92	0.96	-0.2	1.0	-1.5	0.94	0.87	0.80	17.5	14.3	-3.2	10.2
Line 382	2.6	11.0	2.6	25	72	140	0.92	0.95	-1.0	-0.4	-2.7	0.92	0.89	0.79	18.3	14.0	-4.3	10.1
Line 246	2.4	10.5	2.4	21	68	133	0.90	0.94	-2.1	0.5	-1.2	0.94	0.88	0.79	19.4	17.8	-1.5	10.1
Line 031	2.7	10.9	2.7	22	71	137	0.91	0.95	-1.4	0.7	-1.9	0.94	0.89	0.80	19.0	16.3	-2.7	10.1
Line 366	2.6	11.0	2.6	23	68	133	0.92	0.94	-1.6	0.0	-2.2	0.92	0.91	0.78	17.2	15.0	-2.2	10.1
Line 226	3.1	11.3	3.1	23	65	117	0.92	0.95	-0.5	0.7	-2.2	0.93	0.89	0.79	18.0	15.9	-2.1	10.1
Line 239	2.7	10.8	2.8	21	62	116	0.91	0.95	-1.9	0.5	-2.2	0.95	0.91	0.80	18.8	16.2	-2.5	10.0
Line 208	2.8	11.3	2.9	20	65	118	0.92	0.95	-0.5	0.6	-1.8	0.94	0.92	0.81	18.6	17.6	-1.0	10.0
Line 034	2.8	10.4	2.8	21	63	117	0.89	0.95	-2.2	1.2	-1.3	0.95	0.90	0.77	17.7	17.1	-0.6	10.0
Line 060	2.7	10.8	2.8	21	77	138	0.90	0.96	-1.4	0.7	-1.9	0.94	0.87	0.80	17.1	14.3	-2.8	10.0
Line 036	2.8	11.1	2.7	26	80	151	0.92	0.95	-1.3	-0.8	-2.5	0.93	0.92	0.80	17.0	16.0	-0.9	10.0
Line 412	3.0	10.7	2.9	26	78	138	0.92	0.96	-0.5	1.1	-0.9	0.95	0.89	0.78	19.0	16.1	-2.9	10.0
Line 347	2.7	10.0	2.7	21	64	116	0.89	0.94	-2.8	1.2	-1.0	0.95	0.90	0.79	18.5	15.7	-2.9	10.0
Line 184	2.5	10.7	2.4	22	75	144	0.90	0.94	-1.3	0.9	-1.2	0.94	0.89	0.78	19.9	17.2	-2.7	10.0
Line 391	2.5	10.9	2.3	19	61	119	0.92	0.94	-1.4	0.5	-2.3	0.93	0.89	0.79	18.9	15.7	-3.2	10.0
Line 399	2.6	10.7	2.8	25	73	140	0.92	0.96	-0.2	0.9	-2.0	0.94	0.90	0.79	18.3	16.0	-2.2	10.0
Line 158	3.1	10.9	3.0	27	77	135	0.91	0.95	-1.0	0.4	-1.2	0.94	0.90	0.79	18.0	15.8	-2.1	10.0
Parent 1	2.8	10.8	2.8	25	66	130	0.92	0.96	0.1	0.5	-1.6	0.95	0.92	0.79	19.0	15.8	-3.2	9.7
Parent 2	2.5	10.4	2.5	20	71	130	0.88	0.96	-3.4	0.1	-3.5	0.95	0.89	0.80	16.9	14.1	-2.8	9.5

Figure 3.4 – Strategies for yield formation in high grain yielding lines of a double-haploid wheat mapping population grown in the UK in 2015. Traits grouped according to Leaf and Canopy, Biomass, Reflectance and Gas-exchange, measured at stem elongation (SE; Z3.2), booting (BT; Z4.5) and 7 days post-anthesis (PA; 7 days after Z6.5). NDVI, Normalized Difference Vegetation Index; PRI, Photochemical Reflectance Index; WI, Water Index; A, flag leaf net photosynthesis average for all light levels at BT and PA in µmols CO_2 m^{-2} s^{-1}. Leaf and canopy and reflectance traits are dimensionless. Yield in t ha^{-1}. Biomass traits in g. For each trait, values were ranked according to the trait value quartiles: green arrow (upper quartile), yellow arrow (two intermediate quartiles) and red arrow (lower quartile).

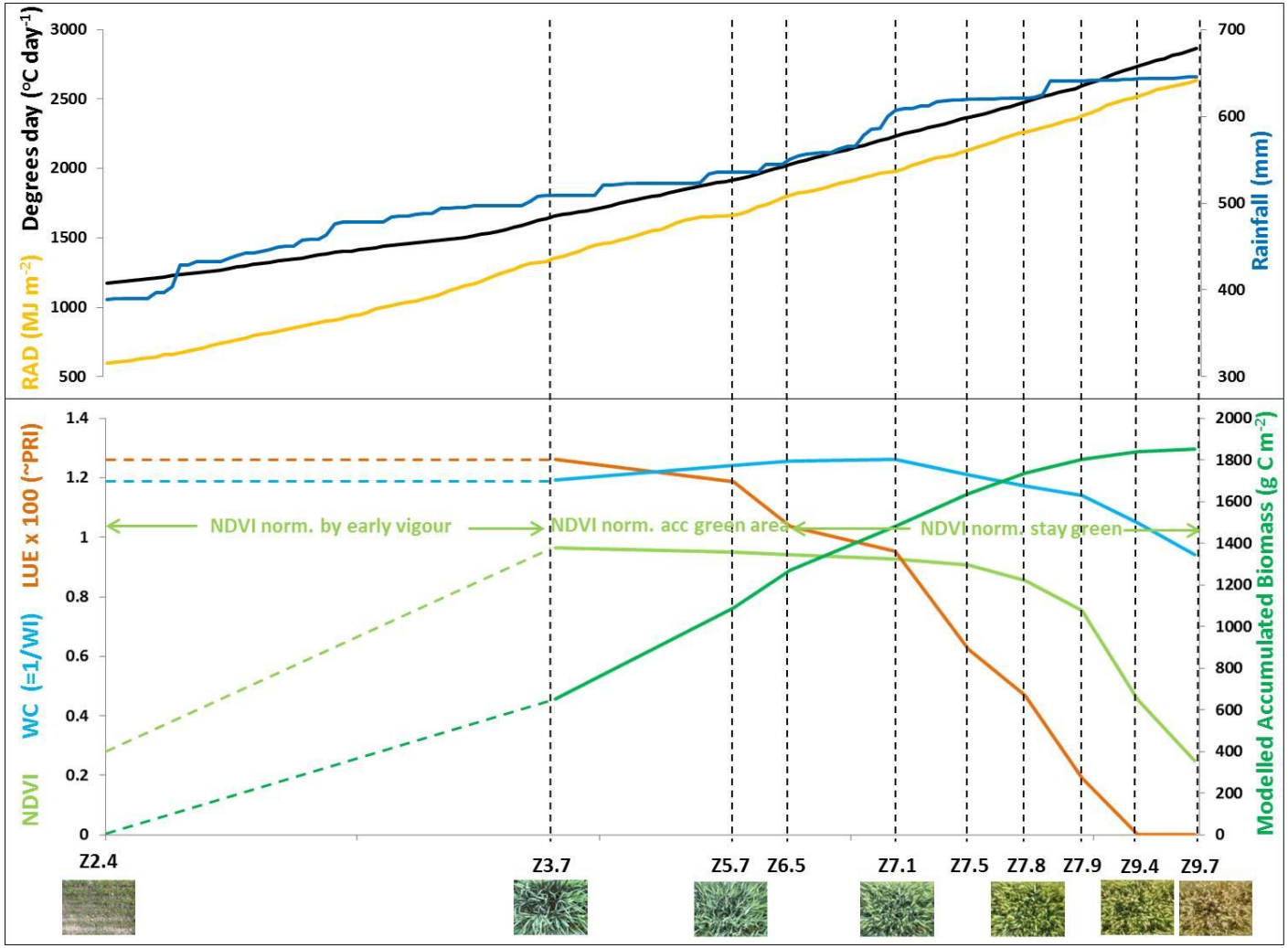

Figure 3.5 – Biomass accumulation response modelled according to RAD, NDVI, WC and LUE for a double-haploid wheat mapping population, grown in the UK in 2016. RAD, sunlight radiation; Rainfall, accumulated rainfall; Degrees day, accumulated degrees day; NDVI, Normalized Difference Vegetation Index; PRI, Photochemical Reflectance Index; WI, Water Index; WC, Water Content; LUE, Light Use Efficiency. Z# corresponds to the developmental stages when reflectance measurements were taken (black vertical dashed lines). NDVI, PRI and WI are dimensionless. Dashed lines from Z2.4 to Z3.7 represent estimated values. Images at the bottom show a representative plot at the indicated developmental stage. Modelled biomass was calculated as described by Formula 2.

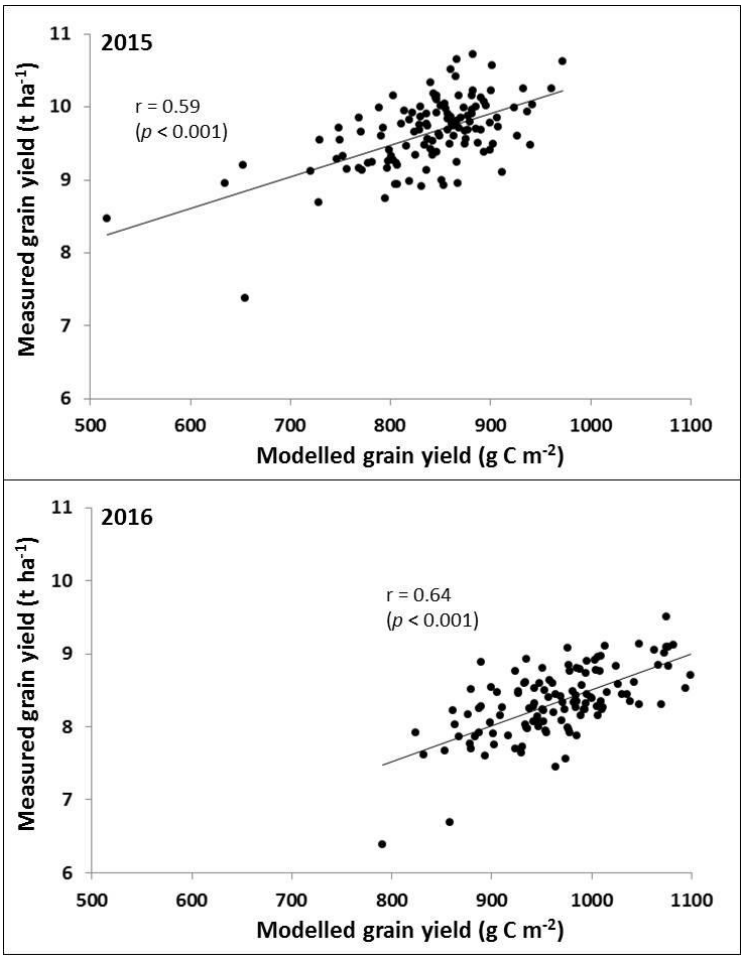

Figure 3.6 – Correlation between modelled and measured grain yield for a double-haploid wheat mapping population grown in the UK in 2015 and 2016. Modelled yield is based on the theoretical yield definition (Formula 1.1).

3.4 DISCUSSION

A double-haploid wheat mapping population was grown at the Rothamsted farm, in the UK, for two consecutive seasons to identify potential grain yield drivers. Grain yield correlated with multiple traits at different levels, from canopy to gas-exchange, with variation along the crop development. Different strategies to reach higher yields were observed in the population. The results are discussed in terms of the multiple correlations between traits and their influence to yield. Potential limitations and improvement to the yield prediction models are also discussed.

3.4.1 Photosynthesis and yield

The selection of a population generated from parents with contrasting photosynthetic characteristics enabled exploiting variability in this trait to understand its influence over yield. For the 2015 season data, the average variability (σ/μ) of photosynthesis in the population was around 5.2% at BT and PA. Other gas-exchange traits presented bigger or smaller variability than photosynthesis, as stomatal conductance (12%) and maximum quantum efficiency of PSII in light adapted leaves (Fv'/Fm') (1.7%).

For the 2015 data, photosynthesis was correlated to yield at BT and PA, with bigger correlations for the first. Correlation between photosynthesis and yield were previously reported in wheat (Sylvester-Bradley, 1990; Sayre *et al*., 1997; Gutierrez-Rodriguez *et al*., 2000; Condon *et al*., 2008). Specific correlations between pre-anthesis flag leaf photosynthesis and grain yield in wheat were recently also reported by Gaju *et al*. (2016) and Carmo-Silva *et al*. (submitted). Flag leaf photosynthesis at BT was generally higher than at PA as also reported by Carmo-Silva *et al*. (submitted) in wheat. The decrease from BT to PA was around 12% at the light levels of 1800, 1000, 500, 8% at 250 and 3% at 100 μmol photons m^{-2} s^{-1}. There was interaction between lines and development stages for photosynthesis, suggesting different patterns for the reduction in photosynthesis from BT to PA between the lines, although the difference was not significantly correlated to yield.

The photosynthesis decay from high to low light levels was similar between lines and there was no correlation between the reduction pattern and yield (Fig. 3.7). Rates of photosynthesis were highly correlated to stomatal conductance at light levels above 250 but the correlation was weaker for the lower light level (Table 3.2). These results agree with the notion that stomatal conductance limits photosynthesis at a range of light levels and biochemical limitations are relatively more prominent as the light reaches low levels (Lawson *et al*. 2012).

The broad-sense heritability estimated for photosynthesis measured at the various light levels was on average 0.51. A similar value has been reported by Ratajczak and Górny (2012). The heritability estimate for photosynthesis decreased with light level (Table 3.1), suggesting a smaller effect of the genetic background in defining the phenotype observed at the lower light level (100 µmol photons m^{-2} s^{-1}). This might explain the lower correlation between photosynthesis and yield at lower light levels, as grain yield was more linked to the genetic background with a broad-sense heritability of 0.73.

