The effect of non-glaucousness, as conferred by *Inhibitor of Wax 1*, on physiology and yield of UK Wheat

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By

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Abstract

As the first barrier to the external environment, the epicuticular waxes have a number of key roles in plant physiology. Although the wheat wild progenitors display a diversity of epicuticular wax phenotypes, the glaucous (visible wax) phenotype dominates cultivated varieties. However, the UK winter wheat variety Shamrock is unusual in that it exhibits a non-glaucous phenotype, conferred by the wild emmer gene *Inhibitor of Wax 1 (Iw1)*. UK field trials with Shamrock associated a yield advantage of 4.15% with *Iw1.* This PhD tests the hypothesis that *Iw1* imparts an advantage for wheat yield and physiology in the UK.

Crossing Shamrock with six glaucous UK winter wheat varieties (Malacca, Alchemy, Hereward, Xi19, Robigus and Einstein) created non-glaucous near isogenic lines (NILs) with *Iw1*. NILs were grown at multiple field trial locations in the east of England over four years. A long-term shade trial reducing incoming light by 40 and 60% was also carried out in 2014. Yield, and various physiological components including water use efficiency (WUE) and spectral properties, were measured.

Iw1 reduced flag leaf photosynthetically active radiation (PAR) reflectance by 15-40% and canopy reflectance by 12-20% ($p<0.05$). Despite this, *Iw1* did not affect flag leaf PAR absorbance or canopy temperature, and conferred no advantage under long-term shading. Furthermore, there was no difference between NILs in photoinhibition following an extended period of high light stress. *Iw1* did not affect WUE or yield. However, non-glaucous Hereward and Alchemy NILs yielded 4.96±1.15% ($p<0.001$) and 2.59±1.01% ($p=0.045$) more than their glaucous counterparts, although this advantage did not map to *Iw1*.

Iw1 offered no advantage to UK winter wheat under normal UK growing conditions, nor under long-term shading. However, the yield advantage associated with the *Iw1* introgression in Hereward and Alchemy is significant within a backdrop of plateauing wheat yields and worth pursuing.

Table of Contents

List of Figures

List of tables

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Chapter 1 Introduction

1.1 Food production

The world population reached 7.3 billion by mid-2015, and by 2050 is projected to rise to 9.7 billion (United Nations, 2015). To keep up with demand, global food production will need to increase by around 70% over the next 35 years (FAO, 2009), equating to around a 2.4% increase in global crop yields per year (Ray *et al.,* 2013). However, a growing population places multiple demands on land. In addition to food crops, biofuel use is growing, more land for meat and dairy production will be required, and greater expansion of urban areas will be necessary. These competing demands mean that, in terms of crop production, increasing yields rather than clearing more land will be the most sustainable way forwards (Foley *et al.,* 2011; Godfray *et al.,* 2010). Sustainable intensification also requires more efficient use of pesticides, fertilizer, and limited resources such as water (Cattivelli *et al.,* 2008; Porter *et al.,* 2014). Yet further strain is being placed on the food supply from the changing climate. Not only are global temperatures increasing, but instances of climate variability and extremes of climate are on the rise (Gourdji *et al.,* 2013; Tingley & Huybers, 2013). These changes are already having an impact on agricultural systems. For example, it has been estimated that climate change has reduced wheat yields by 2.9% globally between 1980 and 2008, when taking account of changes to temperature, precipitation and CO_2 levels (Lobell *et al.,* 2011). As such, an understanding of which traits are beneficial to crop yield under particular environmental conditions is required to adapt agricultural systems as the climate changes.

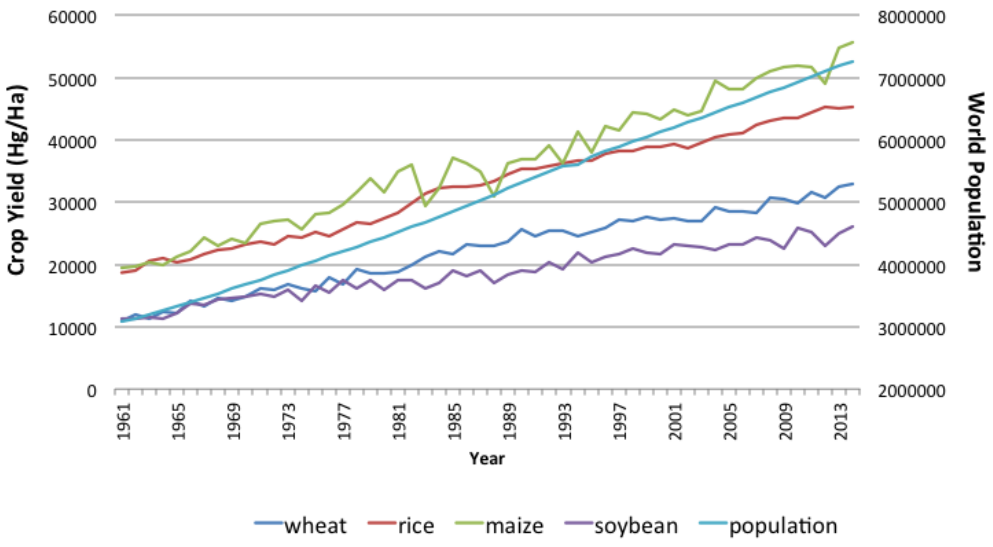

Figure 1. 1 Yield of maize, rice, soybean and wheat between 1961 and 2014

Annual population growth over the same period is also shown. Generated using FAO stat http://faostat3.fao.org/compare/E accessed on 21/02/16

Together, maize, rice wheat and soybean provide nearly two thirds of the worlds calories (Ray *et al.,* 2013). Not only that, but wheat and rice are important sources of dietary protein, providing 21% and 13% of protein respectively (Shiferaw *et al.,* 2013). These crops are therefore important targets for yield improvement. The increase in global yield of wheat, rice, soybean and maize between 1961 and 2014 is shown in Figure 1.1, alongside population growth. The adoption of new varieties and technologies in the green revolution of the 1960s allowed large increases in yield potential of wheat and rice (Evenson & Gollin, 2003). However, yield improvement has since been declining year on year. Between 1966 and 1979 wheat yields increased on average by 3.6% per year, and between 1984 and 1994 by only 2.8% (Dixon *et al.,* 2008). A 2013 study showed that between 1989 and 2008 global rates of yield increase for wheat had fallen to just 0.9% per year. Continuing at this rate, wheat yields will only have increased by 38% by 2050 (Ray *et al.,* 2013). Similarly with rice production, current rates of yield improvement will lead to an increase in production of only 42% over the next 3-4 decades. Furthermore, an in depth analysis of global crop yields by region between 1961 and 2008 indicates that yields are now stagnating in 37% of wheat and 35% of rice growing regions (Ray *et al.,* 2012). The identification of novel ways to improve yields of wheat, rice, and other major food crops, is now of high importance to ensure future food security. Wheat provides 41% of calories and 50% of proteins from total cereal consumption daily (Shiferaw *et al.,* 2013) making it an important target for yield improvement.

1.2 Targets for wheat yield improvement

Grain yield in wheat is related to the proportion of carbon allocated to grain, termed harvest index, and how much biomass the plant can produce (Figure 1.2). Since the 1960s significant increases in wheat harvest index (HI) have been achieved, for example through the use of dwarfing genes (Mathews *et al.,* 2006). This increased allocation of carbon to grain has resulted in great improvements to yield (Shearman *et al.*, 2005). However, whilst genetic variation of HI does still exist in elite germplasm in the range of 0.4-0.55 (Sayre *et al.,* 1997; Shearman *et al.,* 2005), many now argue that we are approaching the maximum possible HI, and scope for further improvement is limited. More recent increases in yield potential have been associated with increasing biomass (Fischer & Edmeades, 2010), suggesting that future efforts should focus on this portion of the yield equation (Furbank *et al.,* 2015; Parry *et al.,* 2011).

The amount of biomass produced is determined in part by crop radiation use efficiency (RUE), which is normalised on the amount of light that can be intercepted or absorbed by the crop and how efficiently this light can then be used (Furbank *et al.,* 2015). Photosynthesis (net canopy carbon gain) in particular is now the target of many projects aiming to increase yield, as research indicates that substantial improvements to photosynthetic efficiency are still possible within elite wheat

germplasm (Long *et al.,* 2006; Zhu *et al.,* 2010). There are over 60 metabolic reactions in photosynthetic carbon metabolism (Zhu *et al.,* 2007). Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is the enzyme that carries out the reaction that fixes CO_2 during photosynthesis. As the primary carboxylase in C3 plants it has a key role in photosynthesis. As such, the genetic modification of this enzyme and its regulators, to improve efficiency of carbon fixation, is currently the focus of many researchers (Parry *et al.,* 2007).

In addition to the carboxylation of CO2, Rubisco catalyses the oxygenation of O_2. Its activity and efficiency of carbon fixation is therefore sensitive to CO_2 concentration. One approach to improve photosynthesis is to engineer Rubisco such that its specificity to CO_2 relative to O_2 is increased. This would decrease photorespiration, potentially increasing photosynthesis (Zhu *et al.,* 2010). A study that simulated canopy photosynthesis found that average Rubisco specificity in C3 crops is not optimised to current levels of atmospheric CO_2 at 400 ppm, finding that it is instead more suited to pre-industrial CO_2 levels of around 220 ppm. Their work suggests that should C3 Rubisco specificity be optimised for the current atmosphere, the same amount of Rubisco could assimilate around 10% more carbon (Zhu *et al.,* 2004). However, it has been found that where the specificity of Rubisco is increased, the rate of carboxylation becomes lower (Bainbridge *et al.,* 1995; Zhu *et al.,* 2004). This trade-off means that how Rubisco is engineered will depend on the target species, environment, and desired result. This could vary even between organs of the same plant and within crop canopy. For example, further down the canopy where photosynthesis is light limited, increased Rubisco specificity to CO_2 could increase photosynthesis. However in the upper leaves that are not light limited, increased specificity could lower the rate of light saturated photosynthesis. Therefore ideally Rubisco with a fast catalytic rate would be present at the top of the canopy, becoming more specific moving down (Zhu *et al.,* 2004).

Improvement of Rubisco specificity could be achieved through the genetic engineering of crop plants to express Rubisco of another species. For example expressing highly specific Rubisco of red alga *Griffithsia monolis* in C3 crops could allow increases in photosynthesis of 27% if expressed in lower regions of the canopy (Long *et al.,* 2006; Zhu *et al.,* 2004). However, genetically modifying a plant to express the Rubisco of another species is a challenge. Each of the subunits of Rubisco is coded from a different region in the genome and expression of each component needs to be coordinated to produce a functional enzyme (Zhu *et al.,* 2010). Therefore this has yet to be applied successfully in the field.

Another option is the introduction of the C4 mechanism to concentrate carbon from crops such as maize and sorghum into C3 crops such as wheat and rice. This results in a reduction or removal of photorespiration, increasing efficiency of photosynthesis. Although to date there has been limited success in wheat, this technique has been demonstrated in oat-maize addition lines. Introduction of maize chromatin into C3 oat plants resulted in the successful expression of C4 photosynthetic enzymes, although limited change was recorded in photosynthesis in the addition lines indicating there is substantial progress yet to be made (Kowles *et al.,* 2008). Other projects have focused on increasing the photosynthetic capacity of non-leaf plant tissue, which can contribute up to 25% of carbon to grain filling (Gebbing & Schnyder, 1999).

Research of this kind into the intracellular processes relating to photosynthesis is important for achieving higher RUE. However, this needs to be done in parallel with research focussed on crop structure and morphology. RUE represents the biomass produced per unit radiation intercepted (Figure 1.2). However, light interception in general has been neglected in the past and not been selected for in breeding programmes (Reynolds *et al.,* 2011). This is therefore an important target for improvement that could allow further increases in yield when applied in conjunction with higher light use efficiency. It should be noted that in sub-optimal environments, resistance to biotic and abiotic stress is of higher priority for yield. For example, in drought prone environments yield is considered to be equal to *water use x water use efficiency x HI* (Reynolds & Tuberosa, 2008). This will need to be a factor taken into consideration increasingly as the climate changes and more regions of the world experience extremes of climate.

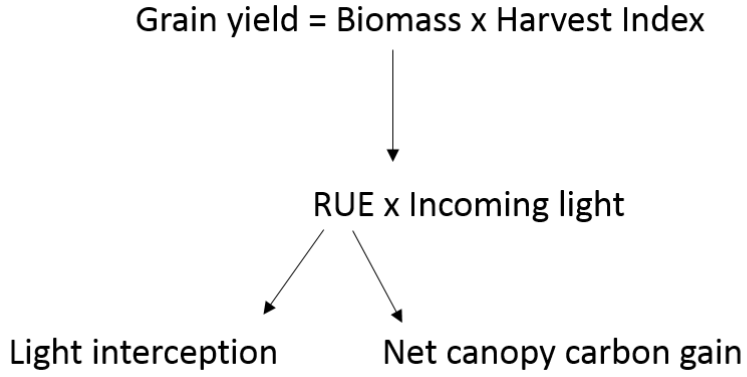

Figure 1. 2 Grain yield components

Grain yield can be improved through increasing the harvest index, or increasing biomass. Adapted from Furbank *et al.,* (2015)

1.3 Advances in wheat breeding genetics

To date, genetic progress concerning yield has primarily been achieved through crossing varieties with favourable traits and then selecting offspring that display a desired phenotype (Reynolds *et al.,* 2011). However, over the past two decades great advances in molecular biology have been made, enabling significant improvements in the understanding of crops at the genetic level (Slafer, 2003). The subsequent development of numerous resources, such as high throughput sequencing and new bioinformatics tools, will assist in identifying beneficial genes for crop improvement. A thorough knowledge of the genes that confer beneficial traits will also enable the more efficient assembly of new genotypes and speed up the breeding process. However, an accompanying understanding of traits at the physiological level is vital for identifying interactions between traits and developing selection criteria (Araus *et al.,* 2002; Jackson *et al.,* 1996; Slafer, 2003). Furthermore, finding a genetic basis for more complex traits remains difficult. In these instances, a clear understanding of plant physiology is of even greater significance. Moving forwards, a fully integrated approach is required, whereby both the underlying genetics and physiology of desirable traits are understood.

1.4 Improving wheat genetic diversity

One issue currently facing wheat breeding is lack of genetic diversity from which to select beneficial traits. The expansion of this gene pool is now a priority for the advancement of wheat yield. This problem is a result of the domestication process, an understanding of which requires knowledge of the history of wheat (Figure 1.3). The cultivated wheat varieties grown today originate from the tetraploid wild emmer *Triticum turgidum* species *dicoccoides* (AABB), which is the result of the hybridization of *T. urartu* with a member of the *Sitopsis* family that is as yet unknown (Haider, 2013). The domestication of wheat from this wild progenitor began around 10,000 years ago in the Fertile Crescent (Nesbitt & Samuel, 1998; Tanno & Willcox, 2006), and tetraploid durum wheat (*T. durum*) was directly derived from selecting wild emmer (*T.dicoccoides*) for desired traits (Haudry *et al.,* 2007). A second hybridization event, whereby *T. dicoccoides* was crossed with a wild diploid grass *Aegilops tauchii* (Jia *et al.,* 2013), resulted in allohexaploid bread (or common) wheat (*T.aestivum)*. Over the past 10,000 years, the refinement of *T. durum* and *T. aestivum* species for their use in agriculture and as a food source has resulted in the selection of a narrow range of traits and physiological divergence from the wild progenitors. For example, reduced seed shattering, free threshing and a soft glume were selected for early on as traits that improved yields and made harvest more efficient (Tzarfati *et al.,* 2013). Subsequently, components of seed size and shape (Eckardt, 2010; Golan *et al.,* 2015) and plant stature (Borojevic & Borojevic, 2005) have also been selected for, amongst other characteristics contributing to yield. It is a side effect of this selection process that potentially beneficial alleles have been lost from the gene pool. Analysis of nucleotide

diversity within the genome of *T. durum* and *T. aestivum* indicate an overall respective loss of genetic diversity of 84% and 69% in comparison to *T.dicoccoides* (Haudry *et al.,* 2007). The loss of diversity is particularly evident within the *T. aestivum* D genome, nucleotide diversity of which has reduced by around 90% due to inability to freely backcross. This presents a problem whereby there is a lack of potential alleles within the gene pool for yield improvement.

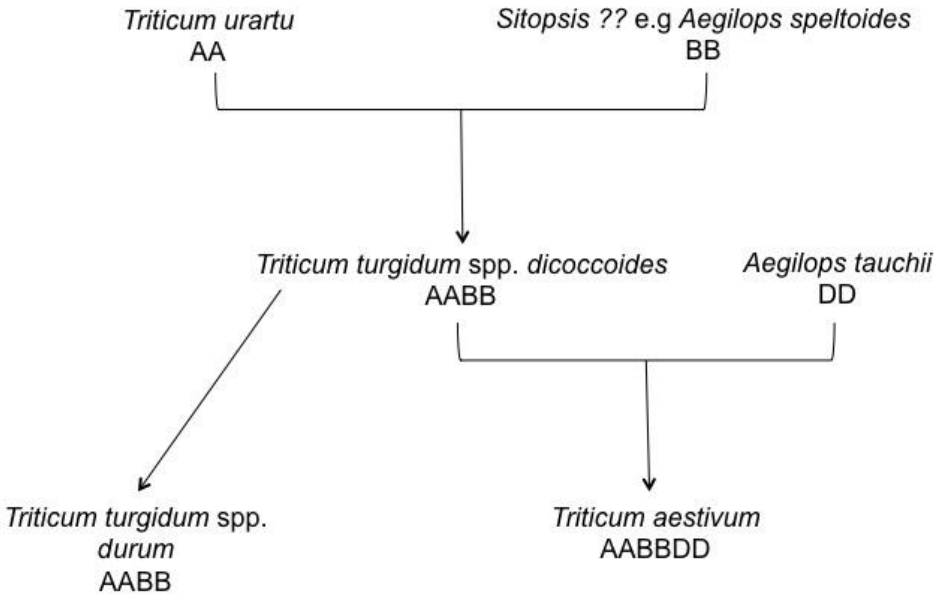

Figure 1. 3 The history of modern wheat

T. durum is a diploid (AABB), and *T. aestivum* is a hexaploid (AABBDD). This polyploidy was a result of hybridization events shown in the schematic.

A solution to this problem is to out-cross modern wheat varieties to other species within the *Triticeae* tribe (Skovmand *et al.*, 2001). This provides a source of novel traits that could increase yields, disease resistance, and resilience within marginal environments (Zamir, 2001). For example, introgressions from the *Secale cereale* (rye) genome, in particular the short arm of chromosome 1R (1RS), have been widely used in breeding programmes since the 1930s (Reynolds *et al.,* 2011). Wheat varieties containing the 1RS translocation have been shown to be higher yielding, drought tolerant and have high nitrogen use efficiency (Ehdaie *et al.*, 2003). A more recent project at the Centre for Wheat and Maize Improvement (CIMMYT), Mexico, has been the development of synthetic wheat varieties. This involved crossing *T. dicoccoides* and *Ae. tauschii* to develop over 1000 synthetic wheat lines (Dreisigacker *et al.,* 2007; Rana *et al.,* 2013; Warburton *et al.,* 2006). These lines can be crossed with elite wheat varieties to expand genetic variation and introduce desirable traits such as improved disease resistance and tolerance to biotic and abiotic stresses. Promising effects on yield, disease resistance and genetic diversity have been demonstrated in Mexico and China after incorporating these synthetic varieties into breeding varieties (Dreisigacker

et al., 2007; Yang *et al.,* 2009). More recently, a new synthetic wheat programme has been developed at the National Institute for Agricultural Botany (NIAB), UK, which targets a distinct set of *Ae. tauchii* accessions not incorporated into the CIMMYT programme.

Whilst the synthetic wheats offer great potential, in particular where the D genome is concerned, the wild ancestors of wheat have been used for some time to introduce advantageous traits into cultivated germplasm. For example, *T. dicoccoides* has already been successfully used to confer resistance to a number of diseases in cultivated wheat including stripe rust (Fahima *et al.,* 1998; Nevo *et al.,* 1986), powdery mildew (Nevo *et al.,* 1985), and stem rust (Nevo *et al.,* 1991). Research has also been carried out into the use of *T. dicoccoides* for improvement of more complex traits such as photosynthetic rates (Carver *et al.,* 1989; Evans & Dunstone, 1970) and increased grain protein content (Gerechter-Amitai & Stubbs 1970; Uauy *et al.,* 2006; Uauy *et al.,* 2006). However, whilst this remains a promising area of research, there has to date been limited success in the field regarding these more complex traits. One trait for which there is high variability within wild wheat progenitors such as *T. dicoccoides* and *Ae. tauchii*, is epicuticular wax type, the focus of this PhD.

1.5 Epicuticular wax

Epicuticular waxes are an important component of the plant cuticle (Edwards *et al.,* 1996) which is primarily composed of cutin and waxes (Figure 1.4). Intracuticular waxes are embedded within the complex polymer matrix of the cuticular layer and cuticle proper, whilst epicuticular waxes coat the external surface (Kerstiens, 2007; Kolattukudy, 1980; Post-Beittenmiller, 1996). The cuticle is the first point of contact between the plant and the external environment and as such has a number of key roles in plant physiology. These include influencing plant-insect interactions (Cervantes *et al.,* 2002; Morris *et al.,* 2000; Ni *et al.,* 1998), providing protection from certain fungal pathogens (Tsuba *et al.,* 2002), reflecting radiation within the photosynthetically active and ultra violet spectra (Holmes & Keiller, 2002) and reducing excessive water loss from the plant surface (Woodward, 1998). Given this importance in modulating the plant-environment interaction, specific adaptations of the cuticle and associated components to particular environmental conditions would be expected to be beneficial for plant fitness. For example, quantity of epicuticular wax has been shown to increase under drought stress (González & Ayerbe, 2010; Uddin & Marshall, 1988), whilst plants in high irradiance environments can display more reflective epicuticular waxes (Close *et al.,* 2007; Richardson *et al.,* 2003). Accordingly, the maintenance of a range of epicuticular wax phenotypes within a species might be expected, as is the case in the wheat wild relatives. However, this is not the case in cultivated wheat, where variation in epicuticular wax is limited. In fact, the same phenotype persists in wheat growing regions around the globe. Given the role of the

epicuticular waxes in plant physiology, in particular their association with water and radiation use, they are a potential target for increasing biomass and adapting wheat to variable environmental conditions. The following sections of this chapter present an overview of epicuticular wax morphology and biochemistry. This will enable understanding of the possible epicuticular wax phenotypes in wheat, and how this trait might be used in breeding programmes.

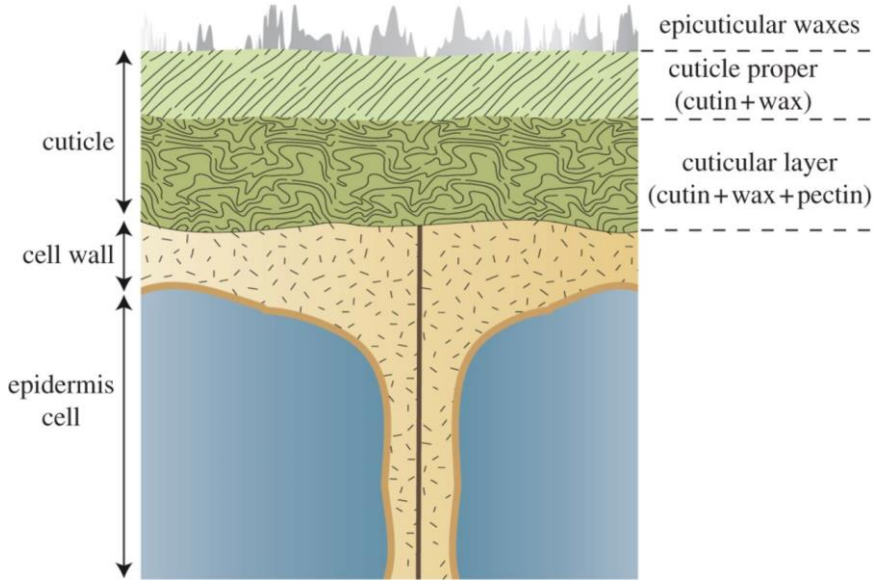

Figure 1. 4 Components of the plant cuticle

The plant cuticle main components are cutin and waxes. Both intracuticular waxes within the cuticle matrix and epicuticular waxes covering the plant surface are a key cuticular component. From Antoniou Kourounioti *et al.,* (2013)

1.6 Epicuticular wax synthesis

There are many different types of epicuticular wax, each varying in biochemistry and morphology dependant on plant ecology. The most common compounds in plant waxes are straight chain, saturated primary alcohols, aldehydes and fatty acids of predominantly even chain length. Secondary alcohols *n*-alkanes, and ketones are also present of largely odd chain length. Hydrocarbon chains can be branched or unsaturated, with odd or even chain lengths between 20-34 carbons. A large number of compounds can make up epicuticular wax, and accordingly a variety of morphological structures have been identified, determined to a large extent by wax composition.

Some examples of these morphologies, including plates, tubules, filaments and crusts, are shown in Figure 1.5 (Koch & Ensikat, 2008).

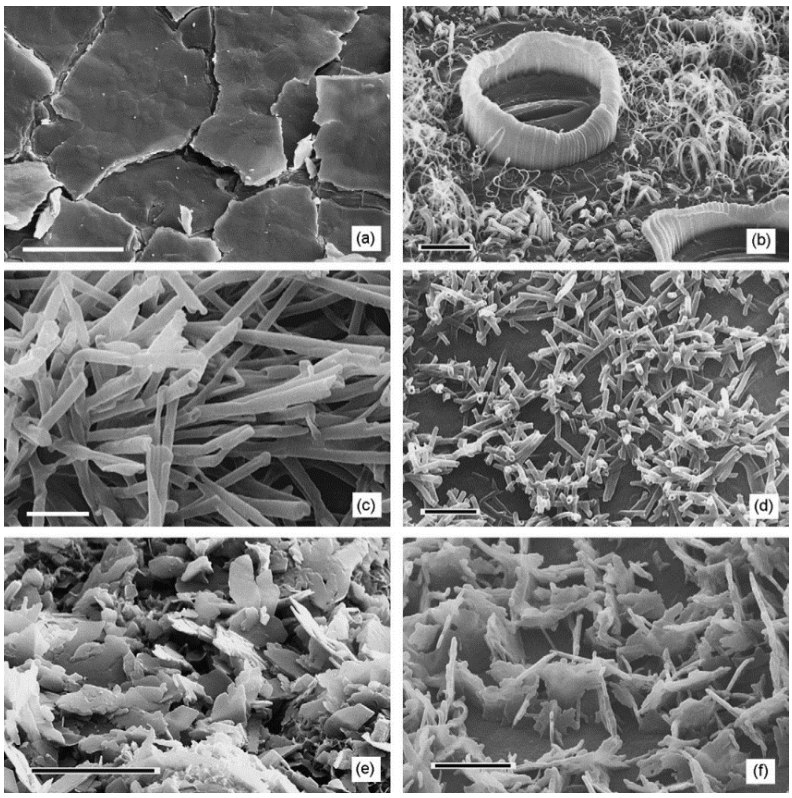

Figure 1. 5 Examples of epicuticular wax morphology

Scanning Electron Microscope images of (a) Crusts (bar = 100 µm), (b) filaments (bar = 10 µm), (c & d) tubules (bar = 1 µm), (e) plates (bar = 10 µm), and (f) platelets (bar = 1 µm). Each structure is normally around 0.2-100 µm in size. Figure from Koch & Ensikat (2008).

Despite the variety of epicuticular wax types across the plant kingdom, the biosynthetic pathways involved in wax production are relatively well conserved across all plant species (Post-Beittenmiller, 1996). The first step of epicuticular wax synthesis takes place in the plastids of the epidermal cells, where the fatty acid synthase multi-enzyme complex adds activated units of malonyl CoA to a carbon acceptor in a cycle of reactions that generates acyl chains of 16 or 18 carbons (C16/ C18) in length. Depending on the tissue and developmental stage, these C16/C18 products will then be used for synthesis of glycerolipids, waxes, cutin or suberin (Post-Beittenmiller, 1996; von Wettstein-knowles, 2012). Partitioning between these various destinations also depends on chain length and saturation. For instance, unsaturated C18 chains are used for glycerolipid, cutin and suberin biosynthesis whilst saturated C18 chains are used for cuticular wax biosynthesis. The mechanisms regulating this partitioning, such as enzyme specificity or substrate availability, are likely to be a key regulatory point controlling epicuticular wax quality and quantity (Kolattukudy, 1980; Post-Beittenmiller, 1996; von Wettstein-knowles, 2012).

The C16/C18 chains destined for cuticular wax synthesis are exported from the epidermal cell plastids and transported to the endoplasmic reticulum of the epidermal tissues (Kolattukudy, 1968; Lessire *et al.,* 1982; Whitfield *et al.,* 1993). Here, very long chain fatty acids (VLCFAs) of C20-C34 are generated by elongase enzyme complexes (Evenson & Post-Beittenmiller, 1995; Whitfield *et al.,* 1993) and serve as precursors to the three parallel pathways generating epicuticular wax components. Figure 1.6 shows a simplified diagram displaying the major steps, products and intermediates of the three pathways (a) decarbonylation, (b) acyl reduction and (c) β-ketoacyl-elongation. Each pathway is responsible for contributing a number of different compound classes to final epicuticular wax composition. A number of other compounds not shown in Figure 1.6 are also produced via these pathways, for example very long chain esters, triterpenoids, flavenoids and phenolics (von Wettstein-knowles, 2012; Post-Beittenmiller, 1996; Koch & Ensikat, 2008). However these are present in much smaller quantities. The compounds synthesised in the endoplasmic reticulum via these three pathways are then translocated to the plasma membrane and eventually to the cell wall and outer surface of the plant (Samuels *et al.,* 2008).

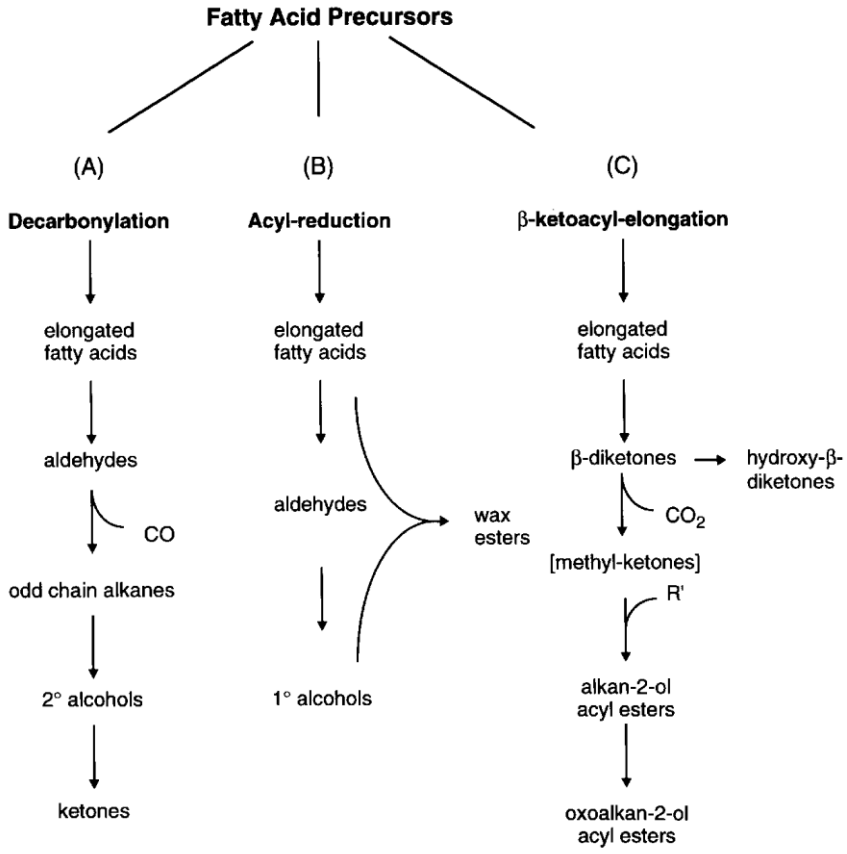

Figure 1. 6 The three major pathways of epicuticular wax biosynthesis

The major steps, products and intermediates in the three epicuticular wax biosynthetic pathways of decarbonylation, acyl reduction and β-ketoacyl elongation. Figure from Post-Beittenmiller, (1996).

It is thought that all three of the biosynthetic pathways detailed in Figure 1.6 are found in the epidermal tissues of most plant species. It is the relative contribution of each pathway that varies between organs and species, creating distinct epicuticular waxes. Additionally, the genetics determining how, when, and where these pathways are expressed within the plant will vary considerably. Minor alterations to wax biochemistry can result in significant changes to visual appearance and potentially modify the properties of the epicuticular waxes.

1.7 Glaucousness in cereal crops

Wheat, and other temperate grasses such as barley, can display a glaucous phenotype, meaning the plants have layer of visible epicuticular wax covering the external surfaces. This gives the plant a distinctive bluish-grey colouring. The opposing phenotype, non-glaucous, is used to describe plants that lack these visible waxes. The resulting appearance is bright green, also termed viridescent (Figure 1.7). The major biochemical difference between these two wax phenotypes is a change in expression of the β- ketoacyl- elongation pathway (Figure 1.6c). The glaucous epicuticular waxes in wheat contain β-diketone and OH- β-diketone compounds, which result in the formation of wax tubules (Figure 1.8b) that protrude from the plant surface (Baker, 1982; Meusel *et al.,* 2000). Non-glaucous waxes lack these products from the β- ketoacyl- elongation pathway, resulting in the formation of wax platelets shown in Figure 1.8a (Bianchi & Figini, 1986; King & von Wettstein-Knowles, 2000). This change in morphology alters light scattering from the plant surface, hence determining visual appearance.

Figure 1. 7 Non-glaucous and glaucous wheat in the field

The non-glaucous wheat on the left lacks visible waxes exhibiting a bright green, viridescent phenotype. On the right are glaucous wheat plants, with a bluish-grey appearance and visible waxes.

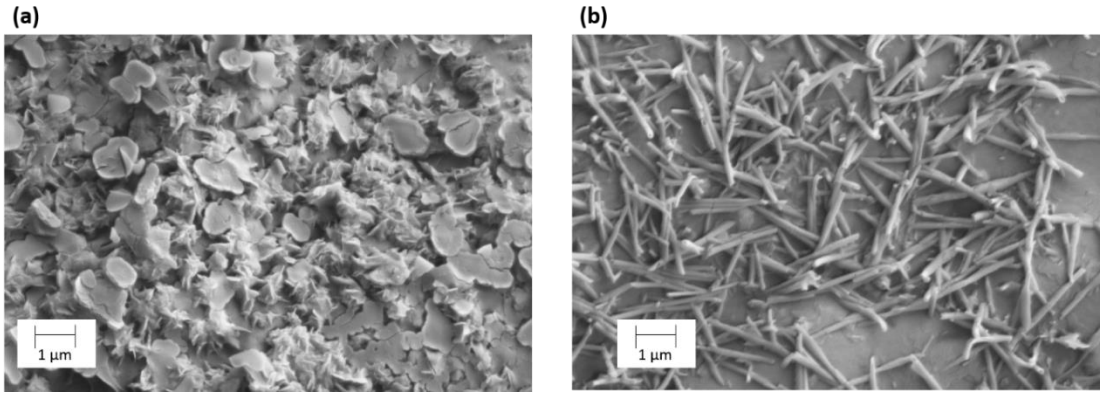

Figure 1. 8 SEM images of wax platelets and tubules

The wheat flag leaf abaxial surface of (a) a non-glaucous plant showing platelet epicuticular wax structures and (b) a glaucous plant with visible wax showing the tubular wax structures that form in the presence of β-diketones.

Wax morphology and composition can also determine visual appearance for other cereal crops such as maize and sorghum. Glossy mutants of maize have a more viridescent appearance than the wild type. In contrast to wheat, rather than complete absence of a compound class, both wild type and mutants contain waxes composed of *n*-alkanes, aldehydes, alcohols and esters, just in differing proportions (Bianchi *et al.,* 1979). Consequently, rather than the distinct morphologies displayed in Figure 1.8, the same wax crystalline structures can be identified in both glossy mutants and wild type maize, but these differ in density and distribution. For example, 40% of the surface of wild type maize has been found to be covered in crystalline wax structures, whereas in glossy mutants this is in the region of 10-18% (Beattie & Marcell, 2002). Another commonly grown crop, sorghum can have a waxy bloom (glaucous), or have a bloomless appearance (non-glaucous). Similarly to maize, no compound class or morphological structure is clearly absent from the epicuticular waxes of bloomless plants, although bloomless plants do tend to have lower quantities of C16-C34 fatty acids, in particular C28 and C30 (Jenks *et al.,* 2000). This comparison across wheat, maize and sorghum demonstrates that different underlying wax biochemistries can result in very similar visual phenotypes.

1.8 Epicuticular wax genetics in wheat

The majority of cultivated wheat varieties display a glaucous phenotype, and non-glaucousness is rare in elite varieties. Conversely, wild progenitors of wheat such as *T. dicoccoides* and *Ae. Tauchii* show wide variation in glaucousness, indicating that genetic diversity for epicuticular wax has been lost during domestication. Five genes are currently known to determine whole plant glaucousness in wheat (Figure 1.9). Given the polyploid nature of wheat, and redundancy in the genome, these

genes are present as homologues on multiple chromosomes, with a number of genes conferring the same phenotype.

WAX-1 (*W1*), on the short arm of chromosome 2B (2BS) is a dominant gene, presence of which results in a whole plant glaucous phenotype (Allan & Vogel, 1960; Tsunewaki, 1964). *Inhibitor of Wax -1* (*Iw1*), also on 2BS, is a dominant inhibitor of glaucousness, and is dominant over *W1*. Presence of *Iw1* results in the absence of β-diketone and OH- β-diketone compounds from all aerial organs, conferring a non-glaucous phenotype (Adamski *et al.,* 2013; Driscoll & Jensen, 1964; Jensen & Driscoll, 1962). A non-glaucous phenotype can arise from having two non-functional copies of *W1 (w1/w1)*, or one copy of *Iw1 (Iw1/W1)*. *Iw1* and *W1* are very close on chromosome 2BS, but Tsunewaki & Ebana (1999) showed recombination between the two loci, proving that they are not allelic. As such the exact interaction between *W1* and *Iw1* appears to be complex and is to date not fully understood. However, *W1* has been recently been cloned in wheat (Adamski & Uauy, personal communication), which should allow further progress in the understanding of this interaction. Homologous genes with the same effect on the epicuticular waxes, *Iw2* and *W2*, are found on the short arm of chromosome 2D (Liu *et al.,* 2006; Yoshiya *et al.,* 2011; Zhang *et al.,* 2013). Both *Iw1* and *Iw2* can inhibit the action of both *WAX* genes, so conferring non-glaucousness in a range of genetic backgrounds (Tsunewaki & Ebana, 1999). However, the *Inhibitor of Wax* genes are thought to be either absent, or present in a non-functional form, in the majority of cultivated wheat germplasm. More recently *W3* has been identified on chromosome 2BS, thought to be closely linked to *W1* and complement the action of *W1* and *W2* (Zhang *et al.,* 2015).

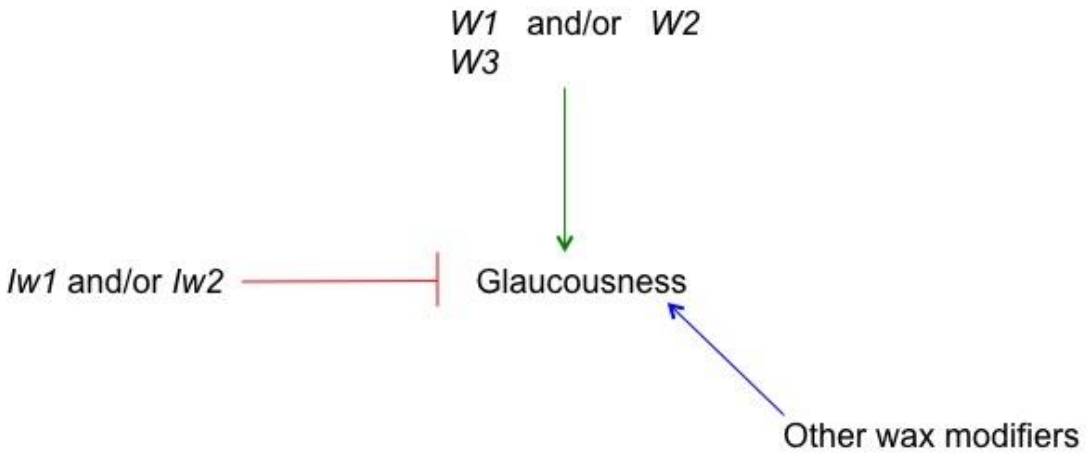

Figure 1. 9 Genes determining epicuticular wax in wheat

W1, *W2* and *W3* confer a glaucous phenotype in wheat, whilst *Iw1* and *Iw2* act as glaucous inhibitors. The presence of these wax-inhibiting genes confers a non-glaucous phenotype. Other genes also modify the epicuticular waxes.

In addition to these wax genes influencing whole plant glaucousness, a number of 'other wax modifiers' (Figure 1.9) have been identified that alter glaucousness of individual organs. For example, *Iw3*, mapped to the short arm of chromosome 1B, only inhibits glaucousness in the spike, with β-diketone compounds still synthesised in all other aerial organs (Wang *et al.*, 2014). Another example is a QTL, named *Qw.aww-3A*, identified on chromosome 3A, presence of which results in visibly more waxy flag leaves (Bennett *et al.,* 2012).

1.9 The effect of glaucousness on wheat physiology and yield

The genetics of glaucousness and precise interaction between the genes is still not fully understood. Much of the research in this area is relatively new and rapidly progressing. However, the effect of glaucousness on crop physiology and yield has been studied for some time in a number of cereal crops including wheat, barley and sorghum. Glaucous plants of a number of crop species have been shown to be between 5 and 30% higher yielding than non-glaucous varieties of the same species (Clarke & McCaig, 1982; Febrero *et al.,* 1998; Jefferson *et al.,* 1989; Johnson *et al.,* 1983.; Merah *et al.,* 2000; Premachandra *et al.,* 1994; Richards *et al.,* 1986). In addition, glaucous plants have repeatedly been found to reflect more incoming light within the PAR, UV and infrared spectra than non-glaucous (Febrero *et al.,* 1998; Holmes & Keiller, 2002; Jefferson *et al.,* 1989; Johnson *et al.,* 1983). This characteristic has been shown to result in cooler canopies (Jefferson *et al.,* 1989; Mondal *et al.,* 2014; Richards *et al.,* 1986), and could provide protection from excessive PAR and UV radiation (Close *et al.,* 2007; Mohammadian *et al.,* 2007; Richardson *et al.,* 2003). However, in some environments this could present a disadvantage in terms of reduced light interception (Febrero *et al.,* 1998). Less consistent results have been obtained regarding water use efficiency (WUE). Work in wheat indicate that glaucous varieties may have higher WUE (Richards *et al.,* 1986), which would allow better growth within a water-stressed environment. However, there is disagreement on this, with some work indicating that over the long term, glaucousness actually reduces WUE (Febrero *et al.,* 1998; Merah *et al.,* 2000; Monneveux *et al.,* 2004), and a few studies reporting no difference in WUE between glaucous and non-glaucous lines (Adamski *et al.,* 2013; Johnson *et al.,* 1983). These multiple effects on yield and physiology will be discussed in more depth within Chapters 3-6.

With potential implications for RUE, yields, and WUE, the effects of glaucousness are important in the context of current wheat breeding. However, there is a gap in this existing literature. A large proportion of the studies regarding glaucousness in cereal crops were carried out in a Mediterranean environment. Cereal crops are grown across the globe in diverse regions. Given the importance of the epicuticular waxes in terms of plant interaction with the environment, a blanket application of epicuticular wax type across growing regions may not be the most productive

approach. For example, more recent work in wheat suggests that glaucousness may only increase yields under water stressed conditions (Merah *et al.,* 2000). In fact, the non-glaucous phenotype has been reported to confer a significant yield benefit under optimum growing environments such as the UK of up to 5% (Merah *et al.,* 2000; Simmonds *et al.,* 2008). An additional problem with existing work regarding epicuticular waxes in cereal crops is lack of defined germplasm. There are many forms of epicuticular wax, with wide variation in biochemistry, morphology and underlying genetics. Each of these factors is likely to affect wax properties and plant physiology, but have often not been specified in past studies. Therefore it is of high priority that the genetics and biochemistry of epicuticular wax are characterised alongside any physiology work. This would allow better comparison between studies and an understanding of under which circumstances specific epicuticular wax genes could be of benefit.

1.10 Research aims

To address these issues, this PhD will focus on the specific *Inhibitor of Wax* gene, *Iw1*, which derives from *T. dicoccoides* (Simmonds *et al.,* 2008). Yield increases of around 2.4 - 5.6% have been associated with *Iw1* in UK germplasm (Simmonds *et al.,* 2008). *Iw1* has also been associated with significantly delayed senescence (Simmonds *et al.,* 2008), and shown not to affect water use efficiency within a UK environment (Adamski *et al.,* 2013). This latter study also characterised the genetics of *Iw1*, and precise action on epicuticular wax biochemistry in UK germplasm (Adamski *et al.,* 2013). This potential to significantly increase yields and the availability of well-defined germplasm makes *Iw1* an ideal candidate to further assess the effect of non-glaucousness on physiology and yield in more detail. Using near isogenic lines with and without *Iw1*, I aim to answer the question: Does non-glaucousness, as conferred by *Iw1*, confer an advantage for yield and physiology of UK wheat?

To answer this overarching question, a number of hypotheses will be addressed across the results chapters:

- In Chapter 3 the *Iw1* germplasm will be defined in terms of epicuticular wax biochemistry, and potential yield effects conferred by *Iw1* will be investigated to address the first hypothesis: (i) *Iw1* increases yield and delays senescence across a wide range of UK winter wheat varieties.
- In Chapter 4 I will address the spectral properties of *Iw1* NILs to explore effects of non-glaucousness on RUE and test the hypotheses that (i) the reduced reflectance of non-glaucous wheat leaves and canopies makes more PAR available to photosynthetic tissues, and that (ii) non-glaucous canopies have a higher temperature in the field.

- Water use efficiency, a trait often associated with glaucousness, will then be addressed in Chapter 5, with the hypothesis that (i) there is no effect of non-glaucousness as conferred by *Iw1* on water use efficiency within a UK (East of England) environment.
- Finally, the role of glaucousness in determining plant interaction with the environment when PAR availability is altered will be explored in Chapter 6. These facets will be studied both through exposure to intense light levels in a controlled environment, and a long term study whereby *Iw1* NILs were exposed to long-term low level irradiance in the field. I hypothesise that (i) the increased reflectance of glaucous epicuticular waxes provides protection when exposed to high levels of light, and that (ii) reduced reflectance of non-glaucous epicuticular waxes would prove beneficial under low solar-irradiance.

Chapter 2: Materials and Methods

Detailed within this chapter are the materials and methodology that have relevance throughout the thesis. Methods specific to each part of the project are described within the relevant results chapters (Chapters 3 – 6).

2.1 Germplasm

2.1.1 Near Isogenic Lines (NILs)

The non-glaucous hexaploid UK winter wheat variety Shamrock is a bread making wheat (Nabim group 1) derived from UK adapted germplasm (CWW 4899/25) crossed with *a T. dicoccoides* derivative (Comp Tig 323-1-3 M). Shamrock was crossed to the glaucous variety Shango, also a hexaploid UK winter wheat, to create a doubled haploid (DH) population containing 87 lines, as detailed by Simmonds *et al.,* (2008). To understand the effect of *Iw1* in multiple genetic backgrounds, a non-glaucous Shamrock x Shango DH line containing around 30% of alleles from Shamrock including *Iw1* was crossed to six hexaploid UK winter wheat varieties that confer the glaucous phenotype: Malacca, Alchemy, Hereward, Xi19, Robigus and Einstein (Table 2.1). These six varieties represent a range of bread and biscuit making wheats with varying properties. Hereward is the lowest yielding of the six varieties, but is of high quality, whilst the highest yielding of the group, Xi19, also has the lowest grain quality. Alchemy is the most disease resistant of the six varieties, with the highest yield in the untreated trial.

Table 2. 1 Quality and yield traits for relevant wheat germplasm

Traits of Hereward, Malacca, Alchemy, Xi19, Robigus and Einstein from the HGCA recommended list 2009/10 (AHDB, 2009)

Variety	Nabim Group	Grain yield as % of control		Quality	
		East of England	Untreated	Protein content (%)	Specific weight (kg/hl)
Hereward	1, Bread wheat	91	74	13.1	78.7
Malacca	1, Bread wheat	93	72	12.4	75
Alchemy	4, feed wheat	101	86	11.6	76.9
Xi19	1, Bread wheat	103	78	11.6	75.6
Robigus	3, biscuit making	101	77	11.7	76.1
Einstein	2, under test for bread making	101	82	12.1	76.9

Near isogenic lines (NILs) were created through selection using six molecular markers (full details of markers are described in Adamski *et al.,* 2013). Shamrock was crossed to each of the six glaucous varieties, and plants that were heterozygous at the *Iw1* interval (non-glaucous) were selected and subsequently backcrossed to the recurrent parent variety (e.g. Hereward). Plants were backcrossed

twice, and those that were heterozygous across the complete region self-pollinated to generate four streams of homozygous BC_2F_2 NILs, 87.5% genetically identical in the background. One stream of BC_2F_2 NILs was taken forwards and two more backcrosses were made. The resulting BC_4F_1 plants were self-pollinated to generate two streams of homozygous BC_4F_2 NILs that were 96.9% genetically identical in the background. Germplasm of both the BC_2F_2 and BC_4F_2 NILs was bulked up at Church Farm in Hege-90 plots (1 m^2) in the 2009-2010 field season for BC_2F_2 and 2011-2012 for BC_4F_2. Throughout this thesis the BC_4F_2 NILs were used for all measurements; both NIL streams were used for yield, and one stream was selected for all other measurements (the streams displayed the same epicuticular wax phenotype). BC_2F_2 NILs were only used in the 2011-2012 yield trials.

The nomenclature used through-out this thesis to refer to the NILs uses *Iw1+* for non-glaucous NILs that contain the Shamrock introgression, and *iw1-* for glaucous NILs that lack the Shamrock introgression. Where NILs of multiple varieties are compared simultaneously a slight variation on this is used. For Malacca NILs, MS (Malacca x Shamrock) is followed by either '+' or '-', donating *Iw1+* or *iw1-*. Accordingly, NILs of the other varieties are named AS+/- (Alchemy), HS+/- (Hereward), XS+/- (Xi19), ES+/- (Einstein) and RS+/- (Robigus).

2.1.2 Recombinant Lines

All non-glaucous (*Iw1+*) NILs contained an introgressed region from Shamrock on the short arm of chromosome 2B. This regions contains an unknown number of genes. Therefore in order to map specific traits more accurately within the introgressed regions a number of recombinant lines were also generated. The recombinant lines were generated at the same time as the NILs. After selfing the BC_4F_1 plants, BC_4F_2 individuals with recombination across the region (as opposed to being homozygous across the complete region as with the NILs) were selected as recombinants and genotyped as explained below.