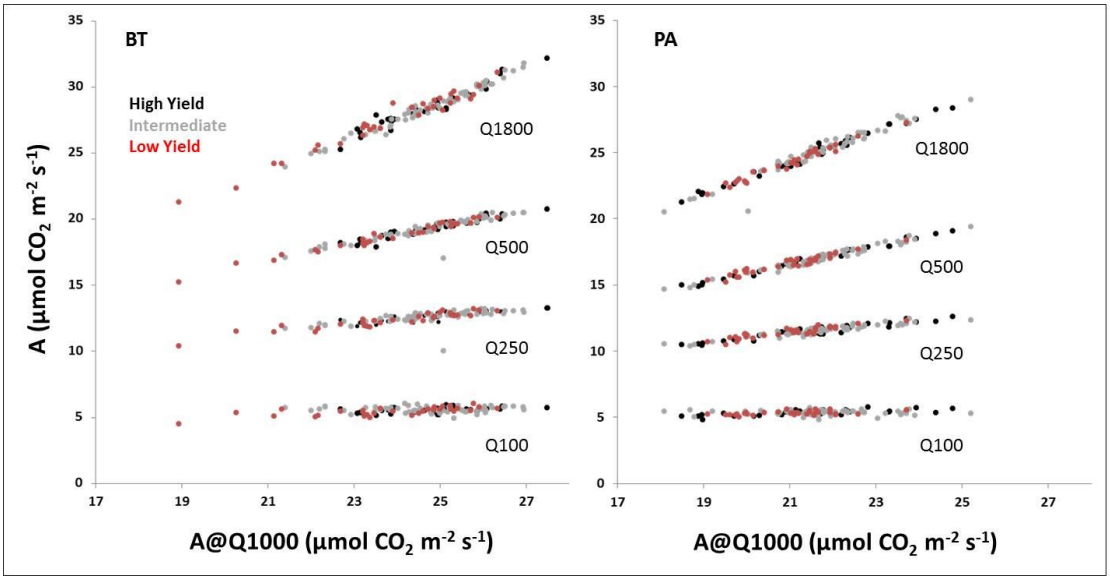

Figure 3.7 – Biplot of flag leaf net photosynthesis (*A*) reduction according to light levels (Q, photosynthetic active radiation in µmol photons m^{-2} s^{-1}) at booting (BT) and 7 days post-anthesis (PA) for a double-haploid wheat mapping population, grown in UK in 2015. Black circles represent higher yielding lines (top quartile); grey circles intermediate yielding lines (second and third quartile); red circles lower yielding lines (bottom quartile).

3.4.2 Canopy vs leaf photosynthesis

Considering its correlation to yield and natural variability, photosynthesis is now a major target trait in breeding for higher yields (Parry and Hawkesford, 2012). Phenotyping leaf gas-exchange traits in detail for large populations under field-grown conditions can be challenging, given the length of time taken to perform the measurements and the plant responses to climatic conditions and the circadian clock. The methodology proposed by Driever *et al*. (2014) has contributed to standardizing measuring conditions and allowed a more reliable method to assess gas-exchange traits in field grown plants. Despite the

improved methodology, measuring gas-exchange at the leaf level, using infra-red gas analysers (IRGAs), is a time-consuming process. Reflectance indices have been proposed as high-throughput estimators of photosynthetic traits at the top of the canopy. For the double-haploid population, flag leaf photosynthesis and PRI presented an average positive correlation at BT ($r = 0.46$, $p < 0.001$) and PA ($r = 0.20$, $p < 0.01$) and *gs* and WI were negatively correlated at PA ($r = -0.19$, $p < 0.05$). Correlations between PRI and yield were higher than the correlations between flag leaf photosynthesis and yield (Table 3.1). As reviewed by Richards (2000), measurements of canopy photosynthesis tend to correlate stronger to yield than leaf photosynthesis.

PRI estimates reflect a response from the top layers of the canopy, which interact with varying light intensities. Flag leaf photosynthesis represents a small leaf portion in specific conditions of light, explaining the relatively low correlation values between the two traits and the difficulties in modelling one based on the other, as also observed by Wu *et al*. (2010) and Peñuelas *et al*. (2011). Flag leaf photosynthesis characteristics cannot be extended to the entire canopy as PRI cannot be used to understand specific changes in the carbon assimilation behaviour at leaf level. Both, gas-exchange and reflectance traits, are valuable to the understanding of plant behaviour and yield formation, at different levels, and should be used together in breeding programs.

3.4.3 Modelling yield using reflectance traits

A model based on the theoretical definition of yield was used to predict biomass production and yield based on canopy reflectance traits. For the 2016 data, the final fitting of the model presented a correlation of 0.64 ($R^2 = 0.40$, $p < 0.001$) between modelled and measured grain yield. Multiple attempts of modelling were made from most simple models with the further addition of traits. The use of the leaf cover indices correction to NDVI improved the model

fitting, mainly considering the lack of measurements in the initial developmental stages where assumptions were made.

Possible sources of error that could have decreased the fitness of the model can be related to the methodology used. NDVI, PRI and WI are influenced by the canopy albedo that can have diurnal and seasonal variations in wheat, as reported by Zhang *et al*. (2013) and Süß *et al*. (2016). PRI can also present rapid variation (from 10 to 30 minutes response) according to weather conditions, as described by Gammon *et al*. (1997) and as observed in the current study by the high values of PRI variability (Table 3.1). Starting reflectance measurements at early developmental stages, measuring at similar weather conditions and time of day, and reducing the time to cover the whole experiment may improve model fitting by reducing experimental error. The use of high-throughput methods as phenotyping platforms (Virlet *et al*., 2017) or drones with multi-spectral cameras could improve the data acquisition and consequently the modelling.

3.5 CONCLUSIONS AND FINAL COMMENTS

Grain yield was strongly correlated to multiple traits related to sunlight interception and conversion, and to biomass allocation, that varied along the crop growth cycle. Early vigour, canopy cover and longevity, biomass accumulation in stem and leaves at post-anthesis, canopy reflectance indices and photosynthesis at pre and post-anthesis were correlated to grain yield. High yielding lines presented different strategies, based in the traits mentioned above, to achieve higher productivity. Due to the complexity of yield formation and collinearity of the measured traits, mathematical models combining traits could not fully predict grain yield. In summary, the measured traits were correlated to yield and were heritable, showing potential to be used as targets for breeding for high yield. An integrated analysis of their interactions would likely improve the understanding and prediction of yield formation.

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CHAPTER 4

EVALUATION OF EXPRESSION OF THE CYANOBACTERIAL ictB *GENE TO IMPROVE PHYSIOLOGICAL AND YIELD TRAITS IN WHEAT*

COLLABORATORS' CONTRIBUTIONS

The author and Elizabete Carmo-Silva designed the research. The author and Rhiannon Page performed research and analysed the data. The author wrote the manuscript.

ABSTRACT

The efficiency of photosynthesis is variable between photosynthetic organisms, but is generally low. Cyanobacteria, C4 and CAM plants are usually more efficient in photosynthesis than C3 plants mainly due to their capacity to concentrate CO_2 to the Rubisco site, limiting RuBP oxygenation and favouring carbon fixation over photorespiration. The cyanobacterial *ictB* gene is associated with HCO_3^- concentration in cyanobacteria and to improvements in photosynthetic rate and biomass production in higher plants. The focus of this study was to assess the effect of *ictB* expression in wheat, a C3 crop. Transformed wheat plants with the insertion of the *ictB* gene were analysed in glasshouse experiments for their gene copy number and *ictB* expression, physiological and yield traits, in comparison to control plants. Gene copy number and *ictB* expression varied between the transgenic lines but were not correlated to each other. No improvement was observed for physiological or yield traits in transgenic wheat compared to the control plants. Unfavourable experimental conditions could have led to a loss of the expected ictB phenotype.

4.1 INTRODUCTION

Wheat is a C3 plant since the first stable product of the internal fixation of CO_2 is a 3-carbon molecule. The 3-carbon product, 3-PGA (3-phosphoglycerate), is formed in the Calvin-Benson

cycle by the carboxylation of RuBP (ribulose 1,5-biphosphate) catalysed by the enzyme Rubisco (ribulose 1,5-biphosphate carboxylase/oxygenase). As the name indicates, this enzyme can catalyse both the carboxylation and oxygenation of RuBP. Photosynthesis is the process that involves the carboxylation of RuBP by Rubisco, using CO_2; photorespiration is the process that recycles the compounds generated by the oxygenation of RuBP by Rubisco (Taiz *et al*., 2014).

While carboxylation of RuBP through photosynthesis is the primordial process in plants correlated to biomass production and accumulation (Long *et al*., 2006), the oxygenation of RuBP creates toxic compounds (glycolate). The recycling of glycolate through photorespiration decreases the resource (energy, nutrient and water) use efficiency of the plant through the loss of pre-fixed CO_2 (Carmo-Silva *et al*., 2015). According to Walker *et al*. (2016), in the current climatic conditions, the losses in wheat yields due to photorespiration are around 20%.

Under conditions of mild drought and heat stress, the internal ratio of CO_2/O_2 concentrations can be decreased due to a stomatal conductance limitation to CO_2 diffusion from the atmosphere, creating conditions to increase photorespiratory activity (von Caemmerer, 2010; Kangasjarvi *et al*., 2012). Although the wheat isoform of Rubisco presents relatively high carboxylation efficiency when compared to other species (Orr *et al*., 2016), including its relatives (Prins *et al*., 2016) and other crops (Hermida-Carrera *et al*., 2016), increasing CO_2 concentration at the Rubisco site could help to achieve higher wheat yields in predicted future meteorological scenarios, contributing to food security (Ainsworth & Ort, 2010).

C4 and CAM plants (Keeley and Rundel, 2003) as well as photosynthetic algae (Giordano *et al*., 2005; Moroney & Inalvez, 2007) and cyanobacteria (Badger *et al*., 2002; Price *et al*., 2008) evolved carbon concentrating mechanisms that mitigate the effects of the low efficiency and the dual-reaction nature of Rubisco (Raven *et al*., 2008). As reviewed by Parry

et al. (2011; 2013), the use of CCMs in C3 plants could be beneficial in multiple aspects related to boosting the concentration of CO_2 at the Rubisco site, leading to an increased CO_2/O_2 ratio and a decrease in photorespiration, increasing the efficiency of C3-photosynthesis. The implementation of C4 and CAM pathways in C3 plants is being studied in crops such as rice (http://c4rice.irri.org/) and poplar (http://cambiodesign.org) where the major challenges that have been faced are the anatomical adaptations and the requirements for multi-gene transfer. The use of specific CCMs from cyanobacteria appears a simpler and more straight-forward option, with fewer genes involved and no need for anatomical adaptations (Hanson *et al*., 2016). Price *et al*. (2011) concludes that including specific CCMs from cyanobacteria into C3 plants could improve photosynthetic performance and water-use efficiency, and McGrath and Long (2014) estimate it could result in an increase in yield in the order of 36 to 60%.

One of the genes apparently involved in the carbon concentration mechanism in cyanobacteria is *ictB* (inorganic carbon transporter B). Bonfil *et al*. (1998) originally identified the ORF467 (open reading frame of 467 amino acids) in a high-CO_2-requiring mutant of *Synechococcus elongatus* strain PCC 7942. In that research wild-type *S. elongatus* was compared to mutants that had the gene encoding ictB knocked down. By comparing the CO_2 and HCO_3^- uptake by the wild-type and mutants in high and low CO_2 conditions, they concluded that the ORF467 region (*ictB*) was related to a Na^+-dependent transporter of HCO_3^-. The hypothesis was reinforced by the fact that the ictB amino acid sequence was homologous to several transport-related proteins from various organisms. Omata *et al*. (1999) highlighted that there are many HCO_3^- transporters and the silencing of one of them would be unlikely to produce a dramatic effect, as reported by Bonfil *et al*. (1998). Since the inactivation of ictB caused such a dramatic reduction on HCO_3^- transport, it would be more likely to have a regulatory role instead of a purely transporter role.

Other researchers highlighted the incapacity to generate a fully segregated *ictB* mutant in *S. elongates* PCC 7942 (Price *et al*., 2002), or a mutant for the homologous gene *Slr1515* in *Synechocystis sp.* PCC 6803 (Shibata *et al*., 2002), hypothesising that the gene knockout would be lethal. Amoroso *et al*. (2003) showed that increased levels of the ictB protein in *S. elongatus* PCC 7942 were correlated to the capability of rapid induction of the high affinity HCO_3^- uptake system in the presence of NA^+. Xu *et al*. (2008) showed that there are only 5 inorganic carbon sequestration systems in *Synechocystis sp.* PCC 6803, and the gene homologous to the *ictB* is not one of them.

Price *et al*. (2012) referred to the findings mentioned above as 'the enigma of the *ictB*' and added that, although there is evidence of increasing HCO_3^- concentration and positive effects in crops, the function of *ictB* remains unknown, but it is clearly not a HCO_3^- transporter. They also highlighted that *ictB* presence is highly conserved in cyanobacterial genomes, suggesting an essential role. *ictB* encodes a membrane protein and the possibility that it may be part of an endoplasmic reticulum stress signalling pathway has been raised. Overexpression of a hydrophobic membrane protein such as ictB could lead to an up-regulation of drought resistance transcription factors, giving resistance to water stress and changing CO_2 transfer resistance at leaf level (Price *et al*., 2012).

Even though the *ictB* function is not well known, it has been genetically engineered into in a range of plants due to its promising effects on carbon concentration in cyanobacteria. The gene has been inserted into the model plants tobacco (Lieman-Hurwitz *et al*., 2003; Piatek, 2015; Simkin *et al*., 2015) and *Arabidopsis* (Lieman-Hurwitz *et al*., 2003), and the crops soybean (Hay, 2012; Piatek, 2015; Hay *et al*., 2017) and rice (Gong *et al*., 2015).