Genotyping recombinant lines

To genotype the Hereward and Alchemy recombinant lines, DNA was extracted from seedlings at around the two-leaf stage for two biological replicates per recombinant. Leaf tissue was harvested and placed in 1.2 mL round collection tubes in a 96 well collection plate (AB-0564, Thermo Fisher Scientific ABgene, Massachusetts, USA), each tube containing 1 x 3 mm tungsten bead (69997, Quiagen, Hilden, Germany). Tissue was then freeze dried for 48 hr, or until completely dry. Sealing mats were then placed over the plates (AB-0674, Thermo Fisher Scientific ABgene, Massachusetts, USA), and samples ground to fine powder in a Spex GenoGrinder 2000 (SPEX sample prep, Stanmore, UK). Samples were ground for 2 min at 160 strokes per min (20 Hz) and spun in a centrifuge (4-15c, Sigma, Dorset, UK) at 2700 rpm for 1 min. Samples were repeatedly ground and spun until powder was sufficiently fine. 500 µl extraction buffer (0.1M Tris-HCl, pH 7.5, 0.05 EDTA

pH 8.0, 1.25% SDS) preheated to 65 °C was added to each tube in the plate and mixed by pipetting. Plates were sealed with clear plastic seals (ABI Thermo Fischer Scientific, Massachusetts, USA) and shaken in the genogrinder at 500-700 strokes per min for about 1 min. Samples were incubated at 65 °C for 1 hr. Subsequently plates were moved to the fridge (4 °C) for about 15 min to cool to room temperature, prior to the addition of 250 µl 6M ammonium acetate at 4 °C to each tube in the plate. Samples were mixed by pipetting and left for 25 min in the fridge. Cooled plates were removed from the fridge and centrifuged for 15 min at 5000 rpm to precipitate proteins and plant tissue. 600 µl of supernatant from each tube within the plate was transferred into a new collection tube containing 360 µl propan-2-ol and mixed by pipetting. Samples were left for 5 min to allow DNA to precipitate, prior to being centrifuged for 15 min at 5000 rpm to pellet the DNA. The supernatant was tipped off, and tubes were inverted to allow the remaining fluid to drain off the DNA pellet. The pellet was washed in 500 µl 70% ethanol, and centrifuged for 15 min at 5000 rpm. The supernatant was again discarded and collection tubes incubated at 65 °C with the lid off to dry. The pellet was re-suspended in 100 µl ddH_2O and sealed with a mat. The plate was then vortexed and left at 65 °C to dislodge the pellet. 1 in 10 dilution plates were made using ddH_2O.

A total of twenty SNP based KASPar markers for chromosome arm 2BS were developed for the Hereward and Alchemy recombinant lines. Primer sequences for KASPar markers are shown in Appendix A1. KASPar assays were performed by James Simmonds on extracted DNA from seedlings of Hereward and Alchemy recombinant lines in 384 well plates. Each well contained 2.5 µL KASP master mix (LGC, Middlesex, UK), 2.5 µL of DNA, and 0.07 µL primer mix. A Mastercycler pro 384 (Eppendorf, Stevenage, UK) was used to carry out the PCR using the following program: Start temperature of 95 ^{o}C maintained for 5 min, followed by ten touchdown cycles, whereby temperature was held at 95 ^{o}C for 20 sec followed by a touchdown at 65 ^{o}C for 25 sec, which was reduced by 1 ^{o}C per cycle. 30-40 cycles of amplification (95 ^{o}C for 10 sec, 57 ^{o}C for 60 sec), followed this. Following PCR, a SAFIRE Fluorescent Scanner (Tecan, Männedorf, Switzerland) was used to read the plates, and output was viewed using KlusterCaller (LGC, Middlesex, UK). Genotypes of all Hereward and Alchemy recombinant lines are shown in Appendix A2 and A3.

2.2 Glasshouse grown material

Where plant material was grown in a glasshouse the following procedure was followed. Seeds were placed on damp filter paper in petri dishes and left for 48 hr at 4 °C to germinate. They were then incubated at room temperature for 48 hr in the light. Once shoots had started to appear they were sown into P60 trays containing peat and sand. This was composed of: 15% grit, 85% fine peat, 2.7 kg m^{-3} Osmocote, 0.5 kgm^{-3} wetting agent (H_2Gro, Everris, Geldermalsen, The Netherlands), 4 kg m^{-3} Maglime (Francis Flower, Somerset, UK), 1kg m^{-3} PG Mix fertilizer (Yara, Lincolnshire, UK, www.yara.co.uk) (Borrill *et al.,* 2015). Plants were vernalized at 16 hr light/ 8 hr dark, 6 °C/ 4 °C, for

6 weeks and then re-potted into 1 L pots containing cereal mix also described by Borrill *et al.,* (2015): 20% horticultural grit, 40% sterilized soil, 40% medium grade peat, 1.3 kg m^{-3} PG Mix 14-16-18 (www.yara.co.uk), 1kg m^{-3} Osmocote Exact Mini, 0.5 Kg m^{-3} wetting agent (H_2Ogro), 3 Kg m^{-3} Maglime and 300 gm^{-3} Exemptor (Bayer, Leverkusen, Germany). Plants were then grown in a glasshouse with the temperature maintained at approx. 20 ^{o}C during the day, 16 ^{o}C night, and humidity at 70%, with 16 hr supplementary light per 24 hr cycle.

2.3 Field Trials

Field trials for yield and physiology were carried out at Church Farm (Bawburgh, Norfolk, NR9; 52.633477, 1.185645) in harvest years of 2012, 2013, 2014 and 2015. NILs were drilled in Hege-90 (6 m^2) plots in a randomized block design, with five blocks, one replication per block. Full field plans for 2013, 2014 and 2015 are shown in Appendix A4. Yield trials were also carried out by wheat breeders at at multiple sites across a number of years in East Anglia to assess yield only. These sites were: Drinkstone (Suffolk, IP30; 52.221036, 0.866089) in 2012, Dukes (Cambridgeshire, CB10; 52.042318, 0.311799) in 2012, Ickelton (Cambridgeshire, CB10; 52.070658, 0.174296) in 2012, Pampisford (Cambridgeshire, CB2; 52.110779, 0.184817) in 2013, Wolferton (Norfolk, PE31; 52.827926, 0.45601) in 2012, 2013 and 2014 and Woolpit (Suffolk, IP30; 52.224758, 0.887937) in 2013. In 2012 Drinkstone and Wolferton used a randomized block design with two blocks only, one replication per block. In all other years and breeder locations a randomized block design with four blocks, one replication per block was used. At all sites (including Church Farm) BC_2 NILs were grown in the 2012 trials, and BC_4 NILs used in all subsequent years.

2.4 Wheat Growth Stages

Throughout this PhD the decimal code for measuring wheat growth is used to identify specific plant developmental stages (Figure 2.1). This decimal code was first described by Zadoks *et al.,* (1974).

Figure 2. 1 The decimal code for wheat growth stages

This system for describing wheat developmental stages was first described by Zadoks *et al.,* (1974) Figure from AHDB (2015)

A number of key growth stages were identified and used as sampling time points throughout this PhD. The epicuticular waxes of both *Iw1+* and *iw1-* NILs are identical during the early stages of development, all with a non-glaucous appearance. Visible, glaucous, epicuticular waxes develop in *iw1-* NILs between GS31, the start of stem elongation where the first node is detectable, and GS39, the final stage of stem elongation just prior to booting. At this point the flag leaf blade is visible. There was slight variation between years in terms of glaucous wax development, but this was generally at GS32 or GS33 (approximately one week after GS31). Therefore any pre-wax sampling was carried out at GS31. The majority of physiological measurements were taken between anthesis (flowering) and 14 days post anthesis (DPA). GS61 is the start of flowering, and flowering is complete by GS69. Exact date of flowering can be difficult to determine accurately, so where appropriate heading date (GS55-GS59) was used as a surrogate, the point at which ¾ of the inflorescence of ¾ of the plot are emerged. Maturation (senescence) was defined as the date when ¾ of the plot are ¾ senesced (between GS87 and GS93). Dates of drilling, heading and maturation for harvest years 2013, 2014 and 2015 are shown in Table 2.2.

Table 2. 2 Key developmental dates during the harvest years of 2013, 2014 and 2015

Dates of heading and maturation. Early indicates the date of the first recorded instance at that stage, and late is the final date at which that developmental stage was recorded.

		Heading		Maturation	
Harvest year	Drilling date	Early	Late	Early	Late
2013	04/10/12	06/06/13	20/06/13	21/07/13	05/08/13
2014	26/09/13	18/05/14	27/05/14	15/07/14	25/07/14
2015	23/09/14	29/05/15	06/06/15	22/07/15	23/07/15

2.5 Weather data

Weather data from Church Farm for 2012-2015 were not available. However, data for temperature, relative humidity and precipitation were obtained from the National Institute of Agricultural Botany (NIAB) farm (Cambridgeshire, CB24 9N2; 52.242727, 0.107642) to provide a broad comparison between years (Figure 2.2). In general 2014 was the warmest year, with the exception of July and August, where 2013 reached the highest average temperature. Prior to May 2013 was the coldest year, reflected in the late heading date for this harvest year. With the exception of July, 2014 was the wettest summer, with the highest total rainfall and average relative humidity (RH).

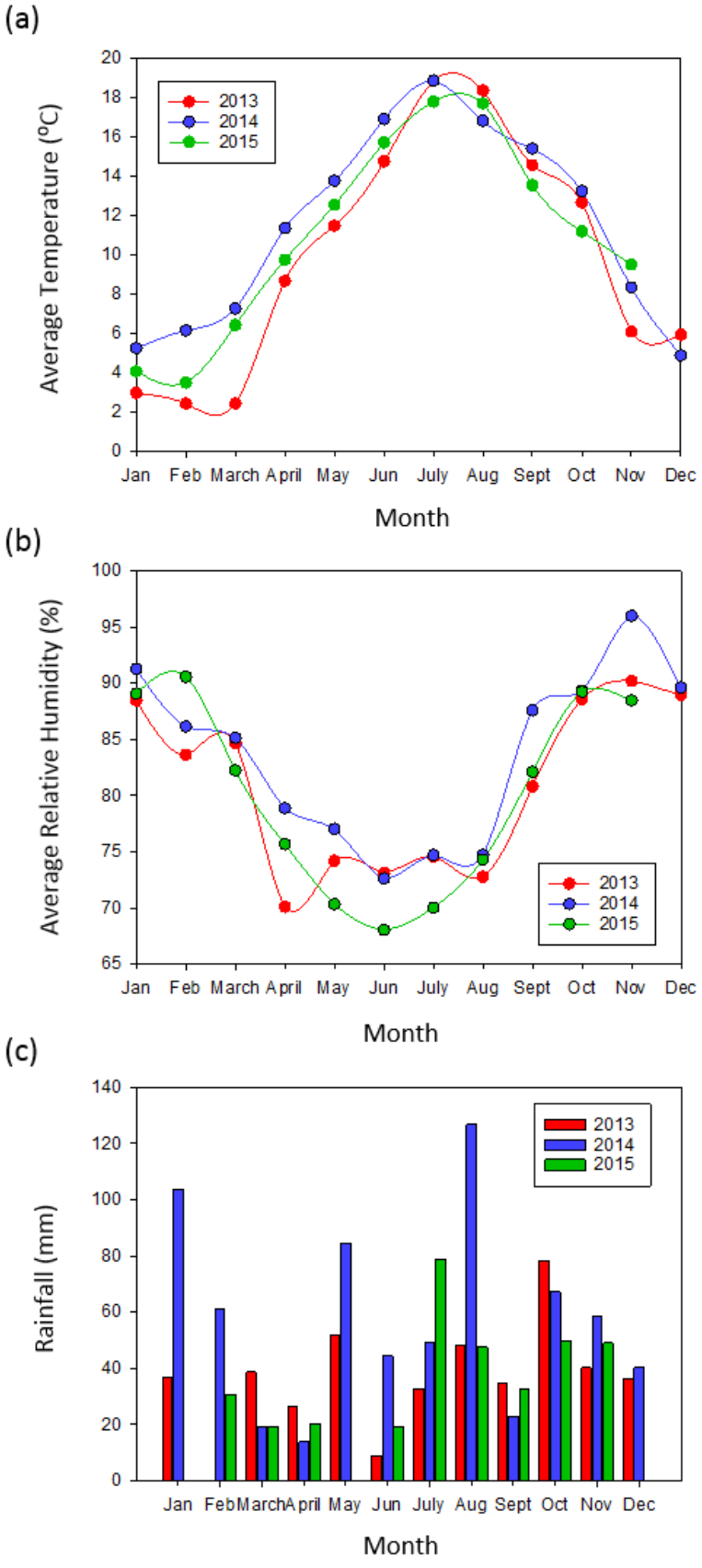

Figure 2. 2 Weather data for the East of England for 2013, 2014 and 2015

Data collected from the NIAB farm weather station (a) average temperature for each month (b) average relative humidity for each month (c) total rainfall for each month.

Chapter 3: Characterising the yield and wax profile of *Iw1* germplasm

3.1 Summary

The majority of UK wheat varieties display a glaucous phenotype during reproductive growth stages, meaning the external surfaces are covered in a layer of visible waxes giving the plant a bluish-grey appearance. Shamrock is an unusual variety in that it displays a non-glaucous phenotype conferred by the wild emmer gene *Inhibitor of Wax 1* (*Iw1*). Recent work with Shamrock suggested that *Iw1* conferred a significant yield advantage. Consequently Shamrock was crossed to a number of glaucous UK wheat breeding varieties to create near isogenic lines (NILs) differing for the presence and absence of *Iw1* and investigate this further.

Field trials using the *Iw1* NILs over multiple years and locations showed that *Iw1* does not confer a yield advantage in the majority of varieties tested. Two varieties, Alchemy and Hereward, did show an average yield increase associated with the *Iw1* introgression of 2.59% and 4.96% respectively. However, further analysis with recombinant lines suggests that this yield benefit is attributable to a closely linked gene rather than *Iw1* itself.

The second component of this chapter describes the composition of the epicuticular waxes of *Iw1* NILs. The meaning of the word glaucousness and the wax composition determining this phenotype will vary between environments, species and varieties, even between plants with similar visual appearance. Therefore specifically characterising the composition of both the *Iw1+* and *iw1-* NILs enables any differences between NILs to be attributed to specific wax components. The major component accounting for around 80% of the epicuticular waxes of both *Iw1+* and *iw1-* NILs was shown to be primary alcohols The glaucous *iw1-* NILs had a significant β- and OH-β-diketone component, which was absent from the *Iw1+* NILs. *n*-Alkanes were present in both *Iw1+* and *iw1-* waxes, accounting for around 50-10% of the waxes. These compounds were slightly upregulated in *Iw1+* NILs compared to *iw1-* NILs.

3.2 Introduction

3.2.4 The UK wheat variety Shamrock has a non-glaucous phenotype conferred by *Iw1*

The UK winter wheat variety Shamrock is a cross between a *Triticum dicoccoides* derivative and adapted UK germplasm. Unusually amongst cultivated wheat, Shamrock lacks the more common visible waxes of domesticated varieties, displaying a bright green, non-glaucous phenotype. In addition to non-glaucousness, Shamrock has a number of traits that lend it to further study for crop improvement. For example it performs consistently over a range of environments in terms of yield, making it resilient to changes to agronomy and environmental conditions (Simmonds *et al.,* 2008). Non-glaucousness in Shamrock is conferred by the dominant gene *Inhibitor of Wax 1* (*Iw1*). *Iw1* is located on chromosome 2BS of wild emmer wheat and introgressed into Shamrock. As an epistatic inhibitor of glaucousness, *Iw1* inhibits the β-ketoacyl-elongation pathway during wax biosynthesis (Chapter 1, Figure 1.6c). This eliminates compounds unique to this pathway (β- and OH-β-diketones) from the epicuticular waxes.

3.2.5 Delayed senescence and higher yields map to *Iw1*

To investigate this non-glaucous trait further, Simmonds *et al.,* (2008) crossed Shamrock with the glaucous wheat variety Shango (Figure 3.1) to create a doubled haploid (DH) population. The population showed 1:1 segregation for the non-glaucous trait, indicating that the trait was controlled by a single gene. Field trials in Norwich, UK, over two seasons (2004-2005 and 2005-2006) showed that non-glaucous lines had significantly delayed senescence by 1.5 days, and a significantly higher yield of 2.4-5.6% compared to glaucous lines. QTL analysis mapped the higher yield and delayed senescence to the same location as the non-glaucous trait (*Iw1*) on chromosome 2B (Simmonds *et al.,* 2008).

Figure 3. 1 Non-glaucous Shamrock and glaucous Shango in the field

Many studies in wheat have reported a positive correlation between delayed flag leaf senescence and yield (Gaju *et al.,* 2011; Hawkesford *et al.,* 2013; Kichey *et al.,* 2007; Verma *et al.,* 2004). Grain filling in wheat occurs between flowering and senescence. Therefore a longer period of photosynthesis after flowering could produce more photosynthates that are transferred to the grain increasing yields. This was suggested by Simmonds *et al.,* (2008) as the mechanism by which yield was increased in the non-glaucous DH lines, although how the epicuticular waxes might determine senescence was not explored. However, there is evidence to show that under certain conditions photosynthesis during grain filling may not be the limiting factor for grain yield (Borrás *et al.,* 2004; Reynolds *et al.*, 2005). A study in wheat found that, whilst the percentage of flag leaf area remaining green 35 days after flowering was correlated with grain yield under both irrigated and non-irrigated conditions, the association was significantly stronger under drought (Verma *et al.,* 2004). More recent research has provided evidence that the relationship between extended photosynthesis and increased yield only exists under stress such as drought, heat (Gregersen *et al.,* 2013; Lopes & Reynolds, 2012) or low nitrogen (Derkx *et al.,* 2012; Gaju *et al.,* 2014). Under optimal conditions yield may be limited not by substrate availability but by sink factors within the plant (Borrás *et al.,* 2004; Borrill *et al.,* 2015; Reynolds *et al.,* 2005). Under these circumstances extended photosynthesis would not be of benefit as the plant cannot use any additional photosynthates. For example, research conducted in a glasshouse has shown that an extension of green canopy duration of 10% resulted in no difference in final grain yield, even though the plants were continuing to photosynthesise (Borrill *et al.,* 2015). This work found that grain yield was limited by activity of the starch synthase enzyme found in the grain, rather than availability of photosynthates, so the additional carbon fixed was not going into the grain. This limiting effect of starch synthase on grain filling has also been reported in field grown material (Yang *et al.,* 2004). The UK could be considered a low stress environment, thus factors other than continued photosynthesis and extended green canopy duration may be more important for increasing wheat yields.

The conclusion that non-glaucousness increases wheat yields is opposing to the majority of past studies, that show non-glaucous wheat and barley plants to be lower yielding than glaucous plants (Febrero *et al.,* 1998; Johnson *et al.,* 1983; Monneveux *et al.,* 2004; Watanabe, 1994). Reductions in yield as high as 26% have been reported in non-glaucous barley plants (Febrero *et al.,* 1998), and reductions of around 5-30% have been associated with non-glaucousness in wheat (Monneveux *et al.,* 2004). However, the bulk of these studies were carried out in a Mediterranean environment characterised by warm and dry summers. Under these conditions wheat often experiences water stress and drought during the growing season. Glaucousness has traditionally been considered beneficial for plant water use efficiency (Richards *et al.,*1986) and thus could confer an advantage to plants in water stressed environments, leading to higher yields (this will be explored further in

Chapter 5). The work in the Shamrock x Shango DH population was carried out in a UK environment where water supply is less limiting. This suggests that in an environment where water stress is not a major constraint, non-glaucous plants could have the advantage. This hypothesis is further supported by the work of Merah *et al.,* (2000), who carried out field trials of glaucous and non-glaucous durum wheat in the Mediterranean over two years. In the first year of measurement, only 285 mm total rainfall fell during the growing season (November – June), with a long drought from February until harvest. Glaucous plants yielded significantly more grain (around 3 g plant^{-1} compared to 1-2 g plant^{-1} for non-glaucous), confirming previous finding that glaucous plants are at an advantage under drought stress. However, in the second year of measurement, total rainfall over the growing season was 933 mm, and no drought occurred until the final two months (May and June). In this case the water stress occurred only after the majority of plant biomass had been formed. Under these conditions, the non-glaucous plants had significantly more biomass than glaucous (around 12-13 g plant^{-1} compared to around 10 g plant^{-1} biomass of glaucous plants), suggesting an advantage to non-glaucous plants under these conditions. There was no difference in final grain yield between glaucous and non-glaucous plants, potentially because grain filling was still occurring when water supply became limiting, thus negating any benefit conferred by non-glaucousness up until that point.

3.2.6 Characterising *Iw1* induced non-glaucousness

Finding novel ways to improve crop yields and feed a growing population is currently of high priority for agriculture (Hawkesford *et al.,* 2013; Thornton, 2012), so further exploration of the relationship between *Iw1* and yield was of importance. Near isogenic lines (NILs) were developed for further study by crossing Shamrock to six glaucous UK wheat varieties. Using marker assisted backcrossing six pairwise NILs were developed, providing a powerful genetic tool to assess the action of *Iw1* in multiple genetic backgrounds. Subsequent work with the *Iw1* NILs fine mapped *Iw1* to a sub cM interval on chromosome arm 2BS and a number of candidate genes were suggested (Adamski *et al.,* 2013). In addition to the fine mapping, Adamski *et al.,* (2013) also fully characterised the wax composition of NILs both with and without *Iw1* over the growing season.

Wax composition of *Iw1* germplasm (including the NILs and the original Shamrock and Shango varieties) grown in the field was assessed at Growth Stage 31 (Adamski *et al.,* 2013) which is during stem elongation, prior to emergence of the flag leaf. At this point in development, plants both with and without *Iw1* cannot be distinguished visually and display a non-glaucous phenotype. Wax profile analysis revealed that there was no difference in epicuticular wax composition or total wax load between tissues of plants with or without *Iw1*; in both cases primary alcohols with a chain length of 28 carbons (C28POH) made up the majority of the wax.

The analysis was repeated on samples collected at Growth stage 47 (during booting, prior to ear emergence and flowering), at which point NILs without *Iw1* (*iw1*-) have developed visible waxes and display a glaucous phenotype, whilst NILs with *Iw1* (*Iw1+*) still have the non-glaucous phenotype. At this point there was still no significant difference between NILs in terms of total wax load indicating that wax load does not alter plant visual appearance, although wax composition at this point was significantly different.

Figure 3.2 shows the typical epicuticular wax composition of (a) glaucous, *iw1*- tissue and (b) non-glaucous *Iw1+* tissue. At a retention time of 17.31 minutes a C28POH peak can clearly be seen and is one of the most abundant wax components in both the *Iw1+* and *iw1*- chromatograms, indicating these compounds are maintained in the waxes in large proportions throughout development. However, in addition to C28POH, other POH compounds of various chain lengths are present, in addition to *n*-alkanes, fatty acids and methylalkylresorcinols (MARs). Peaks for these compounds can be identified in chromatograms of both *Iw1+* and *iw1*- tissue in similar amounts. The major difference between the two ion chromatograms, highlighted in red on Figure 3.2, is the presence of β-diketones and OH- β-diketones in large amounts in the glaucous *iw1*- tissue between a retention time of 18.14 and 20 min. These major peaks are completely absent from the spectra of non-glaucous *Iw1+* tissue.

This same analysis was also repeated at flowering and senescence. Total wax load in both *Iw1+* and *iw1*- plants increased, as did the quantity of all compounds present in the waxes. Furthermore, at these later time points *iw1*- plants did have a significantly higher wax load than *Iw1+*, indicating that wax load increased at a faster rate in the glaucous plants. However, at no point during the time-course were β-diketones and OH- β-diketones recorded in the *Iw1+* plants in any significant quantity, whilst these compounds continued to make up a large proportion of the glaucous *iw1*- waxes (between 12 and 70% dependant on variety).

This characterisation of the epicuticular waxes shows that *Iw1* induces glaucousness through the inhibition of the biochemical pathway that synthesises β-diketones and OH- β-diketones (Chapter 1: β-ketoacyl-elongation pathway, Figure 1.6c). Wax analysis from multiple tissues, including peduncle and flag leaf, demonstrated that the effect of *Iw1* was consistent across all organs measured.

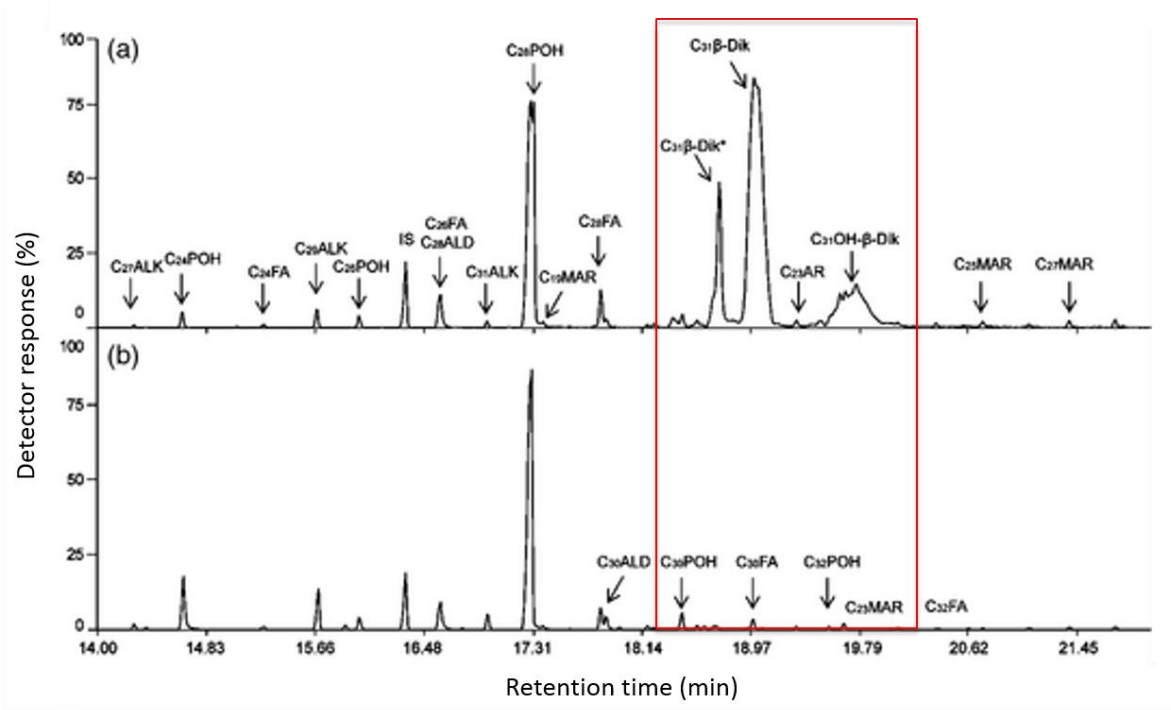

Figure 3. 2 Total ion chromatograms for flag leaf epicuticular wax

From (a) Shango (*iw1-*) and (b) Shamrock (*Iw1+*). The red box highlights the β-diketone and OH- β-diketone peaks present in *iw1-* tissue that are completely absent from the *Iw1+* chromatogram. Chain length is donated by C_{XX}, and compound classes are abbreviated to ALK (*n*-Alkane), POH (Primary Alcohol), FA (Fatty Acid), β-Dik (β-diketone) and ALD (Aldehyde). Adapted from Adamski *et al.,* (2013).

3.2.7 Aims

The *Iw1* NILs provide a powerful genetic resource to explore the effect of *Iw1* in multiple genetic backgrounds, and assess under which conditions the non-glaucous trait could confer a yield advantage within the UK. These NILs are at backcross four, meaning they are 97% identical in the genetic background (with the exception of the introgressed region containing *Iw1*). Any differences observed between NILs can therefore be attributed directly to the introgressed region containing *Iw1*. Furthermore, having a fully characterised wax profile for the *Iw1* NILs is advantageous in terms of interpreting any observed differences between NILs. This chapter aims to:

(i) Test the hypothesis that *Iw1* increases yield and delays senescence across a wide range of UK winter wheat varieties

(ii) Confirm that the wax profile data presented in Adamski *et al.,* 2013 is relevant to the plant material and environments used in the present work.

3.2 Materials and Methods

3.2.1 Field evaluation and phenotyping

3.2.1.1 Location and germplasm

To assess yield and green canopy duration, field trials using near isogenic lines (NILs) were carried out at seven sites across four years in East Anglia as specified in Chapter 2, section 2.3. NILs of Malacca, Alchemy, Hereward, Xi19, Robigus and Einstein were grown. At all sites BC_2 NILs were grown in the harvest year of 2012, and BC_4 NILs used in all subsequent years. Hereward and Alchemy BC_4 recombinant lines were also sown at Church Farm in both the harvest years of 2014 and 2015, and the Hereward lines only sown at Docking in the 2015 harvest year.

3.2.1.2 Phenotyping yield

To phenotype yield weight of grains per plot was measured and normalised to 15% moisture content. Data were standardised across all trials to Tonnes per Hectare.

3.2.1.3 Near isogenic line analysis

Using the Tonnes per Hectare data, yield was analysed by pairwise comparison between NILs at each site. An overall ANOVA for each trial was also carried out. Yield was very variable between years and trials. Therefore to enable better visual comparison between years and locations, within each trial the yield of glaucous *iw1*- NILs of each variety was said to be 100% and the percentage increase in yield associated with *Iw1* was calculated for each variety. A positive value therefore indicates a yield advantage of *Iw1*, whilst a negative value indicates a decrease in yield associated with *Iw1*.

Church Farm was affected by widespread bunt infection in the 2014 harvest year. As such, large amounts of the plot had to be removed and discarded prior to harvest. Bunt affects only the kernels of the plant, converting the kernal into a sorus of black teliospores. This occurred late in development towards maturity after GS71. Plants displaying infected kernals were removed so only healthy plants remained. Physiology work was carried out earlier in plant development at anthesis (around GS61-69) and healthy regions of plot or individuals were selected for measurement. Therefore this data were not affected. However, yield data was measured as number of grains per plot. Due to large amounts of some plots being discarded this resulted in unreliable yield data. Therefore 2014 Church Farm yields were excluded from the overall analysis.

3.2.1.4 Recombinant analysis

To map the yield effect within the introgressed region, QTL analysis using QTL Cartographer (Bioinformatics Research Centre, NC State University, http://statgen.ncsu.edu/qtlcart/) was carried

out using the yield and marker data (Appendix A2 and A3) for each recombinant line. Recombinant yield data were also analysed by ANOVA, to test the single marker significance of the difference between mean yield values of recombinant lines carrying the Shamrock allele and those carrying the Hereward or Alchemy allele at each marker location. Due to the limited data available for the recombinant lines, the bunt-infested 2014 data was included in analysis. However, where possible, plots in the field that were heavily infected with bunt were noted and removed from analysis so as to only use representative data.

3.2.1.5 Phenotyping green canopy duration

In the harvest years of 2013 and 2014 the flowering date for each plot was recorded, defined as the day in which 3⁄4 of the plot had 3⁄4 of their inflorescences emerged. Date of senescence, defined as the day where a loss of chlorophyll was observed in peduncles and leaves for 3⁄4 of the plot, was also recorded. Calculation of the number of days between flowering date and full plant senescence for each plot gave the green canopy duration. Data were analysed by overall ANOVA including both years and all varieties, and by pairwise comparison between NILs within each individual year.

3.2.2 Epicuticular wax profiles

Epicuticular wax extraction and analysis was carried out as described in Adamski *et al.,* (2013) with some minor modifications. As such the full method is detailed below.

3.2.2.1 Extraction of epicuticular wax

Three flag leaves were collected in the field at anthesis, one each from each plot in the field at Church Farm in 2014 for NILs of Malacca, Alchemy and Hereward. Samples were collected in 15 mL polypropylene tubes that had been pre-weighed, and immediately frozen on dry ice for transportation. Tubes were then re-weighed to determine sample fresh weight prior to storage at -80 ^{o}C.

To extract the epicuticular waxes, each flag leaf was placed into a glass tube with a polytetrafluoroethylene screw cap lid. 5 mL chloroform (Analytical Grade, Fischer Scientific) was added in addition to triacontane (Sigma 263842, Poole, UK) as an internal standard (35 µg/mL). Tubes were laid horizontal such that all flag leaf tissue was submerged in the chloroform/ triacontane solution and left for 10 min at room temperature. Tubes were agitated for 10 sec three times during the 10 min period. Subsequently, flag leaves were removed from the glass tubes, and the extracts were dried down in an evaporator (EZ-2 Genevac, Ipswich, UK). Once dry, 1-2 mL chloroform was added to each glass tube to re-suspend the wax extract, which was then transferred to a glass GC-MS vial that had been pre-weighed (Agilent Technologies, USA). Samples were dried

down under nitrogen gas, and any residue evaporated through heating at 80 ^{o}C (approx. 5-10 min). Once completely dry, the vial was re-weighed to determine the total wax load.

3.2.2.2 Gas chromatography-mass spectrometry (GC-MS)

Prior to gas chromatography-mass spectrometry (GC-MS), dry wax samples were dissolved in 100 µL 1:1 pyridine (Cat. No. 270920, Sigma, Poole UK) and TMS-BSTFA (15238, Sigma, Poole UK) and heated to 80 ^{o}C for 1 hr to derivatise. During this time samples were vortexed three times, once at the start and then every 20 min during the hour.

GC-MS was carried out as described in Adamski *et al.,* (2013) on an Agilent GC 6890N gas chromatograph (Agilent Technologies, Wilmington, Delaware, USA) coupled to a 5973 Inert Mass Selective Detector. An Agilent 7683 automatic sampler was used to make automated splitless 3 µL injections. The column was a ZB 5ht (Zebron, 7HG-G015-02, Phenomenex Torrance, CA, USA), 30.0 m x 250 µm x 0.1 µm fitted with a 5m guard column on the front end. Throughout chromatography the inlet temperature was maintained at 250 ^{o}C, Helium carrier gas was maintained at flow rate 0.8 mL min^{-1}, and inlet pressure at 11.06 psi. The initial oven temperature was set to 140 ^{o}C, and this temperature was maintained for 1 min. Subsequently, temperature was ramped up by 10 ^{o}C each min until a maximum temperature of 400 ^{o}C was reached. This final temperature was maintained for 5 min. The manufacturer's recommended default settings were used on the mass spectrometer set to electron ionisation in positive mode (70 eV), quad temperature of 150 ^{o}C and source temperature 230 ^{o}C.

3.2.2.3 Quantification of wax compounds

The *Iw1* germplasm has been characterised previously in terms of wax composition, identifying 53 compounds in total, 26 of these accounting for >95% of the total wax load (Adamski *et al.,* 2013). Here, relative abundances of the 17 most abundant major wax components, including all *n*-alkanes, primary alcohols, Fatty acids, MARs and β-diketones, were quantified using the Agilent GC Chemstation software (D.03.00). Compounds of interest were identified using diagnostic ions, and the areas of total ion chromatogram peaks calculated through automatic integration using the Chemstation Custom Reports function. The known quantity of internal standard was then used to quantify compounds of interest and µg compound per mg leaf tissue calculated. The remaining 9 compounds, including aldehydes and OH- β-diketones, were not quantified but their presence or absence was confirmed. Data were grouped into compound classes to analyse the effect of *Iw1* over all varieties, in addition to pairwise comparison between NILs.

3.3 Results

3.3.1 Yield

3.3.1.1 Yield of near isogenic lines

Yield trials of *Iw1* NILs were grown across five years in multi-site experiments at locations in Norfolk, Suffolk and Cambridgeshire. Yield in Tonnes per Hectare was measured, and the percentage increase in yield associated with *Iw1* calculated for each NIL pair for each trial. Figure 3.3 shows the percentage difference in yield between NILs, with the mean yield increase associated with *Iw1* marked in red. When data were analysed across all six varieties there was no significant effect of *Iw1* on grain yield ($p=0.146$; Figure 3.3). However, there was a significant effect of trial ($p<0.001$) and variety ($p<0.001$) on the percentage yield difference between NILs. The ANOVA showed a significant interaction between trial and *Iw1* ($p<0.001$), but overall no significant interaction between *Iw1* and variety ($p=0.50$). However, despite this, pairwise comparisons for each variety revealed that *Iw1* had a different effect in different genetic backgrounds.

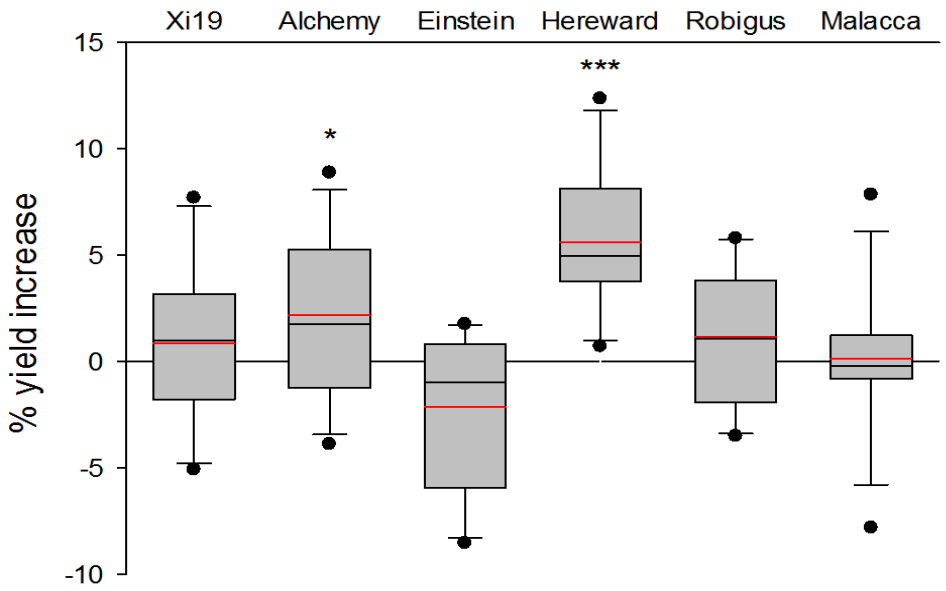

Figure 3. 3 Percentage increase in yield associated with *Iw1*

Increase in yield of *Iw1+* in comparison to *iw1-* NILs of Xi19, Alchemy, Einstein, Hereward, Robigus and Malacca, averaged over field trials across five years from multiple locations. Boxes show the 25th and 75th percentile with the median marked in black. Whiskers show the 10th and 90th percentile, with the highest and lowest data points plotted in black. The mean is shown in red. Significance is indicated according to $p<0.05$ (*), $p<0.001$ (***)

The *Iw1* introgression resulted in a significant yield increase of 4.96% in Hereward (p< 0.001) and 2.59% in Alchemy NILs (p=0.045). In the other four genetic backgrounds (Xi19, Einstein, Robigus and Malacca), the *Iw1* introgression did not have a significant effect on yield. Malacca displayed the most neutral effect, with an average percentage difference between NILs of only 0.17% over all trials.

To explore the yield data in more detail Table 3.1 shows the percentage difference in yield between NILs for each individual trial. Consistent with Figure 3.3, Hereward and Alchemy were the only varieties for which *Iw1* had a significantly positive effect in any one trial. Hereward was unique among the varieties in that in no trial did *Iw1* have a negative effect on yield, although the consistent yield benefit was only statistically significant in 4 out of 10 trials. Only in Einstein did *Iw1* ever have a significantly negative effect on yield, with *Iw1+* NILs yielding significantly less than *iw1-* in two trials during the 2012 harvest year.

Table 3. 1 Percentage increase in yield associated with *Iw1* within each individual trial

Pairwise comparison between NILs of each variety for each trial was carried out on plot yield. Significance is indicated according to p<0.05 (*), p<0.01 (**), p<0.001(***). Significant positive increases in yield associated with *Iw1* are highlighted in green, a significantly negative effect of *Iw1* on yield is highlighted in grey. Trials for which data were unavailable are marked with a dash (-). Trials at Drinkstone and Wolferton during 2012 only had two independent replicates from each NIL. All other trials had at least 4 independent replicates.

Year	Location	% yield increase with *Iw1*					
		Xi19	**Alchemy**	**Einstein**	**Hereward**	**Robigus**	**Malacca**
2012	Church Farm	-1.71%	-0.10%	-8.55% *	10.52% *	-0.06%	-0.32%
	Drinkstone	3.82%	4.10%	-6.05%	0.71%	5.79%	0.50%
	Wolferton	-5.09%	-3.89%	0.64%	12.36% *	-3.51%	-7.82%
	Dukes	2.97%	-2.77%	-5.89% *	-	3.27%	2.09%
	Ickleton	7.69%	8.88% *	-2.87%	8.95%	5.39%	7.85%
2013	Church Farm	1.35%	5.70%	1.76%	1.63%	1.40%	1.05%
	Woolpit	-1.18%	1.69%	1.28%	3.74% *	0.77%	-0.69%
	Wolferton	0.62%	-1.16%	0.44%	5.28%	-2.43%	1.29%
	Whittlesford	2.13%	-1.35%	-0.60%	5.64%	2.86%	-1.07%
2014	Wolferton	-	1.76% **	-	3.89%	-	-0.32%
2015	Church Farm	-	3.75% *	-	4.90% *	-	-

Although yield data were collected at multiple field sites, all other data presented in this thesis were collected at Church Farm only. Therefore it is important that the Church Farm yield data is representative of the overall data. An overall analysis of the Church Farm data from 2012 shows that there was no significant effect of *Iw1* (p=0.933) but borderline significant interaction between *Iw1* and variety (p=0.057), likely a function of the significantly positive yield increase of 10.52% in

the Hereward NILs (p=0.011) compared to a significantly negative decrease of 8.55% in Einstein (p=0.05). Conversely, In 2013 *Iw1* had an overall significant effect (p=0.008) with no interaction with variety (p=0.562); there was no significant effect in Hereward or Alchemy in this year although both are positive. In 2015, where only Alchemy and Hereward were grown, the effect of *Iw1* was significant (p=0.005) with no interaction with variety (p=0.893). Hereward was again the variety that showed the most consistent increase in yield associated with *Iw1* at Church Farm. This effect was significant in 2012 (p=0.011) and borderline significant in 2015 (p=0.059). The effect of *Iw1* on yield in Alchemy was also significant in two of the three Church Farm trials; 2013 (p=0.001) and 2015 (p=0.039). Taking account of all trials it can be concluded that there is a significant yield benefit conferred by the *Iw1* introgression in Alchemy and Hereward. However, this effect was not seen in all varieties leading to the conclusion that the non-glaucousness trait (and *Iw1*) itself is not responsible for the increased yield.

3.3.1.2 Yield of recombinant lines

To better define the genetic interval related to increased yield, Alchemy and Hereward NILs with recombination across the *Iw1* region in both varieties were developed. These lines were derived from the original NILs and carry only a segment of the *Iw1* introgression allowing a more precise definition of the yield effect in Hereward and Alchemy backgrounds. Both sets of recombinant lines were grown at Church Farm (2014 and 2015 harvest years), and the Hereward recombinants were also grown at Docking (2015). The location of *Iw1* relevant to a selection of markers is shown in the chromosome schematic at the base of Figure 3.4. Yield data had a continuous distribution and hence was analysed as a quantitative trait.

For Hereward (Figure 3.4a), the yield effect was associated with the region of chromosome 2B containing *Iw1*, but the effect was only significant for the Church Farm 2015 trial (LOD-score>2.5). In Alchemy (Figure 3.4b) there was no clear effect in 2014, although in this year there was severe bunt infection in the field which may have confounded the data. The QTL from 2015 however does mirror that of Hereward, showing strongest linkage with the portion of the chromosome in which *Iw1* is located, but this was again not significant and tentative at least.

Yield data of recombinant lines were also analysed by ANOVA. This tested the significance of the individual markers for difference between mean yield values of recombinant lines carrying the Shamrock allele and those carrying the Hereward of Alchemy alleles at each marker location (Table 3.2).

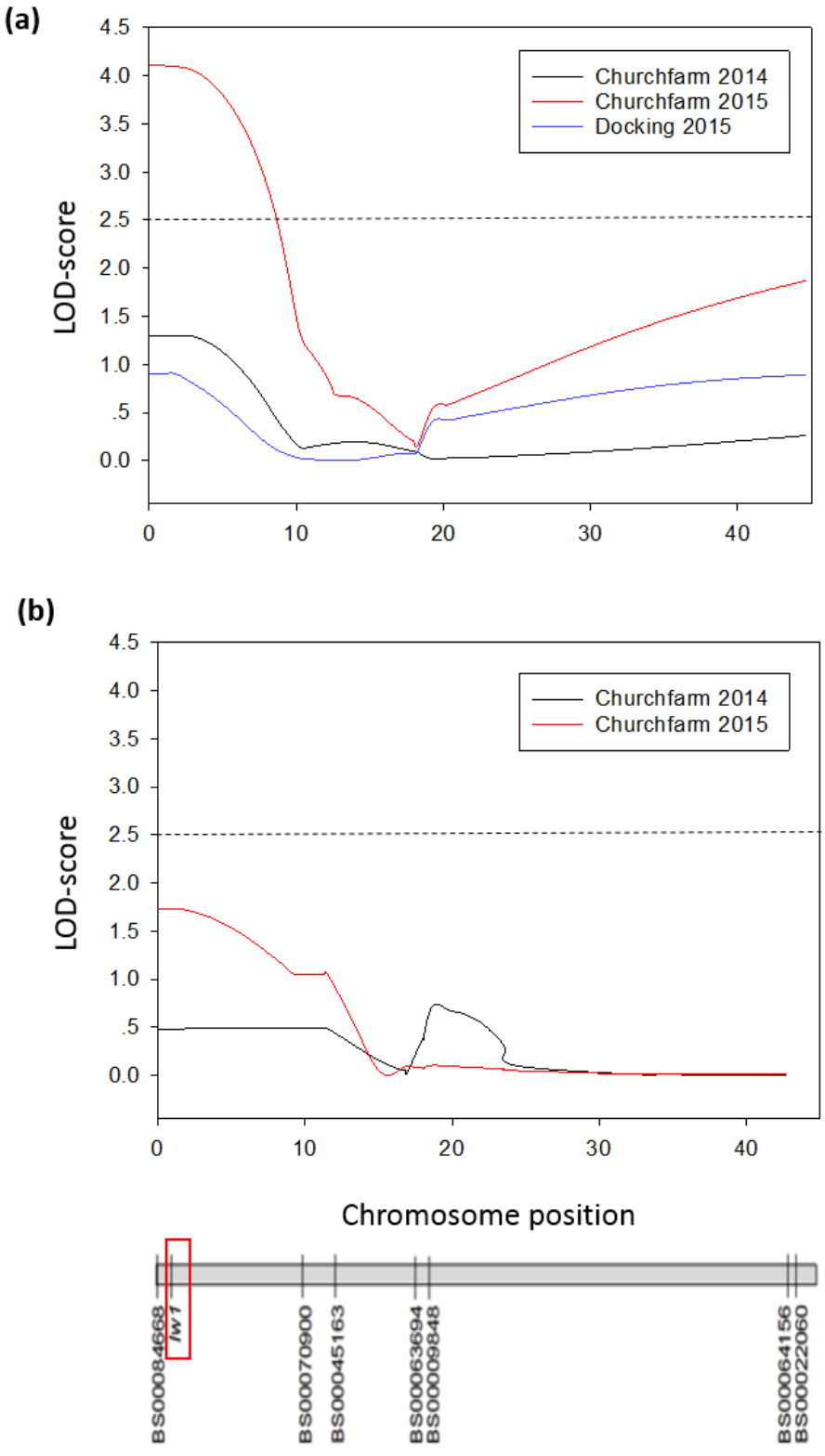

Figure 3. 4 Yield QTL of recombinant lines

Lines of (a) Hereward and (b) Alchemy. The location of *Iw1* is shown on the chromosome diagram (bottom). The y-axis shows the LOD-score test statistic at each chromosome position. A score of 2.5 (shown by the dotted line) or above donates significance.

Table 3. 2 Mapping the yield effect within the *Iw1* introgression

An ANOVA was carried out to test the difference between mean yields of recombinant lines carrying the Shamrock allele and those carrying the Hereward allele at each marker location. For Hereward, data was combined across the three field trials (Church Farm 2014 and 2015, Docking 2014). For Alchemy only data from the Church Farm 2015 is included in the analysis. The relevant p value for each marker is shown in the table. Levels of significance are indicated by p<0.05 (*), p<0.01 (**), p<0.001 (***).

Hereward (all trials)			**Alchemy** (2015 harvest year only)		
Marker	Distance (cM) Chromosome 2BS	p	Marker	Distance (cM) Chromosome 2BS	p
BS00084668	0	<0.001***	BS00084668	0	0.008**
BS00009972	1.15	<0.001***	BS00009972	1.15	0.008**
Iw1	1.77	<0.001***	*Iw1*	1.5	0.008**
BS00070900	10.41	0.022*	BS00010318	9.26	0.038*
BS00010318	10.41	0.022*	BS00070900	9.26	0.038*
BS00045163	12.68	0.087	BS00073542	11.53	0.038*
BS00010637	17.98	0.365	BS00063694	16.83	0.534
BS00065040	17.98	0.365	BS00006788	16.83	0.534
BS00063694	17.98	0.365	Bra1190	17	0.534
Bra1190	18.27	0.365	BS00003719	18.03	0.584
BS00009848	19.18	0.132	BS00076982	18.61	0.523
BS00064156	44.64	0.004**	BS00071995	32.68	0.840
BS00022734	45.19	0.004**	BS00064156	43.49	0.798
BS00022060	45.19	0.004**			
BS00064155	45.19	0.004**			

For Hereward, when data from all three trials were combined there was a significant effect of trial on yield for every marker (p<0.05). However, for no marker was there a significant interaction between genotype (*Iw1*) and trial. This indicates that although overall crop yield was dependent on environmental conditions, the effect of genotype on yield was stable across the various years and locations. In Alchemy, when data from both trials were analysed together the association between yield and each marker was again significantly different between trials (p<0.05). However, in this case there was also a significant interaction between genotype and trial for all markers between 0 and 16.83 cM (p<0.05). This interaction can clearly be seen in Figure 3.6, whereby the QTL curves look very different for the two trials. Furthermore, no significant difference between lines carrying the Shamrock allele and those with the Alchemy allele was found at any location when both trials were analysed together. Due to the widespread bunt infection in 2014 and the effect this could have had on the results, only data collected in the 2015 trial was included in the analysis presented in Table 3.2 and interpretation in the following paragraphs. Due to the absence of interaction in Hereward all three trials were included in the Table 3.2 analysis and subsequent interpretation.

For both varieties there was a significant difference between the yield of recombinants with and without the Shamrock *Iw1* allele (Hereward, p<0.001; Alchemy, p = 0.008). However, there is tight linkage between *Iw1* and the two distal markers on the chromosome arm (BS00084668 and BS00009972), a relationship that can also be seen in Figure 3.6. Therefore the current set of recombinants does not provide sufficient resolution to assess the exact location of the yield effect and to determine if this is due to *Iw1* itself or to a closely linked gene. There was also a significant effect at the markers proximal to *Iw1* (at a distance of 10.41 cM in Hereward, 9.26 cM in Alchemy), although this was less significant than for *Iw1* in both varieties. In Alchemy there was no significant difference between the yield of recombinants carrying the Shamrock allele and those with the Alchemy allele at any other location on the chromosome. However, for Hereward there did seem to be some effect between 44.64 and 45.19 cM, although this was not as significant as the effect recorded at 0-1.77 cM.

3.3.2 Green canopy duration

Green canopy duration is a measure of the number of days between flowering and senescence. This is linked to grain filling period, an extension of which has been reported to have a positive effect on yield and could explain the yield effect of *Iw1* observed in the Hereward and Alchemy NILs. Dates of flowering and senescence were recorded for NILs of Xi19, Alchemy, Einstein, Hereward, Robigus and Malacca over two years at Church Farm. Table 3.3 shows the number of days between these two growth stages for NIL pairs.

Overall there was a significant effect of *Iw1* (p<0.001), year (p<0.001) and variety (p<0.001) on green canopy duration. There was a significant interaction between year and variety (p<0.001), but no interaction between *Iw1* and variety (p=0.330) or *Iw1* and year (p=0.382). This indicates that the *Iw1* introgression did have a significant effect on green canopy duration which was consistent across years and varieties. This can be seen from Table 3.3, where the overall effect of *Iw1* is to extend the green canopy duration. Only Robigus demonstrated a (non-significant) negative effect of *Iw1* in both years. The extended green canopy duration is only a small effect; where *Iw1* was associated with a delay in senescence this was only by around one day. Pairwise comparison revealed that even though the effect of *Iw1* was very significant overall, when considering individual NIL pairs, differences were not always significant. Hereward was the only variety for which the delayed senescence of *Iw1+* NILs was significant in both 2013 with 0.8 days difference (p < 0.05) and 2014 with 1.5 days difference (p = 0.003). Alchemy also had a significant delay in senescence associated with *Iw1* of 0.9 days in 2013 (p=0.004), but showed no difference in 2014.

Table 3. 3 Green canopy duration

Green canopy duration was calculated as the number of days between flowering and senescence. Data were recorded for NILs of all six varieties. Overall *Iw1* significantly increased green canopy duration (p<0.001), although pairwise comparison showed that this effect was not significant between all NIL pairs. Significance is indicated at the level p<0.05 (*), p<0.01 (**).

		2013			2014		
		Average green canopy duration (days) ± S. E	Effect *+Iw1*	p	Average green canopy duration (days) ± S. E	Effect *+Iw1*	p
Xi19	*Iw1+*	44.1 ± 0.5	0.3	0.681	57.6 ± 0.4	1.0	0.024*
	iw1-	43.8 ± 0.5			56.6 ± 0.2		
Alchemy	*Iw1+*	45.0 ± 0.2	0.9	0.004**	55.1 ± 0.3	0.3	0.492
	iw1-	44.1 ± 0.2			54.8 ± 0.3		
Einstein	*Iw1+*	48.4 ± 0.2	0.5	0.137	58.9 ± 0.2	0.8	0.001**
	iw1-	47.9 ± 0.2			58.1 ± 0.1		
Hereward	*Iw1+*	46.7 ± 0.2	0.8	0.05*	57.8 ± 0.4	1.5	0.003**
	iw1-	45.9 ± 0.2			56.3 ± 0.3		
Robigus	*Iw1+*	46.1 ± 0.2	-0.1	0.714	57.5 ± 0.2	-0.2	0.6547
	iw1-	46.2 ± 0.2			57.7 ± 0.4		
Malacca	*Iw1+*	43.9 ± 0.4	0.4	0.478	54.6 ± 0.3	0.5	0.295
	iw1-	43.5 ± 0.4			54.1 ± 0.3		

To understand the relationship between green canopy duration and yield for these field trials, the adjusted yield for each plot was compared with the green canopy duration for that same plot. *Iw1+* NILs are shown in green, and *iw1-* NILs in grey in Figure 3.5.

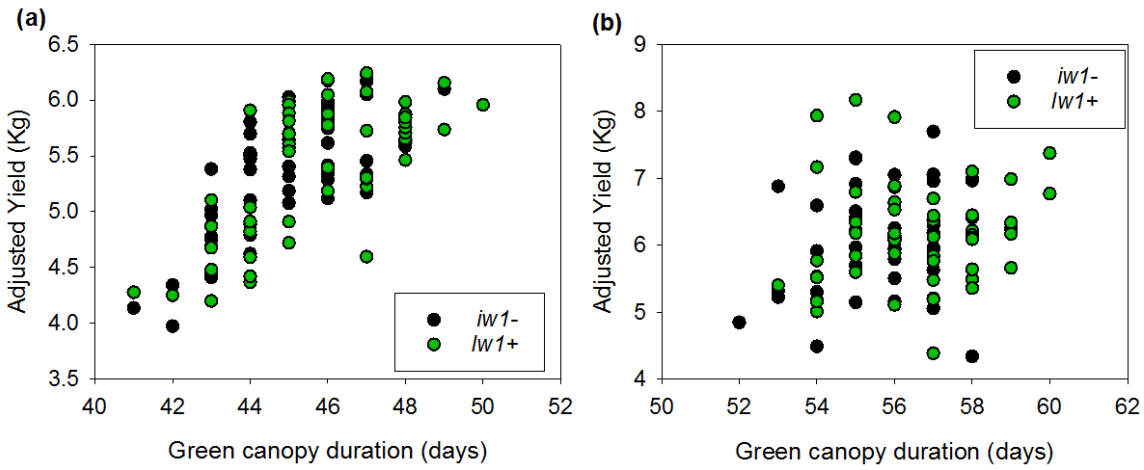

Figure 3. 5 Adjusted yield compared to green canopy duration for *Iw1* NILs

Yield of each plot compared to the green canopy duration for that plot for (a) 2013 and (b) 2014 at Church Farm. Data are included for NILs of all six varieties, *Iw1+* NILs in green and *iw1-* in black. In 2013 (a) there was a significant correlation between green canopy duration and yield (p<0.001) and a significant regression between the two variables (r^2 = 0.486; p<0.001). In 2014 (b) there was no significant correlation between variables.

In 2013 (Figure 3.5a) there was a significant correlation between green canopy duration and yield (Pearsons correlation co-efficient = 0.697; p<0.001). Further analysis with linear regression shows that extended green canopy duration did result in a significantly higher yield (r^2 = 0.482; p<0.001), suggesting that green canopy duration may have been a determinant of yield in these trials. However, there is no clear distinction between the NILs in Figure 3.5a. In 2014 there was no significant correlation between green canopy duration and yield (Pearsons correlation co-efficient = 0.147; p=0.146), showing that for these field trials green canopy duration was not a determinant of yield. However, 2014 was the year of severe bunt infection in the field, so yield data may not be as reliable as in 2013. Again, there is no clear distinction between NILs with and without *Iw1* in Figure 3.5b.

Comparison of the percentage difference in yield between NILs against the difference in green canopy duration indicates that there was no clear correlation between the two parameters (Figure 3.6). Alchemy and Hereward, the varieties that show the most consistent yield benefit of the *Iw1* introgression, did have the biggest extension of green canopy duration associated with the introgression in 2013 (Figure 3.6a), but taking the other four varieties into account there is no clear relationship. Again in 2014 (Figure 3.6b) Hereward had the longest increase in green canopy duration associated with the *Iw1* introgression, but the relationship was absent for Alchemy. Again, this data may be skewed by bunt infection, as can be seen from the Einstein NILs, in which a 23% yield increase was recorded in the *Iw1+* NILs.

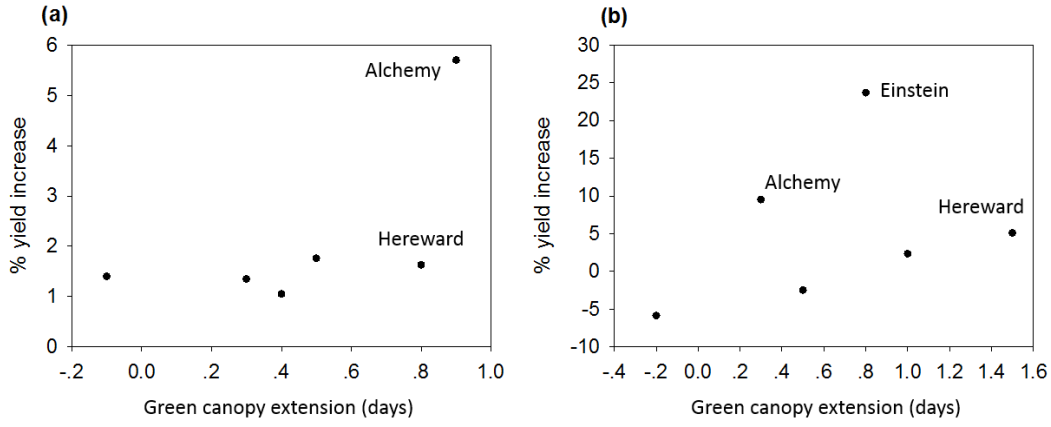

Figure 3. 6 Percentage yield increase associated with *Iw1* compared to green canopy extension for each variety

The increase in yield associated with *Iw1* plotted against the average extension of green canopy duration associated with *Iw1* in (a) 2013 and (b) 2014. Each point on the scatter graph represents the average difference between NILs of one variety for one year.

3.3.3 Wax profile

The *Iw1* germplasm has been characterised previously to assess wax composition, identifying 53 compounds, 26 of which accounted for >95% of the total wax load (Adamski *et al.,* 2013). In this PhD, the bulk of the physiology work was carried out at using BC_4 NILs of the varieties Alchemy, Malacca and Hereward at anthesis. Therefore epicuticular waxes were extracted from flag leaves of field grown BC_4 *Iw1* NILs in the field in 2014 from these three varieties in order to confirm wax composition was as expected and changes to appearance and physiology could be attributed to presence or absence of β- and OH-β-diketones in the epicuticular waxes. Relative abundances of the major wax components *n*-alkanes and primary alcohols (POH), in addition to fatty acids and MARs, were quantified.

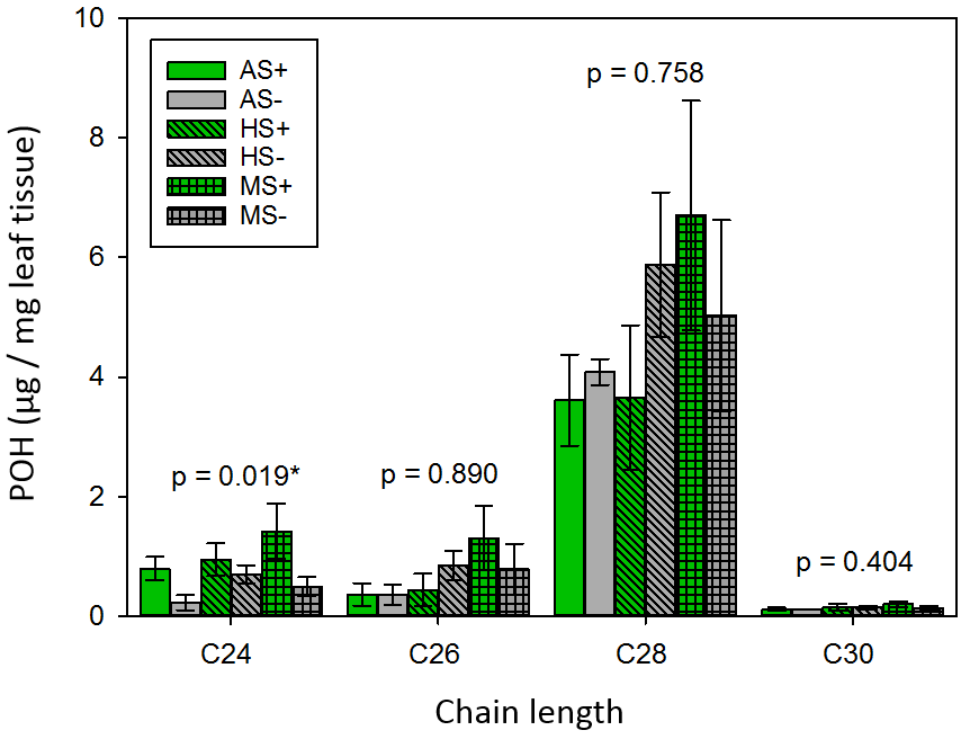

Figure 3. 7 Quantity of primary alcohols (POH) in the epicuticular wax

POH in the epicuticular waxes of *Iw1+* and *iw1-* NILs of Alchemy, Hereward and Malacca. P values for the effect of *Iw1* over all three varieties are shown on the chart. Pairwise comparison showed no significant difference between individual NIL pairs for any chain length. N=3, error bars = S.E.

Overall, POH of chain length C24 to C30 made up around 80% of the epicuticular waxes of both *Iw1+* and *iw1-* NILs (Figure 3.7). Of the four POH compounds present in the wax, C28 POH made up by far the biggest component, ranging from 4 to 8 µg/mg leaf tissue depending on variety. There was no significant effect of *Iw1* on C28 POH quantity (p = 0.758) nor interaction with variety (p = 0.364). In fact the only POH on which *Iw1* had a significant effect was C24 (p = 0.019), of which *Iw1+*

NILs of all varieties had higher quantities than *iw1-*. For C24 POH there was no effect of variety (p = 0.270) nor interaction between *Iw1* and variety (p = 0.150), and pairwise comparison revealed that for no NIL pair was the difference in C24 quantity significant.

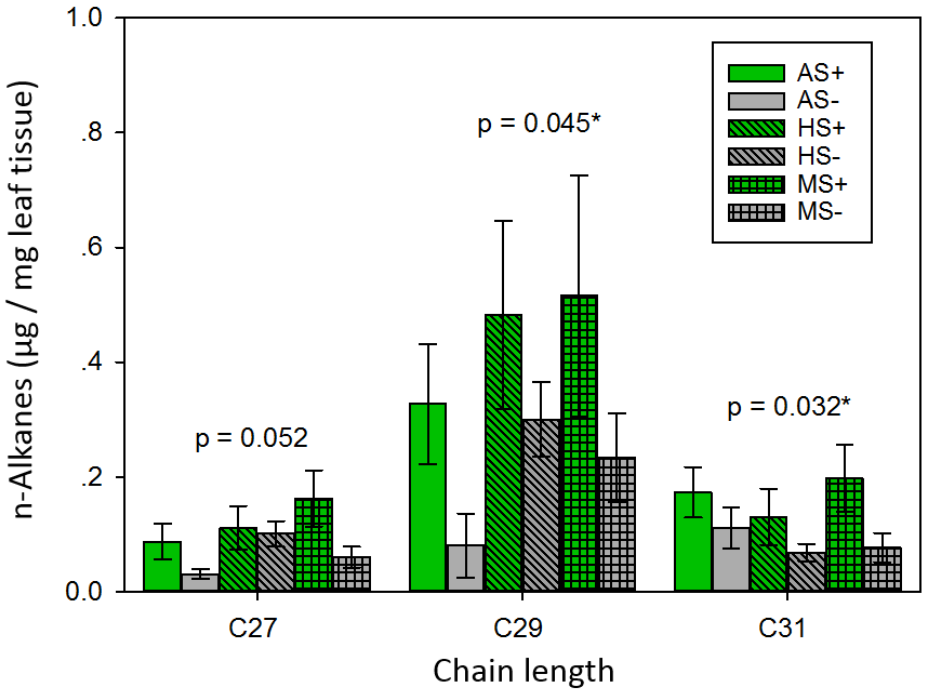

Figure 3. 8 Quantity of *n*-alkanes in the epicuticular wax

Overall p values for the effect of *Iw1* across all three varieties are shown on the chart. Pairwise comparison shows no significant differences between NILs within varieties. N=3, error bars = S.E

After POH, *n*-alkanes were the second major component of the epicuticular waxes present in both *Iw1+* and *iw1-* NILs (Figure 3.8), contributing around 0.1-0.6 µg wax per mg leaf area dependant on chain length. Figure 3.8 shows that *Iw1* had the effect of significantly increasing quantity of *n*-alkanes in the epicuticular waxes for C29 (p = 0.045) and C31 (p = 0.032) and borderline significant for C27 (p = 0.052). For no chain length was there a significant difference in quantity between varieties, and there was no interaction between variety and *Iw1* (C27, p = 0.409; C29, p = 0.934; C31, p = 0.734). However, when analysed by pairwise comparison there was no significant difference between any NIL pair for any of the three *n*-alkanes. In total, *n*-alkanes accounted for around 10% of total wax load in the *Iw1+* NILs, and 3-5% in *iw1-* NILs.

Fatty acids and MARs were also present in the epicuticular waxes in very small amounts, with a combined contribution of around 5% (Figure 3.9 and 3.10). Figure 3.9 shows that there was no significant effect of *Iw1* on any MAR (C19, p = 0.445; C21, p=0.712; C23, p= 0.728; C25, p=0.876; C27, p=0.978).

Similarly, there was overall no significant effect of *Iw1* on C22 (p = 0.718), C24 (p = 0.871) or C28 (p = 0.610) fatty acids (Figure 3.10). However, there was an overall significant effect of *Iw1* on C32 fatty acids (p = 0.001). There were also significant differences between varieties for C32 fatty acids (p=0.008), and the interaction between variety and *Iw1* was significant (p=0.013). However, when interpreting this data it is important to note that the quantity of fatty acids in the waxes was very minor in comparison to *n*-alkanes and POH. In particular, C32 fatty acids were only present in quantities under 1 x 10^{-5} µg/mg leaf tissue so their significance to overall wax properties compared to other components will be small.

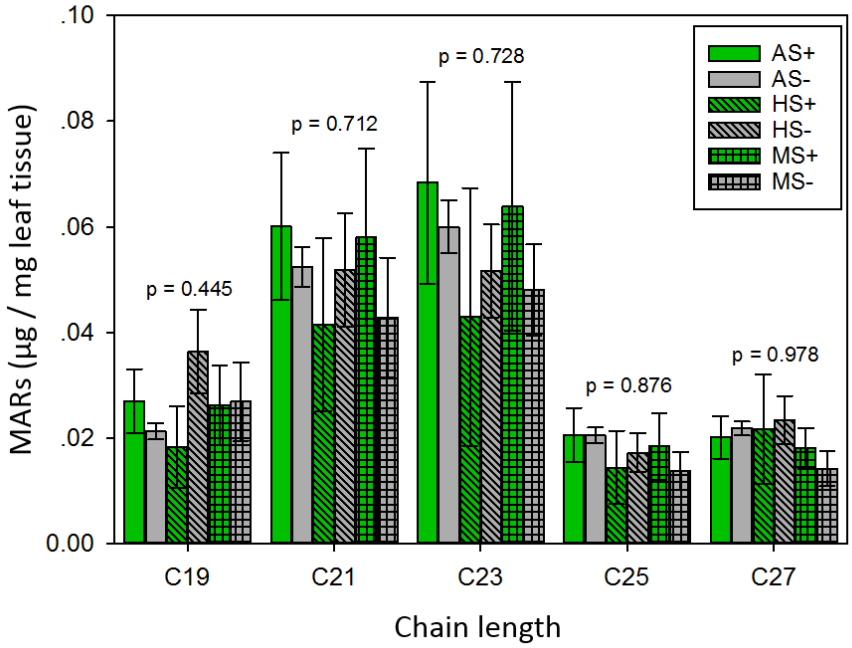

Figure 3. 9 Quantity of C19, C21, C23, C25 and C27 MARs in the epicuticular wax

Overall p values for the effect of *Iw1* across all three varieties are shown on the chart. There is no effect of *Iw1* on quantity of any MAR. N=3, error bars = S.E.

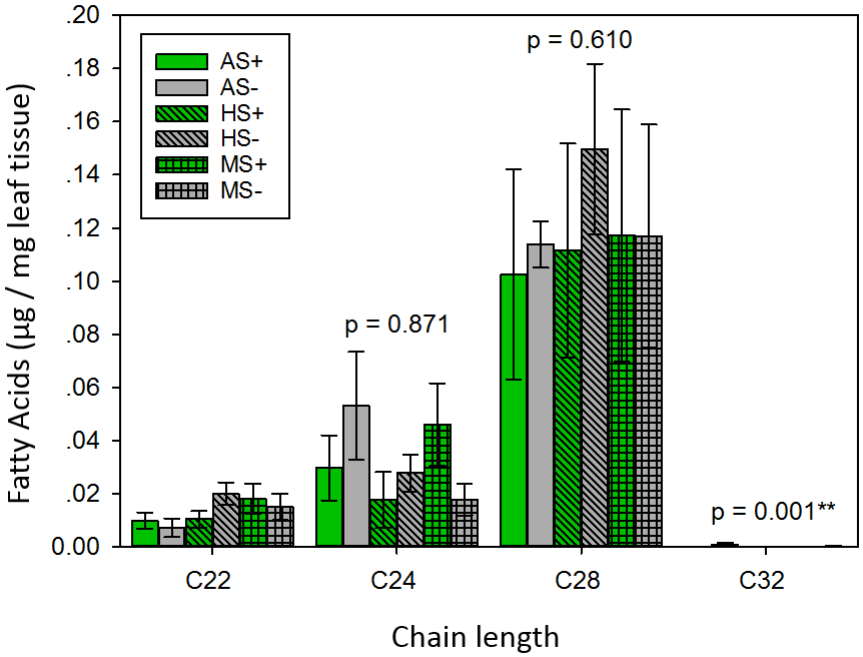

Figure 3. 10 Quantity of C22, C24, C28 and C32 fatty acids in the epicuticular waxes

Overall p values for the effect of *Iw1* across all three varieties are shown on the chart. There is a significant effect of *Iw1* on C32 fatty acids (p = 0.001). n= 3, error bars = S.E.

Table 3. 4 Quantity of β-diketones at anthesis in the epicuticular waxes at anthesis

There was a significant difference between NILs of Alchemy ($p < 0.001$), Hereward ($p < 0.001$) and Malacca ($p = 0.01$). n=3.

		Average β-diketone quantity (µg / mg leaf) ± S.E	p
Alchemy	*Iw1+*	0.0001 ± <0.0000	<0.001***
	iw1-	0.6898 ± 0.1024	
Hereward	*Iw1+*	0.0006 ± 0.0005	<0.001***
	iw1-	0.5446 ± 0.07988	
Malacca	*Iw1+*	0.0002 ± 0.0001	0.01**
	iw1-	0.5313 ± 0.01714	

Table 3.4 demonstrates a significant difference between NILs of Alchemy ($p < 0.001$), Hereward ($p < 0.001$) and Malacca ($p = 0.01$) in quantity of β-diketones; these compounds were present in quantities of around 0.5 - 0.7 µg/mg leaf tissue in *iw1-* leaves, accounting for 5-10% of total wax load, whereas they were only present in trace amounts in *Iw1+* flag leaves. Over all there was no difference in β-diketone quantity between varieties ($p = 0.553$), and no interaction between *Iw1* and variety ($p = 0.551$) indicating that *Iw1* had the same effect on epicuticular wax biochemistry in all three varieties.

The data presented here for epicuticular wax composition are in agreement with that reported in Adamski *et al.,* 2013, indicating that data reported in the 2013 study are applicable to the germplasm and environmental conditions used in the present work. Importantly, it suggests that the effect of *Iw1* on wax composition is stable across years and environments.

3.5 Discussion

3.5.1 *Iw1* did not confer a yield advantage

The initial work that investigated non-glaucousness in Shamrock reported that *Iw1* increased yields by 2.4 to 5.6 % in a UK field environment (Simmonds *et al.,* 2008). This agreed with previous findings that, whilst glaucousness proves beneficial for both water use and yield in water stressed environments (Febrero *et al.,* 1998; Johnson *et al.,* 1983; Monneveux *et al.,* 2004; Watanabe, 1994), non-glaucous plants may have the advantage under well-watered conditions (Merah *et al.,* 2000). However, in the *Iw1* NILs, the *Iw1* introgression did not lead to any significant yield advantage or disadvantage in four of the six varieties wheat varieties assessed. This leads to the conclusion that non-glaucousness and *Iw1* alone do not affect yield in a temperate, UK environment. The *Iw1* introgression did have a positive effect on yield in NILs of two varieties suggesting the action of *Iw1* may depend on genetic background. The use of *Iw1* NILs in the present work, in addition to biochemical wax analysis, allows results to be specifically linked to changes in genetics and epicuticular wax composition. This provides a significant advantage when compared to the work of Merah *et al.,* (2000) who assessed 16 durum wheat accessions of contrasting glaucousness. They did not specifically characterise the underlying genetics or epicuticular biochemistry of these accessions, and as such, the 30 – 50% increase in biomass they observed in the non-glaucous lines under adequate water supply could have been the result of genetic differences between accessions other than presence or absence of visible waxes.

Hereward showed the clearest yield benefit in the *Iw1+* NILs. The yield increase of around 5% in this variety was consistent with the effects seen in the original Shamrock x Shango DH population. Alchemy also showed increased yield in the *Iw1+* non-glaucous NILs, but this was less consistent than in Hereward, and the overall effect (around a 3% yield increase) was less significant. Taking account of yield data from all six varieties, and analysis of the recombinant lines, it seems likely that the yield benefit in both Alchemy and Hereward is coming not from *Iw1* itself, but from a closely linked gene. There are a number of possible explanations why only two of the six varieties demonstrated this effect. It is possible that Hereward and Alchemy contained a deleterious allele within the introgressed region of chromosome 2B that was having a negative effect on yield. Replacing this with the Shamrock allele allowed yield to increase. Alternatively, there could be some interaction between the introgressed region and another gene in the background and only with this specific combination is there a yield benefit. These explanations could also apply to the Shango population initially studied where a yield benefit was recorded. Shango NILs have been created to assess this affect, but to date limited data is available so strong conclusions cannot be drawn.

Yield data is continuous rather than discrete and consequently has to be mapped as a QTL. This means that the trait cannot be defined to a precise genetic interval between two markers. Therefore it was not possible to determine the exact portion of chromosome responsible for the yield advantage. However the 5% yield increase reported in Hereward is significant, and has potential benefit for UK agriculture. It will therefore be important to develop materials and methods to more specifically map this effect and understand which aspect of the *Iw1+* Hereward NILs physiology and genetics is conferring this.

3.5.2 *Iw1* extended grain filling by around 2%

A suggested factor contributing to the increased yield observed in non-glaucous lines of the Shamrock x Shango DH population was delayed canopy senescence that mapped to *Iw1* (Simmonds *et al.,* 2008). In the *Iw1* NILs, the *Iw1* introgression was associated with an overall significantly longer green canopy duration of around one day. This represents a 1.8% increase in green canopy duration in 2013, and a 2.2% increase in 2014. This is comparable (if slightly less) than the 3% increase observed in non-glaucous lines from the Shamrock x Shango population (Simmonds *et al.,* 2008).

Correlation analysis of the Church Farm yield data indicates that, at least in 2013, green canopy duration was a determinant of yield overall, with a difference of around 8 days green canopy duration between the highest and the lowest yielding plots. It has been suggested by a number of studies that delayed canopy senescence may only be of benefit under sub-optimal growing conditions such as drought and heat stress, where supply of substrate does not keep up with sink demand within the plant (Borrás *et al.,* 2004; Gregersen *et al.,* 2013; Lopes & Reynolds, 2012; Reynolds *et al.,* 2005; Verma *et al.,* 2004). This could explain the discrepancy in correlation observed over the two trials. In comparison to 2014, 2013 was generally warmer from June onwards, resulting in the fields drying out very quickly and a shorter green canopy duration overall. Under these circumstances the plants may have been more water stressed. Whilst it would not have been beneficial to delay senescence by too long under these conditions, those plants that went through senescence at the later time point would have had around a week longer to photosynthesise. Under stressed conditions this could have provided enough substrate to enhance grain filling and increase yields. 2014 was cooler and the plants experienced a longer growing season. There was no strong correlation between yield and green canopy duration, potentially because the plants were not stressed, so were limited by sink rather than source factors. Notably, conclusions drawn from this 2014 data should be treated with caution due to the severe bunt infection.

Although green canopy duration could have been a determinant of yield in the trials as a whole, there was no strong evidence that the *Iw1* introgression extends green canopy in a way that increases yield. This suggests that even though *Iw1* may indeed be responsible for delayed senescence, factors other than grain filling period were more important in determining yield differences between NILs in these field trials. Any effect of the non-glaucous trait on extended green canopy duration and yield has not been previously reported within a Mediterranean, or similarly stressful, environment, where substrate availability may be limiting. However it could be that, at least where drought stress is concerned, the benefits of glaucousness on water use could outweigh any small extension on grain filling of the non-glaucous varieties. Alternatively, earlier senescence may be beneficial in a Mediterranean environment, allowing the plant to avoid the terminal drought stress that often occurs in these environments.

3.5.3 The effect of *Iw1* on epicuticular wax was global across three wheat varieties

Analysis of wax composition confirmed that β- and OH-β-diketones were present in glaucous lines of all three varieties lacking *Iw1* and made up a significant proportion of total wax load. These compounds were only recorded in trace amounts in non-glaucous lines with *Iw1*, indicating that synthesis of these compounds was inhibited in the presence of *Iw1*. Additionally, no differences were observed between the three varieties.

As well as this major effect on the β-ketoacyl-elongation pathway, other effects of *Iw1* on wax composition reported by Adamski *et al.,* (2013) were confirmed in the plant material used here. Overall, although *Iw1* did have a significant effect of increasing quantity of *n*-alkanes, there was no difference between NILs in terms of POHs (with the exception of C24), fatty acids or MARs. Crucially, no differences between varieties, or interaction between variety and *Iw1* on any major wax component were recorded. This indicates that within these three wheat varieties genetic background has no effect on *Iw1* action in terms of epicuticular wax biochemistry. This suggests that the global effects of *Iw1* can be studied using these NILs. We would expect that any common difference between NILs of the three varieties could be attributed to the *Iw1* gene itself rather than background genetics.

Characterising the wax composition of the *Iw1* germplasm prior to detailed study is of high importance. The definition of the word glaucousness, in terms of amount of epicuticular wax and exact composition will vary between species and even varieties of the same species with differing genetics. In wheat there are a number of genes that determine epicuticular wax type. Aside from *Iw1* and *W1*, *Inhibitior of Wax 2* and *Wax 2* have also been studied (Liu *et al.,* 2006; Yoshiya *et al.,* 2011; Zhang *et al.,* 2013) in addition to *Inhibitor of Wax 3* and *Wax 3* (Wang *et al.,* 2014; Zhang *et*

al., 2015). Although the phenotypes may visually appear similar, all six of these epicuticular wax genes work via a slightly different mechanism conferring glaucousness (or non-glaucousness) in a variety of ways as detailed below.

For example, the inhibition of glaucousness in wheat by *Iw3* is confined to the spike, with all other organs in an *Iw3+* plant covered in visible β-diketone-containing waxes, whereas in wheat containing *Iw1* β-diketones are inhibited in all organs of the plant (Adamski *et al.,* 2013; Wang *et al.,* 2014). Specifically to wax composition, wheat plants with glaucousness conferred by *W3* have waxes composed of 63.3% diketones, 34% *n*-alkanes and only 0.7% primary alcohols (Zhang *et al.,* 2015). This is quite different from the glaucousness observed in the *iw1*- NILs, in which primary alcohols are by far the largest epicuticular wax component making up around 80% of wax, *n*-alkanes around 5% and β-diketones also around 5-10%. These differing biochemistries could mean that the waxes have quite different properties despite having the same visual appearance. It is therefore vital that all studies into glaucousness specify the genetics and biochemistry associated with their germplasm in order to allow better comparison between studies. This is something that has not always been considered in the past, and thus makes understanding the mechanisms behind any observed effects on plant physiology difficult.

3.5.4 Conclusions

Overall, data from the *Iw1* NILs suggest there is no global effect of non-glaucousness as conferred by *Iw1* on yield within a UK environment. However, there was an associated yield increase observed in Hereward and Alchemy. Although likely not linked to *Iw1*, this was significant, and given the backdrop of plateauing wheat yields combined with population growth, is worth pursuing. However, work with the NILs suggests that it is not an extended grain filling period that is responsible for this yield benefit. Further exploration and identification of exactly what is causing this yield increase could be beneficial for future wheat breeding.

Keeping the yield data in mind, Hereward, Alchemy and Malacca were chosen to take forwards for more detailed study of plant physiology. This provides a contrast; two varieties that demonstrated differing yield advantages associated with the *Iw1* introgression, and Malacca, for which the introgression proved to have no effect. Having detailed wax composition data for NILs of these three varieties will place this work at a significant advantage in comparison to many studies of this type that went before. Any effects of the epicuticular waxes on physiology can be attributed to specific changes to epicuticular wax biochemistry rather than using the terms glaucous or non-glaucous in a general sense based on appearance. Furthermore, we can be certain that the effects of *Iw1* on epicuticular wax biochemistry are the same across all three varieties.

Chapter 4: The effect of *Iw1* on plant and canopy spectral properties and subsequent effect on photosynthesis and temperature

4.1 Summary

This chapter explores the effects of changing epicuticular wax biochemistry and structure on plant spectral properties, and how this impacts on photosynthesis and plant productivity. Glaucous plants are known to have increased reflectance of light within the ultra violet (UV), infrared and photosynthetically active radiation (PAR) spectra. However, It is not understood exactly which components of the epicuticular waxes, or leaf biochemistry, are causing these changes in reflectance. Furthermore, spectral components other than reflectance, such as light transmission, have not been explored. Consequently there is limited understanding of how much impact this change in reflectance has on the amount of light a plant can absorb.

These issues were studied with respect to PAR through measurement of flag leaf reflectance, transmission and absorbance. Canopy light interception was also explored. To further understand how these spectral changes were impacting on plant physiology, components of photosynthesis known to change under altered light availability were measured. Canopy temperature was also briefly studied.

Glaucous plants without *Iw1* reflected more light at both the single leaf and canopy level than the non-glaucous plants with *Iw1*, confirming results of previous studies in other environments. It was conclusively shown that the β- and OH-β-diketone components of cuticular wax cause this increased reflectance, and that waxes without these compounds have no impact on leaf reflectance. However, no evidence was found that this change in reflectance alters PAR absorbed by the leaf, and glaucousness had no effect on photosynthesis or canopy temperature in any of the wheat varieties studied.

4.2 Introduction

4.2.1 Changes to plant spectral properties have implications for plant physiology

The spectral properties of a plant will modulate the amount of light that is absorbed by plant tissues. These properties are determined both by internal factors such as pigmentation, and external components of the plant surface such as epicuticular waxes and pubescent hairs. Variation in these internal and external factors will determine both visual appearance and how the plant uses incoming light.

Figure 4.1 shows the portion of the electromagnetic spectrum of most relevance to plant physiology. Within the visible spectrum (380 – 780 nm), the plant uses light between 400 nm and 700 nm for photosynthesis. As such this light is termed photosynthetically active radiation (PAR). The ability to absorb enough PAR for optimum photosynthetic rates is highly important. However, the amount of PAR absorbed needs to be in balance with PAR that is used during photosynthesis. PAR in excess of the amount that can be used in photosynthesis can cause damage to the photosystems reducing photosynthetic rate (photoinhibition). Exposure to high levels of PAR also results in the production of reactive oxygen species (ROS) which cause further damage to plant tissue and slow the repair of the photosystems after photoinhibition. Prolonged exposure to excessive PAR can cause damage to the photosystems that is irreversible. Therefore mechanisms to protect photosynthetic tissues from high light and limit exposure are important within certain environments (Long & Humphries, 1994; Olascoaga *et al.,* 2014).

Light within the ultra violet (UV) spectrum is also absorbed by plant tissue. Exposure to excessive UV can be very detrimental to plant tissues damaging DNA, reducing plant growth, and lowering photosynthetic rates (Caldwell *et al.,* 1989). However, exposure to levels of UV under typical sunlight conditions is unlikely to cause extreme damage. Furthermore, there is evidence that acclimation to biologically realistic (not extreme) levels of UV-B could be beneficial for the plant. For example, many leaf adaptations to continuous UV-B exposure such as increased leaf thickness (Bornman & Vogelmann, 1991) and higher quantities of phenolic compounds such as flavonoids (Bassman, 2004) can provide protection from photoinhibition under high levels of PAR, thus allowing the plant to be more productive (Wargent & Jordan, 2013). Absorbance of light within the infrared spectrum will influence plant temperature. Temperature is key in regulating a number of key developmental processes such as flowering and grain filling. It also has a role in a number of metabolic processes such as photosynthesis (Acevedo *et al.,* 2002). Therefore adaptations to facilitate either reflection of excess, or absorbance of more heat, depending on conditions, is essential. How a plant responds to its environment in terms of light regulation right across the

spectrum is highly important, and determined by a number of morphological and biochemical adaptations.

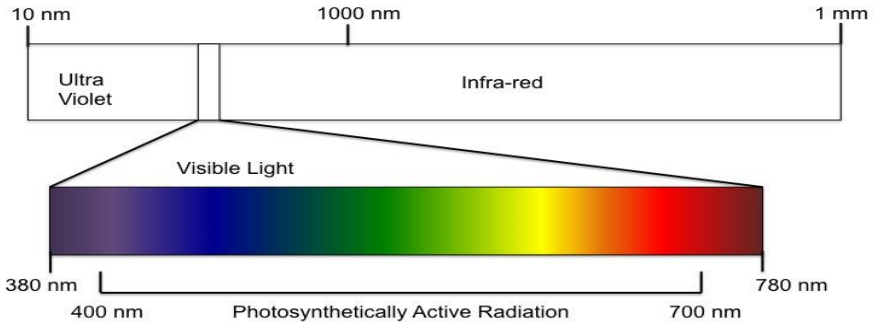

Figure 4. 1 The electromagnetic spectrum between 10 nm and 1 mm

Epicuticular waxes contribute to reflection of excessive radiation within the ultra violet (UV) and infrared wavelengths. Photosynthetically active radiation (400 – 700 nm) is the light within the visible spectrum (380 – 780 nm) that the plant can use for photosynthesis.

At the single leaf level, the total light absorbed can be considered as a function of two processes, reflectance and transmission (Figure 4.2). Of all the incoming light that reaches the plant, a small amount will be reflected directly off the surface. The rest passes into the plant, but a portion of this will be transmitted through the tissues un-used. The total light absorbed will depend on the amount of light lost through each of these processes. Epicuticular waxes cover all surfaces of land plants and have an important role in determining spectral properties. How these spectral properties might influence yield is of key interest in this PhD. As photosynthesis is of high importance for plant productivity, this chapter will mainly focus on components of light absorbance within the PAR spectrum, with some exploration of infrared.

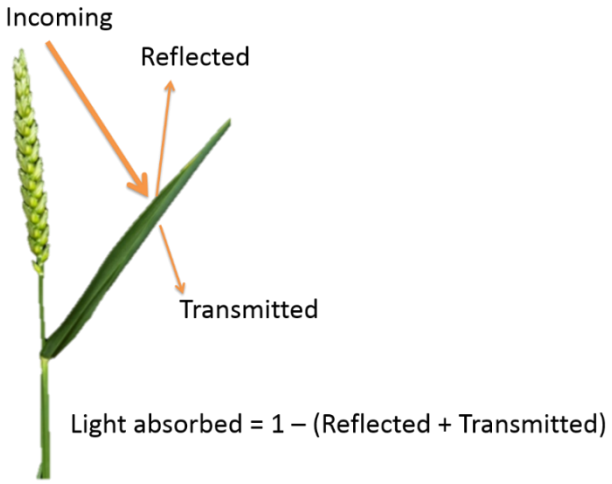

Figure 4. 2 Factors affecting the amount of incoming light absorbed by a plant

How much light is reflected from the surface and how much light is transmitted through the tissue both affect how much light the tissue can absorb. Light Absorbed = 1 – (Reflected + Transmitted).

4.2.2 The effect of epicuticular waxes on spectral properties

The effect of epicuticular waxes on light reflectance, particularly within the PAR spectrum, has been widely investigated. A 2002 study of 45 different species found that epicuticular wax removal significantly reduced leaf reflectance at both 680 nm (PAR) and 330 nm (UV) (Holmes & Keiller, 2002). Furthermore, this study compared glaucousness, pubescent hairs and woolly hairs, and found glaucousness to be the most effective method of light reflectance. Glaucous species reflected around 10-20% more UV and 5-10% more PAR spectra than non-glaucous species with pubescent or woolly hairs. More specifically to wheat, glaucous epicuticular waxes have been repeatedly found to increase reflectance of wheat and barley by around 5-10% at the single leaf level (Jefferson *et al.*, 1989; Johnson *et al.*, 1983) and 20% at the canopy level (Febrero *et al.,* 1998) compared to non-glaucous waxes. However, specifically which properties of these waxes affects the reflective properties has not been directly addressed. For example, PAR reflectance of wheat has been found to increase linearly with quantity of epicuticular wax (Johnson *et al.*, 1983), yet many types of glaucousness are determined by changes to wax biochemistry rather than total wax load.

It has been widely assumed that the increased reflectance of glaucous epicuticular waxes reduces light interception. However, any effect of epicuticular wax type on light transmission has not been studied previously at either the single leaf or canopy level. Consequently the effect of glaucousness on overall light interception is unclear.

4.2.3 Changes to PAR absorbance could impact on leaf level photosynthesis

If the greater PAR reflectance observed in glaucous wheat varieties does indeed lead to lower PAR absorbance, less PAR would be available to the photosynthetic tissues of glaucous plants. Photosynthesis of cultivated wheat is 90% light saturated at PAR levels of 1000 µmol m^{-2} s^{-1}. Full saturation is reached between 1000 and 1500 µmol m^{-2} s^{-1} dependent on variety, at which point light is no longer limiting to photosynthesis (Acevedo *et al.,* 2002). However, prolonged exposure to levels of PAR above this saturation point can damage the photosynthetic machinery, reducing photosynthetic capacity (Monneveux, *et al.,* 2003; Ögren & Rosenqvist, 1992; Yang *et al.,* 2006). Therefore the increased reflectance of glaucous epicuticular waxes could prove beneficial or detrimental to productivity depending on environmental conditions.

Wheat is grown in many high light intensity environments such as Ciudad Obregon in Mexico where light levels are frequently above 1500 µmol m^{-2} s^{-1} for much of the day (Figure 4.3b). The more reflective glaucous epicuticular waxes could be advantageous in this instance, providing protection from high light (this photoprotective mechanism of the waxes will be further explored in Chapter 6). Conversely, in environments such as the UK it is more likely that plants will not be receiving enough light to reach photosynthetic maximum. Figure 4.3a shows a typical sunny day during

summer in Norfolk, UK. The PAR levels are much more variable than for Obregon, only above 1500 µmol m^{-2} s^{-1} for a small portion of the day. The majority of the time plants in this environment are experiencing PAR levels of around 1000 µmol m^{-2} s^{-1} or below. Under these circumstances non-glaucous plants would be at an advantage if their reduced reflectance facilitated greater PAR absorbance, thus allowing for faster photosynthetic rates.

Photosynthesis has long been a target for crop improvement, with many studies claiming that increasing photosynthetic rates is the answer to achieving higher grain yields in wheat (Makino, 2011). If non-glaucous waxes do increase PAR interception this could provide a novel way to increase radiation use efficiency within environments where PAR is regularly below the saturation point. The effect of glaucousness on photosynthesis has been investigated previously at the single leaf level, but there is lack of agreement in the literature regarding the relationship. For example, Johnson *et al.*, (1983) found no difference in light saturated photosynthesis between glaucous and non-glaucous NILs of durum and bread wheat. However, they did not test any light level below saturation. Conversely, a decrease in photosynthesis of 5- 23% has been observed in glaucous sorghum and wheat compared to non-glaucous (Chatterton *et al.*, 1975; Richards *et al.*, 1986). However, these studies do not state the light conditions making interpretation and subsequent comparison of existing studies difficult.

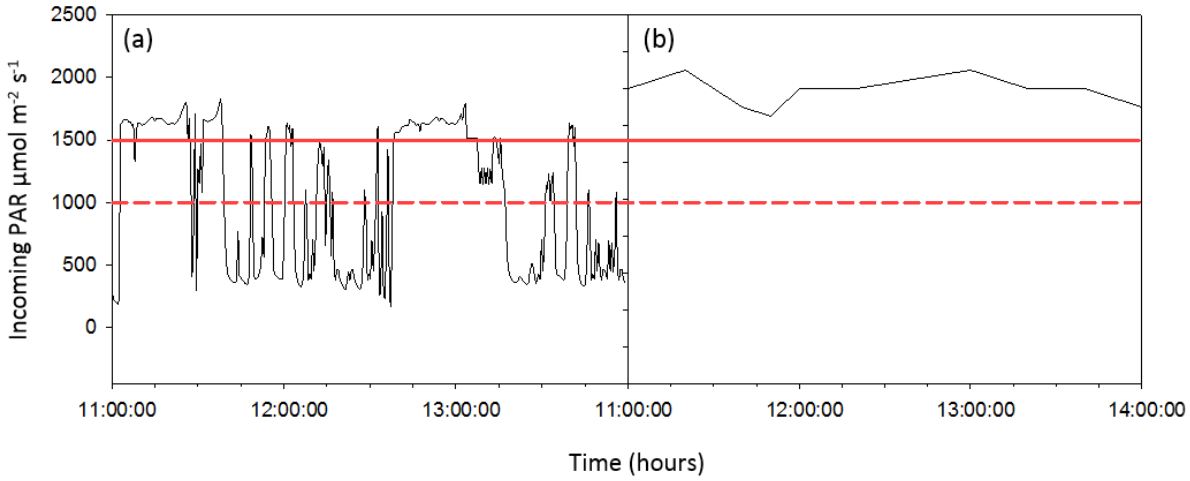

Figure 4. 3 Typical incoming PAR at midday for two contrasting wheat growing regions

The charts show incoming PAR for a clear sunny day in (a) Norfolk, UK, and (b) Ciudad Obregon, Mexico. Obregon data obtained from Monneveux *et al.,* (2003). The dotted red line indicates the PAR level at which photosynthesis is 90% light saturated. The solid red line indicates the PAR level above which extended exposure could cause photoinhibition.

4.2.4 Conclusions drawn at the canopy level may differ from the leaf level

Whilst it is important to study physiology at the leaf level, to fully understand how physiological processes might impact on crop interaction with the environment it is vital that work is translated up to the canopy level. This is because conclusions drawn at the canopy level can be quite different to those from leaf level measurement. For example, green plants are very efficient at absorbing incoming PAR. The exact proportion of PAR absorbed by the photosynthetic tissues will vary between species, but is generally considered to be around 85% (Zarate-Valdez *et al.,* 2015). However, work on a number of common British crops, including wheat and barley, found that in the field the crop canopy only intercepted around 40% of incoming visible light (Monteith, 1977). This was averaged across the season, taking account of all growth stages where canopy structure and ground coverage will vary considerably as the plants develop. These factors cannot be taken into account when looking at individual leaves or organs. Furthermore, the light available to photosynthetic tissues will not be homogenous moving down the canopy, introducing yet further complication in extrapolating results from the individual to canopy level.

Traditionally in wheat, the flag leaf at the top of the canopy has been considered the most important organ in terms of producing photosynthates for wheat grain filling, potentially contributing over 80% of final grain weight (Thorne, 1965). However, values reported in the literature for the contribution of different organs to grain filling vary widely, and depend heavily on wheat variety and environmental conditions. The stem has been frequently reported to contribute 8–25% of photosynthates to final grain yield (Merah & Monneveux, 2015; Zhang *et al.,* 2011), with some studies reporting values as high as 44% (Hannachi *et al.,* 1996). Under water stress the ear and peduncle together have been found to contribute over 73% to grain weight (Zhang *et al.,* 2011), and a recent study that shaded the flag leaf during plant development only recorded a 5% decline in grain weight (Merah & Monneveux, 2015). These studies all indicate that organs other than the flag leaf can have a key role in grain filling. Therefore the amount of light available at different levels of the canopy could have an important influence on final grain yield. The spectral properties of leaves at the top of the canopy could have an impact on the light available to those further down that may usually capture less light. Although an important consideration, this is not something that has been investigated in relation to glaucousness, perhaps due to difficulties associated with studying gas exchange and light interception at this scale.

4.2.5 Changes to spectral properties within the infrared wavelengths will influence canopy temperature

In addition to greater PAR reflectance, glaucous crop plants are also considered to reflect more light at infrared wavelengths and have significantly reduced tissue temperature. This has been shown to be the case in wheat, with photosynthetic tissues of glaucous plants being up to 0.7^{o}C

cooler than non-glaucous under drought in the field, and 0.3^{o}C cooler in a well-watered glass house (Richards *et al.*, 1986). Comparable results in a range of wheat and grass species were later found by Jefferson *et al.*, 1989. A more recent study in wheat even found that cuticular wax production and crop temperature both map to the same QTL on wheat chromosomes 1B, 3D and 5A indicating that wax type is a strong determinant of crop temperature (Mondal *et al.,* 2014).

Temperature is a key regulator of a multitude of plant metabolic processes including photosynthesis (which will be studied in the *Iw1* NILs). Air temperatures of 20 – 25 ^{o}C have been reported as optimum for growth of wheat and barley (Acevedo & Silva, 2002; Hossain *et al.,* 2012; Hossain *et al.,* 2016), and temperatures in excess of this can severely limit yield and photosynthesis (Acevedo *et al.,* 2002). However, studies in rice show that where temperatures are nearing these maxima, only a small decrease in tissue temperature could result in great yield improvement. Conversely, where temperatures are below the photosynthetic optimum, as is often the case in the UK, a small increase could greatly improve yield (Polley, 2002). Therefore, within the UK the non-glaucous phenotype could offer a significant advantage under sub-optimal temperatures if canopies are slightly warmer.

4.2.6 Aims

This chapter will focus on the effects of changing leaf and canopy spectral properties on the availability of PAR to photosynthetic tissues. The effect of glaucousness on canopy temperature will also be explored. This chapter will test the hypotheses that:

(i) Reduced reflectance of non-glaucous (*Iw1+*) wheat leaves and canopies makes more PAR available to photosynthetic tissues.

(ii) Non-glaucous (*Iw1+*) canopies have a higher temperature in the field.

4.3 Materials and Methods

4.3.1 Canopy PAR reflectance measurements

To confirm previous findings that glaucous canopies reflect more PAR than non-glaucous, reflectance of PAR from the top of the crop canopy was measured in the field during the harvest year of 2013 for NILs of Robigus, Xi19, Malacca and Alchemy and 2014 for Hereward, Malacca and Alchemy. All measurements were taken within 14 days of anthesis when the canopy is green and canopy morphology remains approximately stable.

Two hemispherical PAR quantum sensors (SKP215, Skye Instruments, UK) were attached to a pole (SKL 910, Skye Instruments, UK) at a height of 1.8 m above the canopy. One sensor faced upwards logging all incoming light, and a second sensor faced down towards the canopy. This second sensor had a collar that restricted the field of view to 25^{o}, therefore detecting light from an area of 0.5 m^{2}. A bubble on the pole ensured that the sensors were level over the canopy. Both sensors were connected to a Spectrosense + data logger (SKP 215LQ/SS2, Skye Instruments, UK). Measurements were taken at mid-day between 12 and 2 pm. Measurements were taken at 5 locations per plot, with 30 measurements logged at each location. One average value for each of incoming and reflected light was then calculated per plot. Four plots were measured from each NIL. The percentage incoming light reflected up off the top of the canopy was calculated and data were analysed both by overall ANOVA and pairwise comparison within varieties.

4.3.2 Measurement of PAR within the canopy

In order to investigate the effect of glaucousness on fractional interception, the proportion of incoming light that is intercepted by the canopy (Gonias *et al.,* 2012), two 1 m long PAR line quantum sensors (SKP215LQ/SS2, Skye Instruments) with 33 photodiodes spaced at equal distances along the length were used. These sensors were attached to the Spectrosense + data logger alongside the two PAR sensors detailed in section 4.3.1.

In 2013 one line sensor was placed on the floor. Another was placed in line with the base of the second leaf, which is mid-way down the canopy. Measurements on the floor and middle of the canopy were taken simultaneously with the incoming and reflected light measurements described in section 4.3.1 to ensure that data were comparable across all 4 sensors. Figure 4.4 shows placement of all four sensors within the crop canopy. Approximately 30 measurements were taken at 3-5 locations per plot and combined into one average value per plot. For each genotype three plots were measured. All measurements were taken within 14 days of anthesis. Data were collected for NILs of Robigus, Xi19, Malacca and Alchemy. The ratio of incoming light to light at the mid-way

point, and light on the floor was calculated, and from this the percentage incoming light reaching various canopy levels was obtained.

In 2014 the methods were modified slightly. Placement of all four sensors within and above the canopy remained the same, but sensors were left in position for extended periods of time. Sensors were left at a single location in the canopy for one hour, taking a total of 90 measurements. Two locations per plot were measured and data combined to get an average per plot. Three plots per NIL were measured. Data were obtained for three plots per NIL for Malacca and Alchemy and analysed both by overall ANOVA and pairwise comparison within varieties.