Improvements in photosynthetic rates in plants expressing the *ictB* gene, when compared to wild-type controls, were reported in *Arabidopsis* and tobacco (Lieman-Hurwitz *et al*., 2003), soybean (10% increase in glasshouse-grown and 15% in field-grown; Hay, 2012; Hay *et al*.,

2017), tobacco (19% increase; Simkin *et al*., 2015) and rice (15% increase; Gong *et al*., 2015). Conversely, Piatek (2015) reported no difference in photosynthetic rates between *ictB* and wild-type soybean plants growing in glasshouse and field conditions.

In terms of productivity, increases in biomass were reported in *Arabidopsis* (Lieman-Hurwitz *et al*., 2003), soybean (biomass and gran yield) (Hay, 2012; Hay *et al*., 2017) and tobacco (Simkin *et al*., 2015). On the other hand, no improvement was observed in productivity for tobacco (Lieman-Hurwitz *et al*., 2003), soybean (Piatek, 2015) and rice (Gong *et al*., 2015).

The goal of this research was to test the hypothesis that expressing the *ictB* gene, associated with HCO_3^- accumulation in cyanobacteria, would enhance photosynthesis and biomass production in wheat. An evaluation of the *ictB* gene presence and expression and its correlation to gas-exchange traits and wheat productivity was conducted for two genetically-modified plant generations (T1 and T2), to analyse the possible effects of *ictB* expression in this important C3 cereal crop.

4.2 MATERIAL AND METHODS

4.2.1 Construct

A construct to express *ictB* in chloroplasts of leaves of *Triticum aestivum* (Fig. 4.1) was designed and cloned by Dr Steven Driever in the research group of Prof Christine Raines at the University of Essex as part of a CIRC (Crop Improvement Research Club) research project. The monocot-codon-optimized sequence of *ictB* from cyanobacterium *Synechococcus elongatus* PCC 7942 (Bonfil *et al*., 1998) was cloned behind the ubiquitin promoter which was fused to a *Brachypodium distachyon* SBPase (sedoheptulose-1,7-bisphosphatase) chloroplast transit peptide. The gene was followed by the NOS terminator. The original cloning vector with the ubiquitin promoter and NOS terminator was provided by Dr Alison Huttly at Rothamsted and the total size of the final ictB vector was 6899 bp.

Figure 4.1 – Schematic representation of the *ictB* construct.

4.2.2 Generation of wheat plants expressing ictB

Transgenic wheat plants expressing *ictB* were produced by particle bombardment of immature embryos of wheat (Sparks & Jones, 2014), using cultivar Cadenza as background. A particle bombardment machine (Bio-Rad PDS-1000/He, Bio-Rad, Hercules, USA) was used to deliver gold particles coated with the construct to express the gene of interest and the selectable marker gene bar (Ubiquitin + bar + NOS) at 650 psi rupture pressure into immature embryos. The constructs were bombarded in a 1.5:1 equimolar ratio (gene of interest : selectable marker) to skew for the gene of interest and ensure that any surviving selection would also contain this gene. This work was carried out by Mandy Riley and Angela Doherty at the Rothamsted Research Cereal Transformation Group.

Transgenic plants were regenerated *via* somatic embryogenesis and those resistant to glufosinate were potted into soil and analysed by PCR to confirm presence of the gene of interest using the following primers: scr_pRRes-ictB-F (TTTAGCCCTGCCTTCATACG) and scr_pRRes-ictB-R (GATGCAAGAAACTGCTGTGG). The PCR conditions were: denaturing at 94°C for 25 sec, annealing at 60°C for 25 sec and extension at 72°C for 30 sec with a product of 411 base pairs. Positive plants for gene presence (T0 generation) were grown to produce seeds that were used for the subsequent T1 experiment. This work was carried out by Caroline Sparks at Rothamsted Research.

4.2.3 T1 Experiment

4.2.3.1 Plant growth

The T1 experiment was carried out at Rothamsted Research, Harpenden, UK, between September 2014 and February 2015. Seventeen transgenic lines (12 seeds of each) and 2 negative controls (wild-type Cadenza, and plants resulting from bombardment with gold particles without plasmid; 16 seeds of each) were first sown in vermiculite in 48-well plastic trays (0.15 L per well). The initial glasshouse conditions were: a day temperature of 18°C and a night temperature of 15°C; day length of 16 hours. Artificial light was supplemented by banks of Son T 400W sodium lamps (Osram Ltd., Langley, UK) when natural light conditions decreased to below 500 µmol photons m^{-2} s^{-1} inside the glass-house. At Zadoks 2.3 (Zadoks, 1974), plants were screened for gene presence and control and positive transgenic plants were re-potted to 2.5 L pots. The compost composition was: 75% medium grade peat, 12% screened sterilised loam, 3% medium grade vermiculite and 10% 5 mm screened, lime-free grit with added 500 g m^{-3} of nutrient mix (8.5% NH_4, 5.5% NO_3, 16% P_2O_5, 18% K_2O, 0.8% MgO, 0.03% Bo, 0.2% Mo, 0.15% Cu, 0.16% Mn, 0.04% Zn, 0.09% Fe), 3.5 kg m^{-3} of osmocote (8.4% NH_4, 7.6% NO_3, 11% P_2O_5, 11% K_2O, 3% MgO, 0.02% Bo, 0.02% Mo, 0.047% Cu, 0.06% Mn, 0.015% Zn, 0.40% Fe), around 3 kg m^{-3} of lime (until pH 5.5 to 6) and 200 mL m^{-3} of Vitax Ultrawet wetting agent (Petersfield Products, Leicester, UK). After re-potting, the day temperature was changed to 20°C with night temperatures and day length kept as before. The experiment was set in a completely randomised design.

4.2.3.2 Gene presence investigation

The method used for DNA analysis was adapted from Van Deynze and Stoffel (2006). For each plant, a 5 cm long sample was harvested from the youngest fully expanded leaf, 21 days after sowing. Samples were collected into 96 deep-well plates (Life Technologies, Paisley, UK) and placed in a freeze-drier for 48 hours. They were ground using a TissueLyser (Retsch

MM200, TissueLyser, Qiagen, Manchester, UK) with two 5 mm ball bearings added to each well. The ground tissues were suspended in 600 µL of DNA Extraction buffer (100 mM Tris-KOH, pH 9.5, 1 M KCl, 10 mM EDTA, 7.5 g L^{-1} PVP-40 and 3.6 g L^{-1} $NaHSO_3$). The plates were incubated with shaking for 1 h at 65°C and centrifuged for 1 min at 1000 rpm to remove material from the lid. 200 µL of KAc (5 M potassium acetate, pH 5.8, 11.5% glacial (v/v) acetic acid) was added to each well and centrifuged for 10 min at 1000 rpm.

A volume of 300 µL of the supernatant was transferred into blocks containing 165 µL of chilled isopropanol (stored previously at -20°C) and left at room temperature for 10 min. DNA was pelleted by centrifugation for 10 min at 2500 rpm. The supernatant was poured off, 500 µL of 70% ethanol was added to each well. The plates were centrifuged for 5 min at 2500 rpm and the supernatant discarded. DNA pellets were dried at 40°C for 30 min before being re-suspended in 200 µL TER (10 mM Tris, 0.1 mM EDTA, with 0.05 mg mL^{-1} pancreatic RNase), and incubated at 50°C for 1 h. DNA concentration and purity in the samples was analysed by spectrophotometry using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA).

The gene presence was tested by PCR using *ictB* plasmid (Fig. 4.1) as positive control. PCR conditions were: a pre-cycle of denaturing at 94°C for 5 min, annealing at 60°C for 30 s and extension at 72°C for 25 s, followed by 30 cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 25 s, with a final extension step at 72°C for 10 min. PCR was completed in 20 µL reactions (as per manufacturer's instructions; GoTaq DNA Polymerase, Promega, Southampton, UK), with 4 µL of DNA. The oligo sequences (5' to 3') were: scr_*ictB*1-F (GCAACAACTTCCGGATCAAT) and scr_*ictB*1-R (CGGTCGATAGAGCACGGTAT) (Sigma-Aldrich, St Louis, USA).

The PCR products were run in a 1.2% agarose gel with 0.01% SYBR Safe DNA gel stain (Thermo Fisher Scientific, Waltham, USA) and DNA bands were detected with UV light by using the Gel Doc 200 (Bio Rad, Hercules, USA).

4.2.3.3 Gene expression

Samples consisting of 5 cm long pieces of flag leaf were collected into 1.5 mL tubes, frozen in liquid nitrogen and stored at -80°C. Samples were collected 4 to 5 hours after the beginning of the photoperiod. Each sample was then ground to a fine powder in liquid nitrogen, using mortars and pestles, and re-frozen in liquid nitrogen.

RNA was extracted using the NucleoSpin® RNA/Protein Purification Kit (Macherey-Nagel, Duren, Germany). RNA sample concentration and purity was analysed by spectrophotometry using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA). RNA samples were diluted to a 120 ng μL^{-1} concentration by adding sterilized milli-Q water.

cDNA was synthesised in a two-step reaction according to the protocol in the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). Initially, an incubation step, followed by reverse transcription reaction, was run for a 12 µL reaction (9 µL of RNA sample, plus 2 µL of sterilized milli-Q water and 1 µL of oligo (dT)) at 65°C for 5 min. The reaction volume was increased to 20 µL by adding 4 µL of 5X Reaction Buffer, 2 µL of 10 mM dNTP Mix, 1 µL of Revert Aid and 1 µL of sterilized milli-Q water. PCR was run at 42°C for 60 min followed by 10 min at 70°C. cDNA samples were diluted to 1:5 by adding 80 µL of sterilized milli-Q water to the 20 µL after the PCRs.

The RT-PCR was completed in 15 µL reactions: 4 µL of cDNA sample, 3.5 µL of primer mix (forward and reserve primers in a concentration of 2 µM each, Table 4.1), 7.5 µL of SensiFAST SYBR No-ROX Kit (Bioline, London, UK). RT-PCRs were run using a Bio Rad CFX machine and the data was downloaded and analysed using the Bio Rad CFX Manager software (Bio Rad, Hercules, USA). RT-PCR conditions were: initial enzyme activation at 95°C for 2 min, 40 cycles of a three-step amplification at 95°C for 10 s, 60°C for 10 s and 72°C for 15 s, followed by a melt curve at 65°C for 5 s and 100°C for 1 min. The *Ta2291* gene was used as a housekeeping gene (Paolacci *et al*., 2009).

4.2.4 T2 experiment

4.2.4.1 Plant selection for T2 experiment

The selection of the lines for the T2 experiment was done based on the *ictB* gene expression measured through RT-PCR for the T1 plants. Expression data was ranked from highest to lowest levels and the plants with a higher expression of *ictB* were selected (a total of 3 plants for each of the 6 lines with higher expression). This resulted in 18 transgenic plants from the T1 experiment chosen as seed donors for the T2 experiment, plus 9 negative control plants (3 wild-type Cadenza, and 6 gold controls bombarded without plasmid). The lines were named as: Cadenza (wild-type wheat cultivar), Gold (Cadenza plants bombarded with gold particles), Azygous (T2 plants descending from the T1 transgenic plants that lost the gene during segregation) and ictB-1, ictB-2, ictB-3, ictB-4, ictB-5, ictB-6 for the transgenic lines.

4.2.4.2 Plant Growth and experimental design

The T2 experiment was carried out at the Lancaster Environment Centre, Lancaster University, Lancaster, UK, between April and August 2016. Twelve seeds of each of the 27 selected plants (18 transgenic, plus 9 controls) were sown in 12-well plastic trays (0.175 L per well) with the compost described for the T1 experiment (a total of 324 seeds). The glasshouse conditions were: a day temperature of 20°C and a night temperature of 18°C; day length of 16 hours; for the first month of the experiment, lights were turned on when the natural light level decreased to 500 µmol photons m^{-2} s^{-1} with the threshold being changed to 200 µmol photons m^{-2} s^{-1} for the rest of the experiment to avoid excessive warming of the glasshouse. Supplementary light was provided by banks of 600W Plantastar sodium lamps (Osram Ltd., Langley, UK). All the transgenic plants that germinated and almost all the control plants were re-potted to 3 L pots using the same compost described before (a total of 300 plants). At the time of re-potting, roots could be seen coming out of the wells. Despite extreme care, it is possible that the root system could have been damaged during the

transplant procedure. Re-potted plants were arranged in a randomized block design in a total of 10 blocks of 30 plants each. The glasshouse benches were split into sides (1 and 2) and rows (1 to 12) and columns (1 to 20) to facilitate the analysis of spatial influence for the measured traits (Fig. 4.2). Blocks V and VI were not used in the analysis due to the non-uniform light conditions inside the blocks that could have affected plant growth generating biased results.