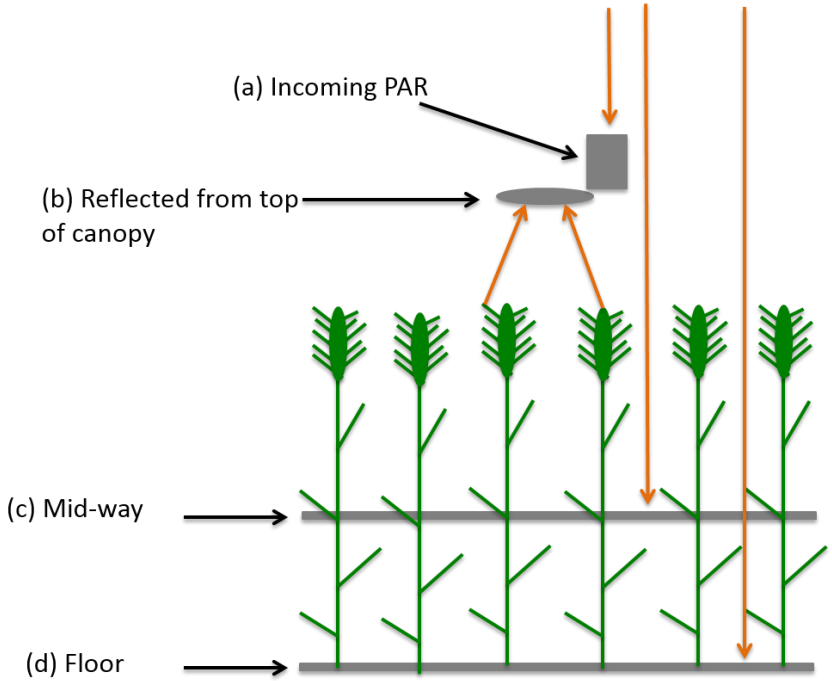

Figure 4. 4 Schematic showing PAR sensor set-up for measurement of canopy light interception

A sensor above the canopy measures incoming PAR (a), and a second sensor above the canopy measures PAR reflected from the top of the canopy within a 25 ^{o} field of view (b). Bar sensors containing 33 equidistant photodiodes were placed within the plot (c and d), one positioned in line with the second leaf (mid-way), and another on the canopy floor. Wheat plants are coloured in green, sensors in grey and PAR in orange.

4.3.3 Integrating sphere measurements

Spectral properties of glaucous and non-glaucous epicuticular waxes were also investigated at the flag leaf level. Flag leaves were sampled from five independent replications in the field at anthesis during the 2014 harvest season at anthesis. Leaves were cut at the base and transported with their ends in water to prevent dehydration. Leaves were kept at 4 ^{o}C with the ends in water until measurement. The spectral properties of flag leaves were analysed using an integrating sphere.

Figure 4.5 illustrates how the sphere works. A halogen projector lamp (15 V/150 W; Philips, Hamburg, Germany) was set up together with a fibre optic illuminator (COLD SPOT, PICL-NEX; NIPPON P-I CO.LTD, Tokyo, Japan) to provide a light source. For each leaf transmission through the leaf was measured as shown in Figure 4.5a. The leaf was placed with the adaxial surface facing the light source. A reflective backing of cardboard covered in silver foil was placed on the back of the sphere. Any light transmitted through the leaf into the sphere was detected by a spectrophotometer, measuring light at every wavelength between 400 and 700 nm. To account for the light lost in the gap between the leaf and light source, in addition to any minor modifications to the sphere between measurements, a blank measurement with no leaf (Q_1) was taken prior to measuring leaf transmission (Q_t). Leaf transmission was calculated as:

$$Transmission = \frac{Q_t}{Q_1}$$

The reflectance of light off the surface of each leaf was also measured, shown in Figure 4.5b. In this case the leaf was placed at the back of the sphere between the sphere and reflective backing, with the light shining through the sphere onto the leaf. Again, the adaxial surface was always facing the light source. Leaf reflectance (Q_r) at every wavelength between 400-700 nm was measured using the spectrophotometer. In order to account for light lost between the light source and leaf, and any minor alterations in integrating sphere set up between experiments a blank measurement (Q_2) with the exact same set up but with no leaf was taken each time. Leaf reflectance was calculated as:

$$Reflectance = \frac{Q_2 - Q_r}{Q_r}$$

From measurement of transmission and reflectance, total PAR absorbed by each leaf was calculated using the formula:

Absorbance = 1- (Transmission + Reflectance)

Absorbance, transmission and reflectance are all given as a proportion. Accordingly a leaf would have an absorbance of 1.0 when transmission and reflectance are both 0. Therefore total PAR absorbance cannot be greater than 1 in these measurements. However, on occasion average absorbance was calculated above 1. This was due to the nature of measurement. The errors associated with the integrating sphere measurement are quite large, and at wavelengths where the proportion of incoming PAR absorbed was high, the error resulted in values over 1.0. This error should be taken into account when interpreting the data presented from these measurements.

For transmission, absorbance and reflectance, data were averaged over 400 –700 nm for each NIL. Data were also divided into red (640-700 nm), blue (425-490 nm) and green (490-550 nm) wavelengths and average for each found. Data were analysed both by overall ANOVA and pairwise comparison within varieties and wavelengths. Certain individual wavelengths were also chosen to assess difference between NILs where the greatest divergence was observed but differences between NILs were not significant.

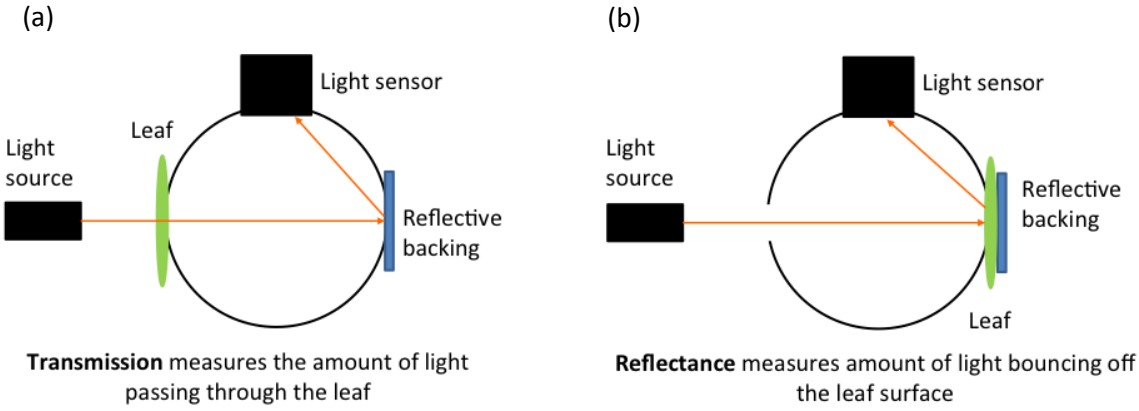

Figure 4. 5 Schematic showing set-up of the integrating sphere for measurement of flag leaf spectral properties

For transmission (a), a fibre optic light source shines light through the leaf into the sphere which is painted completely white on the inside. Light bounces off the sphere internal surface and into a light sensor at the top of the sphere, linked to a spectrophotometer that measures light at every wavelength between 400 and 700 nm. For reflectance (b), light is shone through the sphere onto the leaf at the back of the sphere. Light reflected by the leaf (rather than absorbed) is detected by the light sensor. In both cases leaves were set up such that the adaxial surface was facing the light source.

4.3.4 Wax removal experiment

To specifically understand how the epicuticular waxes affect leaf spectral properties, epicuticular waxes were mechanically removed with gum arabic (Gum Arabic from acacia tree, Sigma Aldrich, UK). Glasshouse grown material of Hereward, Alchemy and Malacca NILs was used during this

experiment grown as detailed in Chapter 2 section 2.2. Flag leaves were sampled at anthesis as described in section 4.3.3.

Gum arabic powder was mixed with water to create a paste thin enough to spread. The paste was spread over the entire surface of the leaf and left to dry. Once dry the hardened gum arabic was peeled off the leaf surface, removing the epicuticular waxes in the process. This process was carried out on both the adaxial and abaxial leaf surfaces prior to measurement. For each NIL, five leaves with mechanically removed epicuticular waxes, and five control leaves (no changes made to the leaf surface) were then subject to the integrating sphere measurements and data analysis described in section 4.3.3. Following measurement with the integrating sphere leaves were imaged using Scanning Electron Microscopy (SEM) to confirm absence of epicuticular waxes in treated leaves, and presence as normal in control leaves.

4.3.4.1 Scanning Electron Microscopy

Cryo SEM imaging was carried out by Kim Findlay and Elaine Barclay in the Bioimaging facility at the John Innes Centre in a manner similar to that described in Adamski *et al.,* (2013). In brief: Immediately after measurement with the integrating sphere, flag leaves were dissected and attached to an aluminium stub using O.C.T compound (BDH Laboratory supplies, Poole, UK). The stub was then snap frozen in liquid nitrogen prior to being placed onto the cryo-stage of a CT1500HF cryo-transfer system (Gatan, Oxford, England). The sample was kept at -95 ^{o}C for 3 min, and then sputter coated with platinum for 135 sec at 10 mA at -110 ^{o}C or colder. The sample was transferred to the cryo-stage of a Philips XL30 FEG SEM (FEI, Eindhoven, The Netherlands). The sample was viewed with the secondary electron detector at 3 kV, kept at -140 ^{o}C throughout imaging. A number of images were taken of each sample to confirm presence or absence of waxes across the surface.

4.3.5 Extraction of photosynthetic pigments

If increased reflectance of glaucous epicuticular waxes reduces the light available to the photosynthetic tissues, photosynthetic pigments might be upregulated to compensate for this. Flag leaf sampling was carried out in the field during the harvest years of 2014 and 2015 for NILs of Hereward Malacca and Alchemy from five independent replications at Growth Stage 31 (prior to visible wax appearance) and Growth Stage 61-69 (anthesis, wax visible). At anthesis flag leaves were collected and at GS31 the newest fully unfurled leaf was taken. Leaves were collected in the field and snap frozen immediately on site in liquid nitrogen. They were then stored in the dark at -80 ˚C until use.

Methods of pigment extraction were adapted from Inskeep & Bloom, 1985. Three discs of 8 mm diameter from each leaf were placed into a pre-weighed light proof 5 mL micro centrifuge tube

(C2500-OB, MTC-Bio, USA). Discs were taken one each from base of the leaf, mid-point and as near the leaf tip as possible, and combined in a single tube (preliminary results showed no significant difference in pigment content between leaf sections). Tubes containing leaf discs were weighed again to calculate total weight of tissue.

3 mL N,N-Dimethylformamide (analytical grade, Sigma Aldrich, UK) was added to each tube. Tubes were then left for 48-64 hr on a horizontal shaker at 4 ˚C until there was no further change in colour. Leaf discs did remain a very pale green colour, but this was consistent across all discs and no more pigment leached out after this point.

Pigment-containing Dimethylformamide was transferred to an optical glass cuvette (6030-OG, Hellma, UK), and absorbance measured in a spectrophotometer (Pharmacia Biotech, Ultraspec 1000E) at 664 (A_{664}), 647 (A_{647}) and 480 (A_{480}) nm. The following equations from Wellburn, 1994 were used to calculate chlorophyll a (chla), chlorophyll b (chlb) and carotenoid content:

$$Chla = 11.65A_{664} - 2.69A_{647}$$

$$Chlb = 20.81A_{647} - 4.53A_{664}$$

$$Carotenoids = (1000A_{480} - 0.89Chla - 52.02Chlb)/ 245$$

Results were then controlled for by both fresh weight and leaf area and analysed both by overall ANOVA and pairwise comparison within varieties.

4.3.6 Light curves

Light curves were taken on flag leaves in the field using a LI-6400XT (LI-COR Biosciences) to investigate whether the reduced reflectance of non-glaucous flag leaves has any effect on photosynthesis. Measurements were taken within two weeks of anthesis. During this period plants are at their photosynthetic maximum and there is little change in photosynthetic capacity (Molero & Lopes, 2012). In order to avoid the post-midday depression of photosynthesis (Xu & Shen, 2005), measurements were taken in the morning, with the first measurement no earlier than 9 am, and the last finishing no later than 12 pm. Four biological repeats were taken on each line measured. Measurements were taken on NILs of Malacca and Alchemy during the harvest year of 2013 and 2014, Robigus and Xi19 in 2013, and Hereward in 2014 and 2015. In 2015 Hereward was explored further through use of the recombinant lines HS17 and HS21 (*iw1-*) alongside HS26 and HS32 (*Iw1+*). Rationale for choice of recombinant lines is detailed in section 4.3.6.2.

Upon placing the leaf in the chamber it was allowed to equilibrate at a PAR level of 1000 µmol m^{-2} s^{-1} for 10 min or until stable. Subsequently, PAR was increased to 1500 µmol m^{-2} s^{-1}, and then reduced through 1000, 750, 500, 400, 300, 200, 100, 50 and 0 µmol m^{-2} s^{-1}. At each light level gas

exchange components including photosynthetic rate were logged after the leaf had equilibrated to the new conditions.

Throughout all measurements, flow was maintained at 300 µmol, reference CO_2 was set to 500 µmol mol^{-1} and the leaf temperature was set to 20 ^{o}C. An effort was made to maintain relative humidity between 50 and 70%. Where this was not possible given variable field conditions, it was allowed no higher than 75% or lower than 45%. Whilst plants for measurement were selected to be representative of the plot, those with flag leaves that were healthy and big enough to fill the leaf chamber were chosen.

Measurements were not corrected for absorbance, and leaf PAR absorbance was assumed to be 85% as per the LI-COR standard settings (LI-COR Biosciences, 2011). Although integrating sphere measurements were taken on the NILs measurements were variable and it was not possible to take these measurements in the field on every leaf to correspond with light curve measurements. However, these results also indicated that there was no significant difference in PAR absorbance between NIL pairs of any variety.

For each variety, differences between NILs in the assimilation data were analysed at each level of PAR by pairwise comparison, and an overall ANOVA that included both years of measurement.

4.3.6.1 Light curve fitting model

Data were fit to the model described in LI-COR Application Note #10 in order to generate a light response curve:

$$A = \frac{\phi Q_a}{\left[1 + \left(\frac{\phi Q_a}{A_{max}}\right)^p\right]^{1/p}} + A_o$$

Where A is assimilation, Q_a gives the absorbed quanta per unit leaf area in the chamber, A_{max} is assimilation under saturating light, A_o is the dark assimilation rate and p is a curvature parameter. Φ is photochemical efficiency at low light levels calculated from the slope of the curve. Using the light response curve generated from the model apparent quantum efficiency (AQE) was calculated. The raw assimilation data were used to calculate light saturated assimilation (A_{max}), dark respiration (A_o) and light compensation point (the intercept on the curve). For each curve parameter data were

analysed by overall ANOVA inclusive of all varieties and years, and by pairwise comparison between NILs.

4.3.6.2 Selection of Hereward recombinant lines for light curve analysis

Recombinant lines with contrasting introgressions within the region were chosen to further investigate the effect of *Iw1* on photosynthesis in Hereward. Table 4.1 shows the genotypes of selected lines in addition to the NILs (HS+ and HS-). The recombinants HS26 (*Iw1+*) and HS17 (*iw1-*) were chosen, in addition to HS21 (*Iw1+*) and HS32 (*iw1-*). Both recombinant pairs have contrasting recombinant fractions to allow trait mapping within the region. The genotypes for all 26 recombinants are shown in Appendix A2.

Marker	Distance chromosome 2BS (cM)	**HS26**	**HS17**	**HS32**	**HS21**	**HS+**	**HS-**
BS00084668	0	B	A	B	A	B	A
BS00009972	1.15	B	A	B	A	B	A
Iw1	**1.77**	**B**	**A**	**B**	**A**	**B**	**A**
BS00070900	10.41	A	B	B	A	B	A
BS00010318	10.41	A	B	B	A	B	A
BS00045163	12.68	A	B	B	A	B	A
BS00010637	17.98	A	B	A	B	B	A
BS00065040	17.98	A	B	A	B	B	A
BS00063694	17.98	A	B	A	B	B	A
Bra1190	28.27	A	B	A	B	B	A
BS00009848	19.18	A	B	A	B	B	A
BS00064156	44.64	A	B	A	B	B	A
BS00022734	45.19	-	B	A	B	B	A
BS00022060	45.19	A	B	A	B	B	A
BS00064155	45.19	A	B	A	B	B	A

Table 4. 1 The genotypes of four Hereward recombinant lines (HS26, HS17, HS32 and HS21) and the NILs

Fourteen markers within the introgressed region were run on recombinant lines. Grey cells containing an A indicate that recombinant has the Hereward allele, whilst a B in a green cell indicates presence of the Shamrock allele. HS17 and HS21, both have the *iw1-* Hereward allele (A) resulting in a glaucous phenotype, whilst HS26 and HS32 are both non-glaucous and have the *Iw1+* Shamrock allele (B). The HS+ and HS- NILs are also shown.

4.3.8 Canopy temperature

Canopy temperature was measured during the 2013 harvest year in the field on NILs of Hereward, Einstein, Robigus and Alchemy, Xi 19 and Malacca. Measurements were taken within 14 days of anthesis on a warm, dry day with clear sky (no cloud cover)

An infrared thermometer was moved slowly along the top of the plot at a constant distance from the top of the canopy for three sec, and average temperature for this time period ^{o}C recorded. This was repeated three times per plot, and an average taken to get a single value per plot. Temperature was recorded for four plots (independent replications) of each line. To minimise the effect of changing air temperature, a single variety was measured at a time, alternating between plots of *Iw1+* and *iw1-* NILs.

Data were analysed by overall ANOVA and pairwise comparison between NILs.

4.4 Results

4.4.1 Canopy spectral properties

4.4.1.1 Canopy PAR reflectance

A major function of the epicuticular waxes is to alter light scattering from the plant surface affecting the spectral properties of the plant. This effect has previously been shown in numerous crop species including wheat, where glaucousness increases reflectance of the crop canopy in the field (Jefferson *et al.,* 1989; Johnson *et al.,* 1983).

To confirm this effect, whole canopy light reflectance was measured across the PAR spectrum (400-700 nm) in the 2013 and 2014 field season. Both incident and reflected PAR was measured and the percentage of incoming PAR reflected calculated. Malacca and Alchemy were measured in both 2013 and 2014, and there was no difference between years. Therefore data from both years have been combined in Figure 4.6.

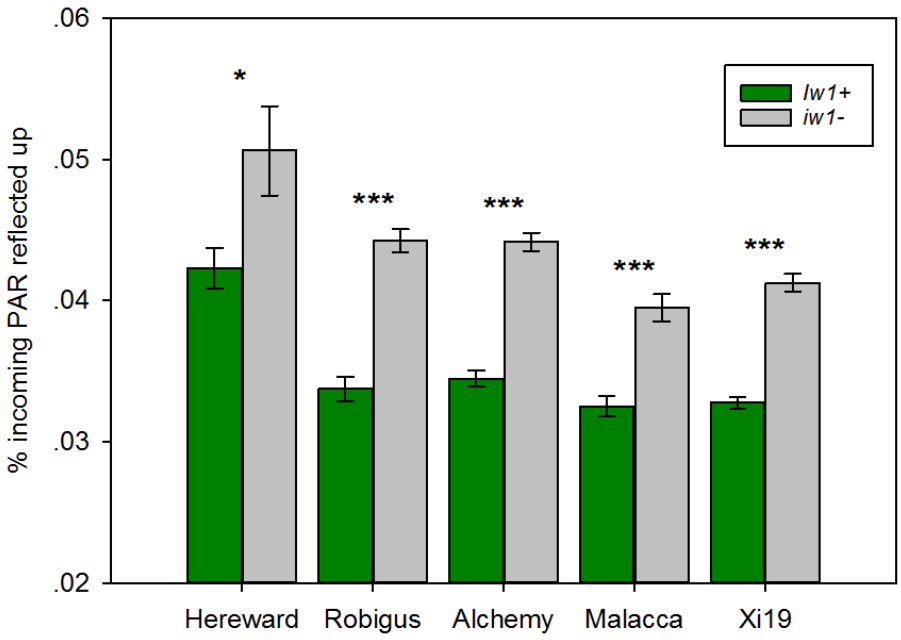

Figure 4. 6 Canopy reflectance of PAR (400-700 nm) in the field at anthesis

Measurements were taken in 2013 and 2014 for Malacca and Alchemy and combined to get an average over both years (n=8). Hereward was measured in 2014 only and Xi19 and Robigus in 2013 only (n=4). Across all three varieties *lw1* significantly reduces reflectance ($p<0.001$). Significance of pairwise comparison within varieties is indicated on the chart $p<0.05$ (*), $p<0.001$(***). Error bars = S.E.

Figure 4.6 shows that consistently across all five varieties, *Iw1+* canopies reflected significantly less PAR than *iw1-* canopies (ANOVA; p<0.001). There were also significant differences in reflectance between varieties (p<0.001) that could have resulted from differences in canopy architecture and morphology. However there was no statistically significant interaction between variety and *Iw1*, or year and *Iw1*, indicating that the reduced reflectance was independent of genetic background and year. When data were analysed within each variety by pairwise comparison, differences between NILs remained significant for all five varieties; Hereward (Independent samples t-test; p=0.043), Alchemy, Malacca, Hereward, Robigus, Xi19 (p<0.001). Notably the proportion of PAR reflected up from the canopy was very low, in the region of 0.03-0.05%.

4.4.1.2 PAR availability within the canopy

To further investigate canopy spectral properties and assess PAR penetrating to deeper levels of the canopy, sensors were placed both mid-way through the canopy and on the canopy floor during the summer of 2013 (Figure 4.7). For direct comparison, measurements at these lower levels were taken simultaneously with PAR reflected from the top of the canopy and incoming PAR.

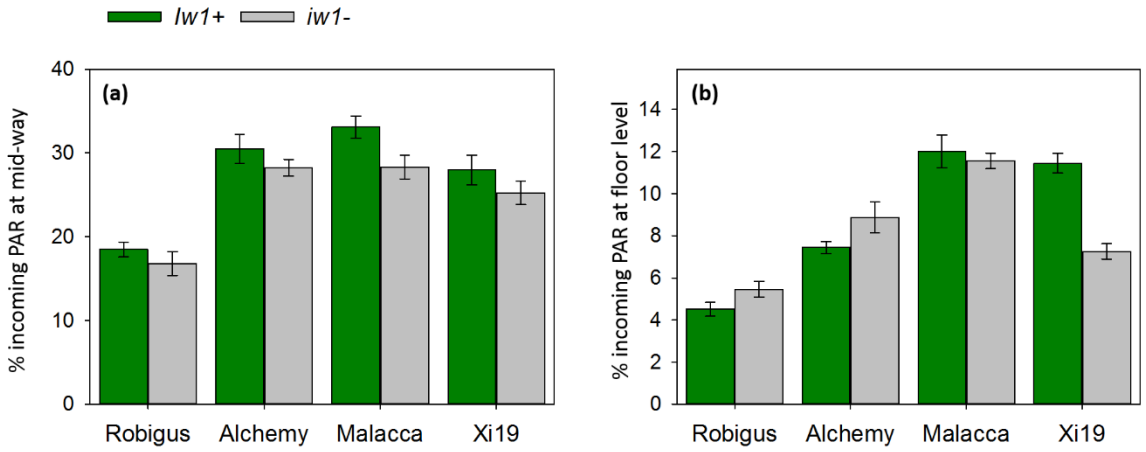

Figure 4. 7 Canopy fractional interception of PAR at anthesis in 2013

PAR was detected (a) within the canopy at the mid-way point and (b) on the floor of the canopy, both expressed as a percentage of incoming light. Measurements were taken at anthesis in 2013. Pairwise comparison showed no significant difference between NILs of any variety. N=3, Error bars = S.E.

Figure 4.7a shows that there was no significant difference between *Iw1+* and *iw1-* canopies in terms of light available at the mid-way point (p=0.136). As with reflectance from the top of the canopy (Figure 4.6), analysis with overall ANOVA indicated that there were significant differences between varieties (p<0.001), but no significant interaction between variety and *Iw1* (p=0.940). Pairwise comparison revealed no significant differences between NILs in terms of light reaching the mid-way point. Figure 4.7b shows the percentage of incoming PAR that reached the floor. There was no significant effect of *Iw1* overall (p=384), but again there was a significant difference between

varieties (p<0.001). The interaction between variety and *Iw1* was significant (p=0.02), likely due to the reversed effects of *Iw1* in Malacca and Xi19 in comparison to Alchemy and Robigus. For all varieties around 25-30% of incoming PAR was detected at the midway point, and around 10- 12% of incoming PAR was detected at floor level. This indicates that although a very small percentage of light was reflected from the top of the canopy, a large proportion of this light was absorbed by the floor.

The same measurements were taken the following year in 2014 using a modified method described in methods 4.3.2 on Malacca and Alchemy NILs, where-by PAR sensors were left at a chosen location for an extended period of time (Figure 4.8). Using the new method, there was overall no significant effect of *Iw1* on light detected at the floor level (Figure 4.8b, p=0.163) or mid-way down (Figure 4.8a, p=0.123) the canopy. Additionally, there was no consistency between the two experiments (Figures 4.7 and 4.8) in terms of overall trends. These data from 2013 and 2014 indicate that there is no effect of *Iw1* on canopy fractional interception of PAR.

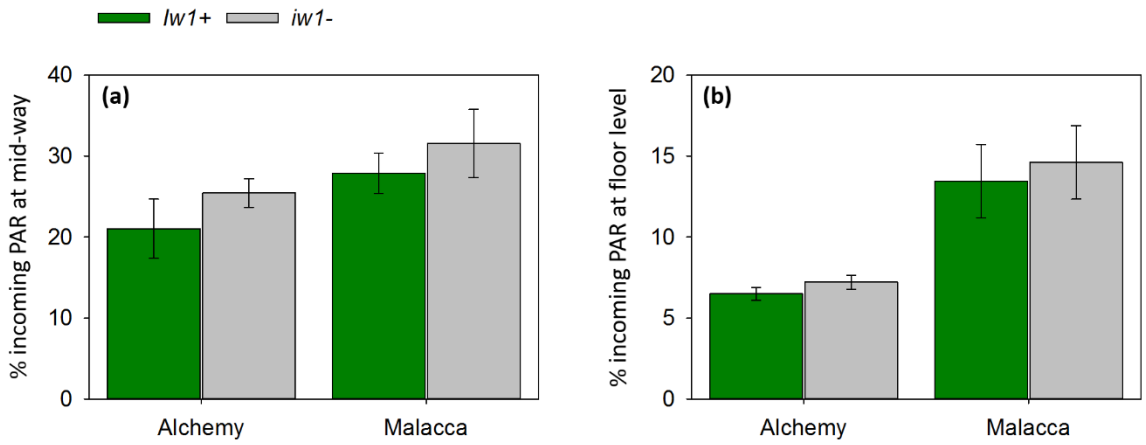

Figure 4. 8 Canopy fractional interception of PAR at anthesis in 2014

Par was detected (a) within the canopy at the mid-way point and (b) on the floor of the canopy, both expressed as a proportion of incoming light. There was no significant difference between NILs of either variety at the floor or mid-way level. N=3, Error bars = S.E.

4.4.2 Leaf spectral properties

4.4.2.1 Spectral measurements on field grown leaves

To support the canopy reflectance measurements, spectral properties were studied at the leaf level using an integrating sphere. PAR reflected from the surface of the leaf and PAR transmitted through the leaf were measured at each individual wavelength between 400 and 700 nm using field grown flag leaves at anthesis. PAR absorbed at each wavelength was then calculated.

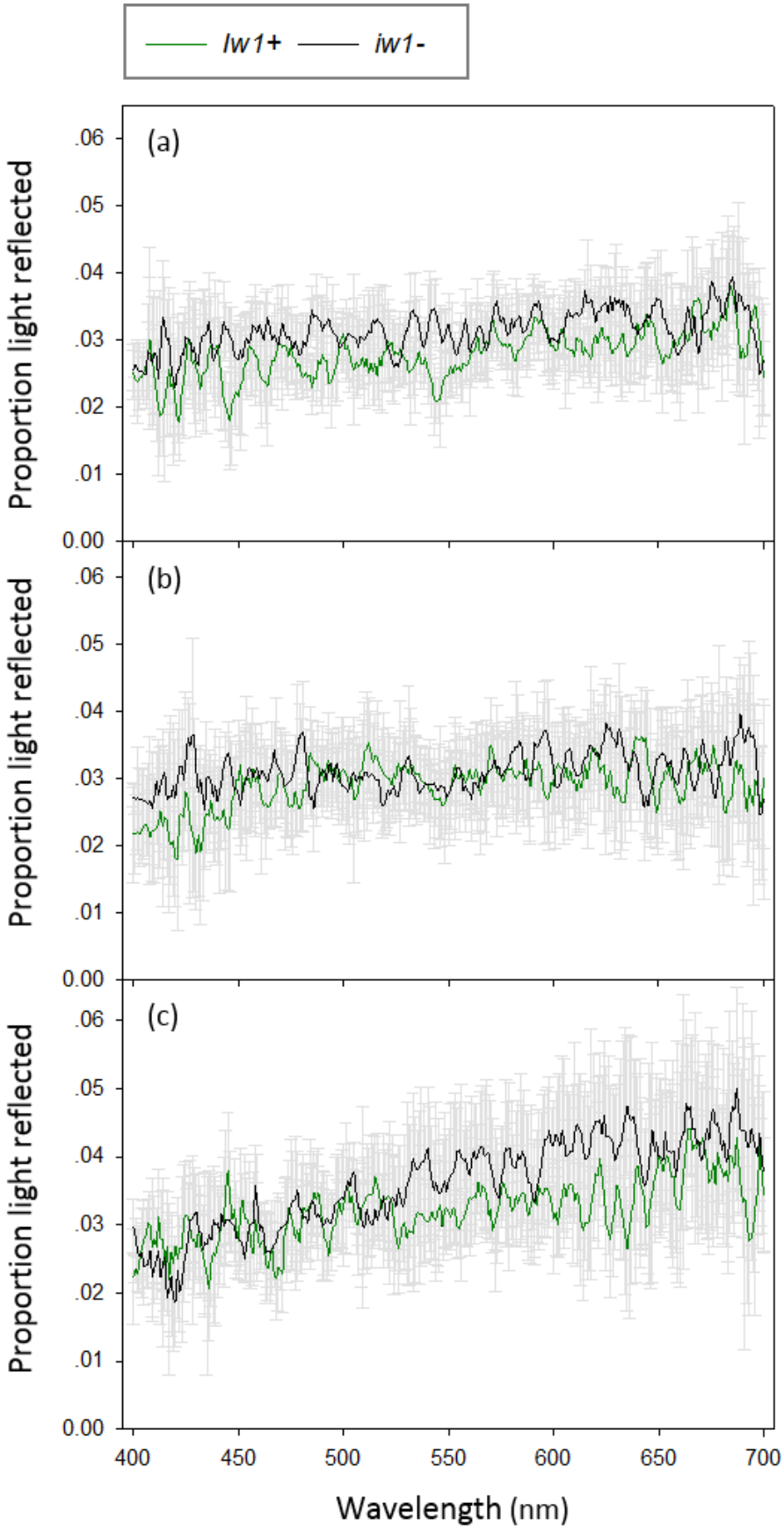

Figure 4. 9 PAR reflected from the flag leaf surface of *Iw1* NILs

Reflectance of (a) Hereward, (b) Alchemy and (c) Malacca NILs measured using an integrating sphere. A moving average was taken across the spectrum to smooth the data. At no individual wavelength is there a significant difference between NILs of any variety. N= 5, error bars = S.E.

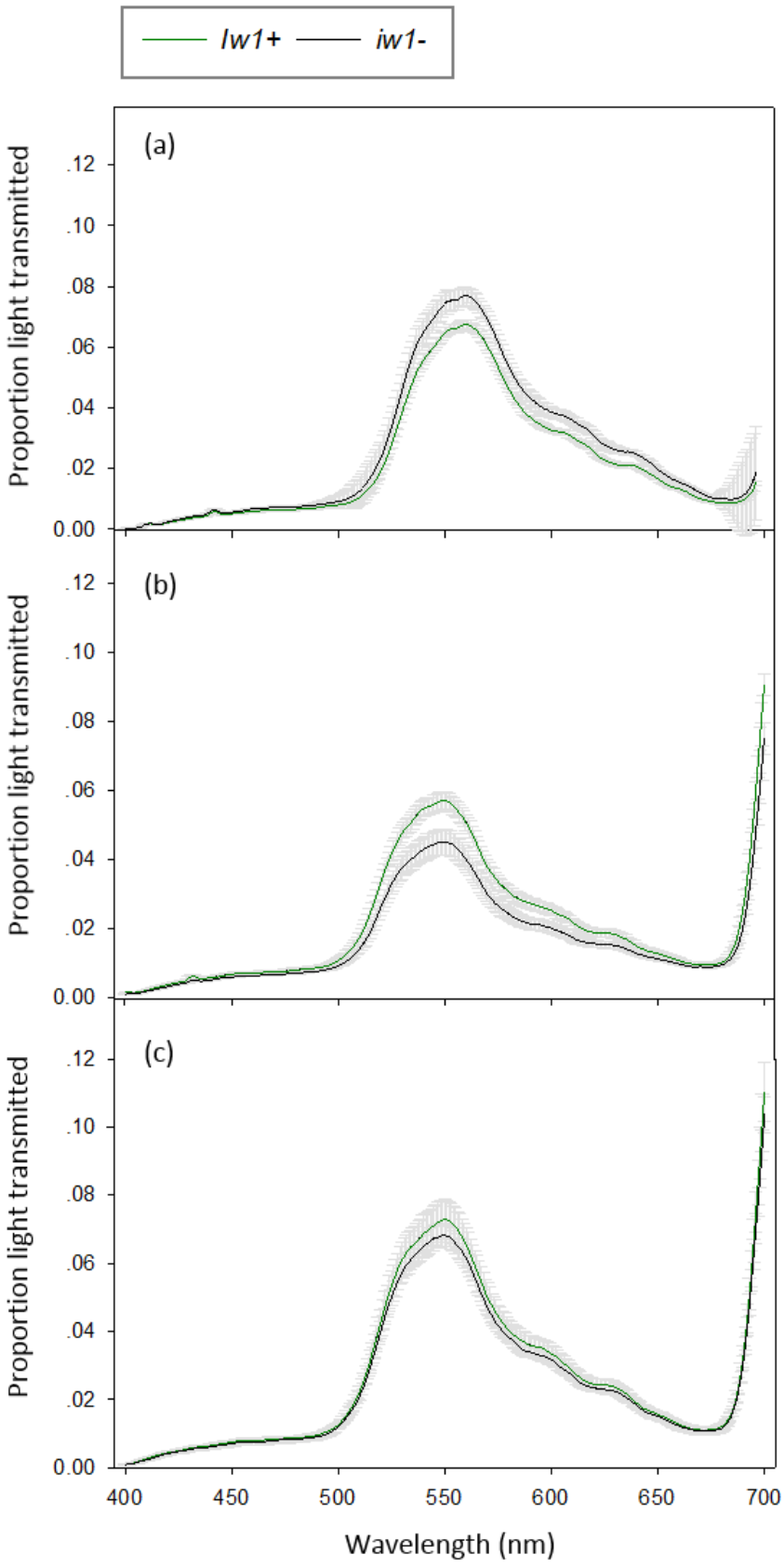

Figure 4. 10 PAR transmitted through the flag leaf

Measured using an integrating sphere bewteen 400 and 700 nmfor NILs of (a) Hereward, (b) Alchemy and (c) Malacca. A moving average was taken across the spectrum to smooth the data. At no individual wavelength is there a significant difference between NILs of any variety. N= 5, error bars = S.E.

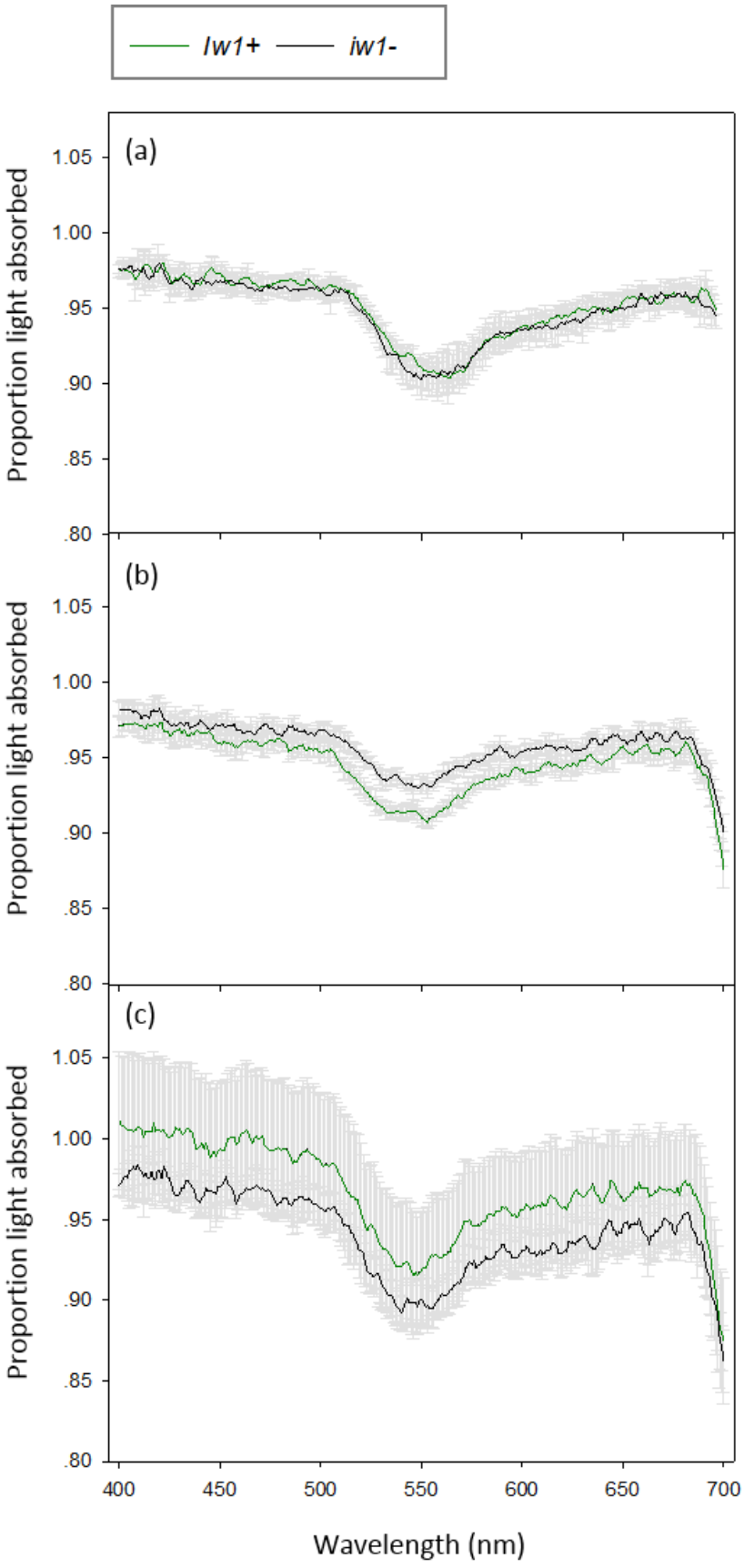

Figure 4. 11 PAR absorbed by the flag leaf calculated from transmission and reflectance measurements

Absorbance was calculated for NILs of (a) Hereward, (b) Alchemy and (c) Malacca. A moving average was taken across the spectrum to smooth the data. At no individual wavelength is there a significant difference between NILs of any variety. N= 5, error bars = S.E.

Figure 4.9 shows reflectance at each individual wavelength across the PAR spectrum for (a) Hereward, (b) Alchemy and (c) Malacca. It is clear that for all three varieties *Iw1+* flag leaves consistently reflected less light than *iw1-* across the PAR spectrum. This difference was less marked in Alchemy than in Hereward and Malacca, with more overlap in reflectance spectra between the NILs. For all varieties there was a lot of variation associated with the measurement, with overlapping error bars. The result of this was that at no individual wavelength was there a significant difference in reflectance.

When PAR was divided into red (640-700 nm), blue (425-490 nm) and green (490-550 nm) light there was a significant difference in reflectance between the three light colours ($p=0.021$). However, there was no statistical interaction between light colour and *Iw1* ($p=0.959$), indicating that the relationship between *Iw1+* and *iw1-* leaves did not change across the PAR spectrum. This constant relationship between the NILs can also be seen in Figure 4.9.

Figure 4.10 shows transmission across the PAR spectrum for all the three varieties. As with reflectance, there was a difference in amount of light transmitted at different points in the PAR spectrum. All flag leaves transmitted significantly more light in the middle of the PAR spectrum ($p<0.001$), which is green light. Furthermore, in terms of transmission, there was limited difference between NILs on the ends of the spectrum (400-500 nm and 600-700 nm), whilst the NILs appeared to diverge in the middle of the spectrum. Again, there was large variation associated with this measurement, so at no individual wavelength was the difference between NILs significant for any variety.

Regarding the difference in green light transmission between NILs, Figure 4.10 indicates that the direction of change was not consistent across all three varieties. The *iw1-* NILs of Malacca and Alchemy transmitted 6% and 25.82% less green light respectively than *Iw1+* NILs, whilst for Hereward *iw1-* NILs actually transmitted 15.15% more green light. There was no statistically significant interaction between light type and *Iw1* ($p=0.729$).

Figure 4.11 shows PAR absorbance across the spectrum. Overall, there was a significant reduction in absorbance of green light ($p=0.032$), but no interaction between *Iw1* and light colour ($p=0.982$). At no individual time point was there a significant difference between NILs. Furthermore, as with transmission, there was no consistent trend across the three varieties in terms of effect of *Iw1* on absorbance.

To more directly compare the leaf level data with the canopy, PAR reflected, transmitted and absorbed by the flag leaf was averaged over the full 400-700 nm spectrum (Figure 4.12).

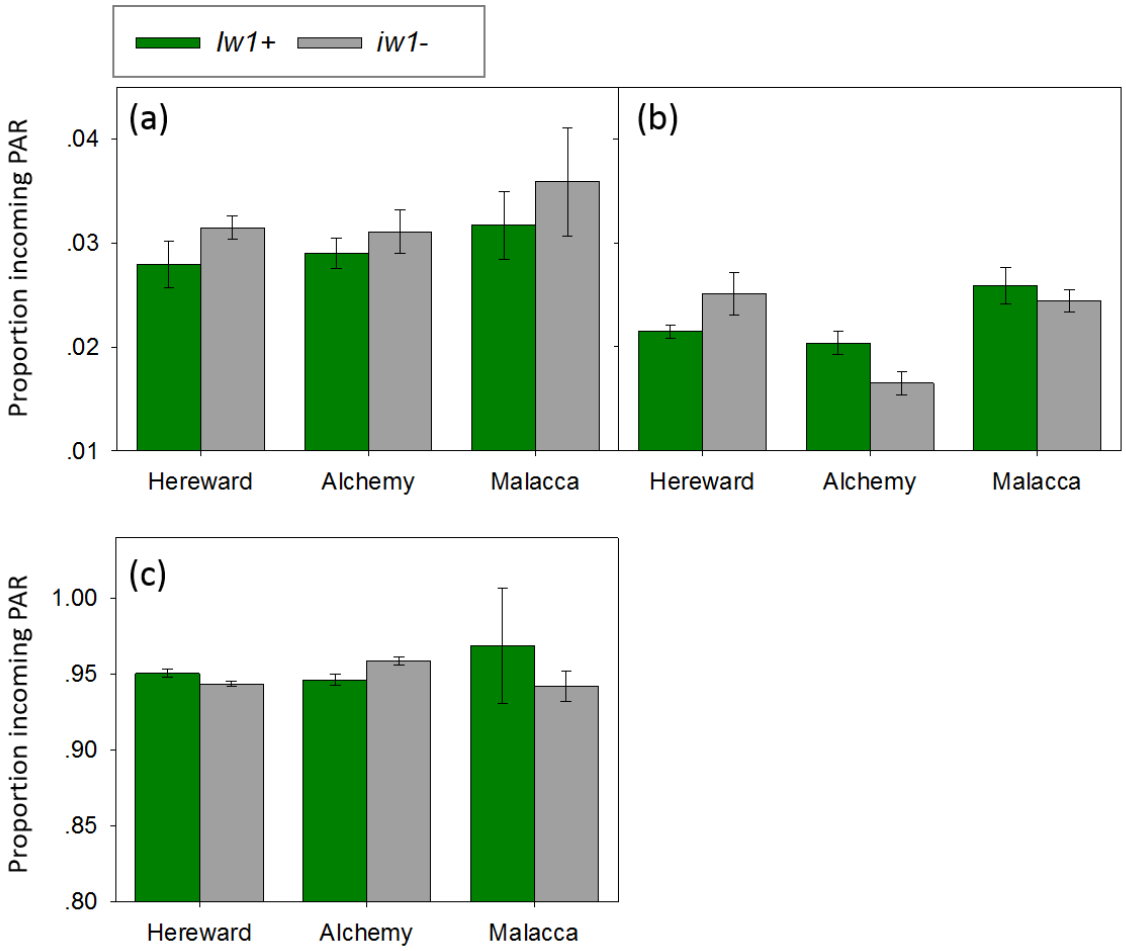

Figure 4. 12 Spectral properties of the flag leaf averaged over the PAR spectrum (400 - 700 nm)

The charts show (a) average reflectance of light from the leaf surface over 400-700 nm (b) average transmission of light through the flag leaf was measured at every wavelength across the PAR spectrum (400-700 nm) (c) average PAR absorbed across the PAR spectrum. There was no significant difference in reflectance, transmission or absorbance between NILs of any variety by pairwise comparison. N=5, error bars = S.E.

From the averaged data it is clear that, as with the canopy, *Iw1+* flag leaves of all varieties consistently reflected less PAR than *iw1-* leaves (Fig. 4.12a). However, overall these leaf level differences were not significant (p=0.150). Figure 4.12b shows that there was no significant effect of *Iw1* on PAR transmission through the leaf (p=0.235). Furthermore, there was no consistent trend across the three varieties as seen in the reflectance data. The primary difference between the epicuticular waxes of the NILs is the presence or absence of β-diketones and OH- β – diketones. Therefore from these data it can be concluded that transmission of light through the leaf is not affected by presence of these compounds in the epicuticular waxes. Although a consistent trend in reflectance was seen across varieties, this, combined with the variable transmission data, resulted

in no significant effect of *Iw1* on PAR absorbance overall (p=0.641). Additionally there was no interaction between *Iw1* and variety (p=0.553). Presence of β-diketones and OH- β – diketones in the epicuticular waxes did not have an effect on PAR absorbance at the leaf level.

4.4.2.2 The effect on flag leaf spectral properties after mechanical epicuticular wax removal

In order to gain further understanding of the role epicuticular waxes play on leaf spectral properties, gum arabic was used to mechanically remove epicuticular waxes from glasshouse grown flag leaves at anthesis. Subsequently PAR transmission and reflectance were measured using an integrating sphere.

A scanning electron microscope (SEM) was used to confirm that the epicuticular waxes had been removed following treatment with gum arabic, and that epicuticular waxes were present as normal in control leaves. Example images are shown in Figure 4.13.

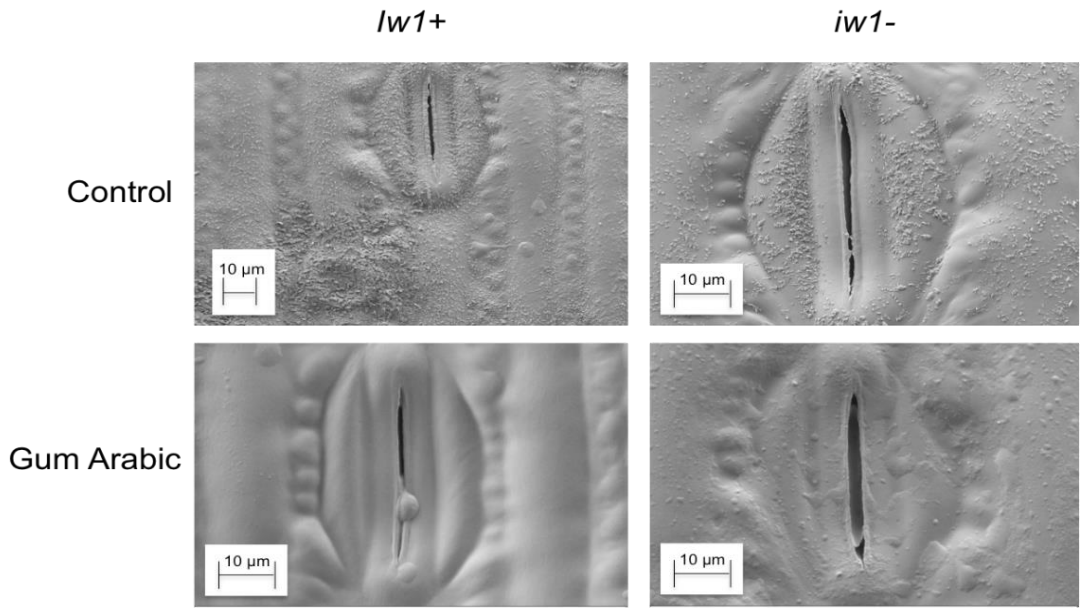

Figure 4. 13 SEM images of *Iw1+* and *iw1-* Alchemy NILs with and without epicuticular waxes

Images show presence of epicuticular waxes around stomata in control leaves of both NILs, and absence of epicuticular waxes after mechanical removal with gum arabic. Due to the destructive nature of SEM imaging the same leaves could not be used in the control and treatment groups.

In Figure 4.13 epicuticular wax structures surrounding the stomata can be seen in both the *Iw1+* and *iw1-* control leaves. These waxes are absent from the gum arabic treated leaves, revealing a smooth leaf surface. Also noted whilst doing this work, prior to wax removal *Iw1+* leaves were bright green in colour, whilst *iw1-* had a bluish-grey bloom. After treatment with gum arabic leaves

of both NILs were green in colour and could not be distinguished visually. This indicated that it was the epicuticular waxes responsible for difference in appearance of glaucous and non-glaucous plants rather than another factor such as pigmentation.