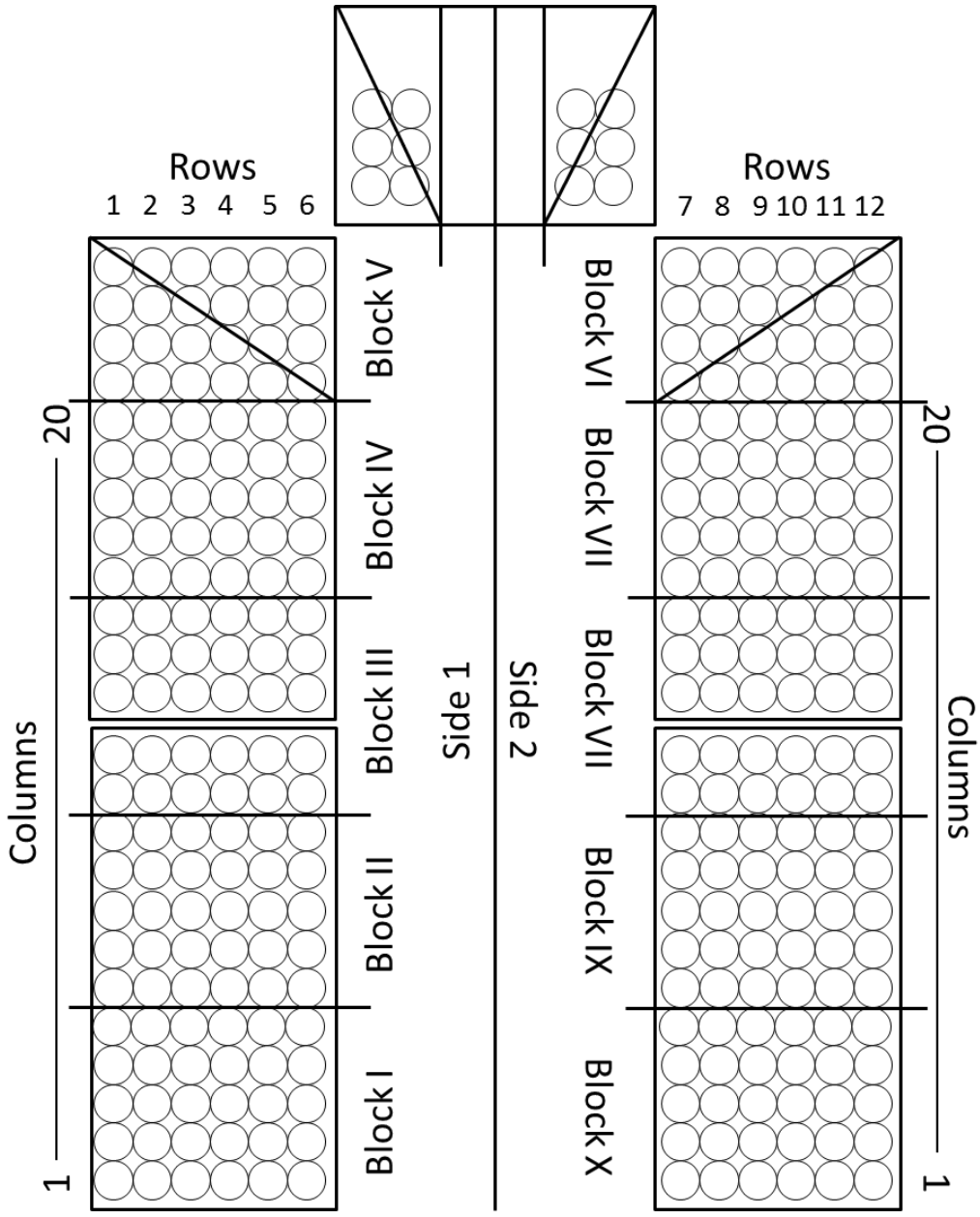

Figure 4.2 – Glasshouse layout and experimental design for T2 generation *ictB* transgenic wheat plants experiment.

4.2.4.3 Gene presence investigation

For the T2 experiment, leaf samples were collected 28 days after sowing. From the initial transgenic plants (grown from seeds of T1 transgenic plants), a number of them were negative for the *ictB* gene due to the loss of the gene during the segregation process. These plants were maintained in the experiment and used as an extra group of negative controls (Azygous). The methodology for gene presence analysis was the same as described for the T1 experiment.

4.2.4.4 Gene copy number and segregation

Analysis of *ictB* gene copy number was done for all the positive plants by IDna Genetics Ltd. (IDna Genetics Ltd., Norwich, UK). At Zadoks 4.0 (Zadoks, 1974), leaf samples were harvested in 1.2 ml round-well block strips (Qiagen, Manchester, UK) and sent to the company on dry ice. The segregation and homozygosity of the lines was investigated by calculating the percentage of positives (the number of plants with the *ictB* gene divided by the total number of plants tested of each line).

4.2.4.5 Gas-exchange measurements and flag leaf characteristics

For gas-exchange analysis, a subset of 119 plants (83 transgenic + 36 controls) was selected from the total 300 plants in the experiment. The selection was randomized, but organized to ensure there were 5 plants of each transgenic line (when available), and 12 plants of each of the controls (Cadenza, Gold and Azygous). For some of the transgenic lines the final number of transgenic plants was smaller than 5 and all the available plants were analysed. The selection accounted for the distribution of plants along the blocks.

Measurements were taken from flag leaves when the plants were around Zadoks 6.0 (spike completely emerged, no flowers visible) (Zadoks, 1974). A portable gas-exchange system was used with the fluorescence chamber (LI-COR 6400 and chamber 6400-40; LI-COR, Lincoln,

USA) and the multiphase flash method was adopted (Loriaux *et al*, 2013). Plants were measured at two ambient CO_2 concentrations of 400 and 200 µmol CO_2 μmol^{-1} air, with light intensity of 1500 µmol photons m^{-2} s^{-1}. As the plants reached the Zadoks 6.0 at different times, gas-exchange measurements were split on different days to ensure plants were analysed at the same developmental stage. After the central area of the leaf was clamped, flag leaf width was measured at both sides of the chamber and these values were used to correct the data for sample area, when the leaf was not wide enough to fully cover the chamber. Flag leaf length and maximum width were also determined using a ruler.

4.2.4.6 Gene expression

The same subset of plants selected for gas-exchange measurements was used for gene expression analysis. Flag leaf samples were collected under the same conditions as explained for T1 experiment, on the day after the gas-exchange measurements were taken. RNA extraction and cDNA regeneration were conducted as described for the T1 experiment.

The RT-PCR was completed in 15 µL reactions: 4 µL of cDNA sample, 3.5 µL of primer mix (forward and reserve primers in a concentration of 2 µM each), 7.5 µL of SYBR Green Precision Plus Master Mix (Primer Design, Southampton, UK). The Mx3005P qPCR System (Agylent Technologies, Santa Clara, USA) was used. RT-PCR conditions were: initial enzyme activation of 95°C for 2 min, 40 cycles of a two-step amplification with denaturation at 95°C for 15 s and annealing and data collection at 60°C for 1 minute, followed by a melt curve at 95°C for 1 min, 60°C for 30 s and 95°C for 30 s. Five housekeeping genes were tested for their stability and primer efficiency (Pfaffl, 2001): *Ta2291*, *Ta2776* and *Ta54227* (Paolacci *et al*., 2009), *Actin* (Giménez *et al*., 2010) and *Succinate dehydrogenase* (recommended by Peter Buchner, Rothamsted Research, unpublished data). The RT-PCRs were run using the *Ta2291* and *Succinate dehydrogenase*. Selection was based on their stability and primer efficiency in the pre-run tests. Data was downloaded and processed using the MxPro software (Agilent

Technologies, Santa Clara, USA). The design of the plates, use of positive and negative controls and calculations of gene expression were made according to Rieu & Powers (2009).

Table 4.1 – Sequences of primers used for RT-PCR.

Primers	5' - 3' sequence
ictB-F	AAGACAGCAGCAACAACTTC
ictB-R	TTGATAGAGGGGATAAACCAGG
Ta2291-F	GCTCTCCAACAACATTGCCAAC
Ta2291-R	GCTTCTGCCTGTCACATACGC
TaSuccDH-F	TTTGCTCTCCGTGGTGCCTTTGG
TaSuccDH-R	GAAGATGTGTAGCTCCTTGCTTGC
TaActin3-F	ATCTCGAAGGGYGAGTATGAYGAG
TaActin3-R	AGAAGACCCAGACAACTCGCAAC
Ta54227-F	CAAATACGCCATCAGGGAGAACATC
Ta54227-R	CGCTGCCGAAACCACGAGAC
Ta2776-F	CGATTCAGAGCAGCGTATTGTTG
Ta2776-R	AGTTGGTCGGGTCTCTTCTAAATG

4.2.4.7 Biomass measurements

Plants were harvested at ripening, Zadoks 9.2 (Zadoks, 1974). Spikes were hand-threshed and the chaff was put in paper bags with the rest of the plant and dried at 80°C until constant weight. Straw weight (at 100% dry matter) was determined. Total fresh grain weight was measured and an aliquot of around 4 g was collected, weighed and dried at 105°C until constant weight. Moisture was calculated and total fresh grain weight was converted to 100% dry mater grain weight. Above ground biomass (at 100% dry matter) was calculated by the sum of straw weight and grain weight, both at 100% dry matter.

4.2.4.8 Statistical analysis

The method of residual maximum likelihood (REML) was used to fit a linear mixed model to each measured trait to test for any statistically significant ($p < 0.05$) variation due to spatial trends over the sides, rows and columns of the glasshouse and the randomised block design. For gene expression and gas-exchange data there was no significance spatial variation and ANOVA for unbalanced designs was used to compare the treatments.

For the biomass and grain yield analyses, the following models were fit:

Total Above Ground Biomass = Constant + Plant_gene + Side.Row + Block + Row.Column + E, where Plant_gene and Side.Row are the fixed effects, Block is a linear random effect and Row.Column are both auto-regressive (AR1) random terms and E is the residual. Plant_gene refers to the treatments were Plant is the name of the line (Cadenza, Gold, Azygous or ictb-1 to ictB-6) and gene is the presence or absence of *ictB*.

Total Dried Grain Weight = Constant + Plant_gene + Side.Row + Block + E, where Plant_gene and Side.Row are the fixed effects, Block is a linear random effect and Row is an auto-regressive (AR1) random term and E is the residual.

The predicted means for each treatment were calculated from the final models and the Wald test was used to compare the treatments.

For the expression data, the Ct values and primer efficiency (Pfaffl, 2001) were used to calculate the relative quantity (RQ) of *ictB* expression. RQ and the normalized relative quantity (NRQ) for the two housekeeping genes was calculated according to Rieu and Powers (2009). Ct is an output of the RT-PCR and represents the number of cycles after which the fluorescence level exceeded the threshold fluorescence. To compare the treatments, ANOVA was applied to the values of $\log_2(1/NRQ)$. NRQ values can be used to inform the expression for different lines but cannot be statistically analysed through ANOVA to compare treatments. NRQ data are usually nonlinear and typically suffer from heterogeneity of variance across biological replicates. The use of log transformation accounts for these problems and allows ANOVA to be used to compare treatments (Rieu and Powers, 2009).

A matrix with the Pearson Product Moment (PPM) coefficients was calculated for all the traits measured to evaluate the correlations between each trait pair. All the statistical analyses were performed using the GenStat 17th Edition software (VSN International Ltd., Hemel Hampstead, UK).

4.3 RESULTS

The results presented in this session relate to the T2 experiment, unless otherwise stated.

4.3.1 Independent **ictB** *lines had gene insertions number from 1 to 16 and segregation ratios from 11 to 100%*

The number of *ictB* copies varied between 1 and 16 (Table 4.2). In diploid organisms, each gene is represented by two copies, thus odd *ictB* copy numbers will by definition correspond to heterozygous plants, whilst even copy numbers may represent homozygous plants. T1 plants, for which all the T2 plants had the same even number of *ictB* copies, are likely to be homozygous. In this way, for line ictB-1, all T2 plants, corresponding to 3 different T1 plants, had two *ictB* copies, representing one single insertion (in diploid state), and are likely to be homozygous. For line ictB-2, the T2 plants originating from T1 plants 34 and 40 had four *ictB* copies, i.e. two insertions, and are likely to be homozygous, the same being observed for line ictB-3 originated from plants 110 to 115, with 16 *ictB* copies, i.e. 8 insertions. Plants of line ictB-4 and -6 appeared to contain 1 to 2 *ictB* gene copies and were less likely to be homozygous. ictB-5 lines varied from 2 to 4 gene copies, i.e. 1 to 2 insertions.

Table 4.2 – *ictB* gene copy number and percentage of positive plants for gene presence.

Line	T0 code	T1 plant	*ictB* copy number	% of positive plants
ictB-1	R5P4a	129	2	77.8
ictB-1	R5P4a	131	2	60.0
ictB-1	R5P4a	132	2	100.0
ictB-2	R10P8c	190	4	80.0
ictB-2	R10P8c	191	2 to 4	75.0
ictB-2	R10P8c	192	4	100.0
ictB-3	R5P2	110	16	100.0
ictB-3	R5P2	111	8 to 16	11.1
ictB-3	R5P2	115	16	100.0
ictB-4	R9P1	58	1	40.0
ictB-4	R9P1	63	2	77.8
ictB-4	R9P1	64	2	100.0
ictB-5	R3P1	78	2 to 4	44.4
ictB-5	R3P1	81	2 to 4	50.0
ictB-5	R3P1	84	2 to 4	55.6
ictB-6	R6P1	34	1	90.0
ictB-6	R6P1	35	1	50.0
ictB-6	R6P1	40	1 to 2	44.4

4.3.2 ictB *gene expression was not correlated to the number of copies of the gene*

Control plants including wild-type, gold bombarded and azygous plants, showed no expression of *ictB* (Fig. 4.3). Transgenic plants from the various ictB lines presented significant ($p < 0.001$) expression of *ictB* when compared to the controls (Tables 4.3 and 4.4) and, of these, lines ictB-2, ictB-4 and ictB-5 had higher expression than the remainder (Table 4.4 and Fig. 4.3). The lines showing greatest expression did not correspond to the lines with greater *ictB* copy numbers, suggesting no association between gene copy number and expression levels (Fig. 4.3).

Table 4.3 – ANOVA table for *ictB* expression ($log_2(1/NRQ)$). df, degrees of freedom; ss, sum of squares; ms, mean square; F, calculated value for the F test; p value, probability to reject the null hypothesis. Block, factor for the block design; Groups, factor for the groups: Cadenza, Gold and Azygous (controls), and ictB-1 to ictB-6 (transgenic lines).

Factor	df	ss	Ms	F	p value
Block	7	32.75	4.68	14.21	<0.001
Groups	8	1170.88	146.36	444.57	<0.001
Residual	91	32.29	0.35		
Total	106	1235.91	11.66		

Table 4.4 – ictB expression for controls (Cadenza, Gold and Azygous) and transgenic lines (ictB-1 to ictB-6). Values are the $log_2(1/NRQ)$ ± standard error of the mean. Gene expression is represented by negative values and smaller values mean higher expression. Normalised relative quantity (NRQ) refers to the *ictB* expression normalized to the two housekeeping genes (*Ta2291* and *Succinate dehydrogenase*).

Lines	*ictB* Expression ($log_2(1/NRQ)$ ± SE)
Cadenza	3.49 ± 0.20
Gold	4.75 ± 0.17
Azygous	4.46 ± 0.20
ictB-1	- 3.56 ± 0.13
ictB-2	- 4.01 ± 0.13
ictB-3	- 2.93 ± 0.20
ictB-4	- 4.00 ± 0.14
ictB-5	- 4.24 ± 0.15
ictB-6	- 2.75 ± 0.14

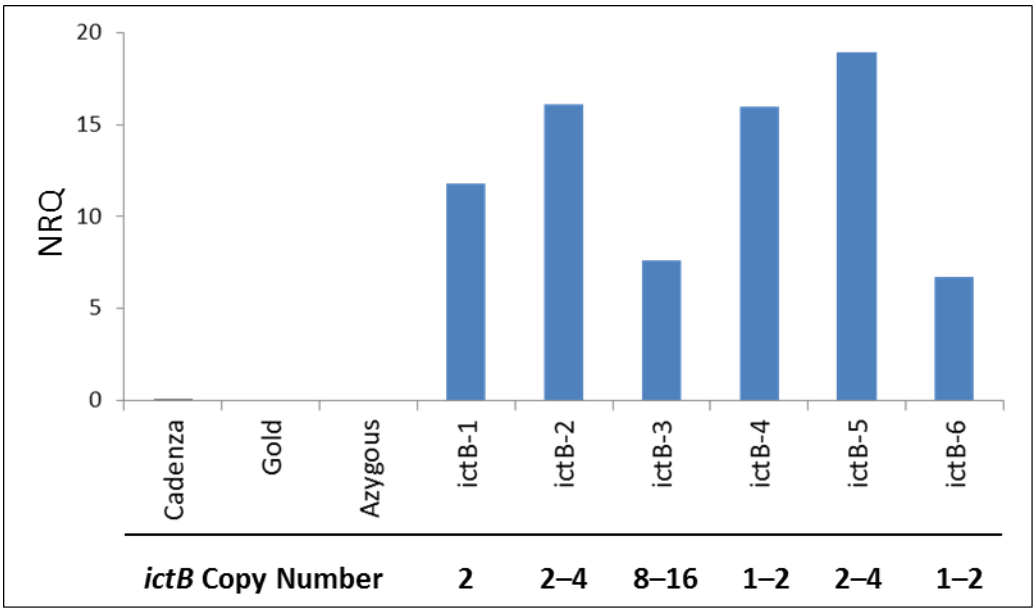

Figure 4.3 – *ictB* gene expression and copy number in glasshouse-grown T2 *ictB* transgenic wheat plants. Normalised relative quantity (NRQ) refers to the ictB expression normalized to the two housekeeping genes (*Ta2291* and *Succinate dehydrogenase*). Cadenza, the wild-type cultivar used as background for genetic engineering; Gold, Cadenza plants bombarded with gold particles without a gene construct; Azygous, transgenic plants that lost the gene during segregation; ictB-1 to ictB-6, transgenic lines expressing ictB. Values are NRQ means for each line. Lines ictB-2, -4 and -5 had significantly higher expression followed by ictB-1, ictB-3 and -6 and the controls (as per Table 2 ANOVA, $F = 444.57$; df = 8; $p < 0.001$ and Table 3).

4.3.3 ictB *expression alters photosynthetic properties at CO_2 levels below ambient*

Photosynthetic traits measured at high light (1500 µmol photons m^{-2} s^{-1}) and atmospheric levels of CO_2 (400 µmol CO_2 $µmol^{-1}$ air) revealed limited differences between the transgenic lines and the respective controls. In brief, the wild-type Cadenza plants had higher photosynthesis and stomatal conductance than the other two controls (Gold and Azygous) and the ictB transgenic lines, with no other differences being observed (data not shown).

Gas-exchange measurements at high light (1500 µmol photons m^{-2} s^{-1}) and low CO_2 levels (200 µmol CO_2 $µmol^{-1}$ air) revealed an effect of *ictB* gene expression on some photosynthetic traits (Fig. 4.4). The wild-type Cadenza had the highest values of photosynthesis (A_{200}), stomatal conductance to water vapour (gs_{200}), intercellular CO_2 concentration (Ci_{200}), and transpiration (E_{200}), compared to all other plant types, suggesting an effect of the transformation procedure, rather than *ictB* expression, on these photosynthetic traits. The lines ictB-1 and ictB-2 had lower A_{200}, gs_{200}, and E_{200} than all the control plants and most of

the ictB lines. For C_{i200} ictB-1 was lower than Cadenza and ictB-3, ictB-4, ictB-5 and ictB-6. Between the transgenic lines, ictB-3, ictB-4 and ictB-5 had similar values for A_{200}, gs_{200} and E_{200}, and these were greater than for ictB-1 and ictB-2. The line ictB-4 had higher C_{i200} than the other lines, being more comparable to Cadenza (Fig. 4.4).

The intrinsic water use efficiency (A_{200}/gs_{200}) was greater in ictB-1 than Cadenza, Azygous, ictB-3, ictB-4 and ictB-5 (Fig. 4.5), due to a smaller relative reduction in A_{200} than in gs_{200} in ictB-1 (Fig. 4.4). The wild-type Cadenza and ictB-4 had the lowest A_{200}/gs_{200} (Fig. 4.5) revealing that their increased levels of photosynthesis were reached at higher gs_{200} and increased intercellular CO_2 concentration (Fig. 4.4).

The quantum yield for CO_2 fixation (ΦCO_2) determined from fluorescence measurements at low CO_2 levels (200 µmol CO_2 µmol^{-1} air) showed a similar pattern to A_{200} (Fig. 4.6). The lines ictB-1 and ictB-2 were lower than the controls and most of the ictB lines, revealing a lower efficiency of using energy from light to fix carbon. Between the transgenic lines, ictB-3, ictB-4 and ictB-5 had higher ΦCO_2 than ictB-1 and ictB-2 (Fig.4.6). No significant differences ($p > 0.05$) were observed for the electron transfer rate (ETR_{200}, data not shown).

Expression of *ictB* did not affect the difference in gas-exchange and fluorescence traits between normal (400 µmol CO_2 µmol^{-1} air) and low (200 µmol CO_2 µmol^{-1} air) CO_2 concentrations. No significant differences ($p > 0.05$) were observed for the increase/decrease of the measured values between the two CO_2 concentrations for the controls and the transgenic lines (data not shown).

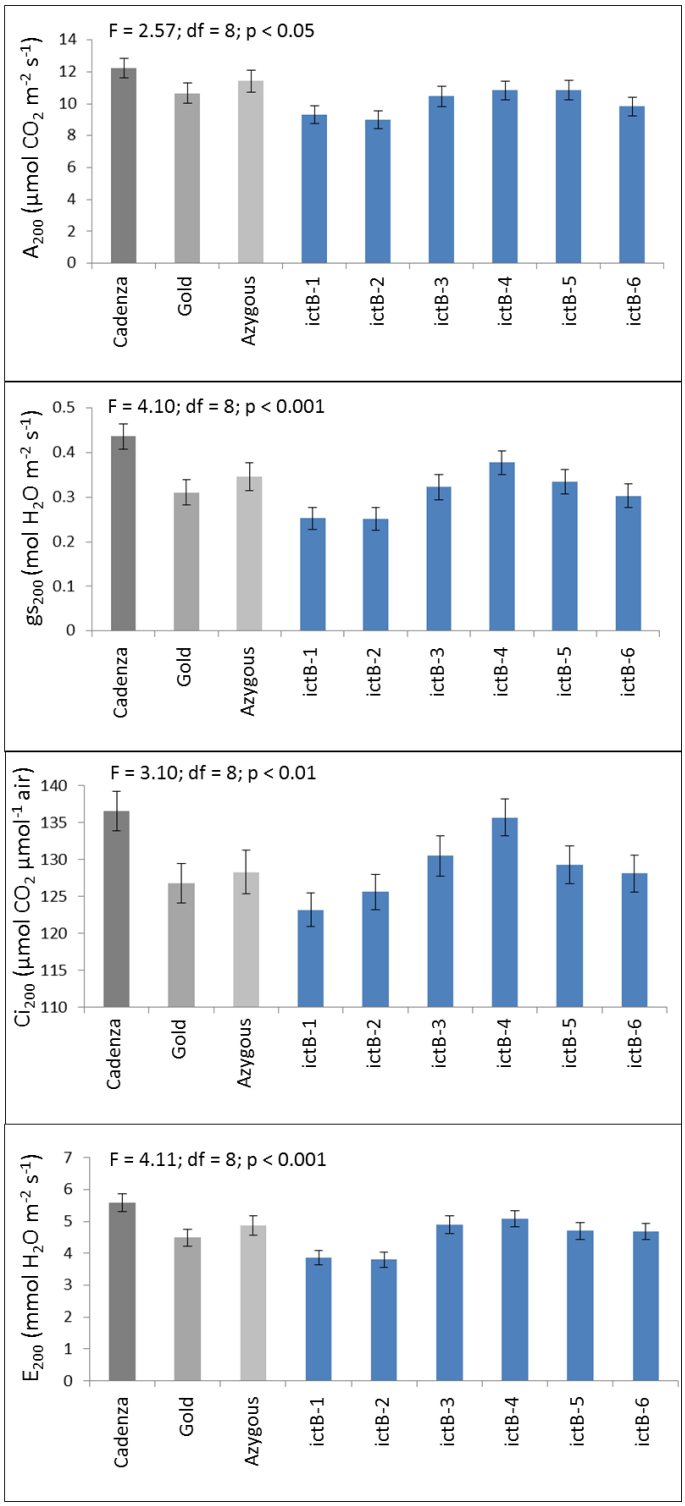

Figure 4.4 – Gas-exchange traits in glasshouse-grown T2 *ictB* transgenic wheat plants. Measurements were performed at light levels of 1500 µmol photons m^{-2} s^{-1}, block temperature of 25°C and ambient CO_2 concentration of 200 µmol CO_2 µmol^{-1} air. A_{200}, net CO_2 assimilation rate; gs_{200}, stomatal conductance to water vapour; C_{i200}, intercellular CO_2 concentration; E_{200}, transpiration rate. Cadenza, the wild-type cultivar used as background for genetic engineering; Gold, Cadenza plants bombarded with gold particles without a gene construct; Azygous, transgenic plants that lost the gene during segregation; ictB-1 to ictB-6, transgenic lines expressing ictB. Values are the means for each line/group ± standard errors of the mean (SE). Differences between the lines/groups were tested by ANOVA to unbalanced designs (F, calculated value for the F test; df, degrees of freedom; p value, probability to reject the null hypothesis).

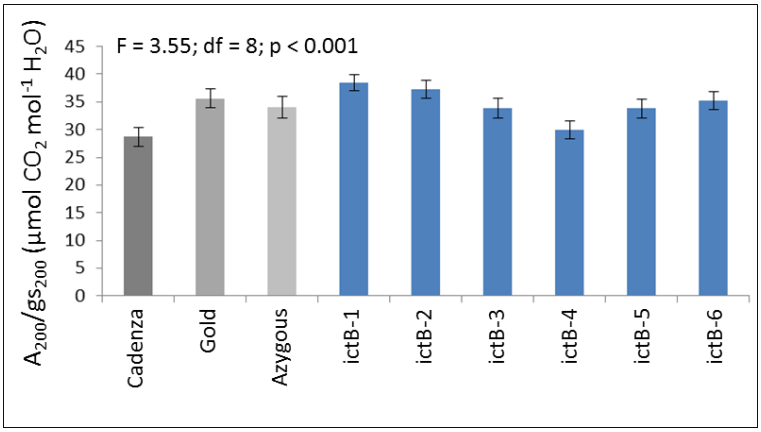

Figure 4.5 – Intrinsic water use efficiency (A_{200}/gs_{200}) in glasshouse-grown T2 *ictB* transgenic wheat plants. Measurements were performed at light levels of 1500 µmol photons m^{-2} s^{-1}, block temperature of 25°C and ambient CO_2 concentration of 200 µmol CO_2 $µmol^{-1}$ air. Cadenza, the wild-type cultivar used as background for genetic engineering; Gold, Cadenza plants bombarded with gold particles without a gene construct; Azygous, transgenic plants that lost the gene during segregation; ictB-1 to ictB-6, transgenic lines expressing ictB. Values are the means for each line/group ± standard errors of the mean (SE). Differences between the lines/groups were tested by ANOVA to unbalanced designs (F, calculated value for the F test; df, degrees of freedom; p value, probability to reject the null hypothesis).

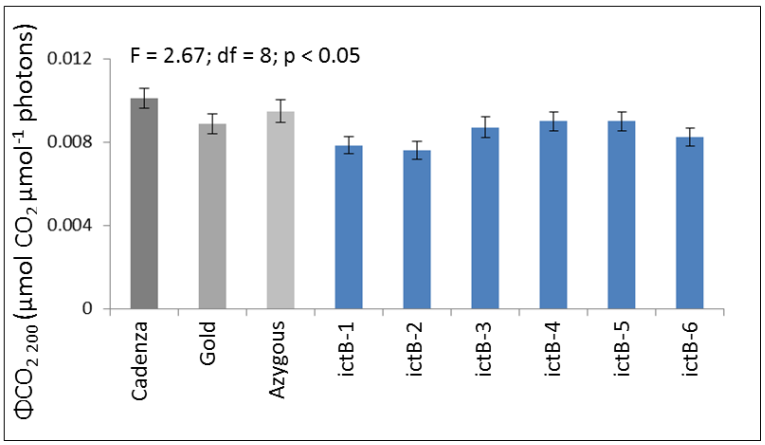

Figure 4.6 – Quantum yield for CO_2 fixation in glasshouse-grown T2 *ictB* transgenic wheat plants. Measurements were performed at light levels of 1500 µmol photons m^{-2} s^{-1}, block temperature of 25°C and ambient CO_2 concentration of 200 µmol CO_2 $µmol^{-1}$ air using the multiphase flash method (Loriaux *et al*, 2013). Cadenza, the wild-type cultivar used as background for genetic engineering; Gold, Cadenza plants bombarded with gold particles without a gene construct; Azygous, transgenic plants that lost the gene during segregation; ictB-1 to ictB-6, transgenic lines expressing ictB. Values are the means of each line/group ± standard errors of the mean (SE). Differences between the lines/groups were tested by ANOVA to unbalanced designs (F, calculated value for the F test; df, degrees of freedom; p value, probability to reject the null hypothesis).