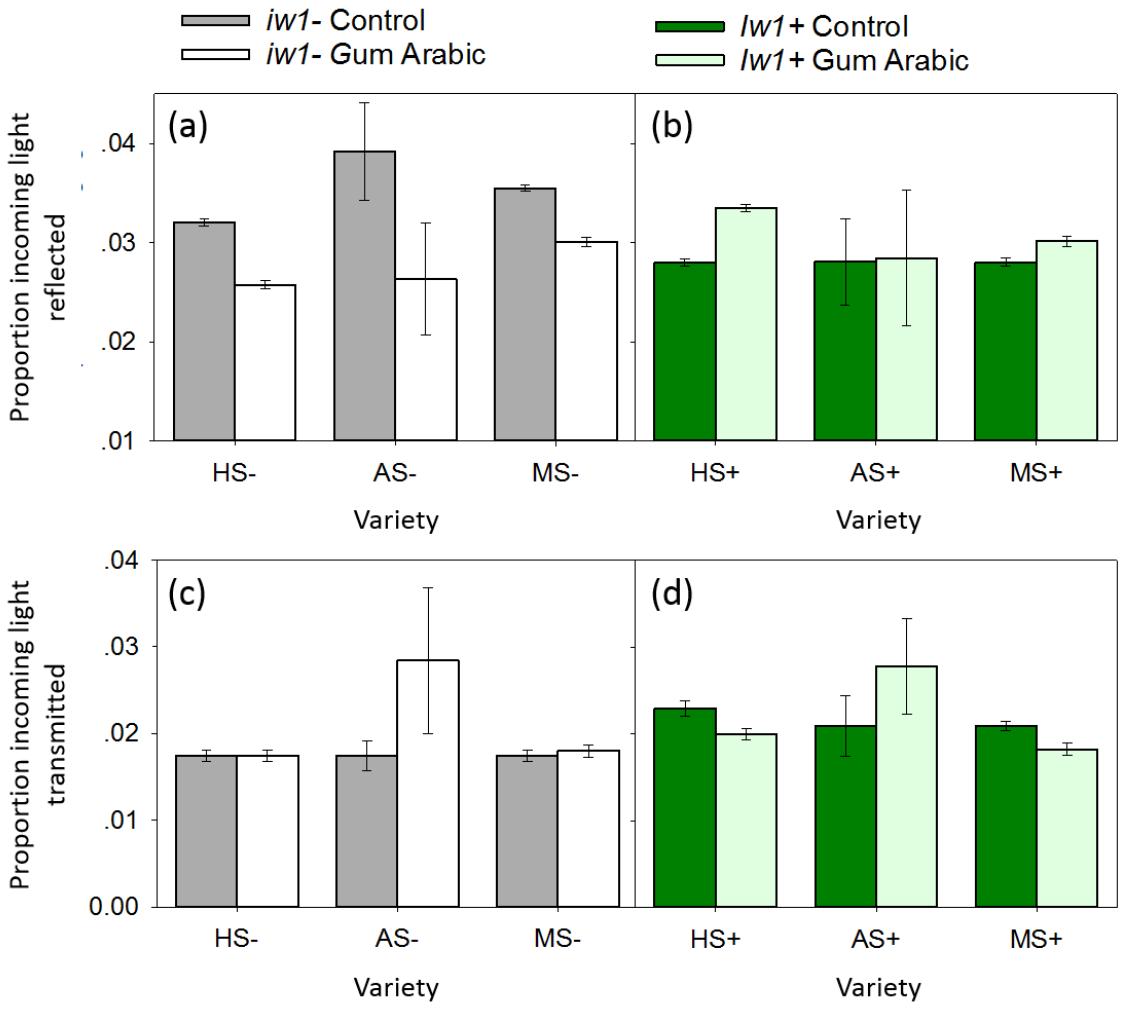

Figure 4. 14 The spectral properties of flag leaves with and without intact epicuticular waxes

Gum arabic was used to mechanically remove epicuticular waxes from Hereward, Alchemy and Malacca NILs. The charts show reflectance of control and gum arabic treated leaves averaged across 400-700 nm measured using an integrating sphere for (a) *iw1-* and (b) *Iw1+* NILs. Flag leaf light transmission across 400-700 nm measured using an integrating sphere for (c) *iw1-* and (d) *Iw1+* NILs. Pairwise comparison between control and treated leaves for each NIL revealed no significant differences. N=5, error bars = S.E.

Figure 4.14a shows that over all three varieties the PAR reflectance of glaucous (*iw1-*) flag leaves treated with gum arabic was significantly reduced compared to control leaves (p=0.001), although when analysed by pairwise comparison within each single variety this reduction was not significant. However, in the non-glaucous *Iw1+* leaves (Figure 4.14b) there was overall no significant change in reflectance after treatment with gum arabic (p=0.345). For Hereward it appears reflectance of *Iw1+*

leaves may even have increased after wax removal, but this increase was not significant, nor was there a significant interaction between variety and treatment (p=0.727). Overall, when *Iw1+* and *iw1-* NILs of all varieties were analysed together, there was a significant interaction between gum arabic treatment and *Iw1* (p=0.003), supporting the contrasting effect of wax removal on PAR reflectance between NILs.

Consistent with previous results from the experiment on field grown flag leaves (Figure 4.12), untreated *Iw1+* flag leaves reflected less light than *iw1-* leaves, although again this difference was not significant (Figure 4.14). In the gum arabic treated group there were no significant differences between NILs. These results provide evidence that β-diketones and OH-β-diketones are responsible for increasing reflectance of *iw1-* leaves, and that waxes lacking these compounds have no or limited effect on PAR reflectance.

Figures 4.14c and 4.14d show PAR transmission of control and treated leaves. For neither *iw1-* nor *Iw1+* leaves was there a consistent change in transmission across varieties or NILs after gum arabic treatment. This provides further support to the conclusion that leaf PAR transmission is not affected by β-diketones and OH-β-diketones in the epicuticular waxes. Furthermore, data indicate that the epicuticular waxes overall in these wheat varieties have no effect on PAR transmission. When the PAR spectrum was divided into red, blue and green, the effect of wax removal remained the same across the spectrum, as there was no significant interaction between light type and treatment for reflectance (p=0.078) or transmission (p=0.828).

4.4.3 Effects on Photosynthesis

4.4.3.1 Photosynthetic pigments

If the amount of PAR available to photosynthetic tissues changes, a plant would be expected to acclimate or adapt to this new light level in order to maximise efficiency of carbon assimilation. Photosynthetic pigments, known to change in amount dependent on light conditions (Valladares & Niinemets, 2008), were extracted from flag leaves collected in the field at anthesis and spectroscopy was used to determine the quantity of chlorophyll a, chlorophyll b and carotenoids. Data were normalised on leaf area.

Figure 4.15a shows flag leaf chlorophyll a content over two years. When pairwise comparisons between NILs were made within individual years there were very few significant effects. However, there was a consistent trend across varieties and years for *Iw1+* flag leaves to have less chlorophyll a than *iw1-* leaves. When analysed over all varieties this effect was significant in 2014 (p=0.040), 2015 (p=0.06), and when both years were combined (p=0.01). Furthermore, there was no

significant difference between years (p=0.734) or interaction between *Iw1* and year (p=0.906). Overall there was a significant difference in chlorophyll a content between varieties (p=0.022), but no significant interaction between *Iw1* and variety (p=0.590).

For chlorophyll b (Figure 4.15b) there was no significant difference between NILs or varieties in either year, although in 2015 there was a significant interaction between *Iw1* and variety (p=0.017), coming from the Alchemy NILs. There was a significant difference between these NILs (p=0.002), but no difference between NILs of the other two varieties. There was no significant effect of *Iw1* on carotenoids (Figure 4.15c) in either year.

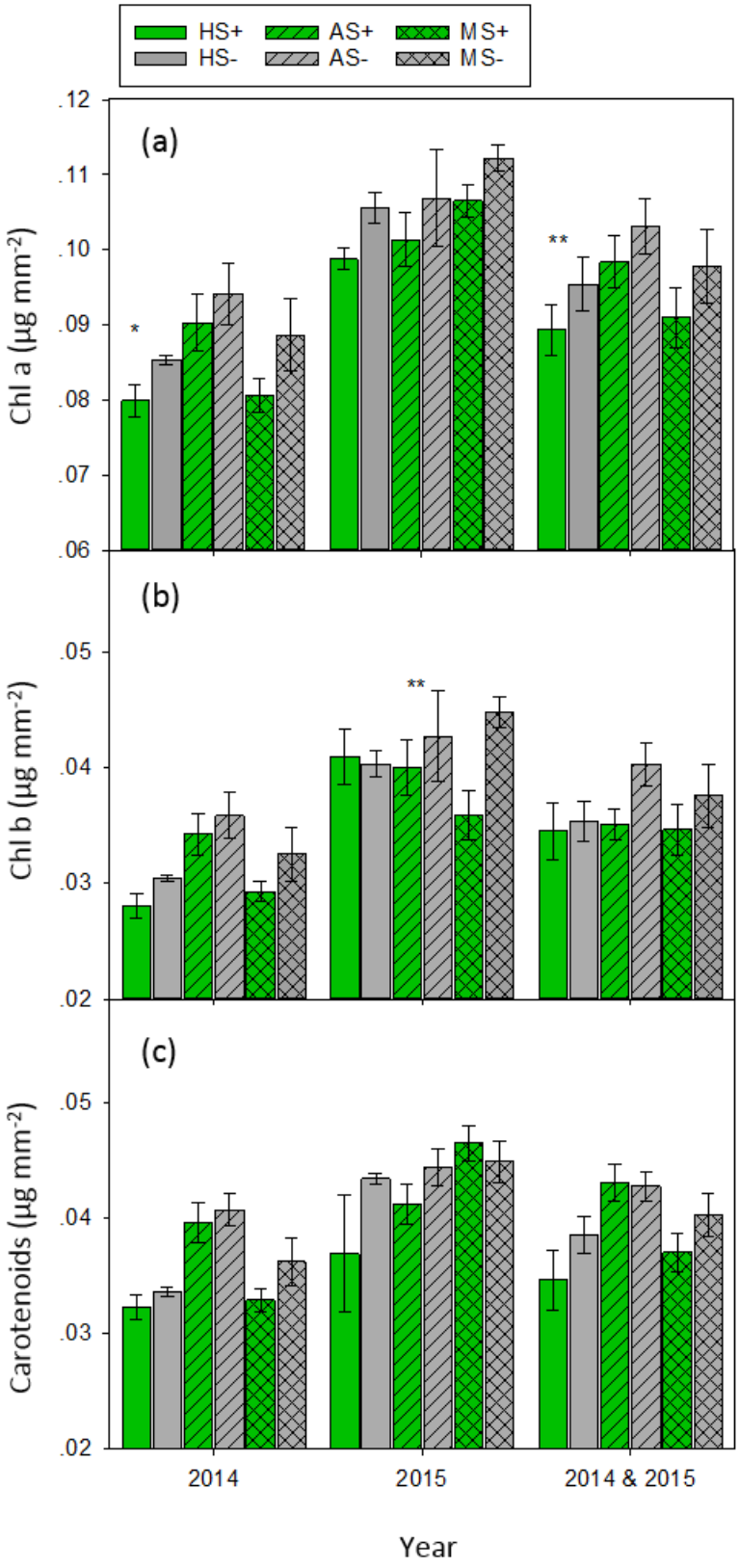

Figure 4. 15 Concentration of photosynthetic pigments per unit leaf area at anthesis

Quantity of (a) chlorophyll a (Chl a), (b) chlorophyll b (Chl b) and (c) carotenoids in µg mm^{-2} leaf area in flag leaves of Hereward, Alchemy and Malacca NILs collected in 2014 and 2015. Significant differences between NILs by pairwise comparison is indicated on the chart $p<0.05$ (*), $p<0.01$ (**). N=5, error bars = S.E.

To investigate further the effect of *Iw1* on photosynthetic pigments, samples were collected at GS31 in 2014. This growth stage is a week prior to synthesis of β-diketones in the waxes, so both *Iw1+* and *iw1-* NILs have non-glaucous waxes with the same chemistry.

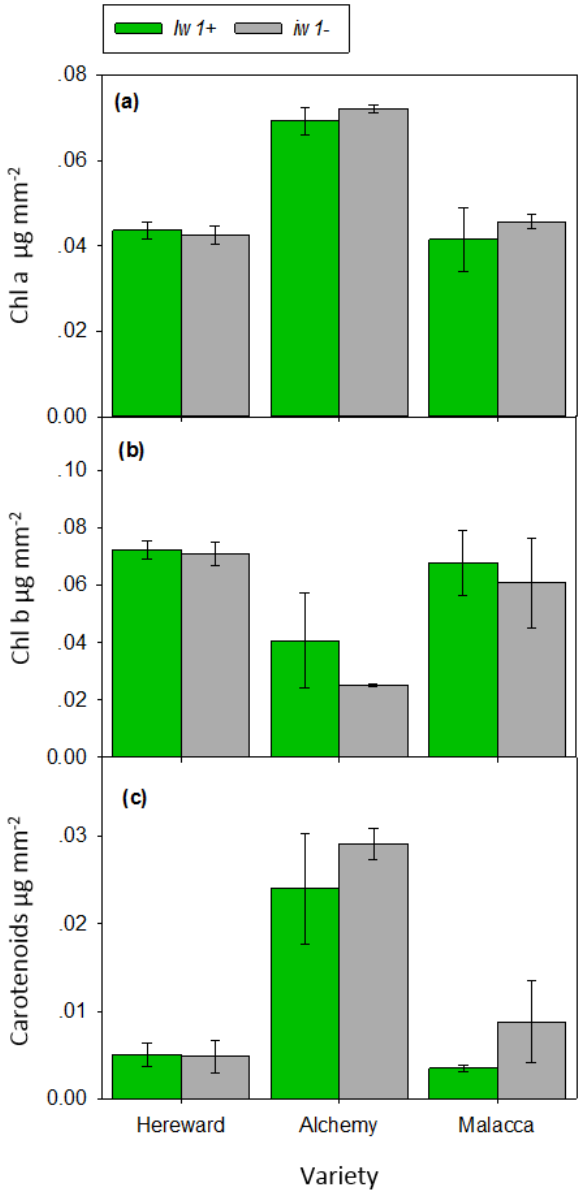

Figure 4. 16 Quantity of photosynthetic pigments per unit leaf area at GS31

Concentration of (a) chlorophyll a (Chl a) (b) chlorophyll b (Chl b) and (c) carotenoids per mm^2 leaf area. The newest expanded leaf at GS31 (prior to visible wax appearance) was collected for Hereward, Alchemy and Malacca. There was no significant difference between NILs of any variety for any pigment. N=5, error bars = S.E.

Figure 4.16 shows that there was no significant or consistent difference in chlorophyll a content between NILs of any variety at GS31. At GS31 β-diketones and OH-β-diketones are absent from both *Iw1+* and *iw1-* NILs, such that all NILs have the same wax profile. Differences in chlorophyll a content were only evident at anthesis, where waxes of *Iw1+* and *iw1-* are biochemically different.

This indicates that it could be differing properties of the waxes at anthesis that cause divergence in chlorophyll a content between NILs.

The chlorophyll a/b ratio is usually approximately 3:1, and reported to increase when a plant is adapted to lower light availability (Dong *et al.,* 2015; Li *et al.,* 2010; Valladares & Niinemets, 2008; Zheng *et al.,* 2011). Table 4.2 shows the chlorophyll a/b ratio for NILs of all three varieties across years at anthesis.

Table 4. 2 The chlorophyll a/b ratio in flag leaves at anthesis

Chlorophyll a and b were extracted from NILs of Hereward, Alchemy and Malacca in 2014 and 2015 at anthesis. Significant differences between NILs in the chl a/b ratio are indicated in the table at the level p<0.05 (*)

		Chlorophyll a/b ratio µg mm^{-2}					
		2014		2015		2014 & 2015	
Variety	*Iw1*	Average ± S.E	p	Average ± S.E	p	Average ± S.E	p
Hereward	+	2.44 ± 0.15	0.185	2.85 ± 0.03	0.277	2.65 ± 0.10	0.433
	-	2.62 ± 0.04		2.80 ± 0.02		2.82 ± 0.10	
Alchemy	+	2.55 ± 0.08	0.896	2.64 ± 0.03	0.025*	2.64 ± 0.06	0.017*
	-	2.53 ± 0.10		2.63 ± 0.04		2.57 ± 0.04	
Malacca	+	3.01 ± 0.17	0.814	2.75 ± 0.01	0.903	2.65 ± 0.05	0.839
	-	2.51 ± 0.07		2.74 ± 0.05		2.71 ± 0.04	

The minor changes recorded in chlorophyll a and b content associated with *Iw1* (Figure 4.15) did not alter the chlorophyll a/b ratio, and when data were analysed overall there was no significant effect of *Iw1* on the chlorophyll a/b ratio (p=0.154). Further analysis with pairwise comparison showed that across two years there was no significant difference in the chlorophyll a/b ratio between NILs for Malacca and Hereward. For Alchemy there was a significant increase in the chlorophyll a/b ratio associated with *Iw1* (p=0.017) when data from both years were combined. This increase was coming from the 2015 data, for which the difference between NILs was also significant (p=0.025). However, there was no difference in the 2014 Alchemy data. This, combined with results from the other two varieties, indicates that there is no effect of *Iw1* on the chlorophyll a/b ratio.

4.4.3.2 Flag leaf carbon assimilation

Further exploration of the changes to carbon assimilation that could result from differences in light availability between NILs was carried out by way of light curves measurement in the field for Malacca and Alchemy in 2013 and 2014, and Hereward in 2014 and 2015 (Figure 4.17).

For Alchemy (Figures 4.17c and 4.17d) there was no difference between the NILs in carbon assimilation at any light level from 0 – 1500 µmol m^{-2} s^{-1} in either 2013 or 2014. Although there was a significant effect of year on carbon assimilation, with maximum assimilation at 1500 µmol m^{-2} s^{-1} being significantly higher in 2014 (p=0.01), there was no interaction of year with *Iw1* (p=0.908). Malacca carbon assimilation in 2013 and 2014 is shown in Figures 4.17e and 4.17f respectively. For Malacca there was no significant difference in assimilation between years and also no significant effect of *Iw1* at any light level. There was a trend for *Iw1+* NILs to have higher assimilation across the entire light curve in 2013. However, at no time point was this difference significant, and this effect was absent in the 2014 data. Hereward is the only variety that displayed a consistent difference in assimilation between NILs over both years of measurement (Figure 4.17a and 4.17b). *Iw1+* NILs had significantly higher photosynthesis than *iw1-* at light levels of 750 µmol m^{-2} s^{-1} and above ($p<0.05$). However, this effect was absent from the other two varieties, so is likely not due to *Iw1*, but potentially a closely linked gene within the introgression.

As there was no significant interaction between year and *Iw1* for any variety, data for both years were combined to calculate the light curve parameters light saturated assimilation rate (A_{max}), dark respiration (Ao) and apparent quantum efficiency (AQE) and light compensation point (Table 4.3). For neither Alchemy nor Malacca was there an effect of *Iw1* on any light curve parameter indicating that *Iw1* and epicuticular waxes do not influence photosynthesis. However, in Hereward *Iw1+* NILs were able to assimilate carbon at a significantly higher rate at higher light levels, achieving a significantly higher A_{max} (p=0.013).

When A_{max} values of *iw1-* NILs are compared across the three varieties, that of Hereward is significantly lower (p=0.006), with an average A_{max} of 18.53 µmol CO_2 m^{-2} s^{-1} in comparison to around 22-24 µmol CO_2 m^{-2} s^{-1} for the other two varieties. However, the *Iw1* introgression increased the A_{max} of Hereward to 21.65 CO_2 m^{-2} s^{-1}, which is more comparable to that of the other two varieties. From this it can be hypothesised that the introgressed region in Hereward has replaced a deleterious allele that was previously reducing light saturated photosynthesis. Light curves were also measured on NILs of Robigus and Xi19 in 2013, and no significant difference in carbon assimilation was found at any light level from 0 – 1500 µmol m^{-2} s^{-1} or at any light curve parameter (see Appendix A4). This further supports conclusions drawn from Malacca and Alchemy that there is no effect of *Iw1* on photosynthesis.

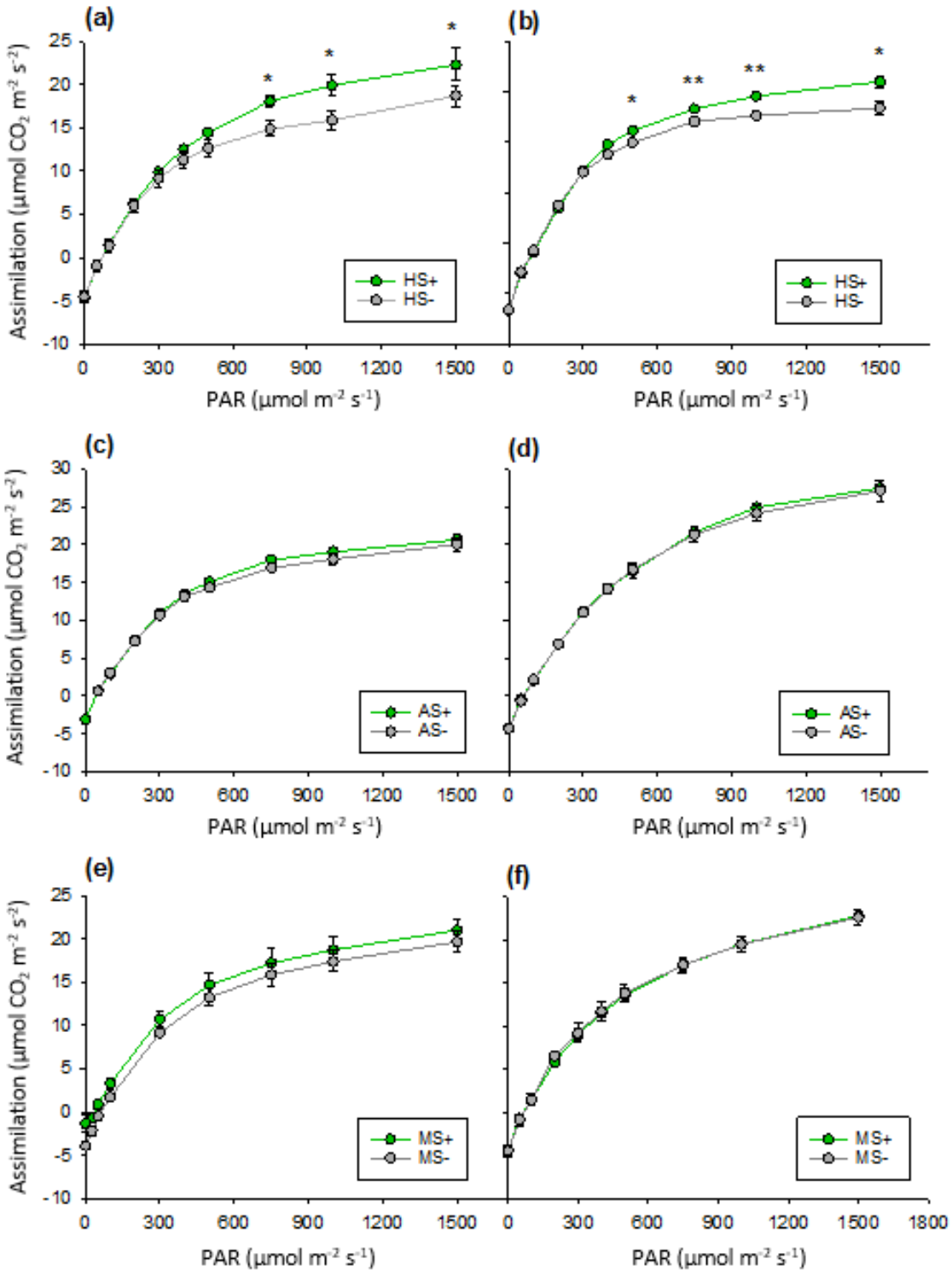

Figure 4. 17 Rate of flag leaf photosynthesis across various levels of PAR

Carbon assimilation between 0-1500 µmol m^{-2} s^{-1} was measured in the field using a LI-COR 6400XT for NILs of Hereward in (a) 2014 and (b) 2015, Alchemy in (c) 2013 and (d) 2014, and Malacca in (e) 2013 and (f) 2014. Significant differences between NILs at each light level are indicated on the chart, $p<0.05$ (*), $p<0.01$ (**). N=4, error bars = S.E.

Table 4. 3 Light curve parameters of field grown flag leaves at anthesis

AQE (apparent quantum efficiency), A_{max} (light saturated assimilation), A_o (dark respiration) and light compensation point for NILs of Hereward, Alchemy and Malacca. Measurements were taken over two years and the average over both years is shown (n=8). There was no difference between light curve of NILs from any variety with the exception of Hereward, for which *Iw1+* flag leaves reach a significantly higher A_{max} (p = 0.013).

		Hereward		Alchemy		Malacca	
		Average ± S.E	p	Average ± S.E	p	Average ± S.E	p
AQE (mol CO_2 mol^{-1} PARi)	*Iw1+*	0.044 ± 0.001	0.206	0.046 ± 0.001	0.272	0.044 ± 0.002	0.744
	iw1-	0.041 ± 0.002		0.045 ± 0.001		0.044 ± 0.002	
Amax (µmol m^{-2} s^{-1})	*Iw1+*	21.65 ± 0.95	0.013*	24.07 ± 1.35	0.138	21.13 ± 0.89	0.464
	iw1-	18.53 ± 0.63		23.58 ± 1.53		21.88 ± 0.67	
Ao (µmol m^{-2} s^{-1})	*Iw1+*	-3.18 ± 0.58	0.760	-3.71 ± 0.23	0.973	-3.92 ± 0.48	0.055
	iw1-	-3.07 ± 0.61		-4.28 ± 0.34		-1.36 ± 0.97	
Light compensation (µmol PARi)	*Iw1+*	3.09 ± 0.62	0.843	2.56 ± 0.35	0.065	1.56 ± 0.37	0.163
	iw1-	3.20 ± 0.76		3.38 ± 0.63		0.78 ± 0.40	

4.4.3.3 Carbon assimilation at different levels in the canopy

Section 4.4.1.2 explored light penetration into the canopy through placing PAR sensors inside the canopy, but this work proved inconclusive. An indirect way to understand this is to measure photosynthesis at various levels within the canopy. If the amount of light penetrating the canopy was altered by *Iw1*, this would have an impact on photosynthesis further down. Therefore carbon assimilation at PAR levels between 0 and 1500 µmol m^{-2} s^{-1} was measured on the second leaf for NILs of Alchemy, Malacca, Xi19 and Robigus in 2013 and compared to the flag leaf. The light curve parameters AQE, light compensation point, A_{max} and Ao were calculated, all of which would be expected to change in a plant adapted to altered light conditions (Table 4.4).

Results from an overall ANOVA indicate that there was no difference between the flag leaf and second leaf for A_o (p=0.127), light compensation point (p=0.361) or AQE (p=0.077). Furthermore there was no interaction between leaf type and *Iw1* for any of these parameters. There was an overall significant difference between the flag and second leaf for A_{max} (p=0.003). This can be seen from Table 4.4 whereby for every NIL, with the exception of Xi19 *Iw1+*, the flag leaf has a higher A_{max} than the second leaf, although this difference was only significant for Robigus *iw1-* NILs (p=0.049) when analysed by pairwise comparison. However, there was no interaction between leaf type and *Iw1* in an overall analysis (p=0.560), nor was there an interaction within any individual

variety. These data suggest that there is no effect of *Iw1* on light conditions within the canopy, at least to the extent that photosynthetic machinery of the plant might be affected.

Table 4. 4 Light curve parameters for the flag leaf and second leaf

Robigus, Xi19, Alchemy and Malacca NILs (n=4). For AQE, light compensation and A_o there was no difference between the flag and second leaf for either *Iw1+* or *iw1-* NILs of any variety. Flag leaves of Malacca *Iw1+* NILs had significantly higher A_{max} than the second leaf (p=0.016), and there was a borderline significant difference between the A_{max} of the flag and second leaf for Alchemy *Iw1+* NILs and Robigus *iw1-* NILs.

	Iw1+			***iw1-***		
	Flag leaf	**Second leaf**	**p**	**Flag leaf**	**Second leaf**	**p**
			AQE (mol CO_2 mol^{-1} PARi) Average ± S.E			
Robigus	0.045 ± 0.001	0.041 ± 0.003	0.221	0.045 ± 0.001	0.043 ± 0.001	0.207
Xi19	0.045 ± 0.003	0.043 ± 0.003	0.746	0.044 ± 0.002	0.040 ± 0.004	0.342
Alchemy	0.049 ± 0.000	0.047 ± 0.001	0.062	0.046 ± 0.000	0.045 ± 0.001	0.444
Malacca	0.049 ± 0.001	0.050 ± 0.000	0.475	0.049 ± 0.001	0.050 ± 0.000	0.487
			Light compensation (µmol^{-1} PARi) Average ± S.E			
Robigus	4.44± 0.87	4.68 ± 0.79	0.847	3.86± 0.73	4.42 ± 0.67	0.592
Xi19	3.64 ± 1.63	3.79 ± 0.44	0.930	4.61 ± 0.51	4.52± 0.14	0.864
Alchemy	3.24 ± 0.17	3.43 ± 0.43	0.691	3.19 ± 0.47	2.63 ± 0.69	0.524
Malacca	1.50 ± 0.63	2.22 ± 0.42	0.374	0.25 ± 0.25	1.52 ± 0.47	0.054
			Amax (µmol m^{-2} s^{-1}) Average ± S.E			
Robigus	21.00 ± 1.97	17.40 ± 1.82	0.229	20.12 ± 0.78	16.85 ± 1.07	0.049*
Xi19	21.24 ± 0.51	21.66 ± 1.07	0.715	21.51 ± 1.21	20.02 ± 1.31	0.434
Alchemy	20.65 ± 0.70	18.67 ± 1.20	0.203	20.05 ± 0.90	17.58 ± 0.77	0.079
Malacca	20.23 ± 0.88	19.12 ± 1.07	0.458	19.67 ± 1.23	17.78 ± 1.71	0.403
			Ao (µmol m^{-2} s^{-1}) Average ± S.E			
Robigus	0.33 ± 0.77	0.78± 0.62	0.667	-0.167 ± 0.75	0.40 ± 0.71	0.601
Xi19	0.50 ± 0.91	0.01 ± 1.00	0.196	0.19 ± 0.71	-0.06 ± 0.77	0.559
Alchemy	-3.14 ±0.55	-2.54 ± 0.24	0.048*	-3.01 ± 0.38	-3.20 ± 0.74	0.825
Malacca	-2.39 ± 1.03	-1.04 ± 0.75	0.503	-3.92 ± 0.48	-1.71 ± 1.03	0.574

4.4.3.4 Exploring Hereward carbon assimilation using recombinant lines

In addition to the NILs, a number of recombinant lines of Hereward were grown in the field in 2015. These were used to further explore the increased A_{max} observed in *Iw1+* NILs and map the effect more specifically within the region. The non-glaucous (*Iw1+*) recombinants HS26 and HS32 were chosen alongside the glaucous (*iw1-*) recombinants HS17 and HS21. A simple schematic of the introgressed region of 2BS for each of these four recombinants is shown in Figure 4.18. Rationale for recombinant selection is detailed in methods section 4.2.7. Portions of the chromosome from Shamrock (*Iw1+*) are green, and those from Hereward (*iw1-*) are grey.

Figure 4. 18 The introgressed region on chromosome 2BS for 4 Hereward recombinant lines

The genotype of recombinant lines HS17 (*iw1-*), HS21 (*iw1-*), HS26 (*Iw1+*) and HS32 (*Iw1+*). Portions of the chromosome from the Shamrock parent are coloured in green, and those from the Hereward parent are shown in grey. The location of *Iw1* itself is marked in red. Data for one marker in HS26 were not available, and this region is coloured in white on the schematic.

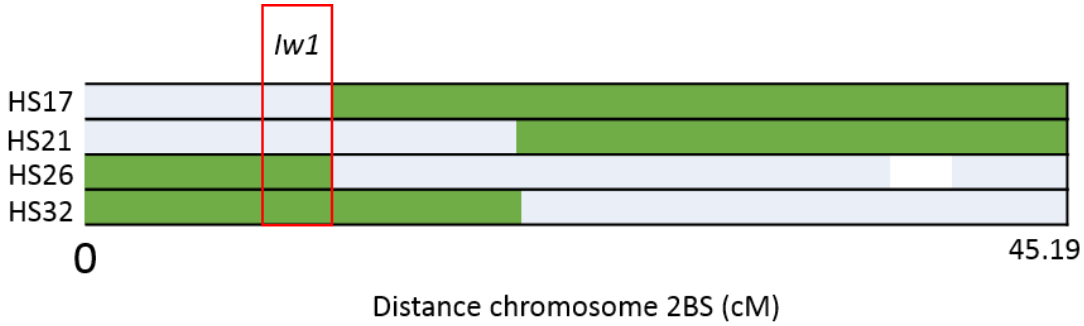

Figure 4.19 shows carbon assimilation at each level of PAR for the four recombinant lines measured together with data for the NILs from 2015. Lines containing the Shamrock *Iw1* allele (non-glaucous) are shown in green, and those without *Iw1* (glaucous) are shown in black. NILs are indicated by solid lines, and the recombinants are dashed. When considering just the NILs, there was a significant difference in assimilation between NILs with and without *Iw1* at 1500, 1000, 750 and 500 µmol m^{-2} s^{-1} ($p<0.05$), data for which was presented in detail in Figure 4.14. When the four recombinants together with the two NILs were included in analysis, the effect of *Iw1* was borderline significant at 1000 µmol m^{-2} s^{-1} ($p=0.044$) and 1500 µmol m^{-2} s^{-1} ($p=0.045$), as indicated in Figure 4.16. However, post hoc testing indicated that this effect was coming from the *Iw1+* NILs. The *Iw1+* NILs had a significantly higher assimilation than *iw1-* NILs and all 4 recombinants, and there was no significant difference between any recombinant line and the *iw1-* NIL.

Table 4.5 shows light curve parameters for the NILs and recombinants. When considering just the NILs (Table 4.3), there was no significant difference between NILs for any light curve parameter with the exception of A_{max}, for which *Iw1+* NILs had significantly higher A_{max} than *iw1-* ($p=0.024$). When all four recombinants and both NILs were included in analysis (Table 4.5), there was a significant effect of both *Iw1* ($p=0.045$) and line ($p=0.044$) on A_{max}. However, post hoc testing showed that this effect was coming from Hereward only, which was significantly different from the other 5 lines. For no other light curve parameter was there an effect of *Iw1* or line when all recombinants and both NILs were included in analysis.

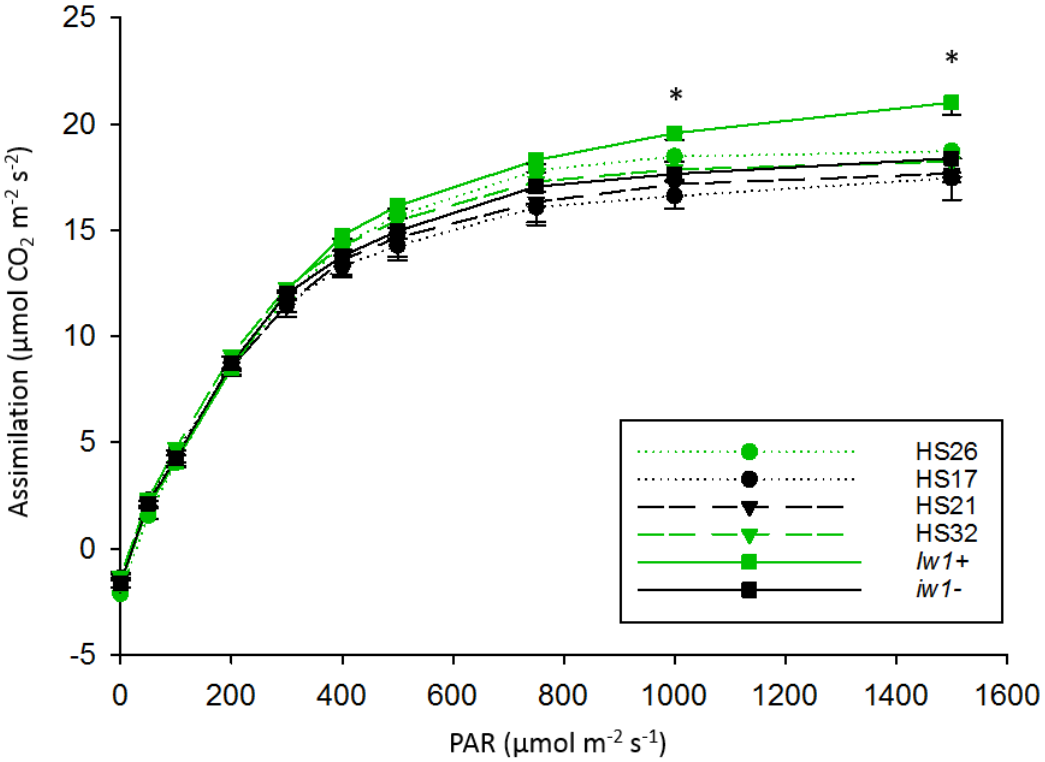

Figure 4. 19 Photosynthesis of 4 Hereward recombinant lines and the NILs at various levels of PAR

Carbon assimilation was measured between 0-1500 µmol m^{-2} s^{-1} in the field on flag leaves at anthesis using a LI-COR 6400 XT in 2015. The chart shows data for non-glaucous (*Iw1+*) recombinants HS26 and HS32, glaucous (*iw1-*) recombinants HS17 and HS21, and 2015 data for the near isogenic lines labelled *Iw1+* and *iw1-*. A significant effect of *Iw1* on assimilation is shown on the graph where p<0.05 (*). N=4, error bars = S.E.

Table 4. 5 Flag leaf light curve parameters for Hereward recombinant lines and NILs

Column 1 shows the name of each of the six lines and column two donates whether that line carries the Shamrock *Iw1* allele (*Iw1+*) or not (*iw1-*). Data was analysed by ANOVA to understand if presence of *Iw1* had an effect on any parameter or if there was a significant difference between the 6 lines. For Amax there was a significant effect of both *Iw1* (p=0.045) and line (p=0.044). For no other parameter was there a significant difference between the 6 lines, or between those with and without *Iw1.* N=4.

Line	*Iw1*	AQE (mol CO_2 mol^{-1} PARi)	Light compensation (µmol PARi)	Amax (µmol m^{-2} s^{-1})	Ao (µmol m^{-2} s^{-1})
Iw1+ NIL	*Iw1+*	0.046 ± 0.001	4.61± 0.19	21.01 ± 0.60*	-1.68 ± 0.17
iw1- NIL	*iw1-*	0.045 ± 0.002	4.82± 0.30	18.38 ± 0.67	-1.64 ± 0.24
HS26	*Iw1+*	0.049 ± 0.000	4.60 ± 0.17	18.71 ± 0.35	-2.11 ± 0.29
HS17	*iw1-*	0.048 ± 0.001	4.84± 0.16	17.46 ± 0.19	-1.39 ± 0.16
HS32	*Iw1+*	0.049 ± 0.000	5.27± 0.12	18.24 ± 0.73	-1.30 ± 0.17
HS21	*iw1-*	0.048 ± 0.001	4.76± 0.32	17.67 ± 1.24	-1.64 ± 0.16
p (*Iw1*)		0.294	0.914	0.045*	0.519
p (line)		0.064	0.367	0.044*	0.154

4.4.4 Canopy temperature

Tissue temperature plays a role in the regulation of a number of important metabolic processes, including photosynthesis which was investigated here. In order to understand if changes to canopy spectral properties resulting from glaucousness are affecting canopy temperature, an infrared thermometer was used to measure the temperature of NILs of five varieties in 2013 (Figure 4.20).

There was no significant difference in temperature between *Iw1+* and *iw1-* canopies overall (p=0.606), and no interaction between *Iw1* and variety (p=0.837). Further analysis with pairwise comparison revealed no significant difference in temperature for any NIL pair within each variety. Calculation of percentage difference in temperature between NILs demonstrated no consistent trend or direction of change in terms of difference in temperature.

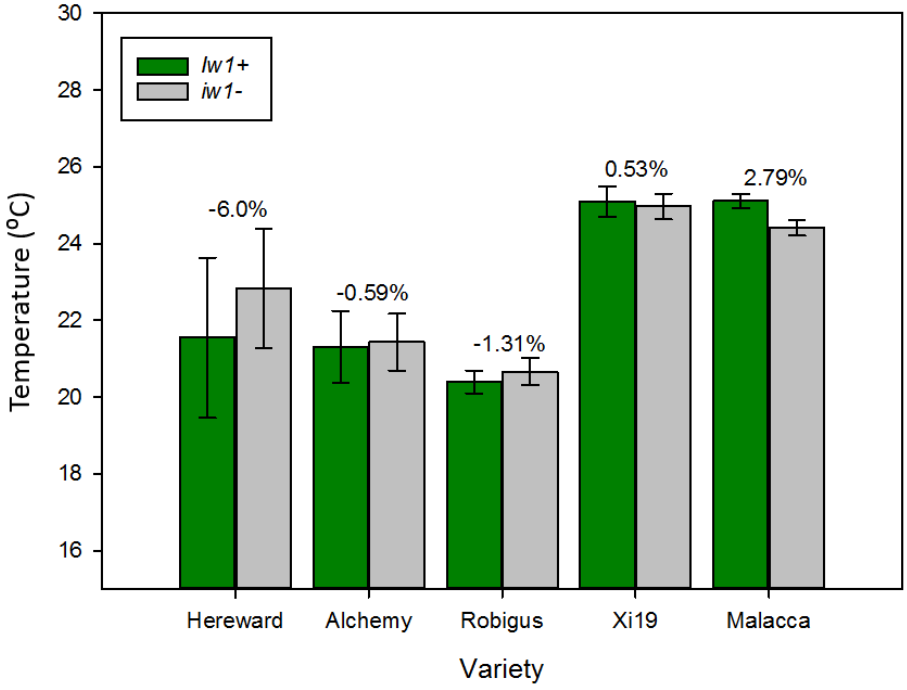

Figure 4. 20 Canopy temperature measured in the field using an infrared thermometer

Each bar represents an average from four plots, with each plot sub-sampled three times. Pairwise comparison revealed no significant difference between NILs of any variety. Percentage increase in temperature of *Iw1+* canopies compared to *iw1-* is shown above each NIL pair. Error bars = S.E.

4.5 Discussion

The biochemistry and structure of epicuticular wax is already known to alter the amount of PAR reflected from the surface of both the plant and canopy, and has been reported to influence canopy temperature. This chapter set out to explore how the spectral properties of five wheat varieties are altered by absence of β- and OH-β-diketones when *Iw1* is present. In addition I tested the hypothesis that reduced reflectance of non-glaucous (*Iw1+*) plants makes more PAR available to photosynthetic tissues and increases canopy temperature, both of which could be beneficial for photosynthesis under UK conditions.

4.5.1 *Iw1* significantly reduced PAR reflectance

The PAR reflected from the crop canopy was significantly reduced in non-glaucous plants by around 12-20% compared to the glaucous *Iw1-* plants. This confirms findings of previous studies of PAR reflectance in wheat and barley canopies who report similar values (Febrero *et al.,* 1998; Jefferson *et al.,* 1989; Johnson *et al.,* 1983). The same trend, although not significant, was also observed at the leaf level consistently across the three varieties.

Although increased reflectance with glaucousness has been widely reported previously, the exact component of the epicuticular waxes leading to this increase has not been clear. Holmes & Keiller, 2002 found that mechanical removal of waxes from glaucous *Eucalyptus* species and *Kalanchoe pumila* species significantly reduced reflectance within the PAR spectrum by around 10-20%. However, they did not have a contrasting non-glaucous genotype or species for comparison. In this study, removal of epicuticular waxes from glaucous *iw1-* leaves resulted in a significant decrease in reflectance of 15 – 40%, whilst there was no effect of wax removal on the reflectance of *Iw1+* non-glaucous leaves. The mechanical method of wax removal ensured that epicuticular waxes were removed without modifying internal leaf chemistry or other structures on the leaf surface and cuticle. Therefore this work provides evidence that it is the presence of β-diketone and OH-β-diketone compounds in the epicuticular waxes that are increasing reflectance in glaucous NILs, and that the platelet waxes of non-glaucous plants has limited effects on leaf reflectance.

Interestingly, in Hereward there was a small but consistent increase in reflectance of *Iw1+* leaves following wax removal. This was not significant, but was seen consistently across all replicates suggesting a biological effect. This should be studied further in the future, to confirm the effect and to understand any particular features of Hereward flag leaves or waxes that may cause non-glaucous epicuticular waxes to actually reduce leaf reflectance.

4.5.2 PAR absorbance of single leaves was not affected by *Iw1*

Simply because the leaf and canopy reflect less light does not mean that more light is available to photosynthetic tissues. At the single leaf (and canopy) level, PAR absorbed also depends on how much light is transmitted through the leaf, something that has not been previously studied in relation to glaucousness. The visible epicuticular waxes of the glaucous *iw1*- NILs are made up of tubular morphological structures, whilst the epicuticular waxes of non-glaucous *Iw1+* NILs form flat platelet structures across the plant surface (Chapter 3). Although these structural differences cause clear changes to visual appearance and PAR reflectance, this work found neither type of wax had any impact on leaf PAR transmission. Some (non-significant) differences in PAR transmission not linked to glaucousness were recorded between NILs, varieties, and before and after treatment with gum arabic. Internal leaf factors such as leaf chemistry and water content can change dynamically according to leaf status and overall condition, and might be expected to vary depending on factors such as sampling time. The most likely explanation is that these internal changes influenced PAR transmission through the leaf during the experiments. This could be confirmed through future experimentation.

Overall, PAR absorbance was not affected by glaucousness in any of the varieties investigated. This suggests that even though *Iw1+* non-glaucous flag leaves reflect less PAR, they do not absorb more, implying a more complex interplay between light reflectance, absorption and genotype than previously thought. However, it is difficult to draw conclusions about canopy light interception based solely on single leaf measurements. For example, a previous study in *Pinus sylvestris* showed that the extent to which glaucousness increased reflectance and altered leaf optical properties varied according to sampling time point within the growing season and location within the canopy (Olascoaga *et al.,* 2014). The work presented here only looked at flag leaves collected at a single time point. Further information on how *Iw1+* is influencing leaf and canopy spectral properties could be obtained by taking leaf spectral measurements at multiple points throughout the growing season and from different levels in the canopy. Additionally, work in maize has shown that canopy light interception is more heavily influenced by crop morphology such as leaf angle, sun angle, leaf distribution and vertical foliage distribution rather than epicuticular waxes (Hatfield & Carlson, 1979; Stewart *et al.,* 2003). These are factors that were not measured in the present work. However, the use of NILs means that morphological differences between NIL pairs should be minimal, if not completely absent, therefore any differences observed between pairs in terms of light interception can be attributed to the waxes (or a closely linked gene within the introgressed region).

In this project, light sensors were placed within the canopy to understand how canopy light interception was changing with glaucousness. However, although various methods were tried,

conclusive data could not be obtained. One major issue with canopy light interception of wheat, and other cereal crops, is that seeds are drilled in uniform rows. Light can directly penetrate through without being affected by surrounding plants, and parts of the sensors remain completely uncovered by plants so just measuring incoming light.

Classical descriptions of canopy light interception such as the Monsi-Saki theory on canopy structure and function (Hirose, 2005) can be applied to analyse canopy photosynthesis and light interception. This theory is based on both light within the canopy and photosynthesis of individual leaves under varying levels of light. It enables analysis of the structure of plant communities and scaling up of photosynthesis from the leaf to canopy level. In addition to the photosynthetic light response of a single leaf, this theory and other classical canopy models place emphasis on canopy structure, leaf arrangement within the canopy (for example horizontal or vertical leaves) and leaf nitrogen distribution. These factors were not measured in the *Iw1* NILs used in this PhD. This could be considered for future work in order to further understand light availability within the canopy and the effect on photosynthesis. However, however the use of NILs that are 96.9% genetically identical in the background ensures that differences in terms of canopy structure and arrangement are minimal if not absent completely. Therefore, the usefulness of these models in regards to the *Iw1* NILs could be limited.

4.5.3 *Iw1* had no effect on photosynthesis

In the absence of definitive data on canopy light interception, one parameter that could be used as a surrogate is photosynthesis, which is heavily dependent on light availability. For example, the concentration of photosynthetic pigments is known to increase in wheat under shade conditions as has the chlorophyll a/b ratio indicating an increase in the number of light harvesting complexes relative to other pigments (Dong *et al.,* 2015; Li *et al.,* 2010; Valladares & Niinemets, 2008; Zheng *et al.,* 2011). This allows the plant to absorb more PAR light over a greater range. Therefore if more PAR is available to non-glaucous *Iw1+* NILs on a long-term basis it might be expected that they would have reduced pigment concentration and a reduced chlorophyll a/b ratio. Confirming this hypothesis, *Iw1+* flag leaves did have significantly less chlorophyll a per unit leaf area than *iw1-*, suggesting some compensation by glaucous leaves experiencing reduced light availability. A study on long term irradiance in winter wheat found that, where light levels are reduced to 60% of maximum plants showed an approximate 30% increase in chlorophyll a (Zheng *et al.,* 2011). Although the difference in the *Iw1* NILs was subtle and not always significant, there was around a 10% increase in chlorophyll a content recorded in some glaucous flag leaves. Over a long period of time, this could be enough to affect plant physiology. However, there was no change in the chlorophyll a/b ratio, indicating no compensation for the reduced chlorophyll a through an increase in light harvesting complexes (chlorophyll b), as would be expected in a shade acclimated leaf.

In addition to pigments, other aspects of photosynthesis are known to change with reduced light availability. A metadata analysis from a variety of species found strong evidence that shade adapted plants have reduced light compensation point, higher quantum yield, lower light saturated photosynthesis and lower dark respiration rate (Valladares & Niinemets, 2008). However, investigation of photosynthesis using light curves in the *Iw1* NILs showed no change in any of these parameters linking to *Iw1*, leading to the conclusion that glaucousness has no effect on photosynthesis. This agrees with previous work that found no effect of glaucousness on light saturated photosynthesis of the flag leaf (Johnson *et al.,* 1983). This conclusion was limited by the fact that these measurements were taken on single leaves, whereby maximum potential carbon assimilation was determined at each light level under optimum conditions. Measurement of whole canopy gas exchange in the field over a period of time could have led to different conclusions. Another possibility is that small differences between NILs in terms of PAR availability have been compensated for by changes to chlorophyll a content, so leading to no difference in overall carbon assimilation. Another limitation of his work on the *Iw1* NILs was that the flag leaf was the only organ investigated. Richards *et al.,* (1986) record an increase in photosynthesis under irrigated conditions of 23% associated with non-glaucousness in wheat ears, whilst they recorded no difference between glaucous and non-glaucous flag leaves. Had it been possible to measure photosynthesis in other organs within the *Iw1* NILs conclusions may have been different.

Not only was there no effect of *Iw1* on flag leaf parameters, there was also no effect of *Iw1* on photosynthesis of the second leaf. This indicates that there was no difference between NILs in terms of light conditions within the canopy. However, when interpreting these results it should be noted that PAR absorbed is only one function of plant energy input and consumption. Multiple additional factors such as efficiency of photosynthetic machinery within the plant, tissue temperature and water status (Olascoaga *et al.,* 2014) all contribute towards how much available PAR is actually used in photosynthesis. It was not possible to measure these parameters in the *Iw1* NILs.

4.5.4 Hereward *Iw1+* NILs had increased light saturated assimilation

The one variety that did show a change in photosynthesis associated with *Iw1* was Hereward; *Iw1+* NILs had significantly increased flag leaf photosynthesis at higher light levels, achieving significantly higher A_{max}. However, this is likely not due to the change in epicuticular waxes as the effect was not seen in any other variety tested. This conclusion is further supported by use of recombinant lines. No recombinant achieved A_{max} levels comparable with the *Iw1+* NILs, suggesting that there may be multiple alleles within the introgressed region that improve photosynthesis and work synergistically. Presence of all Shamrock alleles would be required to have an impact on overall

photosynthesis. However, these data are preliminary, only collected in one field trial using four recombinant lines. Ideally these data need to be collected in future years using multiple recombinants in order to more conclusively understand where the effect in the Hereward *Iw1+* NILs is coming from.

It is also clear from the A_{max} data that Hereward is the lowest performing variety of all those measured in terms of photosynthetic rate. This wheat variety was originally bred for quality rather than quantity (Chapter 2 Table 2.1), and as such higher yields and rates of photosynthesis were not selected for. Addition of *Iw1* brings the A_{max} of Hereward up to the level of the other varieties. From this it can be hypothesised that there may be one, or a number of, deleterious alleles in that region of the chromosome having a negative effect on photosynthesis. By introducing in the *Iw1* introgression, any deleterious alleles are replaced with a beneficial Shamrock allele, allowing for higher light saturated photosynthesis. Previous studies have reported an increase in photosynthesis with glaucousness of up to 23% in sorghum leaves and wheat ears (Chatterton *et al.,* 1975; Richards *et al.,* 1986). However, the use of NILs of multiple varieties in the present work demonstrates that without precise genetic stocks it is difficult to attribute observed effects to the epicuticular waxes, or as suggested here, to another closely linked gene.