4.3.4 Biomass production and grain yield are not affected by the ictB insertion

The biomass and grain yield analyses revealed no effects of *ictB* gene in plant production: none of the ictB lines showed higher grain yield or above ground biomass than the controls

(Fig. 4.7). Between the transgenic lines, ictB-3 and ictB-4 had lower biomass than the others and ictB-5 and ictB-6 presented a higher grain yield than ictB-2 and ictB-3.

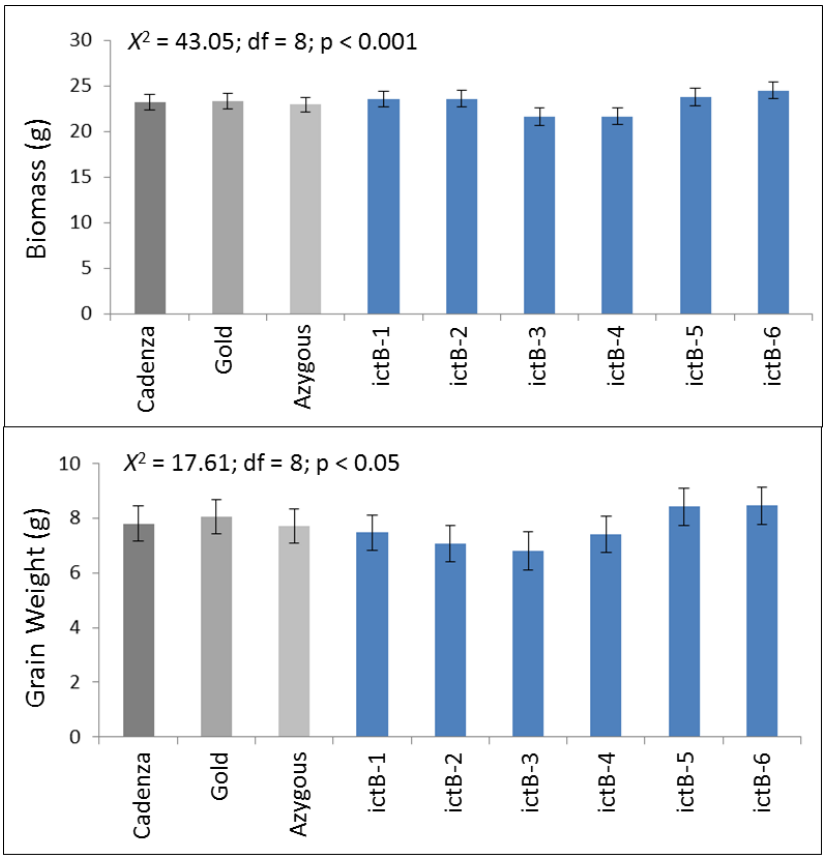

Figure 4.7 – Productivity traits in glasshouse-grown T2 *ictB* transgenic wheat plants. Biomass and Grain Weight corresponds, respectively, to the total above ground biomass and total grain weight at 100% dry matter. Plants were harvested at the Zadoks stage of 9.2. Cadenza, the wild-type cultivar used as background for genetic engineering; Gold, Cadenza plants bombarded with gold particles without a gene construct; Azygous, transgenic plants that lost the gene during segregation; ictB-1 to ictB-6, transgenic lines expressing ictB. Values are the predicted means for each line/group ± standard errors of the mean (SE). Differences between the lines/groups were tested by the Wald test (X^2, calculated value for the chi square test; df, degrees of freedom; p value, probability to reject the null hypothesis).

4.3.5 Physiological and productivity trends are not explained by the ictB expression level in the transgenic lines

The Pearson coefficients did not show significant ($p < 0.05$) correlations between the physiological or plant production traits measured and *ictB* expression levels for the transgenic lines, meaning that different levels of *ictB* expression did not affect the way plants fix carbon from the atmosphere or produce biomass or grains. ictB-5, the line with higher expression of ictB, reached the same A_{200} values as ictB-4, with no difference in gs_{200}, but at lower C_{i200} concentrations (Fig. 4.4).

4.4 DISCUSSION

The cyanobacterial *ictB* gene was successfully expressed in transgenic wheat using the cultivar Cadenza as background. Six ictB transgenic wheat lines were phenotyped for physiological and productivity traits alongside control plants. None of the transgenic lines presented improvements in the measured traits when compared to the wild-type Cadenza control. The results are discussed by comparison with available literature suggesting a beneficial effect of *ictB* expression in other plants. Possible reasons for the lack of an observable phenotype are debated alongside their potential impact for biotechnological wheat improvement studies.

4.4.1 Carbon uptake and biomass production response to **ictB** *expression*

A decrease in photosynthesis by 10 to 20% was observed for the transgenic lines when compared to the Cadenza control at normal CO_2 levels (400 µmol CO_2 μmol^{-1} air; data not shown) and by 11 to 26% at low CO_2 levels (200 µmol CO_2 μmol^{-1} air; Fig. 4.4). These results are not in accordance with previous reports of improvements in photosynthetic rates in transgenic plants with *ictB* insertion: *Arabidopsis* and tobacco (Lieman-Hurwitz *et al*., 2003), soybean (10% and 15% increase in glasshouse and field-grown plants, respectively; Hay, 2012; Hay *et al*., 2017), tobacco (19% increase; Simkin *et al*., 2015) and rice (15% increase; Gong *et al*., 2015). No differences between transgenic and control plants were reported by Piatek (2015) for photosynthetic rate in soybean plants growing in glasshouses and field conditions. The reported increase in photosynthesis was not linked to increases in *gs* in *Arabidopsis* and tobacco (Lieman-Hurwitz *et al*., 2003) and soybean (Hay, 2012; Hay *et al*., 2017). Conversely, an increase in A linked with increased *gs* was reported in rice (Gong *et al*., 2015) and tobacco (Simkin *et al*., 2015), suggesting different patterns of water use efficiency in different plants. Almost all the ictB wheat lines in the present study had lower *gs* and higher water use efficiency than the Cadenza control. An effect of *ictB* expression on the

intercellular CO_2 concentration (Ci) was reported by Hay (2012), with increased C_i for transgenic plants compared to the controls. A contrasting effect was observed with the ictB wheat plants herein. Similarly, while Hay (2012) reported that the increase in the photosynthetic rates in soybean was linked to increases in both ΦCO_2 and J_{max}, a decrease in ΦCO_2 was observed for the wheat transgenic lines compared to the Cadenza at low CO_2 levels (200 µmol CO_2 μmol^{-1} air) and no differences were observed for electron transport rate (J_{max}).

It is important to highlight that there are reports of effects of the *ictB* gene in increasing photosynthetic rates at limiting (Lieman-Hurwitz *et al.*, 2003) and non-limiting (Gong *et al*., 2015; Simkin *et al*. 2015) CO_2 concentrations. If the *ictB* gene is related to carbon concentration in the chloroplast, it is expected to have greater effects at limited CO_2 conditions, increasing carbon at the chloroplast (C_c) and maximum carboxylation rate (V_{cmax}). From the available literature, only Hay et al. (2017) reported an increase in photosynthetic levels at limiting and non-limiting CO_2 conditions with increased mesophyll conductance (*gm*), C_c and V_{cmax} and without changes in stomatal conductance (*gs*), revealing a possible effect of increasing the efficiency of CO_2 concentration.

No differences were observed for biomass and grain yield between transgenic and control wheat plants (Fig. 4.7). Increases in plant productivity were reported in *Arabidopsis* (Lieman-Hurwitz *et al*., 2003), soybean (biomass and gran yield; Hay, 2012; Hay *et al*., 2017) and tobacco (Simkin *et al*., 2015). Other studies showed no improvement in biomass/grain yield for tobacco (Lieman-Hurwitz *et al*., 2003), soybean (Piatek, 2015) and rice (Gong *et al*., 2015).

4.4.2 Could experimental conditions and/or practices have concealed the expected ictB *phenotype?*

During the T2 experiments, the plants were exposed to unexpected biotic stresses due to an aphid infestation and severe powdery mildew infection, from booting stage to the end of the cycle, which could have compromised the photosynthetic capacity and yield of the plants

(Bancal *et al*., 2012; Franzen *et al*., 2008). A possible effect of the insect/disease stress is the noticeable decrease in general grain yield from T1 to T2 of 29% (data not shown). It would be sensible to expect that the biotic stress would affect transgenic and control plants in the same way, depending on the mode of action of the *ictB* gene, the influence of these conditions could be more accentuated in the transgenic plants compared to the controls. It is possible that the biotic stress could be limiting stomatal conductance and decreasing intercellular CO_2 concentration, masking the effect induced by *ictB* gene expression.

The limitation to growth or damage of the root system during early growth and re-potting could also have caused the loss of the expected phenotype (Dr Andrew Simkin, University of Essex, personal communication, January 2016). Although the re-potting process was carried out carefully, there is the possibility that the roots were packed and had their growth limited by the multi-cell tray before being transplanted.

The possibility that improved photosynthetic rates in ictB plants compared to controls were undetectable given the procedure adopted herein can also be considered. Photosynthetic rates of wheat flag leaves decrease from booting to post-anthesis, as leaves age and Rubisco activity and amount decreases (Carmo-Silva *et al*., submitted). The gas-exchange measurements were taken just before anthesis (Zadoks 6.0), when the photosynthetic rates would be starting to decay, as well as the Rubisco activity and amount. The plants were analysed at ambient CO_2 levels of 200 and 400 µmol CO_2 μmol^{-1} air, with an intercellular CO_2 concentration of approximately 130 and 225 µmol CO_2 μmol^{-1} air respectively. According to Sharkey *et al*. (2007) C3 photosynthesis is Rubisco-limited at these intercellular CO_2 concentrations. Differences in the decrease of Rubisco activity or amount between transgenic and control plants could have hidden any effect of *ictB* gene expression at the CO_2 levels used. The plant transformation process could also have impacted on plant performance and masked the effect of *ictB* expression. Although there is no clear effect on

biomass production and grain weight, Cadenza plants presented higher A_{200} compared to the gold control and higher gs_{200}, C_{i200} and E_{200} than the other two controls (Fig. 4.4). Although this possibility cannot be disregarded, it is somewhat more unlikely than a loss of phenotype promoted by the reasons stated above (disease, root damage and conditions of measurement) given that others have reported a phenotype in transgenic lines produced in a similar fashion (Driever, unpublished).

In retrospect, the following actions could improve future experiment conditions: a) reduce the number of plants per area, control the glasshouse humidity and apply more efficient methods to control pests and diseases (such as the use of sulphur burning to prevent powdered mildew or natural predators to control aphids); b) sow seeds direct to 3 L pots to avoid root growth restriction or root damage during re-potting; c) analysis of photosynthesis response to ambient carbon concentration by A x C_i curves to map possible effects of ictB expression in gas-exchange traits at different intercellular CO_2 levels; d) perform gas-exchange analysis around booting stage (Zadoks 4.5) to avoid the influence of senescence and disease on flag leaf performance.

4.4.3 The ictB *protein functionality in the transgenic wheat plants*

The *ictB* gene was present and expressed in the transgenic plants (DNA & RNA analyses), however, there is no assurance that it was translated to functional protein. Attempts to detect ictB protein by western blotting were unsuccessful. In fact, colleagues around the world have to date failed to produce an ictB antibody for the accurate detection of ictB protein. The antibody used by Gong *et al*. (2015) for quantifying ictB in rice plants failed to produce sensible results with the transgenic wheat plants studied here.

To understand and compare the effect of a transgene it is crucial to map its expression from gene to protein level, as gene silencing can happen at different levels (Gallie, 1998). The effect of transgene copy number on gene silencing and expression is controversial with

reports of higher number of copies causing increased silencing (Allen *et al*., 1996; Gallie, 1998; Li *et al*., 2002; Kohli *et al*., 2010), or increased gene expression (Hobbs *et al*., 1993). The position of the transgene is also known to influence gene expression levels (Finnegan & McElroy, 1994).

In addition to the analysis of the final translated amounts of ictB protein, the detection of its location is also important. Gong *et al*. (2015) detected the presence of the ictB antibody in the cytoplasm rather than inside the chloroplast, as expected with their use of the RbcS transit peptide (Lee *et al*., 2006). The authors justify the location of protein in the cytoplasm by the fact that a protein location is intrinsic to its function, which could imply that ictB protein might be involved in transcellular carbon transport to the chloroplast in a similar way to aquaporins (Uehlein *et al*., 2008). For the *ictB* transgenic wheat plants, the SBPase transit peptide was used, which, as above, does not ensure the protein will be located in the chloroplast.

4.5 CONCLUSIONS AND FINAL COMMENTS

The following conclusions can be drawn based on the analysis of the expression of the *ictB* gene in wheat plants:

a) the expression of the cyanobacterial *ictB* gene in wheat plants did not improve the biomass production and grain weight compared to the controls;
b) transgenic ictB wheat presented, in general, lower values for photosynthetic traits compared to the Cadenza wild-type at low CO_2 levels (200 µmol CO_2 μmol^{-1} air);
c) *ictB* expression and gene copy number did not correlate to improvements in physiological and plant production traits;
d) it is possible that unforeseen experimental factors could have masked the *ictB* phenotype in the current study.