4.5.5 Photosynthesis is not the only factor determining yield

Hereward is not only the one variety that demonstrates some improvement in photosynthesis with addition of the *Iw1* introgression, it is also the variety that displays the most significant and consistent yield benefit, with a yield increase of around 5% mapping near to *Iw1* (Chapter 3). Although there is limited evidence of a direct link between *Iw1* and the higher A_{max}, this raises the question of whether or not the effects on photosynthesis in this variety are linked to yield.

Over 90% of biomass of crops such as maize, wheat and rice is thought to be derived from photosynthetic products, and as such a number of studies claim that improvements to photosynthesis are the answer to achieving the higher crop yields we require to feed a growing population (Makino, 2011). A number of studies in wheat have reported a strong correlation between light saturated photosynthesis and grain yield (Fischer *et al.,* 1998; Jiang *et al.,* 2003). However, this is not a simple relationship, and depends heavily on genetics, environment, and the specific limitations to growth within that environment (Fischer *et al.,* 1998; Hubbart *et al.,* 2007; Long *et al.,* 2006; Makino, 2011). No correlation between photosynthesis and yield has been found within environments or genetic backgrounds where improved photosynthesis is not the limiting factor to growth (Evans & Dunstone, 1970). Taking this into account it is possible that substrate availability and increasing biomass production was not a limiting factor within the Church Farm field trials, and as such higher rates of photosynthesis did not result in higher grain yield. Alternatively,

the increased photosynthesis of Hereward *Iw1+* NILs may be contributing to the observed increase in yield. However, if a number of other environmental and genetic factors are also contributing to this, this would explain why no effect or direct link with *Iw1* and yield was observed in the recombinant lines. It should be noted that these measurements of photosynthesis were taken within an IRGA leaf chamber, where conditions such as temperature, CO_2 and RH were constant and optimum for leaf photosynthesis. A previous study of 64 elite wheat varieties in the UK that also used instantaneous gas exchange found no consistent correlation between grain yield and flag leaf photosynthesis (Driever *et al.,* 2014). This suggests a discrepancy between possible A_{max} measured in an IRGA leaf chamber and the reality in the field, where conditions are rarely constant or optimum.

4.5.6 Canopy temperature was not affected by *Iw1*

Temperature is an important factor that can influence the rate of photosynthesis and other metabolic processes. Although materials were not available to directly measure the reflectance and transmission of infrared light from the leaf or canopy as was done for the PAR spectrum, canopy temperature was measured in the field using an infrared thermometer. No difference was found between NILs using this method. This is contrary to previous work which does find glaucous wheat canopies to be up to 0.7 ^{o}C cooler in the field (Richards *et al.*, 1986; Jefferson *et al.*, 1989). However, both of these studies found this difference to be under drought stress conditions. In the UK field environment wheat is not typically heat or drought stressed, which may account for lack of difference between the NILs. A further issue with this work is the practicality of taking these types of measurements in the field. In the UK frequent cloud cover and wind means that the air temperature is constantly changing. Methods of canopy temperature measurement were developed in regions where air temperature is predictable and constant. A better method to use within the UK could be a thermal camera on a drone above the crop, but this would still be affected by frequent fluctuations in air temperature far greater than the 0.5-1 ^{o}C expected temperature difference between NILs.

There is no difference in photosynthesis correlating with glaucousness in the *Iw1* NILs. This indicates that if there is a difference between NILs in terms of temperature, it is not sufficient to have an impact on photosynthesis. Data available from wheat regarding the magnitude of temperature change required to affect photosynthesis indicates that a change of around 10 °C is required to impact on photosynthetic rate (Yamasaki *et al.,* 2002), although some studies in wheat even report no change in photosynthesis after a temperature increase of 15 °C (Alonso *et al.,* 2008). The decrease in tissue temperature previously recorded in glaucous plants compared to non-glaucous is considerably smaller than this (Richards *et al.,* 1986), so any effect on photosynthesis under normal UK conditions, if at all, is probably negligible. Nevertheless, under extremes of temperature

a small change in tissue temperature could have a marked impact. Temperature would also be expected to affect transpiration rate. This, and other components of water use efficiency, will be considered in Chapter 5.

4.5.7 Conclusions

Overall, the work presented in this chapter confirmed that non-glaucous leaves and canopies do reflect less PAR than glaucous canopies, but found no evidence that this lead to increased PAR absorbance by single leaves or increased light interception by the canopy. Furthermore, no evidence was found that changes to leaf spectral properties affect canopy temperature to an extent that would be beneficial to the plant in a UK environment. Glaucousness and *Iw1* was found to have no effect on photosynthesis of leaves at multiple levels within the canopy. However, glaucous (*iw1*) flag leaves were found to have increased chlorophyll a, potentially compensating for any reduced PAR availability these leaves are experiencing.

Iw1+ NILs of Hereward did display increased photosynthesis. Although this does not appear to be linked to *Iw1*, differences were significant, and potentially worth pursuing in terms of crop improvement, particularly given the significant yield increases also seen in *Iw1+* NILs of this variety.

Chapter 5: The effect of *Iw1* on water use efficiency in the UK field environment

5.1 Summary

A primary function of the plant cuticle, of which both epicuticular and intracuticular waxes are a major component, is the prevention of excessive water loss from the plant surface. Water use efficiency (WUE) is defined as the water lost through transpiration per mole of carbon gained in photosynthesis. The development of more water use efficient crops is becoming increasingly important to sustain agriculture under a changing climate.

This chapter explores WUE in the field for *Iw1+* and *iw1-* near isogenic lines of three wheat varieties (Malacca, Alchemy and Hereward). The role of epicuticular waxes in WUE was investigated through measurement of a number of physiological parameters in field grown plant material including stomatal conductance, cuticular conductance, and carbon assimilation parameters. Carbon isotope discrimination was used to integrate all parameters and gain an understanding of water use over the growing season.

Overall, within the field trials in East Anglia where water stress in the field is not a major issue, *Iw1* had no effect on WUE, and β- and OH- β- diketones had no effect on water movement through the cuticle.

5.2 Introduction

The major challenge that faced plants upon colonization of the land was water stress. As such the earliest land plants had a well-developed cuticle composed of cutin and wax (Edwards *et al.,* 1996), a primary function of which was to limit water loss through the plant surface (Woodward, 1998). The fact that all land plants still have this waxy cuticle today is a testament to just how important this function of the cuticle is. However, the cuticle not only acts as a barrier to water vapour, but also to carbon dioxide used during photosynthesis (Woodward, 1998). Stomata, pores within the cuticle through which gases can diffuse, overcome this problem allowing CO_2 into the plant but water vapour can also diffuse out simultaneously. The process of CO_2 and water vapour diffusion in and out of the plant becomes a delicate balancing act. Regulation of this process will determine how much carbon is available for photosynthesis and how much water the plant can conserve.

Supply and demand of water available to the plant is heavily influence by climate (Rosenzweig *et al.,* 2001). It is projected that the rapidly changing climate will have a huge influence on water availability for agricultural production, and indeed is already having a negative effect. In particular the instance of extreme weather events and desertification are both projected to increase in severity over the coming decades with a significantly negative effect on food supply (IPCC 2014). For example, severe drought between 2008 and 2011 in Kenya, where wheat is the second most important agricultural commodity (Monroy *et al.,* 2013), lead to reduced crop yields resulting in economic losses of 1.5 billion dollars (FAO, 2015). This, and other similar events, puts food security and how to increase the WUE of crop plant species firmly in the spotlight. Associated issues are now of great interest to both researchers and policy makers in the UK and around the world. Since the epicuticular waxes are such a key component of the cuticle, their optimisation for the environment in which a plant grows is potentially an effective method that could be employed to increase WUE of major crop plants.

5.2.1 Defining water use efficiency

WUE can be defined by total plant productivity through determining the ratio of biomass produced to overall transpiration rate. Alternatively, a simpler definition which will be used throughout this chapter, is leaf level instantaneous (or intrinsic) WUE:

$$Instantaneous\ Water\ Use\ Efficiency = \frac{Assimilation}{Transpiration}$$

In the above equation, assimilation is the moles of carbon gained in photosynthesis, and transpiration is moles of water used for transpiration (Polley, 2002). Therefore, at the simplest

level, WUE could be improved either through increasing photosynthesis (without a corresponding increase in transpiration) or reducing transpiration during photosynthesis.

5.2.2 Assimilation

The influence of glaucousness on carbon assimilation was explored in Chapter 4, primarily in relation to light availability. In the wheat varieties tested in my work, the presence of β- and OH-β- diketones did not directly link to changes in flag leaf photosynthetic rate at different light levels. Crucially, the methods used did not assess exactly what was happening in the field at the canopy level as all measurements were taken under optimal conditions for leaf photosynthesis. Carbon assimilation is an important component of WUE. As such a number of other parameters linked to efficiency of the plant photosynthetic machinery will be explored in the present chapter.

5.2.3 Transpiration

The second component of instantaneous WUE is transpiration, which is the combination of two processes. Primarily water is lost through open stomata (stomatal conductance), and a smaller contribution is made by water loss through the cuticle (cuticular conductance). Studies measuring overall transpiration report that glaucous wheat plants could have up to 50% lower day time transpiration than non-glaucous (Richards *et al.,* 1986) and equivalent work in sorghum has reported up to a 26% reduction in transpiration of glaucous plants (Chatterton *et al.,* 1975). However, both of these studies were carried out under controlled conditions in a glasshouse, so results may differ significantly from the situation in the field. To fully understand how glaucousness may reduce transpiration, each component of transpiration needs to be studied individually in addition to overall measurement in field grown material.

5.2.3.1 Stomatal conductance

Stomata are the main interface between the internal leaf and the atmosphere (Kaiser, 2009), and as such stomatal conductance is the primary component of transpiration. Guard cells surrounding the stomata control opening and closing of the pores according to internal and external conditions. This process regulates gas exchange, balancing photosynthesis and water loss (Roelfsema & Hedrich, 2005). Many species from drought prone environments confer various epicuticular wax adaptations to limit water loss through stomata such as the formation of a wax roof over the pore (Roth-Nebelsick *et al.,* 2013) or waxy stomatal plugs that reduce maximum conductance (Brodribb & Hill, 1997). However, there are limited studies concerning the relationship between epicuticular waxes and stomatal conductance in temperate environments and cereal crops, perhaps because drought is a less serious concern. Existing work has reported that where tubular (β- diketone containing) epicuticular waxes are present in glaucous wheat and barley plants, they do appear to

be clustered around the stomata as can be seen in Figure 5.1 (King & von Wettstein-Knowles, 2000). This indicates that glaucous epicuticular waxes may have a role in limiting water loss through the stomata. However, the magnitude of this effect, if any at all, remains uncertain. Work in the field in Canberra, Australia by Johnson *et al.,* (1983) showed glaucous wheat lines to have around an 8% reduction in flag leaf stomatal conductance compared to non-glaucous, although this was not significant. The Richards *et al.,* (1986) glasshouse study found reductions in stomatal conductance in wheat ears of 30%-60% associated with glaucousness.

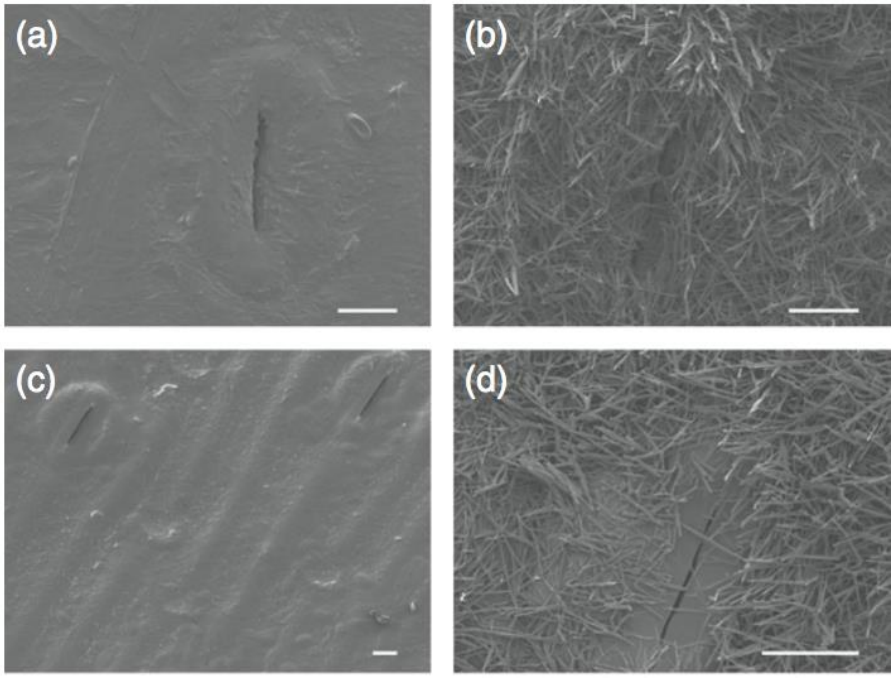

Figure 5. 1 SEM images showing tubular epicuticular waxes clustering around the stomata

Tubular structures are present in glaucous wheat plants without *Iw1* (b and d), whilst in non-glaucous plants that have *Iw1* these tubular wax structures are absent (a and c). Scale bars = 10 µm. Figure adapted from Adamski *et al.,* 2013.

An alternative mechanism of controlling water loss through the stomata is to adapt to environmental conditions and alter the frequency and patterning of stomata in particular organs (Casson & Gray, 2008). Zeiger & Stebbins (1972) were the first to report a possible link between epicuticular wax biosynthesis and stomatal development with their work in barley. The barley *eceriferum-g* mutant not only has a glossy (rather than non-glossy) spike, but also has clustered stomata. A subsequent study in Arabidopsis (*Arabidopsis thaliana*) identified the *high carbon dioxide* (*hic*) mutant which had reduced stomatal density under high CO_2. The *HIC* gene encodes a 3-ketoacyl coenzyme A synthase protein, which is involved in very long chain fatty acid elongation, an important step in wax biosynthesis. Therefore this study again suggested a link between

stomatal development and epicuticular wax (Gray *et al.*, 2000). Since this, a number of other studies in the model plant Arabidopsis have suggested that there could be a relationship between epicuticular wax and stomatal development. However, the exact interplay between these two factors is still not agreed, as different studies report a variety of effects dependent on underlying genetics. For example, in *wax2* mutants the amount of epicuticular wax is decreased, and this corresponds with a reduced stomatal index (Chen *et al.,* 2003), whilst both the gain-of-function *shine* mutants and plants with the gene WIN1/SHIN demonstrate increased leaf cuticular wax correlating with a significant decrease in stomata (Aharoni *et al.,* 2004; Yang *et al.,* 2011). Despite these studies in Arabidopsis suggesting a possible link between stomatal and wax development, any certain relationship remains to be determined. It is likely that these multiple conclusions are a result of the interaction between numerous environmental factors, such as altered permeability of the leaf to water or differences in the absorption of light, and genetics (Casson & Gray, 2008). Furthermore, there have been no studies in cereal crops that report a clear relationship between epicuticular waxes and stomata since the 1972 study in barley.

5.2.3.2 Cuticular conductance

Cuticular conductance is the loss of water through the cuticle, and makes a smaller, yet important, contribution to overall transpiration. Unlike stomatal conductance, water movement through the cuticle cannot be readily controlled depending on environmental conditions. Therefore plants require specific adaptations to their environment in terms of cuticle permeability to water. Epicuticular wax is an important cuticular component and consequently expected to affect cuticular conductance. Leaf water permeability has been correlated to leaf waxiness rather than cuticle thickness in the past, indicating that the wax component of the cuticle is a key determinant of permeability (Schönherr, 1976). Additionally, significantly increased cuticular conductance following complete removal of wax has been recorded in a number of species (Denna, 1970; Hall & Jones, 1961). Numerous subsequent studies have shown that in wheat, barley and sorghum, epicuticular wax load increases under drought and water stress (González & Ayerbe, 2010; Haley *et al.*, 1993; Premachandra *et al.*, 1992; Uddin & Marshall, 1988), suggesting that these waxes play an important role in drought resilience. These studies all support the hypothesis that epicuticular wax, and hence glaucousness, is likely to be an important parameter in cuticular transpiration, thus a potential determinant of WUE.

Exactly which components of the epicuticular waxes affect cuticular conductance is questionable. Observations in barley, maize, sorghum and wheat, show cuticular conductance can increase by up to 75% after complete removal of the waxes, but wax quantity appears not to be correlated with rate of conductance (Araus *et al.,* 1991; Jenks *et al.,* 1994; Larsson & Svenningsson, 1986;

Premachandra *et al.,* 1994; Ristic & Jenks, 2002). This indicates that wax structure and composition could be more important than quantity for determining cuticle permeability to water (Jenks *et al.,* 1992; King & von Wettstein-Knowles, 2000; Premachandra *et al.,* 1994). However, the relationship between wax composition and permeability to water is not yet clear, perhaps due to difficulty manipulating wax structure and composition or inability to find novel ways to thoroughly investigate this aspect of transpiration.

To some extent data from cereals supports a hypothesis that cuticular conductance is affected by glaucousness. A recent study in bread wheat with glaucousness conferred by the *W3* locus found glaucousness to reduce water loss through the cuticle by around 15% compared to non-glaucous (*w3/w3*) plants that had 99% less β-diketones in addition to around a 63% reduction in total wax load (Zhang *et al.*, 2015). Conversely, studies in durum wheat (Merah *et al.,* 2000) and barley (Febrero *et al.*, 1998) have found no difference in cuticle permeability to water between glaucous and non-glaucous tissue. Theses multiple conclusions indicate that the relationship between glaucousness and cuticular conductance is not a simple one. There are many forms of glaucousness specific to species and environmental conditions. As described in Chapters 1 and 3, the visual glaucous appearance can be achieved in a variety of ways, and underlying biochemistry can be quite different, even where the same compounds are present. For example, the pathways synthesising β- and OH-β-diketones are different in wheat and barley than for the same compounds in Arabidopsis (Samuels *et al.,* 2008; von Wettstein-knowles, 2012). It is likely that interplay between a number of factors such as genetics, epicuticular wax composition, study material and growth environment determines the influence of glaucousness on cuticular conductance.

5.2.4 Overall water use efficiency

5.2.4.1 Studies using instantaneous gas exchange

Whilst breaking down water use into individual components is vital, information on both assimilation and transpiration needs to be combined for full understanding. Although there are now accurate methods available to measure these different parameters, such as leaf conductance to CO_2 and water vapour, it can still be difficult to understand how these constituents relate and interact to produce a picture of overall WUE. Two extensive studies on durum and bread wheat under both irrigated and non-irrigated conditions have used gas exchange measurements and biomass to study the effect of glaucousness on WUE (Johnson *et al.,* 1983; Richards *et al.,* 1986). Through measurement of WUE in terms of grams of plant biomass produced per kg water, Richards *et al.,* (1986) showed that glaucous plants were on average 9% more water use efficient throughout development than non-glaucous under moderate drought conditions, but there was no significant

difference overall under irrigated conditions. Gas exchange measurement showed that this increase in WUE was derived more from a reduction in transpiration rather than increased photosynthesis. However, although Johnson *et al.,* (1983) reported increased transpiration with glaucousness, and even recorded the assimilation/transpiration ratio to be 43% higher in glaucous compared to non-glaucous wheat lines on one occasion, overall they find no significant difference between glaucous and non-glaucous lines in terms of WUE. This highlights the problem with reliance on instantaneous gas exchange measurement only. Whilst these measurements are informative, plant water use is likely to change over the course of the growing season as both plant physiology and environmental conditions dramatically change over time. Therefore conclusions drawn from these types of measurement may not be an accurate representation of water use throughout all stages of plant growth.

5.2.4.2 Studies using carbon isotope discrimination

An alternative method to assess plant WUE is through measurement of carbon isotope composition of plant matter. C_3 plants discriminate against ^{13}C during photosynthesis, partly because ^{13}C diffuses through the stomata slower than ^{12}C. In addition to this Rubisco (D-ribulose 1,5 bisphosphate carboxylase/oxygenase), the enzyme involved in the first step of carbon fixation during photosynthesis, has a preference for ^{12}C over ^{13}C. Carbon isotope discrimination ($\Delta^{13}C$) is a measure of the $^{13}C/\ ^{12}C$ ratio of plant biomass relative to that of air (Farquhar *et al.*, 1989; Farquhar & Richards, 1984). Processes determining this ratio are shown in Figure 5.2.

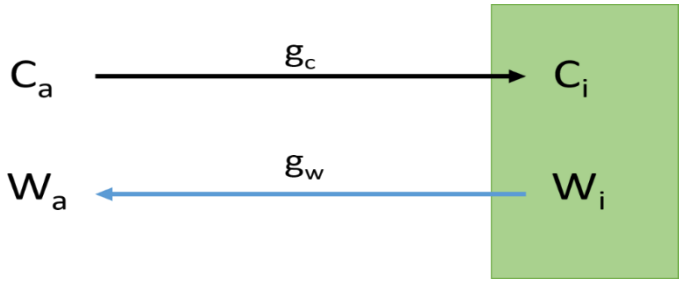

Figure 5. 2 Diffusion of CO_2 and water vapour through the stomata

Carbon (C) moves through stomata by process of stomatal conductance to CO_2 (g_c) from the atmosphere (C_a) to the inside of the leaf (C_i) during photosynthesis. Water vapour also moves through the stomata (stomatal conductance to water, g_w) from the inside of the leaf (W_i) to the atmosphere (W_a).

In Figure 5.2 'g' donates stomatal conductance, C stands for carbon, and W stands for water vapour. Accordingly, the figure shows the process of stomatal conductance to carbon (g_c), as CO_2 diffuses from the atmosphere (C_a) into the leaf (C_i). Farquhar & Richards (1984) showed that $\Delta^{13}C$ increases

when there is a relative increase in the partial pressure of CO_2 inside the leaf relative to that of air. Photosynthesis is also related to the concentration gradient between internal and atmospheric CO_2:

$$Photosynthesis = g_c(C_a - C_i)$$

Water vapour (W) can also diffuse through the stomata. This process occurs together with diffusion of CO_2 into the plant and is termed stomatal conductance to water vapour (g_w, Figure 5.2). This is the primary component of transpiration and can be controlled by the plant. Although cuticular conductance is also a part of transpiration, the contribution is far less and does not depend on stomata being open. Therefore is relation to $\Delta^{13}C$, WUE can be expressed as:

$$Instantaneous\ Water\ Use\ Efficiency = \frac{g_c(C_a - C_i)}{g_w}$$

This indicates that the C_i/C_a ratio is negatively related to intrinsic WUE, and $\Delta^{13}C$ is negatively related to WUE. An advantage of this method of measurement over gas exchange is that it integrates information from plant gas exchange over a long period of time.

Studies investigating the relationship between WUE and glaucousness using carbon isotopes have tended to reach opposing conclusions to those using instantaneous gas exchange measurements. Under water stressed conditions glaucousness in wheat has frequently been associated with higher $\Delta^{13}C$ of around 1-3‰ when measured in a number of tissues including grain and flag leaf (Merah *et al.,* 2000; Monneveux *et al.,* 2004). This indicates that over a long period of time, glaucousness actually reduces WUE. However, this effect is likely to be highly dependent on environmental conditions. Work within a temperate UK environment in wheat has found no effect of glaucousness on $\Delta^{13}C$ (Adamski *et al.,* 2013). Another study found flag leaf $\Delta^{13}C$ to be higher in glaucous that non-glaucous plants only under early water stress (Merah *et al.,* 2000). It should be noted however that a study in barley found a positive correlation between glaucousness and $\Delta^{13}C$ under both irrigated and rainfed conditions (Febrero *et al.,* 1998), a result comparable to the studies detailed earlier carried out under water stress. A draw back of the use of carbon isotopes is that it is not possible to separate out the different physiological processes of assimilation and transpiration that are contributing to the final value. As such $\Delta^{13}C$ is best used in conjunction with other measures such as biomass and instantaneous gas exchange measurement.

5.2.5 Aims

Overall the literature appears to suggest that under drought stress there is some effect of glaucousness on WUE, whether this be positive or negative. However, this relationship may be absent where water availability is not a limiting factor. Therefore, through employment of both gas exchange measurement and carbon isotopes I aim to test the hypothesis that

(i) There is no effect of non-glaucousness as conferred by *Iw1* on WUE within a UK (East of England) environment.

5.3 Methods

5.3.1 Dark adapted chlorophyll fluorescence

Carbon assimilation is one component of instantaneous WUE. To gain understanding of any differences in photosynthetic efficiency between *Iw1+* and *iw1-* NILs, dark-adapted chlorophyll fluorescence parameters were measured using a Handy PEA (Hansatech Instruments, Norfolk, UK) in the field in the 2013 and 2014 harvest years. Measurements were taken within 14 days of anthesis, the point at which wheat reach maximum photosynthetic capacity (Molero & Lopes, 2012) . Measurements were taken on NILs of Malacca, Alchemy and Hereward. Forty flag leaves from independent biological replicates were measured from each line, ten from each of four plots.

Leaf clips were placed on the flag leaves. The clips had a small shutter that could be closed over the leaf when the clip was attached to exclude incoming light. Leaves were left to dark adapt for 40 min, after which point the handy PEA was used to measure chlorophyll fluorescence. Measurement of *Iw1+* and *iw1-* leaves of the same variety were made on the same day simultaneously to minimise possible variation over time.

Minimum fluorescence yield in the dark adapted state (F_o) and maximum fluorescence yield in the dark adapted state (F_m) were measured. Variable fluorescence (F_v) can be calculated from:

$$F_v = F_m - F_o$$

The value of F_v/F_m then gives the maximum efficiency of photosystem II (PSII). Data were analysed by overall ANOVA including all varieties and years, and by pairwise comparison to assess differences between *Iw1* NILs for each variety within each year.

5.3.2 Measurements using infrared gas analyser (IRGA)

The LI-COR 6400XT (LI-COR Biosciences) infrared gas analyser (IRGA) system was used to take measurements on flag leaves within 14 days of anthesis on flag leaves of Malacca and Alchemy NILs in 2013 and 2014, and Hereward NILs in 2014 and 2015. Four biological replicates were measured for each line.

The leaf, upon being placed in the leaf chamber was allowed to equilibrate at a photosynthetically active radiation (PAR) level of 1000 µmol m^{-2} s^{-1} for 10 min or until stable. Subsequently, PAR was increased to 1500 µmol m^{-2} s^{-1}, and then reduced through 1000, 750, 500, 400, 300, 200, 100, 50 and 0 µmol m^{-2} s^{-1}. Throughout all measurements flow was maintained at 300 µmol, reference CO_2

was set to 500 µmol mol^{-1} and the leaf temperature was set to 20 °C. An effort was made to maintain relative humidity between 50 and 70%. Where this was not possible given variable field conditions, it was allowed no higher than 75% or lower than 45%. Whilst plants for measurement were selected to be representative of the plot, those with flag leaves that were healthy and big enough to fill the leaf chamber were chosen. Various parameters were logged at each light level as detailed in sections 5.3.2.1 - 5.3.2.2.

5.3.2.1 Light adapted chlorophyll fluorescence

Actinic light was set to 'on'. At each light level from 1500 to 0 µmol m^{-2} s^{-1} a rectangular flash of fluorescence with 10% blue light was applied to the leaf. F_s' (steady state chlorophyll fluorescence in the light adapted state) and F_m' (maximum fluorescence yield in the light adapted state) were logged at each light level. The quantum yield of Photosystem II in the light-adapted state (ΦPSII) was calculated using the equation:

$$\phi PSII = \frac{F_m' - F_s'}{F_m'}$$

Like F_v/F_m, ϕPSII provides an indication of and how well the fundamental photosynthesis machinery is functioning (E H Murchie & Lawson, 2013). Data were analysed by pairwise comparison at each light level for each NIL within each year individually. In addition to an overall ANOVA at each light level by variety to compare across the two years.

5.3.2.2 Gas exchange CO_2 and water vapour

The IRGA system was also used to measure components of instantaneous WUE. Gas exchange components of CO_2 and water vapour including photosynthetic rate, internal CO_2 concentration, transpiration and stomatal conductance were logged at each light level. Data for carbon assimilation at each light level were presented in Chapter 4 section 4.4.3.2. The ratio of stomatal conductance to assimilation was calculated at each light level and data for the NILs analysed by pairwise comparison.

5.3.2.3 Calculation of water use efficiency from gas exchange measurement

WUE at each level of PAR between 0 and 1500 µmol m^{-2} s^{-1} was calculated from the gas exchange data according to the equation:

$$Water\ Use\ Efficiency = \frac{Assimilation\ (mol\ CO_2\ m^{-2}\ s^{-1})}{Transpiration\ (mol\ H_2O\ m^{-2}\ s^{-1})}$$

where Assimilation is the moles of carbon gained in photosynthesis, and Transpiration is the moles of water used for transpiration (Polley, 2002).

5.3.3 Transpiration components

Overall transpiration was measured using the IRGA detailed in section 5.3.2. However, transpiration has a number of components: cuticular conductance and stomatal conductance. These parameters were measured in field grown flag leaves in the *Iw1* NILs.

5.3.3.1 Cuticular conductance

Flag leaves were collected at anthesis from independent replications in the field. In 2013 and 2014 NILs of Malacca and Alchemy were sampled with 15 and 5 replications respectively. Flag leaves of Hereward BC_4 NILs were collected in 2015 across 5 replications.

Flag leaves were left overnight at 4^{o}C in the dark with the ends in water. This saturated the leaves with water and ensured stomata were shut. The following day, water saturated leaves were weighed (Satorius analytical balance BP61S), called time point 0, and then placed in the dark at ~50% relative humidity and 25 ^{o}C to dehydrate for the full duration of the experiment. After the first weighing (time point 0), flag leaves were weighed every 20 min for 120 min, and then again at 240 and either 260 or 280 min. The leaves were then freeze-dried for 48 hr, and the dry weight recorded.

The following equation was used to calculate water content (g) per unit dry weight (g):

$$Water\ content\ (g\ g^{-1}) = \frac{Fresh\ weight - Dry\ weight}{Dry\ weight}$$

Data from time points 0 and 20 min were discarded as leaves were still equilibrating to conditions during this time. Water content per unit dry weight at 40 min was treated as 100%, and water content at all other subsequent time points was calculated as a percentage of 40 min. Percentage water content between NILs was compared at each time point for each variety by pairwise comparison. A regression line was fitted between 40 and 120 min to confirm a linear relationship between water loss and time. Overall rate of water loss (calculated as the gradient of the regression line for each leaf) was then analysed using overall ANOVA including all varieties and years.

5.3.3.2 Stomatal density

Flag leaves were collected from independent replications in the field and stored with their ends in water, 3 biological replications per NIL. On the same day, leaf impressions were made of both the adaxial and abaxial leaf surfaces using Xantopren L-blue dental putty (Heraeus Kulzer International,

Germany) and Universal Activator (Heraeus Kulzer International, Germany) to create a negative impression of the leaf. Clear nail varnish was then spread onto the negative impression and peeled off once dry using clear sellotape to create a positive impression. Each positive impression was stuck onto a microscope slide. Three positive impressions per side of each leaf were created; one each from the top, middle and bottom of the leaf.

A light microscope (M205 fluorescent stereo, Leica Microsystems, UK) was used to take an image of 9 fields of view per positive impression. The field of view was 2.89 mm, magnification 80 x. Any deviation from this was recorded and controlled for accordingly. The number of stomata within each field of view were counted, and stomata per mm^2 calculated to get stomatal density. Average stomatal density over all 9 sub-samples was taken to give stomatal density per positive impression. No difference in stomatal density was found between the three portions of the leaf. Therefore all 27 measurements were combined into a single number to get mean stomatal density per adaxial or abaxial surface.

Statistics were therefore done on three values per NIL per side of each leaf. Data were analysed both by overall ANOVA combining all varieties and years, and pairwise comparison for each variety within each year.

5.3.5 Bulk $\delta^{13}C$ measurement and calculation of $\Delta^{13}C$

Flag leaves were sampled from three independent replications in the field at anthesis and 40 DPA. Tissue collected in the field was immediately frozen on dry ice and stored at -80 ^{o}C. Samples were freeze dried, and then each freeze dried sample was cut up and placed into a 1.5 mL eppendorf tube (Eppendorf, UK) with 1 x 3 mm tungsten bead (Quiagen 69997). Samples were ground to fine powder using a genogrinder (Tissue Lyser, Quiagen). For each sample, 2 x ~0.5 mg powder was weighed out into individual silver foil parcels, and run on a Thermo Finnigan Deltaplus XP isotope ratio mass spectrometer interfaced to a Costech Elemental Combustion System CHNS-O 4010 as described in Adamski *et al.,* (2013) to measure $\delta^{13}C$. ~0.5 mg of in-house collagen and casein standards were run every 20 samples.

Carbon isotope composition is expressed relative to Vienna PeeDee belemnite standard (VPDB). The change in $\delta^{13}C$ of the collagen standard during the run was used to control for drift and correct sample $\delta^{13}C$ values accordingly. $\Delta^{13}C$ was then calculated using the following formula from Farquhar *et al.,* (1989):

$$\Delta = \left(\frac{\delta a - \delta p}{1000 + \delta p}\right) x\ 1000$$

In this equation δa is the ambient CO_2, value, assumed to be -8 ‰, and δ p is the sample $\delta^{13}C$ value. As each sample was run in duplicate an average of the two was taken to get one value per sample.

5.4 Results

5.4.1 Carbon assimilation

Carbon assimilation is one component of WUE. Chapter 4 explored the effect of *Iw1* and epicuticular wax type on photosynthesis at light levels between 0 and 1500 µmol m^{-2} s^{-1} and found no effect of *Iw1* on carbon assimilation. This chapter aims to ascertain whether there are any differences in function and efficiency of the photosynthetic machinery between *Iw1+* and *iw1-* NILs and thus further explore photosynthetic efficiency.

5.4.1.1 Dark adapted chlorophyll fluorescence

Measurement of chlorophyll fluorescence allows assessment of the performance of photosynthetic machinery and pathways within a plant. Certain fluorescence parameters were measured in the field at anthesis on dark adapted flag leaves. Table 5.1 shows values for minimum (F_o) and maximum (F_m) fluorescence yield in the dark adapted state. F_v (variable fluorescence) / F_m is the quantum yield of photosystem II (PSII) in the dark adapted state.

Table 5. 1 Dark adapted chlorophyll fluorescence for field grown flag leaves at anthesis

F_o (minimum fluorescence yield in the dark adapted state), F_m (maximum fluorescence yield in the dark adapted state), and F_v/F_m (quantum yield of PSII in dark adapted state) using a Handy PEA. Measurements were taken on flag leaves in the field at anthesis for NILs of Malacca, Alchemy and Hereward. N=40. P values for pairwise comparison between NILs for each variety are shown.

			F_o		F_m		F_v/F_m	
			Average ± S.E	p	Average ± S.E	p	Average ± S.E	p
Malacca	2013	*Iw1+*	420.57 ± 3.83	<0.001***	2917.43 ± 33.23	<0.001***	0.856 ± 0.001	<0.001***
		iw1-	538.29 ± 5.50		3112.69 ± 32.04		0.826 ± 0.003	
	2014	*Iw1+*	491.55 ± 4.58	0.775	2524.95 ± 36.14	0.101	0.804 ± 0.004	0.292
		iw1-	493.79 ± 6.39		2618.72 ± 43.67		0.809 ± 0.004	
Alchemy	2013	*Iw1+*	473.36 ± 6.12	0.049*	2778.25 ± 43.08	<0.001***	0.829 ± 0.002	<0.001***
		iw1-	490.03 ± 5.64		3125.20 ± 35.63		0.843 ± 0.002	
	2014	*Iw1+*	468.63 ± 4.59	0.945	2650.18 ± 28.05	0.703	0.822 ± 0.002	0.884
		iw1-	469.05 ± 4.10		2666.55 ± 32.22		0.823 ± 0.003	
Hereward	2014	*Iw1+*	466.10 ± 4.85	0.436	2585.44 ± 49.59	0.843	0.817 ± 0.004	0.717
		iw1-	460.65 ± 5.01		2572.86 ± 38.39		0.819 ± 0.003	

An overall ANOVA that taking accounts of all varieties and years indicated that there was no significant effect of *Iw1* on F_v/F_m (p= 0.317), but the effect of *Iw1* was dependent on both year of measurement (p=0.014) and variety (p<0.001). These interactions are clear from the F_v/F_m data in Table 5.1, which shows that there was a significant difference between NILs of Malacca (p<0.001) and Alchemy (p<0.001) in 2013 only. However, the direction of this effect was not consistent between the two varieties; Malacca *Iw1+* flag leaves had higher F_v/F_m than *iw1-*, yet in Alchemy this effect was reversed. Furthermore, for all flag leaves the F_v/F_m values were within the healthy range,

approximately 0.83 (Björkman & Demmig, 1987), indicating that PSII of all flag leaves tested was fully functioning. No difference in F_v/F_m between NILs of any variety was recorded in 2014.

Taking account of all years and varieties there was a significant effect of *Iw1* on *Fo* ($p<0.001$) and F_m ($p<0.001$). However, for both of these parameters there was again a statistically significant interaction of *Iw1* with year (F_o, $p<0.001$; F_m, $p<0.001$), and for F_o an interaction with variety ($p<0.001$). Pairwise comparison revealed that, as for F_v/F_m, there were only significant effects in 2013, and the direction of change was inconsistent between varieties.

In conclusion, *Iw1*, and absence of β- and OH-β-diketones from the epicuticular waxes, has no effect on chlorophyll fluorescence parameters. Although significant, minor changes observed in 2013 were not seen in any other year.

5.4.1.2 Light adapted chlorophyll fluorescence

The quantum yield of PSII can also be measured in the light adapted state to further understand efficiency and function. Light adapted ΦPSII was measured at levels of PAR between 0 and 1500 µmol m^{-2} s^{-1} using a LICOR-6400XT in the field over two years at anthesis (Figure 5.1).

An overall analysis combining the two years for Hereward NILs (Figure 5.1a and 5.1b) showed that *Iw1+* flag leaves had significantly higher ΦPSII than *iw1-* at 1500 µmol m^{-2} s^{-1} ($p = 0.01$) and 1000 µmol m^{-2} s^{-1} ($p = 0.036$), and there was no interaction between year and *Iw1* (1500 µmol m^{-2} s^{-1}, $p = 0.710$; 1000 µmol m^{-2} s^{-1}, $p = 0.870$). However, pairwise comparison of Hereward NILs within each year individually revealed no significant differences, although the overall trend is clear. Figure 5.1c shows Alchemy NILs in 2013. *Iw1+* flag leaves had significantly higher ΦPSII than *iw1-* at 1500 µmol m^{-2} s^{-1} ($p = 0.030$) and 1000 µmol m^{-2} s^{-1} ($p = 0.024$). However this was not the case at any other light level in 2013, and no difference between NILs was recorded in 2014 (Figure 5.1d). For Malacca NILs (Figure 5.1e and 5.1f) there was no effect of *Iw1* on ΦPSII in either year.

The subtle increases in ΦPSII observed in Hereward and Alchemy *Iw1+* NILs indicates there is potentially some small effect of the introgressed region containing *Iw1* on ΦPSII. However, it is unlikely that this is due directly to the epicuticular waxes. There is no difference in the wax profile of the three varieties (Chapter 3) and the effect was not seen at all in the Malacca NILs, nor consistently in Alchemy. Furthermore, whether or not these small increases would make a significant difference to plant productivity in the field is questionable.

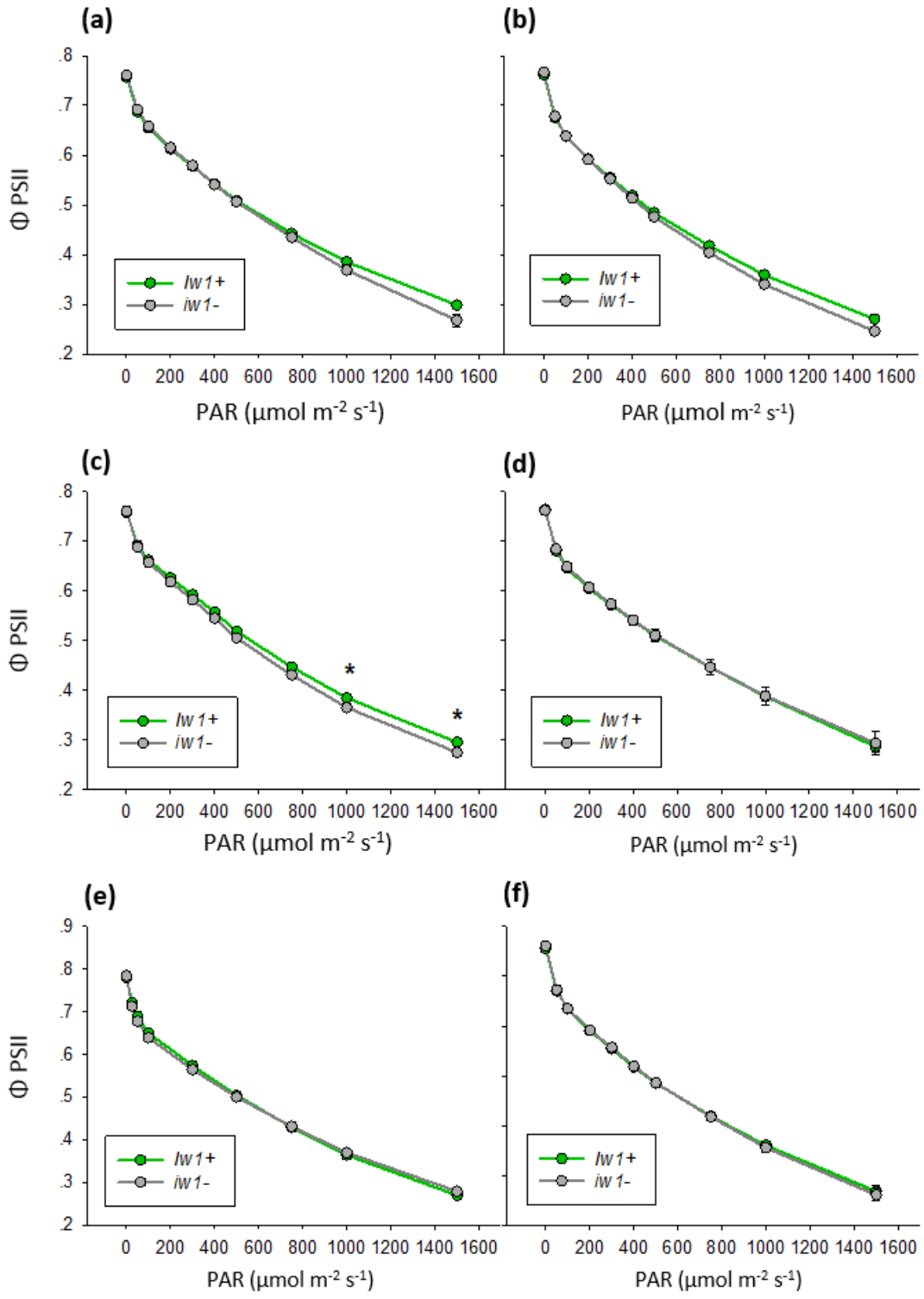

Figure 5. 3 Light adapted chlorophyll fluorescence measurement of flag leaves in the field

ΦPSII (F_v'/F_m') at light levels between 0 and 1500 µmol m^{-2} s^{-1} was measured for flag leaves at anthesis for Hereward in (a) 2014 and (b) 2015, Alchemy in (c) 2013 and (d) 2014 and Malacca in (e) 2013 and (f) 2014. Significance by pairwise comparison is indicated at the level $p<0.05$ (*). N=4, error bars = S.E.

5.4.2 Transpiration

The second component of WUE is transpiration, a combination of cuticular and stomatal conductance. In order to understand how inhibition of β- and OH-β-diketones within the epicuticular waxes affects water loss, various methods were used to measure water loss through the stomata and cuticle.

5.4.2.1 Cuticular conductance

Water movement through the cuticle when the stomata are closed is often reported to be affected by epicuticular waxes (Monneveux *et al.*, 2004; Zhang *et al.,* 2015). To assess the effect of *Iw1* on cuticular conductance, water saturated flag leaves were dehydrated in the dark for 120 min, and fresh weight measured every 20 min for field grown NILs of Malacca, Alchemy and Hereward (Figure 5.4).

Pairwise comparison between NILs within each year showed that for no variety was there a significant difference in percentage water content between NIL pairs at any time point over the two hr time period. Additionally, an overall ANOVA revealed no effect of *Iw1* on rate of water loss ($p=0.853$), although there was an effect of year on the rate of water loss ($p<0.001$). This is clear from the comparison between 2013 and 2014 for Alchemy (Figure 5.4a and 5.4b) and Malacca (Figure 5.4c and 5.4d). The summer of 2013 was very dry with little precipitation and low relative humidity in comparison to 2014 (Chapter 2 Figure 2.2). These differences in weather conditions between years could be responsible for changes to development of the cuticle and thus permeability to water. However, there was no interaction between year and *Iw1* ($p=0.385$), indicating that the differences between NILs were not influenced by changes in environmental conditions between years.

After 120 min, leaves were left to dehydrate in the dark for a further 2 hr, and weighed again at 240 min and then at either 260 or 280 min (Table 5.2). Pairwise comparison showed that for all three varieties there was still no difference between NILs in percentage water content at any of these final time points. This shows that NILs of all varieties were losing water at the same rate across the full four hr time frame of this experiment.

These results indicate that presence of OH- β- and β-diketones in the epicuticular waxes does not affect water loss through the flag leaf cuticle in these wheat varieties.

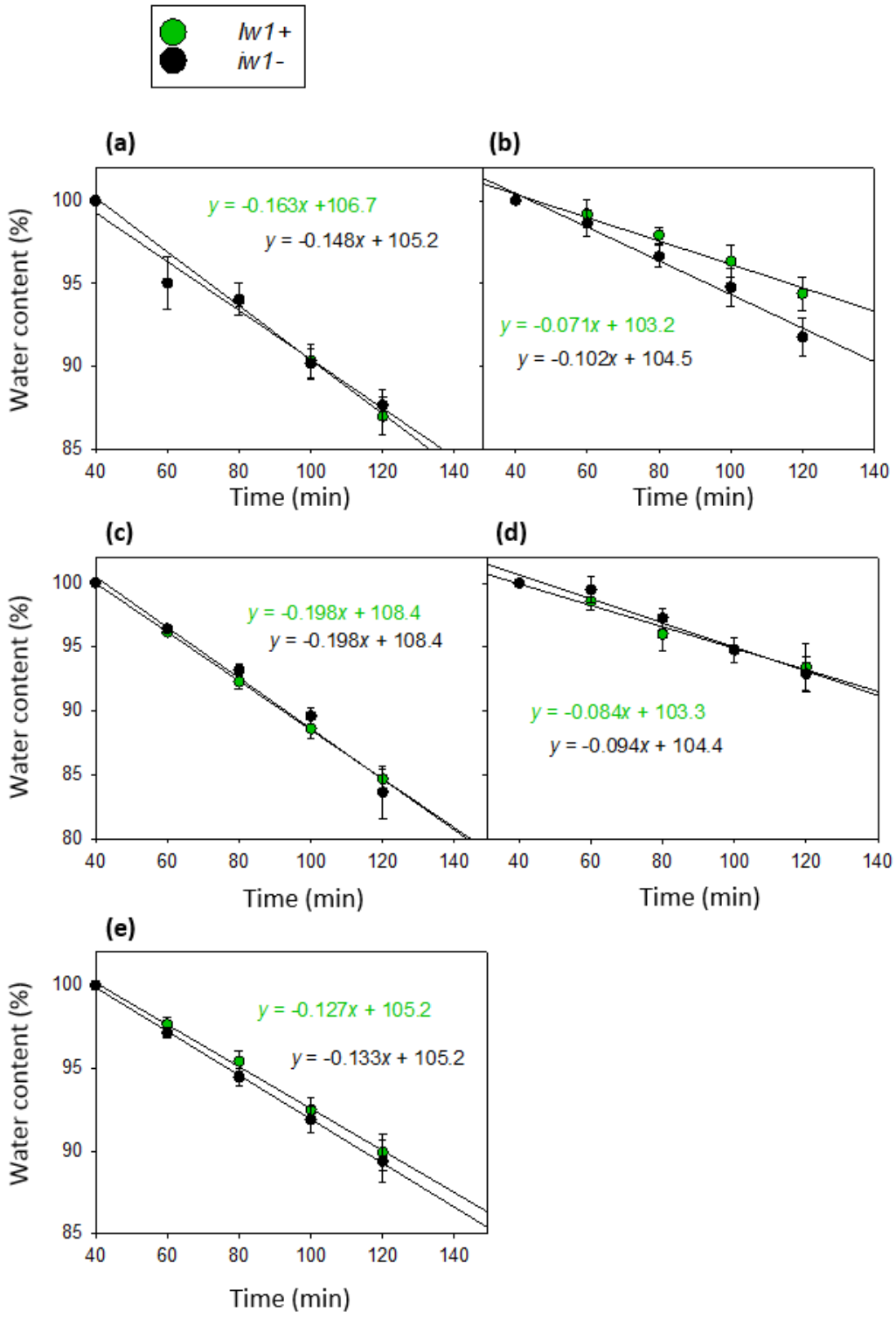

Figure 5. 4 Cuticular conductance of flag leaves at anthesis

Water loss through the cuticle when stomata were shut was measured over a period of 120 min. Water content at 40 min was taken as 100%, and water content at each subsequent time point calculated as a percentage of this. This was done for field grown flag leaves of Alchemy NILs in (a) 2013 and (b) 2014, Malacca NILs in (c) 2013 and (d) 2014, and (e) Hereward NILs in 2015 only. Equations of regression lines are displayed on each chart; *Iw1+* in green and *iw1-* in black. For no variety or year was there a significant difference between NILs in terms of water content at any time point, or rate of water loss over the 2 hr. N= 15 in 2013, 5 in 2014, 5 in 2015. Error bars = S.E.

Table 5. 2 Percentage water content of flag leaves after 240 - 280 minutes of dehydration

Dark adapted flag leaves were dehydrated in the dark for four hours. Fresh weight was recorded after four hours (240 min) and then again at either 260 or 280 min. Dry weight was used to calculate water content at both these time points as a percentage of water content after 40 min of dehydration (100%). This was done for field grown flag leaves of Malacca and Alchemy NILs over two years, and Hereward NILs in 2015 only. There was no significant difference at any time point in terms of water content between NILs. N= 15 in 2013, 5 in 2014, 5 in 2015.

			Water content (%)					
			Malacca		Alchemy		Hereward	
Year	Time point	*Iw1*	Average ± S.E	p	Average ± S.E	p	Average ± S.E	p
2013	240	*Iw1+*	74.78 ± 1.49	0.535	75.90 ± 3.02	0.166		
		iw1-	76.08 ± 1.43		80.44 ± 1.10			
	280	*Iw1+*	74.92 ± 3.96	0.335	72.36 ± 4.07	0.207		
		iw1-	69.81 ± 3.41		77.70 ± 1.23			
2014	240	*Iw1+*	82.87 ± 0.78	0.282	85.33 ± 0.86	0.507		
		iw1-	85.19 ± 1.65		84.40 ± 1.03			
	260	*Iw1+*	83.71 ± 1.38	0.838	84.15 ± 0.93	0.215		
		iw1-	84.24 ± 1.88		82.74 ± 0.82			
2015	240	*Iw1+*					78.89 ± 2.45	0.778
		iw1-					77.95 ± 2.10	
	260	*Iw1+*					76.68 ± 2.55	0.811
		iw1-					75.75 ± 2.18	

5.4.3.2 Stomatal conductance

Stomatal conductance relative to assimilation at various light levels between 0 and 1500 µmol m^{-2} s^{-1} was recorded in the field at anthesis using a LI-COR 6400 XT (Figure 5.5). The ratio of stomatal conductance to assimilation was calculated at each PAR level, and analysed by pairwise comparison.