This study highlighted a number of experimental factors that should be taken into account in future biotechnological efforts to improve wheat photosynthesis and crop yields.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

GENERAL DISCUSSION

The present study focused on wheat crop physiology and growth to investigate carbon uptake and allocation patterns in relation to wheat productivity and resilience to environmental pressures. Two main approaches were explored: exploiting the natural genetic variation of traits related to photosynthesis and yield and mimicking strategies of other species by transgene expression.

The study of 12 elite wheat cultivars over three seasons in the UK has shown that lower rainfall during the growth cycle has shortened the grain development stage and impacted in yield patterns. Yield stability was related to the resilience of the cultivars to the shortage in grain development duration and this resilient behaviour was partially explained by the genetic background of the cultivars.

The phenotypical analyses of a wheat mapping population generated from parents with contrasting carbon uptake rates, leaf characteristics and biomass production revealed lines with different strategies to achieve higher yields. Grain yield was correlated to traits related to light interception and conversion as well as biomass allocation with variable patterns along the crop growth cycle.

The insertion in wheat of the *ictB* gene, related to carbon concentration in cyanobacteria and to improved photosynthetic rate in transgenic plants, allowed an investigation of the possible effects of the gene in carbon uptake and wheat yields. The *ictB* insertion apparently did not improve carbon concentration, photosynthesis or yield in wheat in two generations, although possible unforeseen experimental conditions could have masked the expected phenotype.

This general discussion is focused in the potential techniques and target traits for breeding for a combined high and stable yield in wheat. The natural genetic variation of photosynthesis and the possibilities of high-throughput phenotyping of traits related to carbon assimilation are also discussed in the scope of the use of photosynthesis as a target trait for breeding. The use of transgenic wheat, the methodological and ethical issues related to its use and the possible alternatives for future generation of genetically improved plants are also discussed. The discussion is supported and motivated by research results presented in the previous chapters (Chapters 2, 3 and 4) and methodological limitations.

5.1 BREEDING FOR FOOD SECURITY: WHAT TO BREED FOR?

The breeding of new plant genotypes with increased production capacity and resilience is one of the strongest cornerstones for addressing the current and future food security challenge faced by humanity (Flavell, 2017). Although the plant breeding process was successful in improving crop productivity in the past and in current days, the exponential increases in food needs predicted for the next decades are forcing scientists and breeders to adapt and innovate breeding technologies and strategies. It is expected that more food will have to be produced in the next 40 years than has been produced in the whole history of humanity, to meet world demand.

One of the most important questions to be answered in what concerns breeding is “what to breed for”? Higher yield and yield stability are commonly the main targets for crop improvement. It is often thought that yield stability in sub-optimal conditions is linked to yield penalties in years of optimal conditions, but it is not a rule (Tester and Langridge, 2010). The yield patterns observed for Humber, Savannah and Soissons, always at or above ERYCC panel average yield (Chapter 2), under different conditions of water availability, suggests the possibility of combining high yield and yield stability. It is crucial to understand which traits are linked to higher yields or to yield stability and mainly to define traits that are important

for both at the same time. It might help to select high yielding genotypes that are not penalized in optimal or stressful conditions.

Although the recommended lists of cultivars for specific countries, such as the AHDB list for the UK (AHDB, 2017), is a strong base for the decision of which cultivars to be used, the specific climatic conditions of each season can dictate the production by a particular cultivar. Plants cannot predict the environmental constraints they will face during the growth cycle, but will respond to them. The main limitations to wheat grain yield are related to non-ideal growth conditions at the late stages of crop cycle, during flowering and grain filling, as observed for the ERYCC panel experiments (Chapter 2). These conditions are predicted to become more limiting in future climatic conditions (Semenov *et al*., 2014). Traits that give plants developmental advantages in multiple environments and that confer better fitness might increase flexibility and improve the response under normal and sub-optimal conditions. Higher water use efficiency during the growth cycle could allow a better water balance besides saving water in the soil to be used during later critical growth stages, such as grain filling (Araus *et al*., 2002). A longer and branched root system to reach water in the deeper soil layers, fast canopy cover to avoid soil water evaporation combined with more efficient stomatal control would allow plants to perform well in normal conditions but also take advantage of extra water in case of a combined heat and drought stress at later growth stages in rainfed conditions.

Another strategy can be related to increased photosynthetic rates based on a more efficient Rubisco, which could decrease the requirements of nitrogen storage in leaves' proteins without limiting the source capacity of the plant (Parry *et al*., 2013). A bigger nitrogen storage in other plant organs (as the stem) can be achieved if less nitrogen is required in leaves, leading to a more efficient remobilization of nitrogen from stem to the grain, at the grain filling stage, without requiring early senescence of flag leaves (Madani *et al*., 2010).

This could be beneficial in normal growing conditions as well as conditions where the duration of grain filling is supressed, as reported for the ERYCC panel experiments (Chapter 2). A more efficient Rubisco could also contribute to increase the ratio of carboxylation/transpiration, improving water use efficiency.

Plants with higher resource use efficiency (water, light, nutrients) could potentially perform within average in optimal conditions, but be more flexible and robust when facing non-optimal conditions.

5.2 THE YIELD FORMATION MODEL

From its theoretical definition, yield can be defined as an energy conversion model, as stated by Formula 1.1. In the conversion pathway from the radiative energy from sunlight to the chemical energy in the grains, there are three main steps related to the efficiency in intercepting sunlight, converting it into biomass and partitioning biomass to the grain (Long *et al.*, 2015). It is important to highlight that this general model considers the other growth conditions as ideal or non-limiting. There are similar equations for yield definition according to conditions of limited water (Passioura, 1977) or nutrient availability. Although the traits related to any of the efficiencies in the energy conversion model will potentially improve yields, their interaction with other traits in resource-limited conditions will be also crucial to define targets for yield and yield stability improvement.

Results from the ERYCC Panel experiments (Chapter 2) highlighted the importance of the grain development stage to yield. Traits related to source and sink capacity, as well as biomass partitioning (to the grain), play important roles in the yield potential and also in yield stability. Cultivars' different behaviour in terms of grain yield in contrasting environmental conditions, such as rainfall accumulation, suggests the importance of understanding these traits in different growth conditions.

The extensive phenotyping of the mapping population (Chapter 3) has presented a number of traits that correlate with yield. Traits presented on Table 3.1 are correlated to each of the three main efficiencies mentioned in Formula 1.1. Early vigour, accumulated green area, highest cover and stay green are all related to canopy cover and to light interception. As they are calculated based on green area cover, they are also related to the conversion efficiency and to the total carbon assimilation during the season. From the reflectance indices, NDVI is related to light interception and energy conversion, and PRI and WI to energy conversion. The gas-exchange traits are also related to energy conversion and carbon balance. The biomass accumulated in leaves, stems and in the whole plant is related to the carbon storage and to the partitioning capacity.

When selecting target traits for a breeding program aiming improve yields, the correlation to yield is an important factor. However the genetic variability of the trait and its repeatability (or heritability) are also crucial. The variability measures the range of variation in the population for the specific trait and reflects how much it can be exploited. For the mentioned traits, the ones related to light interception as accumulated green area, highest cover and NDVI presented low variability, from 1 to 3%. Early vigour and stay green, related to light interception but also to an extended energy conversion period presented variability of 9%. According to Table 3.1, PRI presented huge variation, although it is related to the method of measurement. When the values are converted to light use efficiency, the variability is around 5%. It accords with the levels of variability of 5% for the flag leaf photosynthesis (Table 3.1), which is reassuring as PRI and the photosynthesis are both related to energy conversion. The biomass accumulation traits presented variation from 8 to 10%. This value is bigger than the variability for harvest index, around 4% (data not presented). It suggests that the population presented different patterns in allocating the biomass stored at post-anthesis to the grain and that this trait could be better investigated. The variability for grain yield for the population was 5%.

In terms of the heritability, the traits related to light interception presented values between 0.71 and 0.77 with the exception of early vigour (0.50). For the traits related to energy conversion there is a bigger gap between PRI (around 0.9) and flag leaf photosynthesis (around 0.60 and decreased for the lower light levels). For the biomass allocation traits, heritability is around 0.42, although harvest index presented a heritability of 0.70 (data not presented). It is important to highlight that heritability in a single environment has a limited interpretation for breeding purposes. It is not a simple task to choose one specific trait from a multitude of possible target traits, but the combined analysis of their correlation to yield, variability and heritability can be a good indicator.

Of course there is a theoretical limit for the improvement in each of these traits and hence final yield. For instance, covering the area quickly at the beginning of the cycle would be beneficial to intercepting and converting light and to biomass storage, but may be limited by water and nutrient availability. Similarly, increases in photosynthetic rates would be beneficial to conversion efficiency but require a bigger investment in leaf nitrogen and higher stomatal conductance.

Key traits to improve yield seem related to a more efficient use of resources, mainly light, carbon, nutrient and water. Integrated improvements in resource-use efficiency may be a more reliable way of improving yield potential and stability than improvements in single traits. The technology proposed for the *ictB* wheat (Chapter 4) may work by increasing the conversion efficiency by an enhanced efficiency of carbon, light, nutrient and water use. It could bring improvements in the dry matter accumulation without requiring extra nutrient inputs or water loss. However, there is a need to better characterise and phenotype the lines to reveal the potential of the technology, since benefits have previously been reported in other crops.

5.3 THE GENETIC BASIS OF PHOTOSYNTHESIS' CONTRIBUTION TO YIELD

The change in breeding strategy from entirely phenotype-based to combined phenotype-genotype interaction, by the emergence of marker-assisted selection (MAS) technology, has increased greatly the understanding of genes underlying metabolic and physiological processes and plant behaviour (NAS, 2016). Yield, as a complex quantitative trait, is influenced by multiple genes, which makes the process of finding specific QTLs for yield a complicated process (Saint Pierre *et al*., 2016). Revealing the traits that can contribute to yield in multiple environments and mapping QTLs for these may help to solve the puzzle of yield formation and variation. Photosynthesis is recognized as one of the main drivers of plant biomass production and revealing its genetic regulation could lead to higher chances of improving yield formation and stability. Mapping QTLs for photosynthetic capacity and efficiency was one of the initial aims of this study and, although it is not reported in this document, it will be part of the further outcomes of this research as part of the studies reported for the double-haploid mapping population (Chapter 3).

Flood *et al*. (2016), analysing photosynthesis in *Arabidopsis*, revealed that the influence of some QTLs to the analysed phenotype varied along the growth cycle and that trait heritability could vary greatly according to the time of the day. The study of yield drivers in a wheat mapping population (Chapter 3) showed that traits' correlations to yield and heritability varied over the growth season reinforcing the dynamism of the genetic basis of yield formation. Combined screening of genotypic and phenotypic data will help to build a detailed map of interactions and enlighten possible strategies for reaching high and stable yields. Given the dynamism of the QTLs and genes response, the frequency of the screening process needs to be increased. The screening of genotypic information has evolved greatly in the last decades and the phenotyping process at short-time scales is now the main limitation for the mentioned approach (Araus and Cairns, 2014).

Advances in high-throughput phenotyping are required to fill in the gap between genomics and phenomics capacity (Araus *et al*., 2014). In terms of photosynthesis phenotyping, the use of chlorophyll fluorescence evaluation methods has emerged as a fast screening strategy for a high number of plants, although the results need to be carefully interpreted depending on the methodologies used and the assumptions linking the photochemical and carbon fixation reactions (Flood *et al*., 2011; Furbank *et al*., 2013; Murchie and Lawson, 2013). New technologies of screening photosynthetic membranes are also promising as high-throughput methods of photosynthetic parameters measurements (Liu and Scheuring, 2013). For gas-exchange measurements, the use of whole plant chambers for the intermittent evaluation of plant photosynthesis (Kölling *et al*., 2015), as well as new technologies based on rapid and simple measurements (Bellasio *et al*., 2014; Stinziano *et al*., 2017), were reported recently. New features of recently released IRGAs have impacted positively on the quality, stability and speed of gas-exchange measurements, although it is still far from the required technology to monitor large numbers of genotypes several times a day to assess possible influences of the circadian clock in gas-exchange, mainly in field-based experiments. For the acquisition of gas-exchange data for the wheat mapping population presented in this manuscript (Chapter 3) a total of 8 days of work by 3 researchers was required for each of the developmental stages analysed.

Other approaches for the study of gene function and its effect in plant phenotypic response have been reported and raised interest in the last years. The use of chemical genetics has shown great potential (Toth and van der Hoorn, 2009), mainly with the involvement of new technologies of high-throughput phenotyping (Burrell *et al*., 2017).

The analysis of genetic similarity based on gene presence for the ERYCC panel subset has revealed the influence of the presence/absence of specific genes in phenotypic behaviour, especially for yield stability (Chapter 2) highlighting the potential use of genetic modelling.

Mapping genes for photosynthetic traits and adding gene function information to phenotypic models can improve the understanding and prediction of photosynthesis influence in plant performance and save time and money in the breeding process (Chapman *et al*., 2002). Wilczek *et al*. (2009) managed to model the flowering time of *Arabidopsis* using genetic modelling with a 92% of fitness. The complexity of genetic background defining photosynthetic efficiency and its influence to yield formation and stability in wheat might be bigger than the one defining flowering time in *Arabidopsis*, however the addition of multiple genes and their interaction may improve the modelling process.

5.4 EXPLOITING PHOTOSYNTHESIS' GENETIC VARIATION FOR BREEDING

Variation is the key point for breeding and the science behind crop improvement is focused on the discovery, creation and manipulation of genetic variation of target traits, followed by selection (Francis *et al*., 2017). The natural genetic variation of photosynthesis is high but unexplored for breeding purposes (Flood *et al*., 2011). Despite the importance of photosynthesis to productivity, the breeding for yield improvement in the last decades has not delivered big improvements in photosynthesis and photosynthetic efficiency if compared to the improvements in light interception and biomass allocation (Long *et al*., 2015). This is related to the fact that, although the genetic variation in photosynthesis is high, it is smaller than the variation observed for leaf cover traits and harvest index (Long *et al*., 2015). In modern commercial cultivars, such as the parents of the mapping population reported in Chapter 3, harvest index variation is not expected to be high, as it was the biggest target of improvement during the Green Revolution.

For the wheat mapping population (Chapter 3), variation was around 5% for photosynthesis for booting and post-anthesis, 4% for harvest index and 10% for leaf area index. For the 64 cultivars of the ERYCC panel (Chapter 2), variation was around 6% for photosynthesis at booting, 7% for harvest index and 12% for leaf area in 2012 and 8% and 11% for

photosynthesis at booting and post-anthesis, respectively, 9% for harvest index and 19% for leaf area for 2013. The variation for all the three traits tended to be bigger for the ERYCC panel than for the mapping population which can be explained by the diversity of cultivars in the panel and the fact that all the lines in the mapping population were generated from the same parents. The bigger difference in harvest index between the panel and the mapping population can be also explained by the presence of cultivars with old release date that did not benefit of the harvest index improvements from the Green Revolution. The higher variation in all the traits for 2013, compared to 2012, can be related to the reported environmental constraints faced by the plants in this season (Chapter 2).

Mapping QTLs, and potentially genes, for traits related to photosynthetic efficiency is an important step in understanding the genetic basis of the process. The use of reverse genetic techniques, such as Targeting Induced Local Lesions IN Genomes (TILLING), may help to explore the limitations of genetic variability in genes that control the photosynthetic process (Parry *et al*., 2009), unlocking unrealized crop yield potentials (Kissoudis *et al*., 2016). Improvements in leaf photosynthesis related to stomatal conductance increases using the TILLING technology were reported in rice (Kusumi *et al*., 2012) and the technology can also be used in wheat despite of its polypoid character (Krasileva *et al*., 2017).

5.5 THE USE OF TRANSGENIC TECHNOLOGY FOR CROP IMPROVEMENT

The use of transgenic plants has been successful and impacted greatly in agricultural activity, mainly in enhancing and protecting crop yields. However, the process of generating genetically modified plants, testing the impact of the modification in glasshouse conditions and validating in field-grown experiments can be very long. Also, the use of genetically modified organisms in open field experiments or commercially is limited in many countries around the world, mainly by political and bioethical issues (Tester and Langridge, 2010). For instance, in the UK, the use of genetically modified organisms in experimental fields is

regulated by the Department for Environment, Food and Rural Affairs (DEFRA) and involves a laborious application process. In 2016, DEFRA approved the sowing of a GM trail at Rothamsted Research to test, at field scale, a transgenic wheat line whose positive effects in enhancing photosynthesis had been established in glasshouse experiments. Although this represents an advance for transgenic research, a lot of other potential GM approaches to improve yields are denied to be tested at field scale, which makes the process of releasing germplasm at commercial level much more complicated and delayed. Since 2010 only 6 GM field trails have been approved in the UK: 4 to Rothamsted Research and 2 to The Sainsburys Laboratory. Three of them were approved in 2016 to be grown in the current year, one of them being the above mentioned wheat trail for photosynthesis improvement (DEFRA, 2017).

A second and more technical issue with transgenic use in crops is related to the gene transfer process. The commonly used gun-mediated transformation methods do not allow a complete control of the location and number of insertions of the transferred gene. The number of copies of the gene and its location can influence the gene expression and the phenotype of interest and also the viability of plant growth in case of interference with important genes during the bombardment process. For the cyanobacterial *ictB* gene insertion in wheat reported herein, gene copy numbers for the studied lines varied greatly, from 1 to 16 copies (Chapter 4). Although gene copy number was not statistically correlated to plant behaviour, it influenced the selection of homozygous lines for further steps of the transgene evaluation. Surprising differences were observed in photosynthetic and yield traits between wild-type cultivar Cadenza and plants bombarded with gold particles without the plasmid presence (Chapter 4). Moreover, some plants in one of the lines were not able to produce spikes or produced spikes with a small number of tiny seeds. The differences between controls and the incapacity to produce spikes/seeds could suggest an unexpected influence of the bombardment process in the plant phenotype.

The use of New Breeding Technologies (NBTs) of genome editing as Site-Directed Nucleases (SDN, e.g. ZFNs, TALEs and CRISPR/Cas9), could tackle the above mentioned limitations. Firstly, crops generated using the CRISPR/Cas9 technology were not regulated as genetic modified by the USDA and were released normally to farmers last year in the United States (Waltz, 2016), although the possible restrictions to the use of these technologies are still being debated (Waltz, 2015). Secondly, the use of the specific and site-directed nucleases offer the ability to create variation by modifying existing genes and target transgenes to specific sites in the genome which could avoid the problems of multiple gene copies or damage to crucial parts of the DNA responsible for plant development (Francis *et al*., 2016). These technologies could also substitute the TILLING technology, although, in the case of the SDNs, being regulated as GM, TILLING could be favoured, as it is considered conventional breeding (NAS, 2016).

5.6 MAKING SCIENCE MORE EFFICIENT AND INCREASING THE SUCCESS RATE

Beddington *et al*. (2012) highlighted the important role of science and scientists on finding sustainable solutions to the problems related to future food shortage. The progress in basic science and technology in recent years has brought enormous improvements, mainly in what concerns the resolution, quality and speed of equipment and computational capacity. The advances in genetic screening and genome mapping of important species have increased the range of possible solutions for improving food production. The main challenge is to make good use of tools and build complementary skills in research groups. There are important points in science that need to be discussed to increase the efficiency of the process of using the current and future technology to solve real world problems. Some of these points are: the restrictions of published work and data, the need of collaborative work and respective recognition of individuals contribution, the availability of funding, the partnership between

companies and institutes/universities and the collaboration between researchers/breeders and farmers.

As a first point, it is crucial to open the access to methods, publications and data from research groups around the world, as recently required by European science leaders (Enserink, 2016). This would allow a quick flow of information, mainly to parts of the world where research capability needs to be improved, as in developing and poor countries.

The requirement of multidisciplinary and collaborative work is clear, on a way to build groups of researchers with diverse expertise and increase the capacity to solve problems in multiple areas (Beddington *et al*., 2012). In this scope, initiatives such as the RIPE (Realizing Increased Photosynthetic Efficiency) project (www.ripe.illinois.edu) are crucial in combining renowned scientists with the influence to lead impactful scientific efforts. Collaborative research can also save money by allowing an integrated use of resources and equipment, which is required in scenarios of decrease in funding opportunities for agricultural sciences.

International collaborations are a scape valve for the problem of lack of funding as the ups and downs in funding opportunities tend to vary across the globe. It can also help to increase the quality of science in developing and poor countries, giving them options to tackle food security problems at a local scale, as food insecurity is greater in these regions. The "BBSCR-FAPESP joint funding program" and the "EU-Africa Research & Innovation Partnership on Food and Nutrition Security and Sustainable Agriculture" are good examples of international collaborative efforts. These links can also facilitate the process of understanding the environmental pressures in crops around the world, improving the quality of breeding and speeding up the selection process, as highlighted by Borlaug (2007).

In terms of a worldwide science network, the PhotosynQ (www.photosynq.org) project led by Prof David Kramer, is a clear example of initiatives that can lead to a greater impact of science on real life problems. The project aims to collect phenotypic data in real time from

across the world using a simple and relatively cheap technology for measuring physiological traits of plants. As a result, a massive open database is being generated for assessing the impact of different environmental conditions in the growth of multiple species around the globe, with the possibility of integrating phenotypic and genotypic data.

Collaborations between companies and institutes/universities can be mutually beneficial and help to alleviate the funding shortening, as well as combining different skills and increasing the chances of research success. However, it is important that the research goals and expected outcomes, as well as the role of each of the parts, are well defined at the beginning of the partnership, for a final achievement of mutual interests. In countries where this partnership is limited or even not allowed, the reduction in research capacity and loss in research quality and impact are clear.

Another important point in making research more efficient and impactful is the need of contact between researchers and farmers. This can be achieved by understanding the real life problems faced by the farmers as well as providing them with technologies that will make the difference in their daily activity and result in more efficient and sustainable food production. Spending money and energy to find solutions for humanity problems will only make sense if this meets the needs of the population. The success of the Grameen Danone Foods Limited highlights the viability of social business that could improve the delivery of solutions and greatly increase research impact (Yasmin, 2016). There are extension projects between agricultural companies/institutes/universities and smallholder farmers but there is still need for a better integration in technology delivery or the development of simpler solutions that fit in the budget and knowledge of the people that feed the world from the backstage.

5.7 CONCLUSIONS AND FINAL CONSIDERATIONS

The main objective of this research project was to study wheat crop physiology and growth, with a special focus on energy conversion and carbon uptake through photosynthesis and its influence over productivity. An additional aim was to evaluate photosynthetic traits as targets for breeding and selection of potential elite genotypes.

The study of the ERYCC panel over three field seasons (Chapter 2) allowed an integrated analysis of genotype x phenotype interaction and effect on plant performance and yield patterns under different environmental conditions. The main findings were related to the effect of sowing date and reduced water availability in shortening the grain development period and the influence on yield. Different yield stability in the conditions leading to a short grain filling period was related to strategies of avoiding the shortage or improving grain filling rate. These strategies could be partially explained by genetic similarities between the cultivars. The use of grain yield heritability to explain the effect of the environmental pressures in the expression of the genetic potential of the cultivars was also reported. The main contribution of this research chapter is related to the report of specific farm practices (date of sowing) and climatic conditions (mainly accumulated rainfall and degrees day) that can affect wheat growth, development and yield, and the identification of different strategies to yield resilience. The use of combined methods of analysis, including stress indices and multivariate statistics, shed light on simple techniques that allow a fast and integrated evaluation of large numbers of cultivars in multiple environments, which is crucial to breeding processes.

The use of a wheat mapping population (Chapter 3) derived from parents with contrasting photosynthetic and biomass performance revealed multiple interactions between yield and traits related to light interception and use efficiency and biomass allocation in field-grown wheat plants. Different strategies of high yield formation between the population lines were

also identified. One of the main contributions of this research chapter is related to the report of the correlation to yield and broad-sense heritability for an extensive list of phenotypic traits along the wheat growth cycle. Although there are reports of traits' correlation to yield and heritability, the advantage of this research is to bring condensed information of a big range of diverse traits, mainly in what concerns the carbon uptake capacity and efficiency, in field-grown wheat. The report of different strategies to reach high yields can also be important to the selection of traits that could confer enhanced crop flexibility, mainly in contrasting environmental conditions, improving the breeding of high and stable yields. The mapping of QTLs for photosynthetic capacity and efficiency may be a further outcome of this research and has the potential to improve the understanding of photosynthesis genetic control, opening a wide range of possibilities for improving photosynthesis and crop yield.

The study of transgenic wheat with the insertion of the cyanobacterial *ictB* gene (Chapter 4) revealed an unexpectedly similar performance between control and transgenic plants for biomass production and yield patterns. Transgenic plants tended to present a decrease in some of the photosynthetic traits which was also unexpected. Methodological issues and non-ideal growth conditions could have affected the expected genotype. As the *ictB* mode of action is still unclear and the reports of its insertion in other crops is controversial, with both positive and null effects being reported, further studies are required to understand if it can be of benefit for crop improvement. The work developed during this project has highlighted unwanted conditions that can be avoided in the future for plant growth in glasshouse experiments. Some of the techniques related to sowing and pot size, substrate selection, number of plants per area, disease and pest control, gene presence and expression analysis and harvest procedures are already in use in current follow-on experiments with the *ictB* lines as part of an IWYP-funded project and have improved the experimental practices. The research reported here was also key in selecting a sub-set of *ictB* lines for further investigation of the gene function and effect. Homozygosity and gene copy number, as well

as photosynthetic and yield traits of the various lines studied here, were used to inform this selection. After two generations, the outcomes of this research have allowed the selection of 4 ictB lines from a total of 17 available lines at the start of the screening process.

In summary, this research project has generated a large volume of information in what concerns wheat growth, development and yield patterns and the influence of environmental conditions and multiple traits – related mainly to plant physiology, architecture, development and productivity – to plant behaviour. The outcomes support photosynthesis as a potential target for breeding elite genotypes due to its influence in determining yield and heritability, although the effect of multiple environments requires further investigation. It has also highlighted the time limitation of gas-exchange phenotyping for photosynthesis and the needs and new possibilities of high-throughput methods to keep pace with genotyping. The possible further results in QTL mapping may contribute greatly to empower the use of photosynthesis in breeding higher-yielding wheat.

5.8 FUTURE WORK AND RESEARCH OUTCOMES

For the ERYCC panel analysis, the main outcome of this research will be a paper to be submitted in the near future, based on the information shown in Chapter 2. Other papers have already been published (Driever *et al*., 2014) or submitted (Carmo Silva *et al*., submitted) based on the data collected in the 2012 and 2013 seasons, respectively. The present work focused on the use of available data from those two first seasons and the 2014 trial, for which data was collected as part of the work presented herein. In terms of future work, the mapping of genes related to control of plant development or resilience to stresses could be used to improve the understanding of plant performance in the three seasons analysed. The genotypic diversity of the map in terms of parentage, origin, habit and release date would be beneficial for this type of analysis. Although there are genetic markers

mapped for the panel, the use of GWAS technologies for mapping QTLs is limited by the low number of individuals.

For the mapping population the outcomes of the research will be dependent on the agreement between Syngenta and Rothamsted Research. The data presented in Chapter 3 has the potential to be published and could be of great use in the field of research. The mapping of QTLs and possible genes related to photosynthesis could also be a strong outcome of this research. In terms of future work, in addition to the QTL mapping, there is an opportunity for improvement of the modelling based on reflectance indices which could help in the predicting of farm yields, mainly if coupled to high-throughput phenotyping.

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