In 2014 for Hereward (Figure 5.5a) and Alchemy (Figure 5.5d) *Iw1+* flag leaves tended to have higher stomatal conductance than *iw1-* leaves at equivalent levels of carbon assimilation. However, these differences between NILs in terms of stomatal conductance relative to assimilation were not statistically significant for either Hereward or Alchemy. Unfortunately the 2013 Alchemy (Figure 5.5c) and 2015 Hereward data (Figure 5.5b) show very low levels of stomatal conductance so could be unreliable and have been excluded from interpretation. However, the trend for *Iw1+* flag leaves to have higher stomatal conductance is still present within both of these data sets. For Malacca (Figure 5.5e and 5.5f) this trend was absent in both years and there were again no significant differences between NILs.

Overall, these data indicate that stomatal conductance is not significantly affected by presence of OH- β- and β-diketones in the epicuticular waxes. NILs of all varieties were equally efficient in terms of water lost through stomatal conductance relative to carbon assimilation.

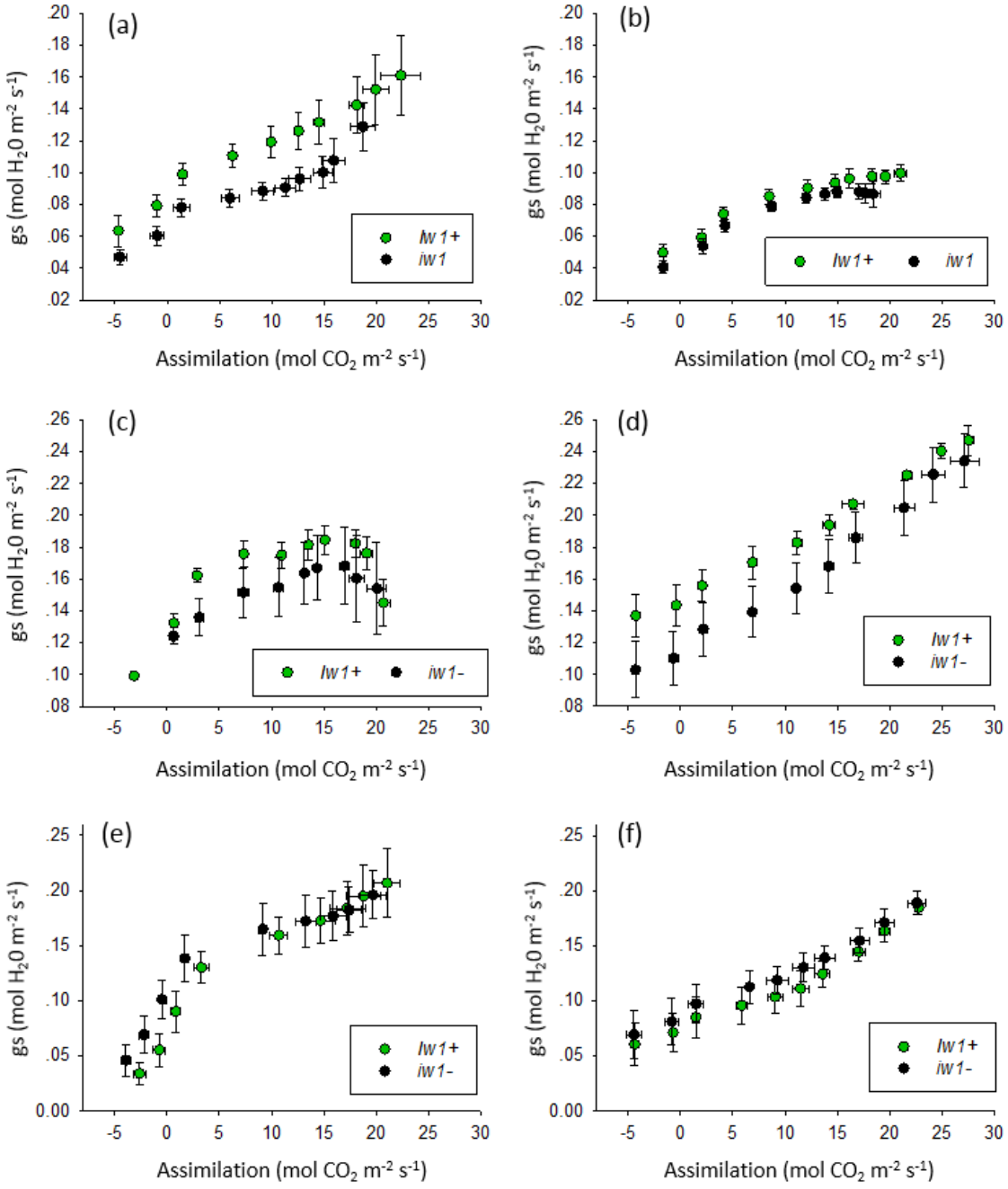

Figure 5. 5 Stomatal conductance (g_s) and carbon assimilation at a range of PAR levels

Measurements were taken between 0 and 1500 µmol m^{-2} s^{-1} in the field at anthesis for Hereward in (a) 2014 and (b) 2015, Alchemy in (a) 2013 and (b) 2014 and Malacca in (e) 2013 and (f) 2014. The ratio of stomatal conductance to assimilation was calculated at each light level and analysed by pairwise comparison between NILs. There was no significant difference between NILs of any variety in any year in terms of stomatal conductance relative to assimilation at any light level. N=4, error bars = S.E.

5.4.2.3 Stomatal density

To further understand how stomatal development and function might be affected by *Iw1*, the stomatal density of flag leaf samples collected in 2014 and 2015 was investigated. Stomata were counted on both the adaxial and abaxial leaf surfaces and the number of stomata per mm^2 leaf area was calculated (Figure 5.6).

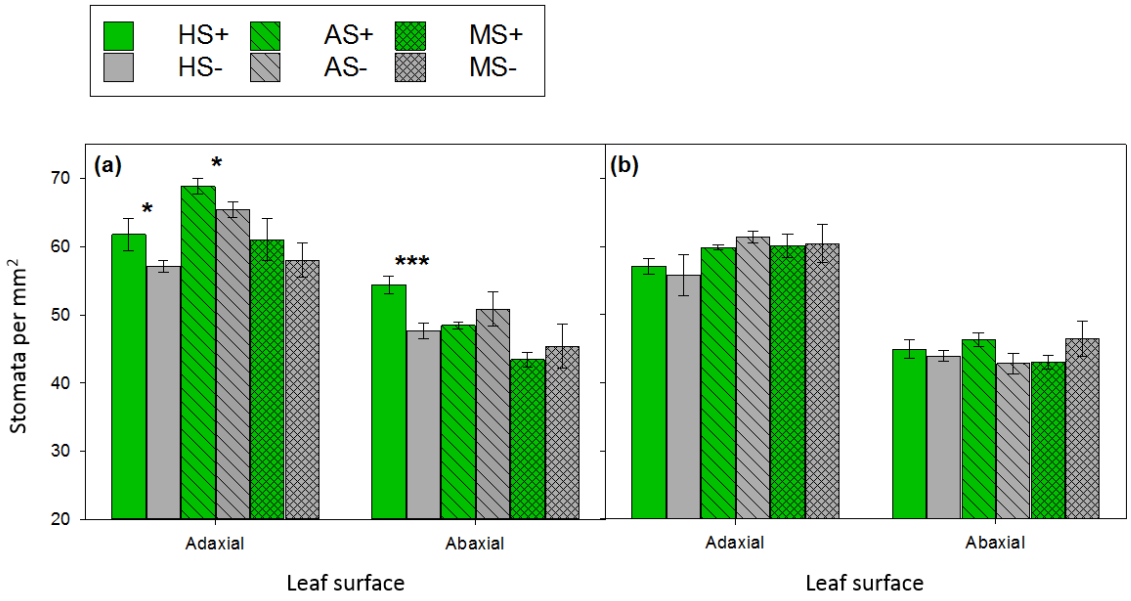

Figure 5. 6 Stomatal density on the abaxial and adaxial surfaces of the flag leaf

Number of stomata were counted per mm^2 at anthesis for NILs of Hereward, Alchemy and Malacca NILs from samples collected in (a) 2014 and (b) 2015. Significant differences by pairwise comparison are indicated at p<0.05 (*), p<0.001 (***). N=3, error bars = S.E.

In 2014 on the adaxial surface there was an overall significant difference in stomatal density between NILs (p=0.001); *Iw1+* NILs had more stomata per mm^2 than *iw1-*, significantly so in Hereward (p=0.021) and Alchemy (p=0.044). There was also an overall significant effect of *Iw1* on the abaxial leaf surface (p<0.001). However, in this case only for Hereward did *Iw1+* flag leaves have significantly more stomata per mm^2 than *iw1-* leaves (p<0.001). For the other two varieties there was no significant difference between NILs and the effect was actually reversed, as supported by the significant interaction between *Iw1* and variety (p=0.003).

In 2015 there was overall no significant difference in stomatal density between NILs on the adaxial surface (p=0.255), and no interaction with variety (p=0.663). There was also no significant effect of *Iw1* on the abaxial surface (p=0.844). Pairwise analysis of 2015 data revealed no effect of *Iw1* within any variety on either surface.

Overall, taking both years into consideration, it appears that the epicuticular waxes do not consistently affect stomatal density. Although there was a small effect in 2014 on the adaxial surface, this was not observed consistently in all years or varieties. This effect could have been due to natural variation in the plants, or a very specific plant interaction with the environment in that year.

5.4.3 Overall water use efficiency

WUE is a function of transpiration and carbon assimilation, both of which have been explored individually in relation to glaucousness. Combining both factors together into a single value is essential to understand overall water use of the plant.

5.4.3.1 Transpiration versus Assimilation

Carbon assimilation at PAR levels between 0 and 1500 µmol m^{-2} s^{-1} was measured in the field at anthesis over two years (data are presented in Chapter 4). Transpiration, the sum of water loss through both the stomata and cuticle, was recorded simultaneously. WUE at each light level was then calculated using the formula WUE = Assimilation/ Transpiration.

Figure 5.7 shows that there was no significant difference in instantaneous WUE between NILs of any variety in either year of measurement. It seems the significantly higher carbon assimilation recorded for Hereward *Iw1+* NILs in Chapter 4 did not translate into significantly higher WUE. These data indicate that *Iw1* does not affect WUE in these varieties within a UK environment.

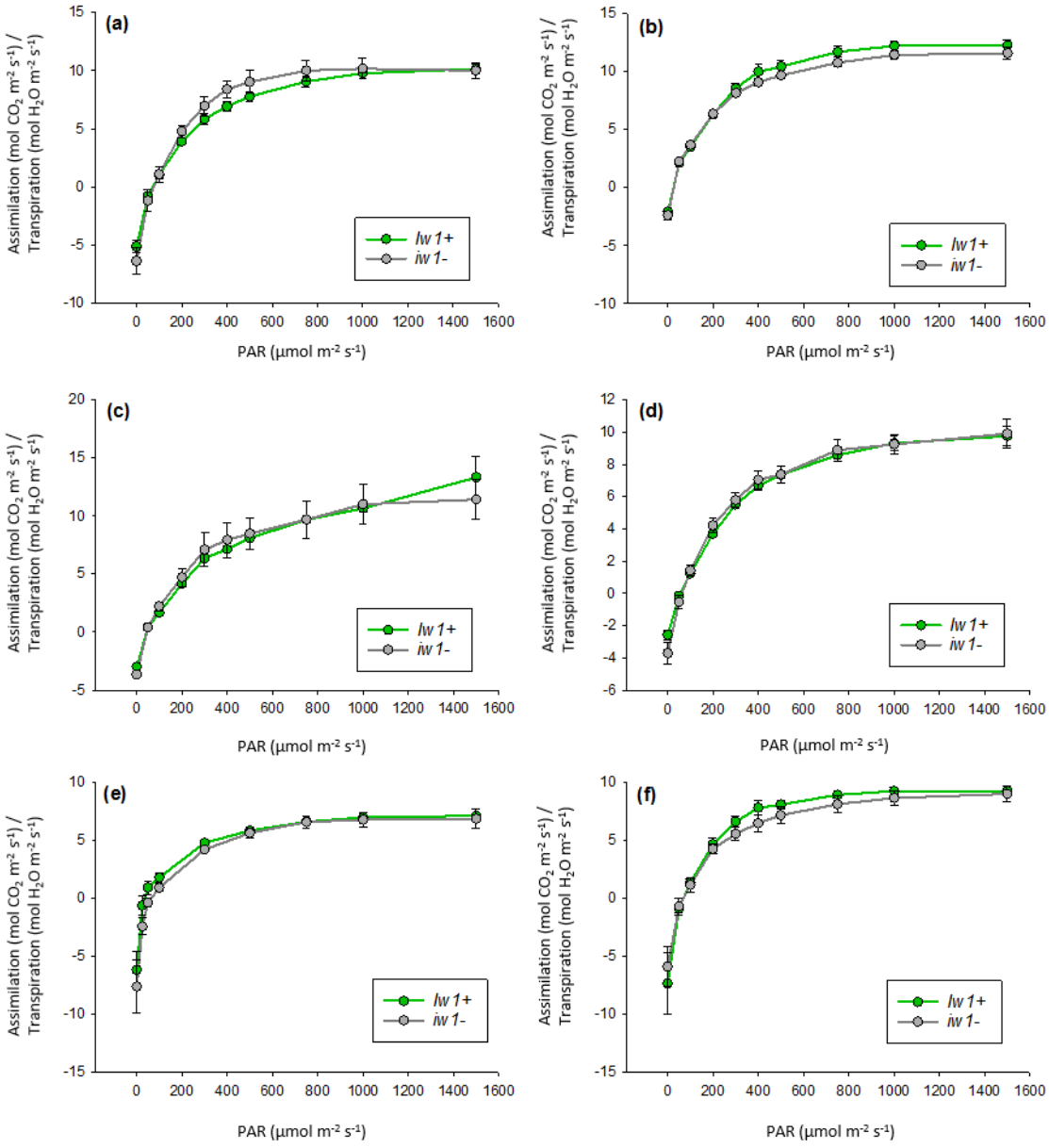

Figure 5. 7 Instantaneous water use efficiency for flag leaves at anthesis

Carbon assimilation/ Transpiration at PAR levels between 0 and 1500 µmol m^{-2} s^{-1} was measured in the field for NILs of Hereward in (a) 2014 and (b) 2015, Alchemy in (c) 2013 and (d) 2014 and Malacca in (e) 2013 and (f) 2014. Analysis by pairwise comparison shows that there was no significant difference between NILs of any variety in terms of WUE at any light level. N=4, error bars = S.E.

5.4.3.2 Carbon isotope discrimination ($\Delta^{13}C$)

Measurement of assimilation and transpiration using gas exchange shows potential WUE at one time point in the growing season. However, environmental conditions and plant physiology fluctuate over the growing season. $\Delta^{13}C$ provides an estimate of WUE that takes into account many factors including major components of transpiration and carbon assimilation. Furthermore these

factors are integrated over the growing season, allowing understanding of what the plant is actually doing in the field.

$\Delta^{13}C$ of flag leaves sampled at anthesis and 40 days post anthesis (DPA) was measured in 2014 (Figure 5.8). Overall there was no effect of *Iw1* on $\Delta^{13}C$ at anthesis p= 0.145 (Figure 5.8a), neither was there an effect of variety (p=0.139). When analysed by pairwise comparison within varieties there were no significant differences between NILs of any variety. Similarly, at 40 DPA (Figure 5.8b) there was no effect of *Iw1* on $\Delta^{13}C$ (p=0.562).

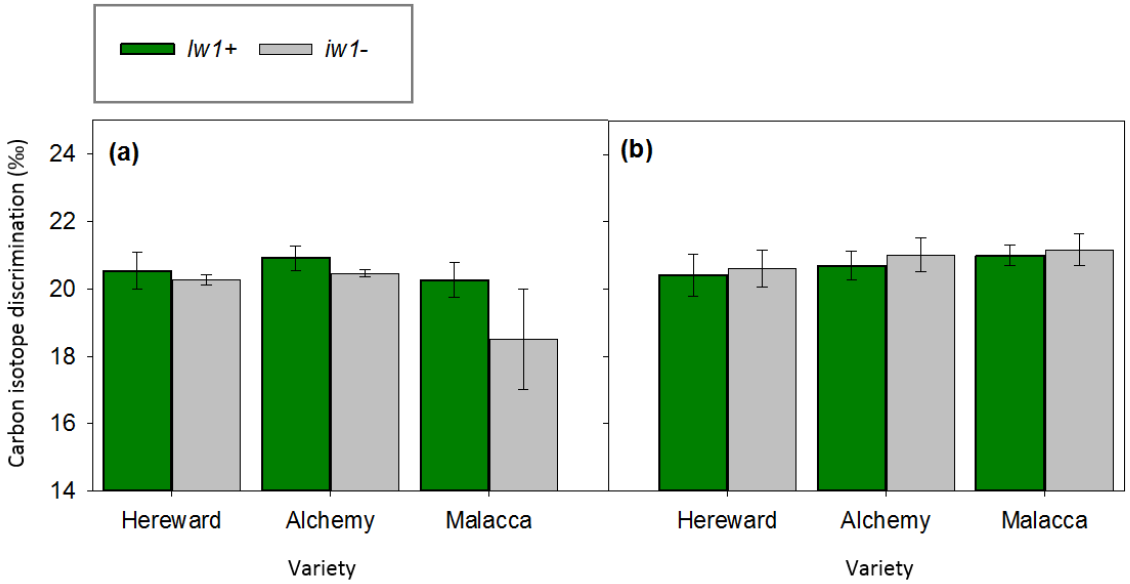

Figure 5. 8 Carbon isotope discrimination of field growth flag leaves

Measured at (a) anthesis and (b) 40 days post anthesis for NILs of Hereward, Alchemy and Malacca in 2014. For no variety was there a significant difference between NILs at either sampling time point. N=3, error bars = S.E.

$\Delta^{13}C$ of the Malacca and Alchemy *Iw1* NILs has previously been measured in various tissues at anthesis and senescence (GS93), and no difference was found between NILs (Adamski *et al.,* 2013), a result that has been verified by the 2014 field data presented here. To further confirm the result in Hereward, a variety not previously studied, flag leaf samples were also collected in the field at anthesis in 2015. *Iw1+* NILs had an average $\Delta^{13}C$ of 19.03 ± 0.04 ‰, and *iw1-* a value of 19.11 ± 0.10 ‰. This difference between the NILs of 0.08 ‰ was not significant (p = 0.487). Together with measurement of instantaneous gas exchange, these data provide evidence that there is no effect of non-glaucousness as conferred by *Iw1* on WUE over the growing season in these wheat varieties in a UK environment.

5.5 Discussion

Through measurement of a number of gas exchange and chlorophyll fluorescence parameters in the field in addition to use of stable carbon isotopes, this chapter set out to address the hypothesis that non-glaucousness, as conferred by *Iw1*, does not affect WUE within an environment such as the East of England. This hypothesis was opposing to the findings of many previous studies regarding glaucousness, which found a difference between glaucous and non-glaucous plants in WUE both when using gas exchange and carbon isotopes. However, many of these past studies were carried out in Mediterranean climates, where plants experience frequent water stress under the hot and dry conditions. This is quite different to UK conditions, where water supply during the growing season is usually adequate and drought is not currently a major issue.

5.5.1 *Iw1* had no effect on carbon assimilation

Chapter 4 investigated carbon assimilation of the flag leaf at varying light levels, and found that absence of β- and OH-β-diketones from the epicuticular waxes had no effect on photosynthesis. The chlorophyll fluorescence parameters investigated in this chapter further support this conclusion. There was no correlation between glaucousness and chlorophyll fluorescence for either the dark or light adapted measurements, indicating that there was no effect of *Iw1* on PSII capacity. Even within 2013, where significant differences were recorded between NILs for F_v/F_m, all flag leaves were within the range of a healthy plant (Björkman & Demmig, 1987). This indicates that all NILs were responding in the same way to the environment in terms of stress, and capacity of PSII was not compromised by presence, or absence, of *Iw1*.

Interestingly Hereward *Iw1+* NILs did have significantly higher ϕPSII at higher levels of light. This corresponds to the Chapter 4 carbon assimilation data, for which *Iw1+* NILs had higher photosynthetic rate at these same light levels. It is possible that increased ϕPSII capacity could be a contributing factor to the observed increase in photosynthesis. However, as discussed before, it appears that this increase in photosynthesis is not related directly to *Iw1* itself, but is a result of one, or several closely linked genes within the *Iw1* introgression region.

The next step to ascertain the underlying mechanism causing the increased photosynthesis of Hereward *Iw1+* NILs would be A_{Ci} curves; measurement of carbon assimilation at varying levels of carbon dioxide whilst light is held at a constant, saturating value. If the increased photosynthesis of Hereward NILs is not due to an effect of epicuticular waxes on light availability, A_{Ci} curves could shed light on other possible contributing factors such as maximum electron transport rate and catalytic activity of the enzyme Rubisco (Farquhar *et al.,* 1980). These measurements were

attempted during the summer of 2015, but data collected were not useable due to issues with stomatal conductance. A_{Ci} curves should be a priority for future work on the Hereward NILs.

5.5.2 *Iw1* did not affect water movement through the flag leaf cuticle

A key function of the cuticle is widely recognized to be prevention of excessive water loss through the plant surface. The Malacca, Alchemy and Hereward varieties used in the present work showed no difference between glaucous and non-glaucous NILs in terms of cuticular conductance across a four-hour experiment. This leads to the conclusion that the presence of β- and OH-β-diketones in the epicuticular waxes does not affect water movement across the cuticle of the flag leaf.

This is the opposite conclusion to that drawn in a recent study in wheat specifically linking presence of β-diketones to a 15% increase in water loss over a 4 hour dehydration of the flag leaf-sheath (Zhang *et al.,* 2015). This study focussed on bread wheat with wax locus *W3*, which confers glaucousness through the presence of OH- β- and β-diketones. The non-glaucous plants in this 2015 study, rather than conferring a functional copy of *Iw1* or *Iw2,* had two non-functional copies of *W3* (genotype *w3*/*w3*). Where glaucousness is conferred in the absence of *Iw1* in the present study, primary alcohols are the most abundant compound, followed by β-diketones and then *n*-alkanes (Chapter 3). However, although appearing visually similar, the most abundant compound in the *W3* plants of Zhang *et al.,* (2015) were reported to be *n*-alkanes contributing 63.3% to total wax load, followed by β-diketones, with only trace amounts of primary alcohols present. The opposing conclusions reached regarding cuticular conductance could be a consequence of these differing wax compositions. There could be some interaction between β-diketones and *n*-alkanes that results in reduced cuticle permeability that doesn't occur when primary alcohols are the major component. An alternative explanation could be that cuticle permeability is affected by wax load. The non-glaucous plants lacking functional *W3* had significantly reduced wax load as no other compound was upregulated to account for loss of β-diketones. In contrast, the difference in wax load between the *Iw1* NILs is less dramatic because β-diketones make up a smaller proportion of the glaucous waxes of *iw1*- NILs, and upregulation of *n*-alkanes in *Iw1+* slightly compensates for the loss of diketone compounds.

In addition to the Zhang *et al.* (2015) study, a number of others have investigated glaucousness in relation to cuticle permeability in wheat and barley with varying results. For example Merah *et al.* (2000) and Febrero *et al.* (1998) find no effect of glaucousness on cuticle permeability supporting the result in the *Iw1* NILs, whilst Monneveux *et al.* (2004) find water movement through the cuticle to decrease with glaucousness. An issue with interpreting the results of these earlier studies is that they do not provide a precise biochemical profile of the epicuticular waxes in question. Because

there can be many forms of glaucousness, this makes it difficult to determine the reasons for any discrepancy between studies. However, the relationship between epicuticular wax biochemistry and water permeability has been studied in detail in species other than cereal crops.

Work using Brazilian species from the Caatinga and Cerrado, in addition to a study in *Jatropha mollissima*, show that *n*-alkanes and tripterpenes are the most effective barriers to water loss (Figueiredo *et al.,* 2012; Oliveira *et al.,* 2003). However, in the *Iw1* NILs, non-glaucous *Iw1+* NILs of all varieties had significantly higher quantities of *n*-alkanes than glaucous *iw1*- NILs (See Chapter 3 and Adamski *et al.,* 2013). This indicates that these additional quantities of *n*-alkanes were either not enough to affect cuticle permeability, or that in wheat n-alkanes do not have an important role in cuticular conductance. A study in barley found poor correlation between cuticular conductance and quantity of a number of wax components including *n*-alkanes, aldehydes, primary alcohols, fatty acids and esters (Larsson & Svenningsson, 1986).

A second finding of these studies using the Brazilian species was that plants with significantly reduced cuticular conductance had compounds with longer than average chain length in their epicuticular waxes (Figueiredo *et al.,* 2012; Oliveira *et al.,* 2003). This is supported by the work of Macková *et al.,* (2013), who found that the epicuticular waxes of *Lepidium sativum* plants subjected to simulated drought stress had aliphatic compounds with significantly longer chain lengths than those not under stress. Fatty acid, alcohol and *n*-alkane chain lengths of C26 and longer were up-regulated under stress, whilst chain lengths shorter than this were down-regulated. Presence of longer chain lengths could lead to higher hydrophobicity of the cuticle and decrease cuticular water loss. In the *Iw1* NILs, whilst there were some differences in quantity of various compounds between NILs, compound classes of the same chain length were present in all NILs, which could explain the lack of difference in terms of cuticular conductance. This is a theory that has not yet been explored in cereal crops.

Further work is required to ascertain if differences in underlying wax biochemistry are causing these differing conclusions regarding glaucousness and cuticle permeability in wheat and barley. An alternative explanation could be that the extent to which cuticular conductance is affected by epicuticular waxes varies according to tissue type and organ studied. For example, in the *Iw1* NILs only the flag leaf was considered whereas Zhang *et al.* (2015) studied the flag leaf sheath. There is some evidence in wheat that where the flag leaf is concerned epicuticular waxes play only a minor role in cuticular transpiration, with other factors being more important (Araus *et al.,* 1991; Johnson *et al.,* 1983; Richards *et al.,* 1986). For example, Araus *et al.,* (1991) suggest that deposition of silica in the cuticle is important in the upper leaves of cereal crops and may be a key factor in leaf water

permeability. However, this conclusion regarding the role of epicuticular wax in flag leaf permeability to water has been drawn using plants where glaucousness only affected the abaxial surface of the flag leaf. In the *Iw1* NILs both the adaxial and abaxial leaf surfaces have the same type of wax. It would be of interest to investigate cuticular conducatance in organs other than the flag leaf in the *Iw1* NILs to confirm if results vary between different tissues. There is also evidence to suggest that intracuticular waxes are in fact more important than epicuticular waxes in terms of functioning as a barrier to transpiration, whereas epicuticular waxes are more involved in wettability and light reflection (Koch & Ensikat, 2008; Svenningson & Liljenberg 1986). This is something that should be further investigated in the future using the *Iw1* NILs as in the bulk of work to date no distinction has been made between the intra or epicuticular waxes.

Some of the discrepancies between studies with regards to cuticular conductance could be a function of the methodology adopted by different research studies. Separating out the contributions of stomatal and cuticular conductance to overall water loss is not easy. Cuticular transpiration is defined as transpiration at maximum stomatal closure, so the term can only be correctly applied when stomata are totally closed, or shut so tightly that their contribution to transpiration is negligible (Kerstiens, 1996). However, measurements of transpiration under dark conditions, in which stomata should be closed, show that epidermal conductance may be correlated with stomatal density (Muchow & Sinclair, 1989) indicating that stomata do not fully close (although not all studies agree on this (Araus *et al.,* 1991). Therefore, results of cuticular transpiration need to be interpreted with caution due to uncertainty over the extent of stomatal contribution, which could vary between studies and methods used. For example, the work of Zhang *et al.* (2015) does not state that measurement of cuticular conductance was carried out in the dark.

5.5.3 There was no consistent effect of *Iw1* on stomata

To assess whether non-glaucousness conferred by *Iw1* has any impact on regulation of plant water loss via the stomata, both stomatal conductance under optimal conditions and stomatal density of field grown flag leaves were measured.

5.5.3.1 Stomatal conductance

Overall there was no significant effect of *Iw1* on stomatal conductance. Particularly with regards to Malacca, both *Iw1+* and *iw1-* NILs had identical levels of stomatal conductance at equivalent levels of carbon assimilation. In Alchemy and Hereward however, there was a (non-significant) trend over both years for *Iw1+* flag leaves to have around 10-20% higher stomatal conductance relative to *iw1-*, which might indicate that the tubular waxes of glaucous plants are functioning to subtly reduce stomatal conductance as has previously been suggested (King & von Wettstein-Knowles,

2000). This is comparable to the findings of Johnson *et al.,* (1983), who found a non-significant increase in stomatal conductance of around 8% associated with non-glaucousness in wheat. Much greater increases in stomatal conductance of 30-60% were associated with non-glaucousness by Richards *et al.,* (1986), although this study was carried out within a glasshouse. Further hard evidence of any link between glaucousness and stomatal conductance or the mechanism by which this may happen, is lacking from the literature. The distinction seen here between the Malacca NILs and the other two varieties in terms of stomatal conductance in relation to glaucousness provides an opportunity and resource to study the link between epicuticular waxes and stomatal conductance. Investigation of the distribution and concentration of biochemicals comprising epicuticular wax (Chapter 3) showed that *Iw1* functions the same within all three varieties, and there was no statistical interaction between *Iw1* and variety for any major wax component. It is therefore not differences in wax composition between varieties that is affecting stomatal conductance. However, a detailed study of exactly how wax structures are arranged across the surface of the flag leaf (and other organs) in the *Iw1* NILs has not been carried out. For example, one possibility is that glaucous flag leaves of Alchemy and Hereward have tubular epicuticular waxes more heavily clustered around stomatal openings than Malacca flag leaves.

5.5.3.2 Stomatal density

The relationship between stomatal density and epicuticular wax genes has been investigated in the literature more than any link with stomatal conductance. However, whilst studies in Arabidopsis show that the type of epicuticular wax may be linked with stomatal patterning and development, a large variety of effects have been observed and no clear conclusion about this relationship has been reached (Aharoni *et al.,* 2004; Gray *et al.,* 2000; Yang *et al.,* 2011; Zeiger & Stebbins, 1972). Moreover, there is limited evidence of any link in cereal crops. In keeping with this, data from the *Iw1* NILs in 2015 shows no effect of glaucousness on stomatal density on either the abaxial or adaxial surface. In 2014 (on the adaxial surface only) *Iw1+* NILs overall had significantly more stomata than *iw1-*. However, the change was subtle and it is possible that this 2014 effect was simply due to natural variation in the plants. Alternatively there could be some genotype x environment interaction whereby *Iw1* increases stomatal density only under particular conditions present in the field in 2014 and not 2015. For example 2014 overall was warmer and more humid than 2015 (Chapter 2), a set of conditions that might influence action of *Iw1*. This work on stomatal density on the *Iw1* NILs is by no means comprehensive; it provides a preliminary conclusion that any effect, if at all, of *Iw1* on stomatal numbers is subtle and inconsistent. Other components of stomatal development such as stomatal size and clustering have not yet been assessed.

Any potential effect of *Iw1* on stomatal development does not appear to affect water loss through the stomata; the patterns observed across varieties and years in stomatal conductance did not appear to correlate with stomatal density. For example, the significantly increased stomatal density in 2014 for *Iw1+* NILs did not translate to a significant increase in stomatal conductance. There is both evidence that stomatal density correlates with cuticular conductance (Muchow & Sinclair, 1989) , and that there is no link between the two (Araus *et al.,* 1991). No difference was observed in terms of cuticular conductance for the *Iw1* NILs, indicating that any minor changes in stomatal density were not affecting water movement across the cuticle.

5.5.4 *Iw1* did not affect water use efficiency within a UK environment

Measurement of transpiration in relation to photosynthesis through gas exchange revealed no difference between NILs of any variety. From this I conclude that *Iw1* induced non-glaucousness does not affect instantaneous WUE within a UK environment. Even for Hereward, where *Iw1+* had significantly higher carbon assimilation than *iw1-* (Chapter 4), there was no difference in water use, indicating that *Iw1+* NILs also increased their transpiration. Comparable work using gas exchange measurements has found glaucous wheat to be more WUE than non-glaucous only under drought stressed conditions (Richards *et al.*, 1986). A later study measured WUE in 4 lines of Sorghum, each with a normal (glaucous) and bloomless (non-glaucous) mutant. They only found glaucousness to confer an advantage in one line under irrigated conditions, with no effect of glaucousness on WUE in the other three. However, under drought conditions 3 out of the 4 lines demonstrated significantly increased WUE associated with glaucousness (Premachandra *et al.,* 1994). This would explain why no difference between *Iw1* NILs was observed in the present work. In Norfolk, where these plants where grown, plants were not subject to severe water stress during growth.

Gas exchange measurement provides only an instantaneous measure of WUE at the single leaf level, and conclusions drawn could be quite different from the reality in the field. However, conclusions from the gas exchange work were confirmed by $\Delta^{13}C$, which provides an integrative measurement of WUE right across the growing season in the field. No difference in $\Delta^{13}C$ was recorded in flag leaf tissue between NILs of any variety either at anthesis, at which point photosynthates from the flag leaf start to fill the grain, or just prior to senescence when the plant is at physiological maturity and grain are fully formed. This confirms finding from previous work using these same *Iw1* NILs, in which no difference in $\Delta^{13}C$ of flag leaf, spike or grain was found between NILs (Adamski *et al.,* 2013) . The combination of the data presented by Adamski *et al.,* (2013) and the data collected in 2014 and 2015 field trials conclusively shows that there is no difference in WUE in the field between *Iw1* NILs. This supports the evidence from studies using

both gas exchange (Premachandra *et al.,* 1994) and carbon isotope measurements (Merah *et al.*, 2000) that suggest glaucousness only effects WUE under drought stress.

5.5.5 Conclusions

The data presented in this chapter provide evidence that there is no difference between *Iw1* NILs in terms of WUE within a UK environment. Currently, the literature is lacking in studies investigating glaucousness and water use within the UK with regards to glaucousness. Additionally there is a lack of specific information on the genetic and biochemical basis for the types of glaucousness presented within existing studies. The combination of these factors makes it difficult to ascertain whether the lack of difference in WUE observed here is due to environmental conditions, the specific type of non-glaucousness conferred by *Iw1*, or a combination of the two. To understand this, glaucous Mediterranean wheat varieties with glaucousness could be used to generate *Iw1* near isogenic lines. These NILs could be grown under water stress in a Mediterranean environment and WUE assessed.

Chapter 6: The role of glaucousness in modulating plant – light interaction

6.1 Summary

Iw1 inhibits β- and OH-β-diketones in the epicuticular waxes. In Chapter 4 the β- and OH-β-diketones were shown to be responsible for an increase in PAR reflectance of the flag leaves of 15-40% from the flag leaf surface. In the field the crop canopy of *iw1*- NILs reflected around 12-20% more PAR than *Iw1+*. It has been hypothesised that this difference could alter the way glaucous and non-glaucous plants respond to environmental conditions in terms of light availability. The increased reflectance of *iw1*- NILs may provide photoprotection, enabling glaucous plants to withstand higher levels of light stress, whilst the reduced reflectance of non-glaucous *Iw1+* NILs may provide an advantage when light levels are sub-optimal.

Many wheat growing regions frequently experience light levels in excess of the light saturation point for wheat (1000-1500 µmol m^{-2} s^{-1} PAR). Extended periods of exposure to these conditions can cause photoinhibition and reduce photosynthesis. To investigate the possible photoprotective properties of glaucous epicuticular waxes, excised flag leaves were subject to 1500 µmol m^{-2} s^{-1} for a three hour period. However, no difference in amount of post-stress photoinhibition was found between *Iw1+* and *iw1*- NILs as measured using dark adapted chlorophyll fluorescence. This indicated that epicuticular waxes containing β- and OH-β-diketones do not offer increased photoprotection. Furthermore, no effect of *Iw1* was found in dark adapted chlorophyll fluorescence parameters 20 hours post stress, indicative of no differences in their ability to recover from high light stress.

Sub-optimal light conditions below saturation point could also cause problems for wheat productions. This may become a particular problem in the future as wheat growing regions shift to more Northern climes, and aerosols and pollutants cause global dimming. Selection for traits that allow optimum plant function under these conditions could be of benefit. Response of plants to low level irradiance was tested in the field in 2014. *Iw1* NILs of Hereward, Alchemy and Malacca were subject to 3 months shading reducing incoming light by 40% and 60% from GS39 (the start of stem elongation) until harvest. Various physiological parameters were measured over the course of the growing season to assess acclimation to shade conditions. This included factors contributing to photosynthesis, water use and yield. Overall NILs yield was significantly reduced by both levels of shading ($p<0.05$). However, there was limited difference in other physiological parameters measured. Furthermore, there was no difference in the response of *Iw1+* and *iw1*- NILs indicating no advantage attributable to non-glaucousness under low level irradiance.

6.2 Introduction

6.2.1 High light causes photoinhibition

The photosynthetic rate of plants growing in the field varies throughout the day. On a cloudy day with weak sunlight photosynthesis generally follows the diurnal pattern shown in Figure 6.1, curve 1. Photosynthesis increases through the morning until peak sunlight at midday. After this point photosynthetic rate declines throughout the afternoon as light levels drop. However, on a clear day with more intense sunlight, peak sunlight can exceed the amount of light that can be absorbed by the photosynthetic machinery. In wheat photosynthesis generally becomes light saturated between 1000 and 1500 µmol m^{-2} s^{-1} of PAR (Acevedo *et al.,* 2002). At this saturation point light is no longer limiting to photosynthetic rate. Absorption of excess light by the chlorophyll for prolonged periods of time damages the reaction centre subunits of photosystem II (PSII), most notably the D1 protein (Ohnishi & Murata, 2005). The D1 protein can be repaired by process of biodegradation and *de novo* protein synthesis (Aro *et al.,* 1993), and as long as the repair process exceeds the rate at which the reaction centres are being damaged, photosynthetic rate can be maintained. However, when the rate of damage exceeds repair, photosynthetic capacity is reduced in a process termed photoinhibition (Monneveux, *et al.,* 2003; Ögren & Rosenqvist, 1992; Yang *et al.,* 2006). In the field this photoinhibition is exhibited as a 'mid-day depression' of photosynthesis. The pattern of photosynthesis for a plant experiencing this depression can be seen in Figure 6.1, curve 2. In this circumstance photosynthesis increases until just before mid-day. At this point there is a fast drop in photosynthetic rate due to photoinhibition caused by intense peak sunlight. However, repair of the D1 protein allows re-generation of the PSII reaction centres and a second peak in photosynthesis can occur in the late afternoon (Figure 6.1, curve 2). The time spent in this mid-day depression, and how severe the reduction in photosynthesis, will depend on the relative rate of protein repair compared to damage.

Figure 6.1, curve 3, shows the scenario for a plant suffering a more serious mid-day depression in a very high light environment. Excessive light levels become a problem for the plant when the repair process cannot keep up with the rate at which the reaction centres are damaged. In addition to direct damage to PSII, absorption of excess light can cause production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and singlet oxygen (Chen *et al.,* 2011). These ROS species exacerbate photoinhibition through the inhibition of protein synthesis and repair of the D1 protein (Murata *et al.,* 2007). Within this very high light environment, peak photosynthesis occurs much earlier in the day as photoinhibition occurs even before maximum sunlight levels are reached. This is followed by a gradual decline in photosynthetic rate. The damage done to photosystems early on is so severe that they do not recover and no second peak in photosynthetic rate occurs.

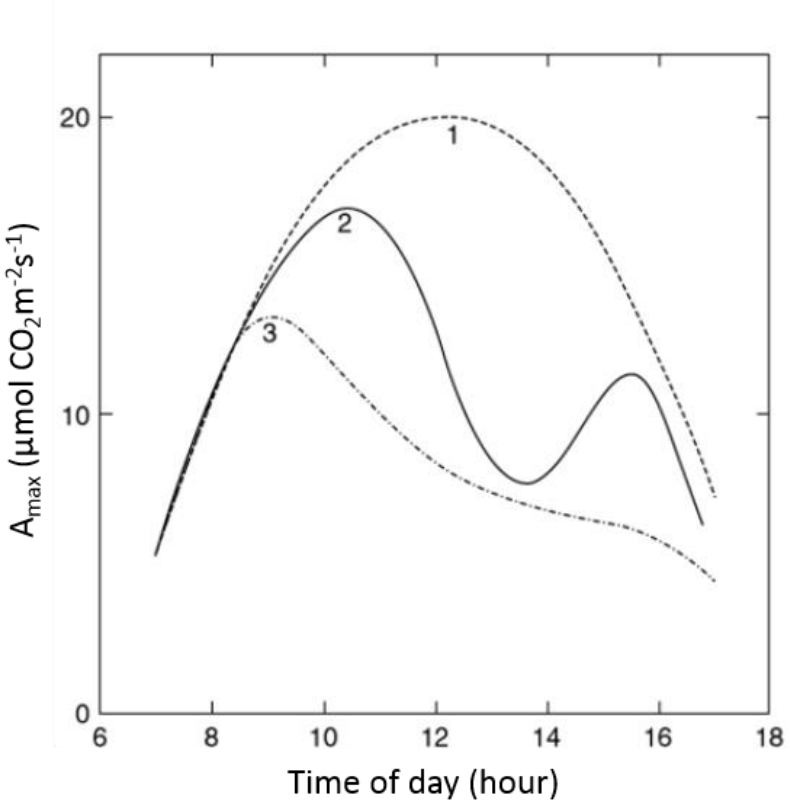

Figure 6. 1 Light saturated photosynthesis (A_{max}) over the course of a typical day

Three scenarios that occur in the field between 6 am and 6 pm. Line 1 shows the situation on a dull cloudy day where no photoinhibition occurs. Line 2 shows photoinhibition at noon leading to a mid-day depression of photosynthesis and subsequent recovery that can occur on a clear sunny day. In environments with very intense sunlight photosynthetic machinery may be unable to recover from the initial photoinhibition, a situation shown in Line 3. Figure from Xu & Shen, 2005.

Although excessive sunlight is a contributing factor to photoinhibition, the reduction in photosynthetic capacity is usually a result of multiple abiotic stresses, the effects of which cannot be distinguished. Extremes of temperature, salt stress and oxidative stress can further intensify the effects of photoinhibition by supressing the repair of photodamaged PSII (Xu & Shen, 2005). It is thought that this could be via a mechanism whereby ROS levels within the cells are increased under stress, which inhibit the translation factors required for PSII repair. Additionally, temperature stress can destabilize translation machinery required for D1 protein synthesis (Takahashi & Murata, 2008). In the absence of these multiple stresses, the mid-day depression observed in the field is more likely to result from a reduction in in the quantum yield of CO_2 rather than the reductions to photosynthetic capacity and light saturated photosynthesis associated with photoinhibition (Murchie & Niyogi, 2011).

6.2.2 A number of protective mechanisms prevent excessive photoinhibition

A reduction in the quantum yield of CO_2 can result from a number of photoprotective processes that occur under high light levels in order to prevent excessive photoinhibition. At the sub cellular level, a common mechanism of photoprotection is the dissipation of excess light energy from the PSII antennae complexes as heat in the process of non photochemical quenching (NPQ). This process is stimulated by the PsbS subunit of photosystem II (Kiss *et al.,* 2008; Li *et al.,* 2000) alongside the carotenoid pigments zeaxanthin and violaxanthin formed under excess excitation energy by the xanthophyll cycle (Chen *et al.,* 2011; Demmig-Adams & Adams, 1992; Gilmore, 1997; Niyogi, 1999). In addition to NPQ, electron transport can be altered to protect photosynthetic machinery from excessive light levels. One mechanism by which this is thought to happen is by cyclic electron flow, whereby electrons flow around PS I in a process that results in ATP synthesis only, and electrons are not passed to a terminal electron acceptor. Exactly how this process assists in photoprotection is still unclear, but it is thought that it has the effect of lowering cell PH, stimulating processes that feed into NPQ (Kramer & Evans, 2011). Another method by which electron transport is altered is through the Mehler reaction. During this reaction one molecule of O_2 to two molecules of H_2O becomes reduced at the reducing side of PSI. This is via electrons that were generated from two H_2O molecules at PSII. The overall result of this process is the scavenging of ROS species hydrogen peroxide and superoxide, in addition to the dissipation of PSII excitation energy (Foyer & Shigeoka, 2011).

In addition to the photoprotective mechanisms detailed above that can reduce the quantum yield of CO_2, plants have a number of other strategies to cope with high light. Antioxidant enzymes are present in the plant to counteract ROS formation under high light (Chen *et al.,* 2011; Noctor & Foyer, 1998), and many plants synthesise the red anthocyanin pigments in response to abiotic stress such as drought, low temperature and UV radiation. A strong correlation has been found between photoprotection and concentration of anthocyanin, thought to maintain PSII activity under extended high light levels (Gould *et al.,* 2010). Other morphological and physical adaptations to avoid absorbing excess light include a) protective pubescent hairs and epicuticular waxes that reflect light (Holmes & Keiller, 2002), b) increases in leaf thickness (Sims & Pearcy, 1992), and c) changes to leaf angle under unfavourable conditions (Bonos & Murphy, 1999). Many growing environments for wheat, for example Ciudad Obregon (see Chapter 4, Figure 4.3), have intense sunlight for much of the day. As such it is important to identify traits that might enable reduced photoinhibition and increased productivity under high light levels.

6.2.3 The effect of low light levels on crop production

In contrast to Ciudad Obregon, other wheat growing environments, such as the UK, experience sub-optimal light intensities for much of the growing season. Annual solar radiation available in the UK

under normal current circumstances is shown in Figure 6.2. Maximum light levels of around 25-30 MJ/m^2 are reached between May and July, equivalent to around 1700-1800 µmol m^{-2} s^{-1} PAR (Boelee *et al.,* 2012)[1]. These light levels are enough to cause photoinhibition and a mid-day depression of photosynthesis. However, cloud cover can reduce the incoming radiation by up to two thirds to around 5-10 MJ/m^2 light (300-600 µmol m^{-2} s^{-1} PAR), well below light saturation point for photosynthesis of 1000 – 1500 µmol m^{-2} s^{-1} (AHDB, 2015). Under these conditions physiological adaptations to maximise light interception would be of benefit to growth and development.

The number of wheat growing regions with sub-optimal light conditions is likely to increase in the future. Global levels of irradiation are decreasing annually, thought to be a result of increasing aerosols and pollutants in the atmosphere from human activity (Ramanathan & Feng, 2009). A metastudy that combined data from 39 individual sites, each with a minimum of 20 complete year's data, concluded that global radiation has been decreasing on average by around 2.7% per decade. The greatest declines in solar radiation were recorded in Hong Kong, where light levels have been dropping by 1.05% every year. In the UK (measurements taken at Aberporth, Wales), radiation was found to be decreasing by around 0.32% every year (Stanhill & Cohen, 2001). In parallel with these changes, wheat growing regions are expected to shift in the coming decades. For example, wheat is currently grown up to 55^{o} North in North America. Due to the warming climate some modelling scenarios project that these growing regions will shift Northwards up to 65 ^{o}N by 2050, and similar changes are expected in Northern Eurasia (Ortiz *et al.,* 2008). Moving North, the amount of light available generally reduces. This, in combination with global dimming means it is important to understand the effect of reduced radiation on crop production, and investigate how cropping systems and varieties could be adapted to cope with new conditions.

[1] Boelee *et al.,* (2012) provide details of conversion of MJ/m^2 to PAR within Appendix A. This method was used to estimate PAR in Figure 6.3 to illustrate PAR available throughout the UK growing season.

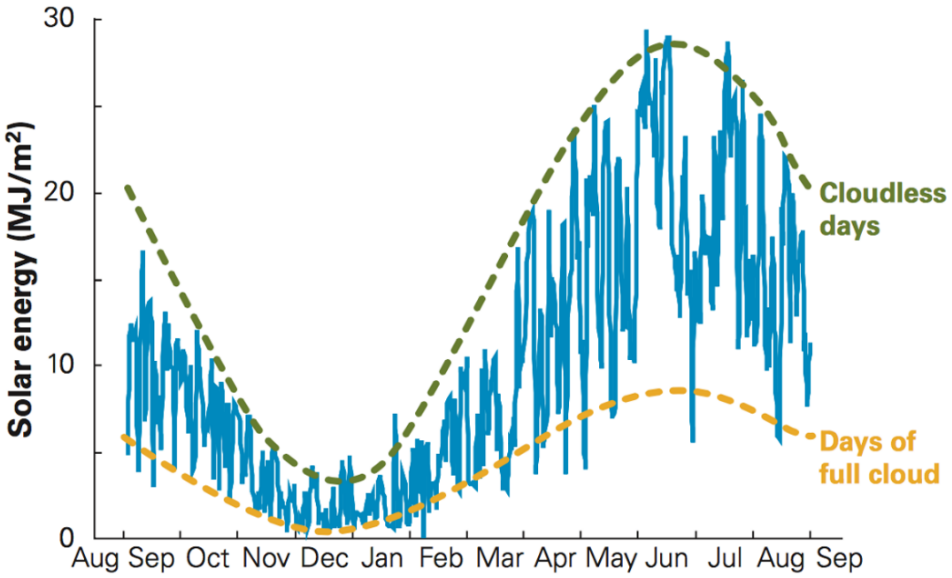

Figure 6. 2 Annual solar radiation received by crops in the field in the UK

The green line shows clear, cloudless days, and the orange line shows solar energy under full cloud. Figure from AHDB (2015) Wheat Growth Guide

6.2.4 Low solar radiation reduces yield

Many studies into the effect of low light conditions on wheat production have shown grain yield to significantly decrease upon application of shade both prior to and after anthesis (Beed *et al.,* 2007; Estrada-Campuzano *et al.,* 2008; Mitchell *et al.,* 1996; Slafer *et al.,* 1994; Wang *et al.,* 2003*)*. This work has been carried out in a range of germplasm from the USA, South America, UK and China, demonstrating consistent effects in wheat varieties across the world. However, many of these studies were carried out with short term shade applied for around 20 days at various growth stages (Beed *et al.,* 2007; Estrada-Campuzano *et al.,* 2008; Slafer *et al.,* 1994; Wang *et al.,* 2003). Although it is important to understand the effect of reduced light at different developmental stages, an issue with these short-term shade studies is that plant acclimation to long term changes in environmental conditions cannot be investigated. Furthermore, some studies were carried out under controlled conditions in pots (Mitchell *et al.,* 1996). Glasshouse conditions are quite different from those in the field, and as such there can be large discrepancy between results.

A number of longer term shading trials in the field have been carried out in winter wheat in China. In China, levels of solar radiation are decreasing by around 6% per decade (Che *et al.,* 2005) which is significantly faster than the global average, highlighting the significance of this type of research for maintaining high levels of wheat production in that country. These long term experiments applied shading a month prior to anthesis, with shading covering the plots for a total of two months.

Decreases in the quantum yield of PSII (ΦPSII), electron transport rate (ETR), the chlorophyll a/b ratio, and light saturated photosynthesis (A_{max}) were observed in plants grown under various levels of shade ranging from reductions of incoming light from 8–80 % (Li *et al.,* 2010; Mu *et al.,* 2010; Zheng *et al.,* 2011) . All these features have been previously reported as characteristics of shade tolerant species (Valladares & Niinemets, 2008), indicating the plasticity of Chinese winter wheat to acclimate to shade conditions. In the majority of these studies grain yield was also decreased in relation to shade. Reducing incoming light by 22% consistently resulted in yield reductions of 5-10% dependent on variety (Li *et al.,* 2010; Mu *et al.,* 2010), and reducing incoming light by 30% resulted in a 16-25% yield reduction (Mu *et al.,* 2010). In every study the percentage loss in yield was less than the corresponding light reduction, even in the less shade tolerant varieties, indicating some physiological compensation by plants subject to shade. One study even found that plants subject to reductions in incoming light of 8% and 15% even exhibited a corresponding increase in yield compared to control of 2% and 1.3%, respectively (Li *et al.,* 2010).

6.2.5 The role of epicuticular waxes in modulating plant-light interactions

Specific adaptations to prevailing light conditions are required for optimum plant growth. The epicuticular waxes are the first barrier between a plant and its environment, and their biochemistry can determine the spectral properties of the plant surface (Chapter 4). Therefore the optimisation of epicuticular waxes both in terms of wax load and biochemistry could be one way in which a plant can adapt to its light environment. For example, a study in *Quercus velutina* (black oak) sampled leaves from upper positions in the canopy regularly exposed to full sunlight in addition to leaves from the lower 3 m of the canopy that were permanently in the shade. Shade leaves were found to have thinner cuticle membranes on both the adaxial and abaxial leaf surface, with reduced quantities of all cuticle components including epicuticular waxes (Osborn & Taylor, 1990). Another study in *Tradescantia pallida cv. Purpurea* (Rose) found leaves from low light intensity environments had a lower density of epicuticular wax platelets than those grown under high light (Sousa Paiva *et al.,* 2003). Similar findings were observed in a study on the rhizome herb *Valeriana jatamansi.* Plants grown in the field under 50% shade demonstrated a 50% reduction in total wax load compared to plants grown under natural light (Pandey & Nagar, 2002). All of these studies suggest that epicuticular wax load is an important component of acclimation and adaptation to sun or shade.

A study using the South African flowering plant *Leucadendron lanigenum* found that the epicuticular waxes in this species increased PAR reflectance from the plant surface by around 4%. These epicuticular waxes were mechanically removed from the leaves, and leaves both with and without intact epicuticular waxes were exposed to two hrs of full sunlight. After exposure, the dark-adapted chlorophyll fluorescence parameter F_v/F_m, indicative of PSII efficiency, was reduced by 10%

in leaves with intact waxes. However, those with the waxes removed demonstrated around a 20% reduction in F_v/F_m indicating greater damage to PSII. This indicates that the epicuticular waxes could have a significant role in photoprotection (Mohammadian *et al.,* 2007). However, similar studies in other species, including a study in the tree species *Juniperus thurifera* (Esteban *et al.,* 2014) and a study into flowering plants of the family Bromeliceae (Pierce *et al.,* 2001), found no difference in F_v/F_m after exposure to high light levels between plants with and without intact epicuticular waxes. The extent to which the ability to withstand extreme light levels is determined by epicuticular waxes is therefore questionable and likely to be dependent on species and wax type.

The presence of *Iw1* inhibits β- and OH-β-diketones in the epicuticular waxes. This change in biochemistry results in a decrease in PAR reflectance of around 15-40% from the flag leaf surface, and around 12-20% from the canopy in the field (Chapter 4). A comparable decrease in reflectance of non-glaucous plants compared to glaucous had been reported previously in a range of species (Holmes & Keiller, 2002; Jefferson *et al.,* 1989; Johnson *et al.,* 1983) and it has been suggested that the epicuticular waxes have a role in regulating light availability to the plant (Koch & Ensikat, 2008). However, the literature is lacking information regarding glaucousness in wheat in response to extreme light conditions. A study by Close *et al.,* (2007) was carried out on the tree *Eucalyptus urnigera*, which exists in both glaucous and non-glaucous forms on Mount Wellington in Tasmania, Australia. Glaucous phenotypes are found on the upper portion of the cline, where conditions are very open, and particularly in winter, reflectance of light from snow covering can create high light conditions. In contrast, non-glaucous phenotypes are found at the lower sites, where the canopy is closed and available PAR is lower. It is thought that the reflective glaucous waxes provide photoprotection at higher sites, whilst lower reflectance of non-glaucous tissues at the lower sites allows increased interception of incoming light, and greater ability to compete with other species for light. Similar results were obtained in a study into Alaskan *Picea monana* (black spruce) and *P.glauca* (white spruce) on an elevation gradient from 610-1050 meters. In both species surface reflectance of the leaves was found to increase with altitude, consistent with greater levels of irradiance. This corresponded with a reduction in chlorophyll content and increase in yellow colouring, both of which are known stress responses (Richardson *et al.,* 2003). Both of these studies suggest that leaf surface reflectance could be a strong determinant of ability to withstand environmental stress.

6.2.6 Aims

I hypothesise that the increased reflectance of glaucous (*iw1*-) epicuticular waxes could provide protection from excessive light levels to the crop canopy and reduce the amount of PAR that reaches the photosystems. Conversely, the reduced reflectance of non-glaucous epicuticular wax could enable greater PAR absorption by the crop canopy under low light conditions. This chapter

will explore the response of *Iw1* NILs to variable light conditions to understand under which conditions the glaucous (or non-glaucous) phenotype could be most beneficial. This chapter aims to test the following hypothesis:

(i) Increased reflectance of glaucous *iw1*- NILs provides protection from photoinhibition upon exposure to high light.

(ii) The reduced reflectance of non-glaucous *Iw1+* NILs provides an advantage under long-term low solar irradiance.

Within hypothesis (ii), an advantage attributed to non-glaucous NILs under low solar irradiance could be assessed in a number of ways. As such, to test hypothesis (ii) the following sub-hypotheses will be addressed:

(a) *Iw1+* NILs will yield higher under low-level light than *iw1*- NILs.

(b) *Iw1*- NILs will display greater acclimation to reduced light levels in terms of changes to photosynthesis or the plant cuticle

6.3 Materials and Methods

6.3.1 Flag leaf response to high light stress

To test the hypothesis that glaucous (*iw1-*) epicuticular waxes provide greater photoprotection than non-glaucous (*Iw1+*) waxes, excised flag leaves of Hereward, Alchemy and Malacca NILs were exposed to saturating light for an extended period of time to assess levels of PSII inhibition.

6.3.1.1 Plant material

NILs of Hereward, Alchemy and Malacca were grown under glasshouse conditions as described in Chapter 2 (2.2). A week prior to measurement (GS55-59) plants were transferred to a controlled environment room (CER) to acclimate to environmental conditions: Lighting was on an 8 hr dark, 16 hr light cycle, with PAR levels maintained between 200-300 µmol m^{-2} s^{-1} during hours of light. Relative humidity (RH) was maintained at around 70%, and the temperature was 20 ^{o}C during hours of light and 15 ^{o}C during dark hours.

6.3.1.2 Light stress group

Measurements were taken between anthesis (GS61) and 14 days post anthesis (DPA). To measure the effect of light stress, flag leaves were cut from the plant to a length of 12 cm. Oil paint was used to mark each leaf at the tip on the adaxial surface such that it could be identified throughout the experiment. 10-12 biological repeats were used for each NIL. Directly after excision from the plant leaves were dark adapted for 40 min using leaf clips, and during dark adaptation the ends of the leaves were kept in water so that leaves would remain saturated and not suffer water stress. After 40 min pre-stress chlorophyll fluorescence measurements were taken using a Handy PEA (Hansatech, UK). Leaves were subsequently placed in a water bath (6 cm deep) with the adaxial surface facing upwards below a high pressure sodium (HPS) light. The water temperature was maintained at 20-24^{o}C throughout. A PAR sensor at the level of the water bath was used to check plants were receiving 1500 µmol m^{-2} s^{-1}, and this level of light was maintained for 3 hr. After 3 hr, leaves were removed from the water bath, and returned to ambient light level (200-300 µmol m^{-2} s^{-1}). Leaf clips were immediately placed on the leaves, and left to dark adapt for 40 min with the ends in water. Post-stress measurements of dark adapted chlorophyll fluorescence were then taken.

6.3.1.3 Control group

Leaves in the control group were also excised from the plant, labelled with oil paint and pre-stress dark adapted chlorophyll fluorescence measured in the same manner as the stress group. Leaves were then placed into a 6 cm deep water bath which was maintained at 20-24^{o}C in the same room as the stress group for the same 3 hr period of time. However, the HPS light was not used, so leaves

were experiencing ambient light of 200-300 µmol m^{-2} s^{-1} for the full 3 hr duration. After 3 hr leaves were removed from the water bath, and leaf clips placed on the leaves with their ends in water. Post stress dark adapted measurement was taken in the same way as for the stress group.

6.3.1.4 Recovery from light stress

Recovery post stress was measured for NILs of Alchemy and Malacca. Leaves from both the control and stressed groups of these varieties were placed back in the water bath after post stress dark adapted chlorophyll fluorescence measurement. Water bath temperature was maintained at room temperature and leaves were left to recover under CER conditions (8 hr at 200-300 µmol m^{-2} s^{-1}, 16 hr dark) for 20 hr. Dark adapted chlorophyll fluorescence was measured periodically using the method described previously.

6.3.1.5 Data analysis

At all time-points of measurement (pre-stress, post-stress and recovery) F_o, F_m and F_v/F_m were recorded and averaged over 10-12 biological replicates per NIL. An overall ANOVA was carried out for each chlorophyll fluorescence parameter, and pairwise comparison between NILs of the same variety carried out.

6.3.2 Response to long term low level irradiance

To test the hypothesis that *Iw1+* NILs would be at an advantage under low light levels, NILs of Hereward, Alchemy and Malacca were grown in the field under long-term shading.

6.3.2.1 Experimental design

NILs of Hereward, Alchemy and Malacca were grown at Church Farm, Bawburgh, in 2014 in Hege-90 (6 m^2) plots to assess the effect of long-term low irradiance. All three NILs were grown in three blocks in the field. Within each block 5 independent replications of each NIL were arranged in a randomized block design with 1 replication per block. Figure 6.3 shows this experimental design in the field. Each block had a buffer of 1 Hege-90 plot of the variety Soissons around the main experiment, and four Soissons plots were drilled between each of the three blocks. The full field plan is shown in Appendix A7.

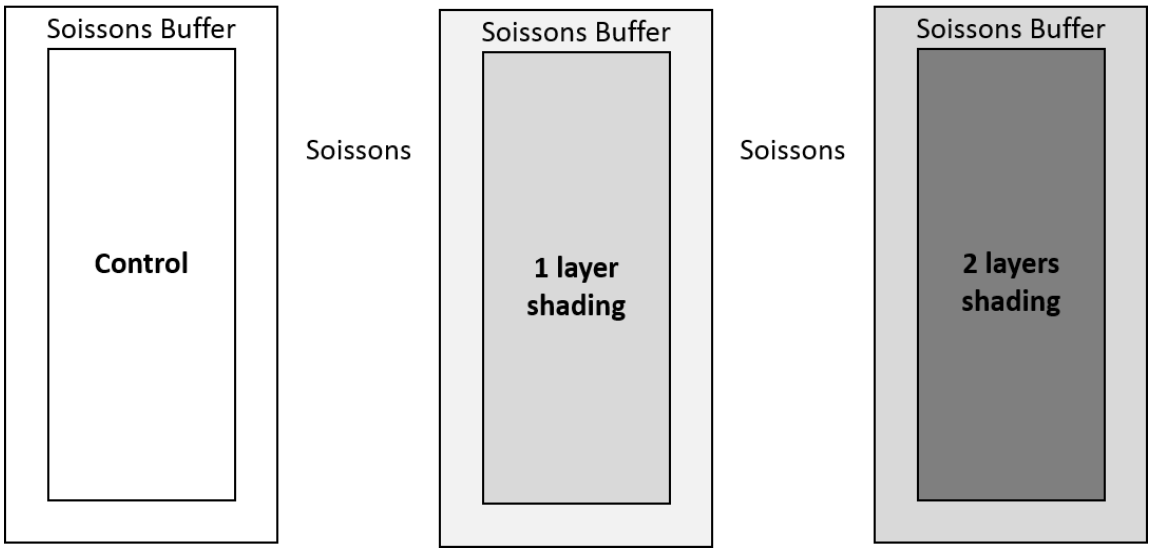

Figure 6. 3 Field trials layout for the low level irradiance trial

Each of the three blocks has 5 independent replications of each NIL of Hereward, Alchemy and Malacca surrounded by a buffer zone of Soissons. Five blocks of Soissons were drilled between each of the three blocks.

All three blocks were drilled on the 16th October and grown under natural light (no shade). On 9th May one block was left as a control under natural light, one layer of spectrally neutral polyolefin net (Svensson Solaro, Farmtek, USA) was erected over the second block, and a double layer of netting over the third block. Nets were erected at a height of 2.13 m (7 feet) to allow for circulation of air above the canopy to continue as normal. At this point the plants were around GS33. Visible epicuticular waxes had developed on the *iw1*- NILs around the 1st May. All plants reached heading between 24th May and 1st June, and reached maturity between 16th July and 2nd August. This large range of dates for both heading and maturity was a result of the shading, with plants under the shade heading 1-2 weeks later than control plants.

According to the manufacturer specifications the single layered netting transmitted 73% PAR, and the double layer transmitted 49% PAR when tested using an integrating sphere. In order to confirm this in the field simultaneous measurements of PAR were made under shade and control plots, with PAR sensors placed at the level of the crop canopy (Figure 6.4). Under the double layered nets PAR levels were reduced by 65% compared to control at the highest light level measured (control PAR 1700 µmol) and 54% at the lowest PAR level measured (control PAR 76 µmol). There was less of a range under the single layer nets, with a reduction of incoming light of 42% at the highest light level measured (control PAR 1833 µmol) and 38% at the lowest light level (control PAR 185 µmol). Therefore the two shade environments will be referred to as 60% and 40% shade throughout this chapter, as an average across the spectrum.

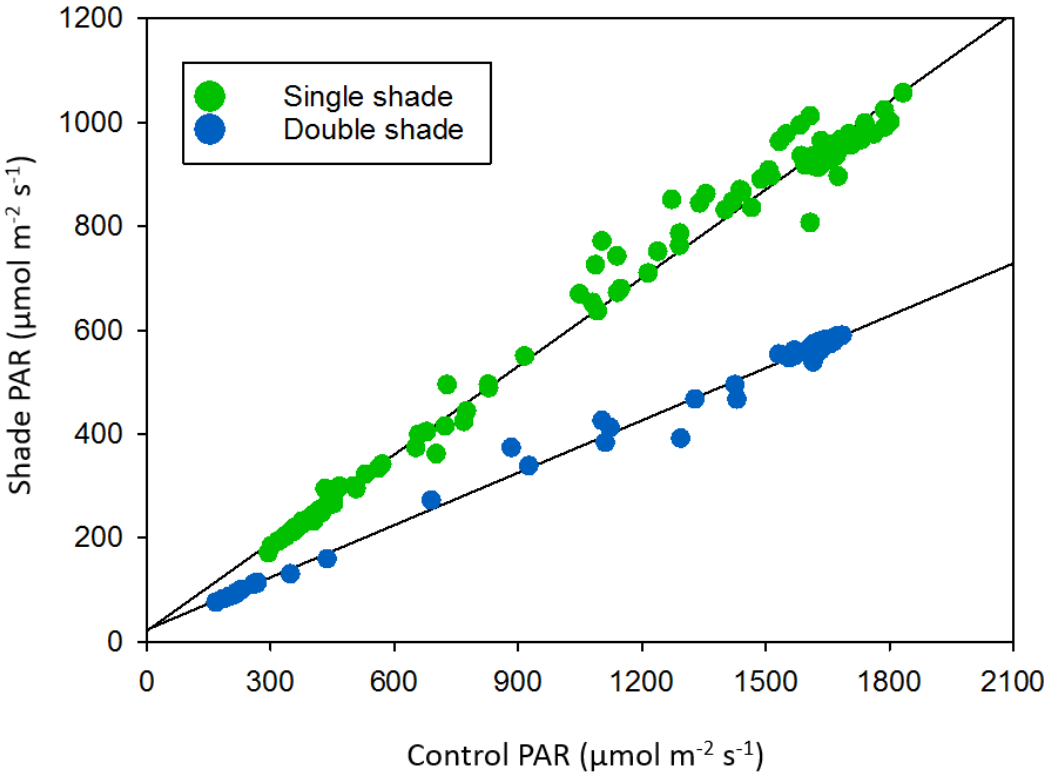

Figure 6. 4 PAR levels under the shade plots compared to control

PAR was simultaneously measured under the double netting and control, and under single netting and control. Regression lines shows a linear relationship between control PAR levels and those for both shade environments

Within this chapter, all yield data (6.4.2.2) was collected within this experimental design as was physiology data from 40 and 60% shade. However, due to limited resources, control (no shade) physiology data detailed within results sections 6.4.2.3., 6.4.2.4 and 6.4.2.5 was collected from the main Church Farm experiment detailed within Chapter 2 section 2.2 and has been presented before within Chapters 4 and 5. Where 'control' is stated within these sections it is therefore referring to the main experiment. The shade trial and main experiment were located in close proximity within the same field. Therefore environmental conditions would have been comparable across both.

6.3.2.2 Field environmental conditions under shade

Environmental conditions other than light availability can affect plant development and might have been affected by the netting placed over the trial. Therefore to understand differences in environmental conditions between the three blocks, temperature and RH data loggers (Tinytag, West Sussex, UK) were placed within each of the three experimental blocks on 21st May. Data for both parameters were logged 9 times per hr continually until data loggers were removed on 8th August when plants were harvested.

Daytime temperature and relative humidity were defined as all values between 7 am and 6 pm. RH values recorded at times that coincided with rain (100% RH under control conditions) were excluded from analysis. The average per day was then calculated for each of the two parameters.

6.3.2.3 Phenotyping yield

According to the hypothesis that *Iw1+* NILs would be at an advantage under low level irradiance *iw1-* NILs would be expected to show a greater decrease in yield under shade than *Iw1+* NILs. In order to test this, yield was measured in all three treatment blocks as weight of grains per plot and normalised to 15% moisture content. Average yield per NIL was calculated per treatment block and analysed both by pairwise comparison and overall ANOVA.

6.3.2.4 Photosynthesis

Any advantage conferred by *Iw1+* under low level irradiance may also present in differences between NILs in terms of acclimation to lower light levels. To test this, various components affecting photosynthesis known to change in plants exposed to long term shading were assessed.

6.3.2.4.1 Extraction of photosynthetic pigments

Concentration of carotenoids, chlorophyll a and chlorophyll b in flag leaf tissue from Hereward, Alchemy and Malacca NILs at anthesis was quantified. Five flag leaves of each NIL were collected from each treatment block, one from each independent replication within the block. Full details of sampling method, extraction and analysis are available in Chapter 4 section 4.3.5.

6.3.2.4.2 Carbon assimilation

Carbon assimilation at various levels of PAR between 0 and 1500 µmol m^{-2} s^{-1} was measured in flag leaves of Hereward, Alchemy and Malacca at anthesis from the control and 60% shade blocks. For each of the environments four flag leaves of each NIL were measured, one from each independent replication 1-4 within the block. Light curve parameters were calculated and averaged over all four flag leaves. Full details of method of measurement and analysis are available in Chapter 4 section 4.3.6.

6.3.2.4.3 Chlorophyll fluorescence

Both light and dark adapted chlorophyll fluorescence were measured from control and 60% shade environments using methods detailed in Chapter 5 sections 5.3.1 (dark adapted) and 5.3.2.1 (light adapted. NILs of Hereward, Alchemy and Malacca were measured. For each of the two treatment blocks forty flag leaves of each NIL were measured, ten each from four independent replications within each block.

6.3.2.5 Effects on the cuticle

Cuticular components are also known to change in plants exposed to varying light levels. Differences between NILs in terms of epicuticular waxes, stomata, and transpiration were measured to test the hypothesis that *iw1*- display greater acclimation to low level irradiance.

6.3.2.5.1 Epicuticular waxes

Epicuticular wax biochemistry was assessed in NILs of Hereward, Alchemy and Malacca at anthesis and 40 DPA. Five flag leaves of each NIL were collected from each treatment block, one from each independent replication within the block. Epicuticular waxes were extracted in chloroform, the biochemical profile of wax extracts analysed through GC-MS and the 17 most abundant wax components quantified. Full methods for wax extraction and analysis are detailed in Chapter 3 section 3.2.2.

6.3.2.5.2 Stomatal density

Flag leaves were collected from the field at anthesis from NILs of Hereward, Alchemy and Malacca from control, 40% shade and 60% shade blocks. Three flag leaves were collected from each treatment block, one each from three independent replications within each block. Stomatal density per mm^2 were quantified according to methods detailed in Chapter 5 section 5.3.3.2.

6.3.2.5.3 Cuticular conductance

In 2014 cuticular conductance was measured in NILs of Malacca and Alchemy grown under control, 40% shade and 60% shade at anthesis. Five flag leaves were collected from each treatment block, one from every independent replication within each block. Full methods of measurement and analysis are detailed in Chapter 5 section 5.3.3.1.

6.3.2.5.4 Stomatal conductance

Stomatal conductance at various levels of PAR between 0 and 1500 µmol m^{-2} s^{-1} was measured in flag leaves of Hereward, Alchemy and Malacca at anthesis from the control and 60% shade blocks. For each of the two treatment blocks, four flag leaves of each NIL were measured, one from each of four independent replications within the block. Full details of measurement and data analysis are available in Chapter 5, section 5.3.2.

6.3.2.6 Overall water use efficiency

Both photosynthesis and transpiration affect water use efficiency (WUE). Furthermore, netting over the shade blocks affected temperature and RH, both of which may affect plant water use. Therefore

to understand how differences in acclimation between the NILs was affecting WUE both instantaneous gas exchange and carbon isotopes were used.

6.3.2.6.1 Instantaneous gas exchange

Instantaneous gas exchange measurements of carbon assimilation and transpiration were taken on flag leaves of Hereward, Alchemy and Malacca NILs from control and 60% shade at anthesis. For each of the two experimental blocks, four flag leaves of each NIL were measured, one from each of four independent replications within the block. Gas exchange measurements were then used to calculate overall WUE Full details of methods and analysis can be found in Chapter 5 sections 5.3.2.

6.3.2.6.2 Bulk δ13C measurement and calculation of ^{13}C discrimination

Flag leaves of Hereward, Alchemy and Malacca NILs were sampled from control, 40% shade and 60% shade at anthesis and 40 DPA. Three flag leaves of each NIL were collected from each treatment block, one from each independent replication within the block. Full of details of methods and analysis can be found in Chapter 5 section 5.3.5.

6.4 Results

6.4.1 The role of epicuticular waxes in photoprotection

The increased reflectance of PAR by glaucous epicuticular waxes has been suggested to have a role in photoprotection. Reflecting excess PAR from the plant surface could reduce the photoinhibition that can occur to PSII under high light levels. To explore this effect, excised flag leaves from both glaucous and non-glaucous NILs were exposed to 1500 µmol m^{-2} s^{-1} PAR for 3 hr and dark adapted chlorophyll fluorescence parameters recorded before and after stress. A control group of excised leaves were exposed to ambient light (200-300 µmol m^{-2} s^{-1}) for the 3 hr (Table 6.1).

Overall, flag leaves that were exposed to the light stress had significantly increased F_o ($p<0.001$), decreased F_m ($p<0.001$) and decreased F_v/F_m ($p<0.001$) after the three hour period. However, a significant change in all three parameters was also recorded in control leaves of Hereward and Malacca ($p<0.05$), and in Alchemy a significant change in F_m and F_v/F_m was observed ($p<0.05$). In all cases the recorded change in control leaves was not as great as the change observed in stressed leaves. This difference in response is reflected in the statistically significant interaction between treatment group and change in chlorophyll fluorescence ($p<0.001$).

Table 6. 1 The difference between dark adapted chlorophyll fluorescence measurement before and a period of light stress

The difference in the dark adapted chlorophyll fluorescence parameters F_o, F_m and F_v/F_m before and after exposure to light stress calculated as pre stress – post stress. For no variety does *Iw1* have a significant effect on the change in any chlorophyll fluorescence parameter after stress. N=10-12.

			Change in chlorophyll fluorescence after light stress					
Variety	**Treatment Group**	***Iw1***	**F_o**	**p (*Iw1*)**	**F_m**	**p (*Iw1*)**	**F_v/F_m**	**p (*Iw1*)**
Hereward	Stress	*Iw1+*	+80.00 ± 11.40	0.671	-660.00 ± 65.23	0.402	-0.110 ± 0.012	0.522
		iw1-	+73.62 ± 9.49		-773.23 ± 115.74		-0.101 ± 0.009	
	Control	*Iw1+*	+42.20 ± 13.52	0.484	-282.60 ± 81.24	0.166	-0.044 ± 0.010	0.231
		iw1-	+31.18 ± 8.92		-163.00 ± 48.72		-0.026 ± 0.005	
Alchemy	Stress	*Iw1+*	+135.20 ± 28.39	0.893	-1218.20 ± 91.44	0.543	-0.228 ± 0.029	0.552
		iw1-	+129.55 ± 29.82		-1124.55 ± 117.44		-0.202 ± 0.031	
	Control	*Iw1+*	+19.64 ± 27.95	0.864	-264.73 ± 51.05	0.905	-0.028 ± 0.009	0.977
		iw1-	+43.00 ± 11.98		-325.17 ± 89.89		-0.046 ± 0.009	
Malacca	Stress	*Iw1+*	+132.73 ± 11.91	0.960	-744.45 ± 118.10	0.758	-0.199 ± 0.021	0.408
		iw1-	+133.85 ± 12.47		-705.08 ± 59.76		-0.181 ± 0.009	
	Control	*Iw1+*	+68.50 ± 18.45	0.272	-250.33 ± 87.19	0.706	-0.057 ± 0.014	0.946
		iw1-	+90.80 ± 15.52		-167.80 ± 155.63		-0.066 ± 0.024	

An overall analysis that was inclusive of all three varieties, both the pre and post stress time points, and control and light stressed leaves shows that there was no significant effect of *Iw1* on F_o ($p=0.413$), F_m ($p=0.074$) or F_v/F_m ($p=0.167$). There was no significant interaction between *Iw1* and time point of measurement for any parameter, indicating that the lack of difference between NILs

in chlorophyll fluorescence parameters remained the same when measured both before and after the 3 hour light stress period. This conclusion was further supported by pairwise comparison between NILs seen in Table 6.1. Furthermore there was no significant interaction between *Iw1* and treatment group, indicating that the difference between NILs in chlorophyll fluorescence parameters was the same in both the control and treated groups. In conclusion these data show that under these experimental conditions glaucous epicuticular waxes do not provide photoprotection.

In the field, the total reduction in carbon assimilation caused by photoinhibition will be determined not only by a plant's ability to withstand high light levels at mid-day, but also the ability to recover normal levels of photosynthesis post exposure. To assess rate of recovery, after the three hour exposure to 1500 µmol m^{-2} s^{-1}, Malacca and Alchemy flag leaves were moved to ambient light conditions (200-300 µmol m^{-2} s^{-1}), and dark adapted F_v/F_m measured again after 20 hours. Leaves in the control group were maintained at ambient light throughout.

For both Alchemy (Figure 6.5a) and Malacca (Figure 6.5b) there was a significant increase in F_v/F_m of stressed leaves after 20 hours in recovery compared to immediately post stress ($p<0.001$). For Malacca there was a significant decrease in F_v/F_m of control leaves after 20 hours ($p=0.003$), but the same significant decrease was not observed in Alchemy ($p=0.925$) indicating that the Alchemy leaves could better maintain PSII function after excision from the plant. At no time point was there a significant difference between NILs of Malacca or Alchemy, indicating that not only does *Iw1* have no effect on F_v/F_m after high light exposure, but additionally that *Iw1* does not affect ability to recover from high light stress in these wheat varieties.

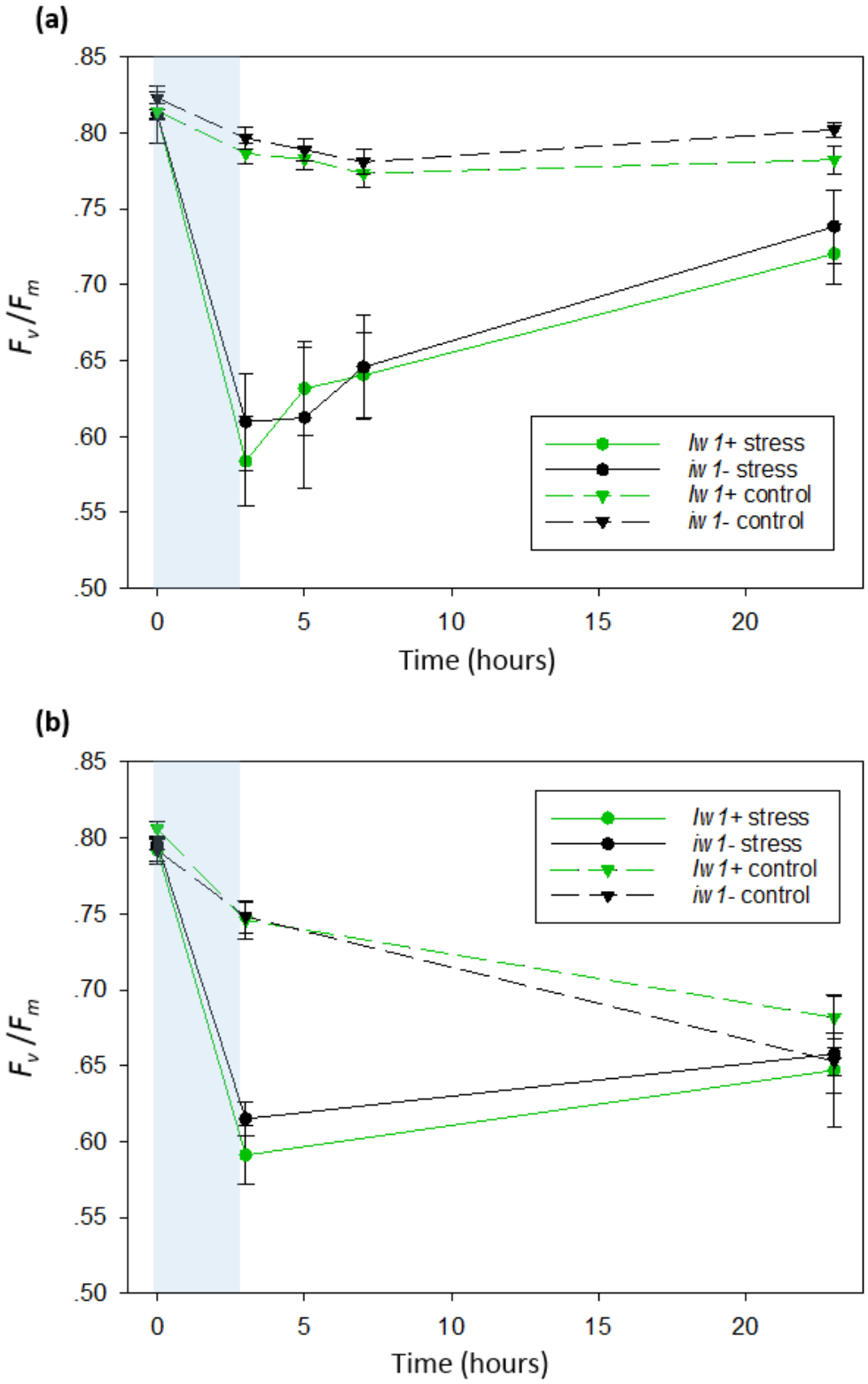

Figure 6. 5 Recovery of flag leaves after a 3 hour period of high light stress

NILs of (a) Alchemy and (b) Malacca were measured before and after light stress. Time point 0 hours indicates the pre stress F_v/F_m measurement. The stress period is shown shaded in blue. Post stress measurements were taken at 3 hours. Leaves were left under ambient light to recover and F_v/F_m measured again after 20 hours. N=10-12, error bars = S.E.

6.4.2 The effect of epicuticular waxes on plant response to long term low radiation

To explore any effect of glaucousness on the physiological response to long term low level radiation in the field, NILs of Hereward, Alchemy and Malacca were grown in 2014 under polyolefin covers that reduced incoming light by 40% and 60%. A control trial with no covering was also grown.

6.4.2.1 Field environmental conditions

Temperature and RH were monitored within each trial to understand the effect of the shading on environmental parameters other than light. Unfortunately long term solar radiation data were not available, although the reduction in PAR under shading across a variety of light levels was measured to confirm consistency (see Figure 6.4).

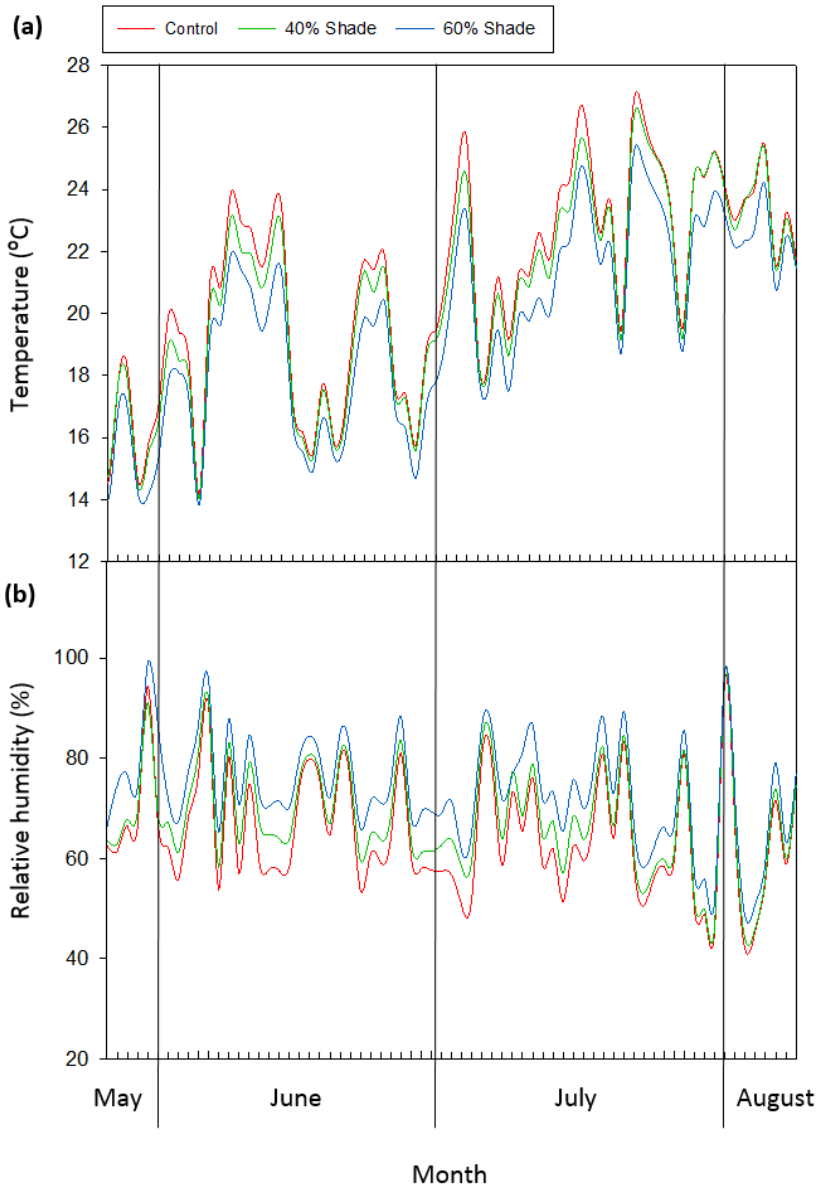

Figure 6. 6 Average daily (a) temperature and (b) RH under 40% and 60% shade trials and control

Data were measured 9 times per hour from the 25th May until 8th August (harvest). The chart shows the daily average (6 am – 7 pm).

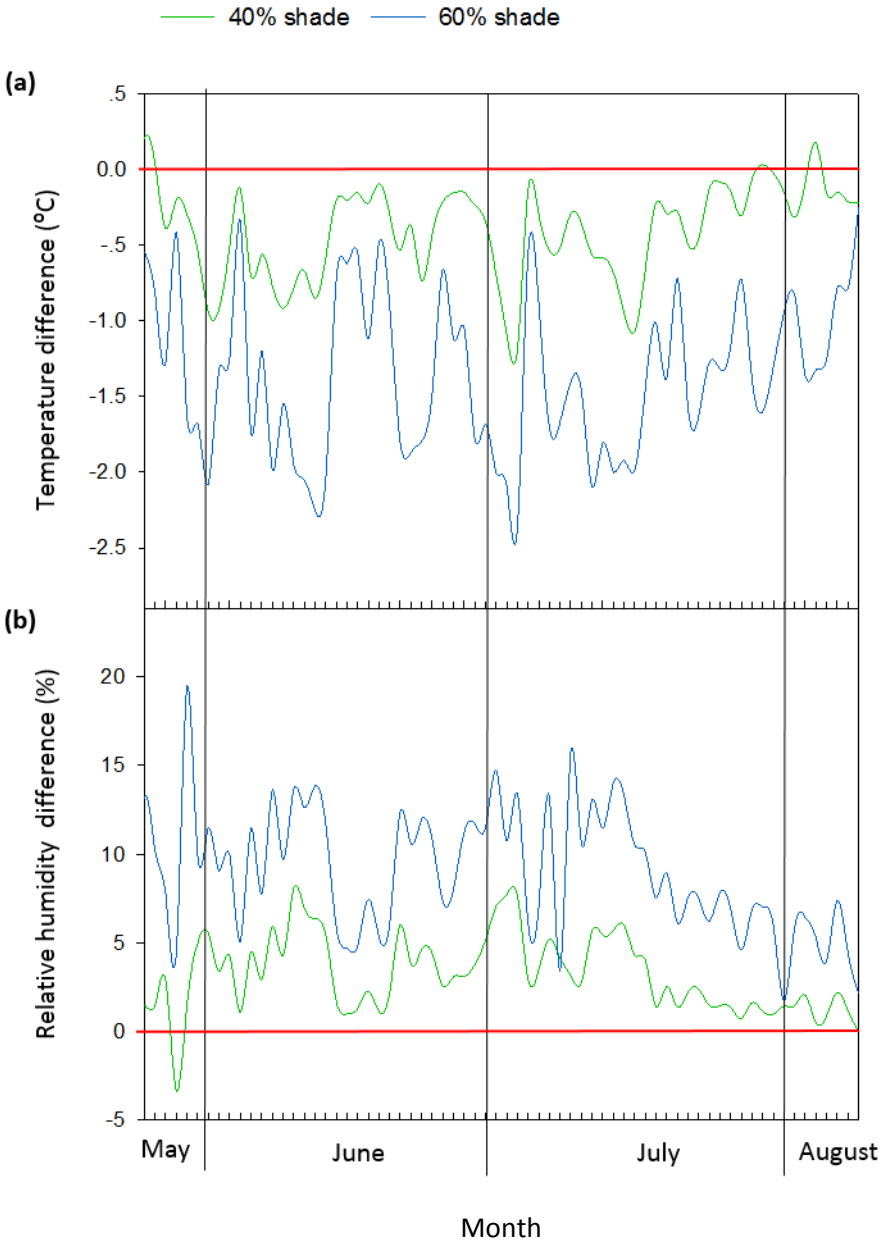

Figure 6. 7 Difference in temperature between control and shade plots

The difference was calculated as shade – control for (a) temperature and (b) RH. The red line at 0 indicates the point where there is no difference between the shade and control environments. Any values above the line indicate higher values recorded within the shade compared to control, whilst values below the line indicate that higher values were recorded in the control plot.

Figure 6.6 shows the average daily temperature and RH for the control, 40% shade and 60% shade plots. Figure 6.7 shows the difference in conditions between the shade and control for both 40 and 60% shading. Overall, on the warmest days the control trial tended to be around 2 ^{o}C hotter than the 60% trial and 0.5 - 1.0 ^{o}C hotter than the 40% shade trial (Figure 6.6a and 6.7a). On cooler days the temperature difference between the control and shaded trials was reduced if not absent completely for 40% shade. The opposite trend was present when considering RH (Figure 6.6b). The

60% shade trail had the highest RH at all time points, particularly on less humid days where the 60% shade trial had up to 10-15% higher RH than the control. On overall more humid days this difference between the control and shade trials was reduced, with little difference in RH between control and 40% shade, but around a 5% increase in RH under 60% shade. Temperature and RH are important environmental parameters affecting plant physiology. Therefore it will be important to take these general trends into account when considering any differences in physiology seen between the three shade environments.

6.4.2.2 Grain yield

Grain yield provides a measure of overall plant performance. To understand if *Iw1+* plants were at an advantage in the shade in terms of productivity, yield was measured for NILs of Malacca, Hereward and Alchemy in the control trial and the 40% and 60% shade trials.

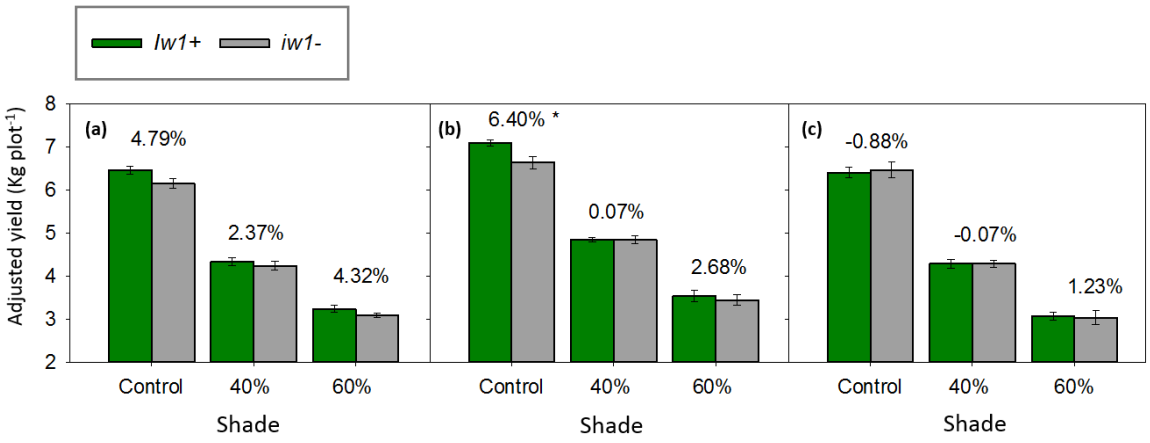

Figure 6. 8 Adjusted yield of *Iw1+* and *iw1-* NILs under shade and control conditions

Average yield for (a) Hereward (b) Alchemy and (c) Malacca under 40% shade, 60% shade and control conditions. Percentage increase in yield of *Iw1+* compared to *iw1-* is shown above each pair. Significant differences between NILs are indicated by * ($p<0.05$).

Figure 6.8 shows the difference in yield between NILs in each of the three environments. In Hereward (Figure 6.8a) the effect of *Iw1* on yield was not significant overall (p=0.093), and pairwise comparison within each of the three environments indicated that there were no significant differences between NILs. However, in the control and 60% shade environments there was a respective 4.79 and 4.32% increase in yield associated with *Iw1*, which is consistent with data previously reported in the *Iw1* yield trials (Chapter 3). There was a significant interaction between shade and *Iw1* (p=0.027) as under 40% shade the difference between NILs was reduced. Under these intermediate conditions *Iw1* only conferred a 2.37% yield advantage. A similar trend was recorded in Alchemy (Figure 6.8b). In Alchemy *Iw1* had a significant effect on yield overall (p=0.016), with a significant 6.40% yield increase associated with *Iw1* under control conditions (p=0.024), and

a (non-significant) 2.68% yield increase under 60% shade. However, the difference between NILs was only 0.07% under 40% shade, although the interaction between *Iw1* and yield in this case was not significant (p=0.168). Malacca consistently displayed a much smaller difference between NILs in all three environments (Figure 6.8c), again consistent with conclusions from the yield trials (Chapter 3), and there was no significant effect of *Iw1* on yield overall (p=0.390).There was even a negative effect of *Iw1* under control and 40% shade conditions, which again is consistent with data reported previously in Chapter 3. There was no statistically significant interaction between *Iw1* and shade for Malacca (p=0.570).

Table 6.2 shows the percentage loss of yield for plants grown in the shade compared to control. There was a significant difference in yield between all three environments (p<0.001), and the effect of shade was also significant for all three varieties individually (p<0.001). Yield was consistently reduced by around 30% under 40% shade compared to control, and by around 50% under 60% shade. In conclusion, although the yield of all three varieties was significantly affected by light availability, non-glaucous plants with *Iw1* had no advantage under the low light.

Table 6. 2 Percentage yield loss of shade grown plants compared to control

NILs of Hereward, Alchemy and Malacca. Yield within all three shade environments is significantly different (p<0.05) but there is no difference in response between NILs.

	Percentage yield Loss against control					
	Hereward		Alchemy		Malacca	
	+Iw1	*-Iw1*	*+Iw1*	*-Iw1*	*+Iw1*	*-Iw1*
40% Shade	32.9%	31.2%	31.7%	27.1%	33.2%	33.7%
60% Shade	50.0%	49.8%	50.0%	48.1%	52.0%	53.0%

6.4.2.3 Photosynthesis

Photosynthesis is highly dependent on light availability and as such plants adapted to long term low light levels have a number of adaptations to maximise efficiency of carbon assimilation (Valladares & Niinemets, 2008). If non-glaucous *Iw1+* plants are at an advantage under shade conditions, *iw1-* material may be expected to show greater acclimation to the shade in terms of photosynthetic parameters.

6.4.2.3.1 Photosynthetic pigments

Carotenoids, chlorophyll a and chlorophyll b were extracted from flag leaves of Hereward, Alchemy and Malacca NILs grown in the field under control, 40% shade and 60% shade conditions (Figure 6.9). Pigment quantity per mm^2 leaf area was quantified by spectroscopy.

Overall analysis incorporating all three shade environments and varieties showed that statistically there was no effect of *Iw1* (p=0.333) or shade (p=0.197) on quantity of chlorophyll a (Figure 6.9a, b and c). Furthermore there were no significant interactions between *Iw1*, shade and variety. Under control conditions *Iw1+* NILs of all varieties had consistently higher quantities of chlorophyll a than *iw1-* (p=0.040) as reported previously in Chapter 4. This same effect was also seen under 40% shade in Hereward and Alchemy although overall there was no effect of *Iw1* on chlorophyll a (p=0.184). Comparison of control and 40% shade in Hereward and Alchemy NILs (Figures 6.9a and 6.9b) shows that there is a (non-significant) increase in the difference in chlorophyll a content between NILs under 40% shade compared to control. For Hereward *iw1-* NILs had 0.005 µg mm^{-2} more chlorophyll a under control conditions, whilst under 40% shade *iw1-* had 0.007 µg mm^{-2} more chlorophyll a. In Alchemy this trend was also present. Under control conditions there was a difference of 0.004 µg mm^{-2} between NILs, whilst this increased to 0.009 µg mm^{-2} under 40% shade. This might suggest that *Iw1+* NILs did have an advantage in the shade and *iw1-* NILs had to compensate more for the reduced light availability. However, this trend was absent in Malacca and did not extend to 60% shade in any of the three varieties. Overall under 60% shade there was no effect of *Iw1* on chlorophyll a (p=0.386) although Malacca *iw1-* NILs still had (non-significantly) increased chlorophyll a quantity. However, in Hereward and Alchemy the trend was actually reversed under 60% shade, with *Iw1+* NILs having higher quantity of chlorophyll a. This interaction between variety and *Iw1* however was not significant (p=0.219).

Similar trends were present for chlorophyll b quantity although overall there was no significant effect of *Iw1* (p=0.093) or shade (p=0.185) on chlorophyll b. When analysed at the variety level there were no significant effects of *Iw1* or shade. Trends in the carotenoid data were slightly different. There was overall no significant effect of *Iw1* on carotenoid concentration (p=0.149) but there were significant differences both between varieties (p<0.001) and shade environments (p=0.002). This can be seen in Figure 6.9g- 6.9i, whereby across all varieties there appears to be a positive relationship between carotenoid content and light level. However, these differences were quite subtle, and when analysed at the variety level, the effect of shade was no longer significant.

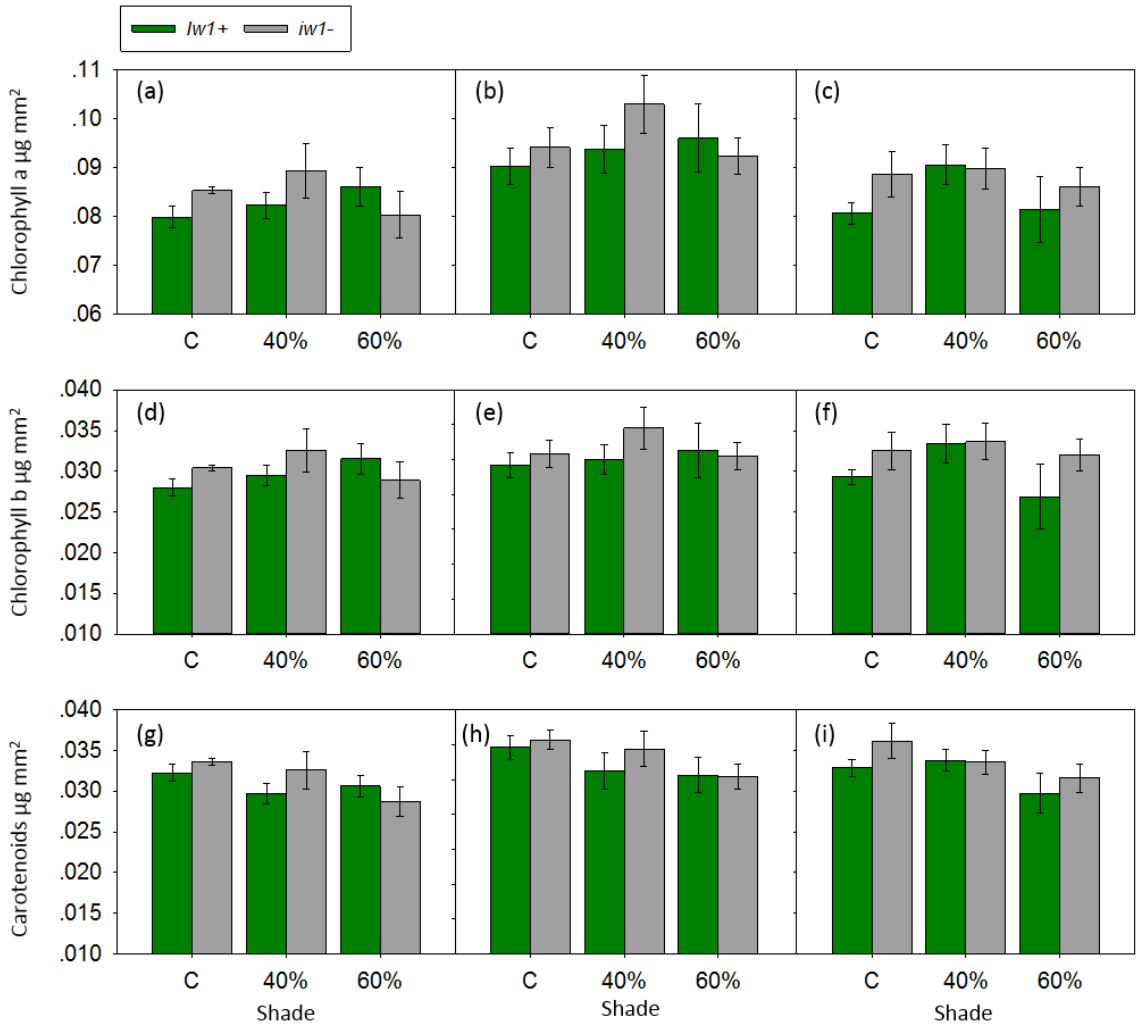

Figure 6. 9 The effect of shade on chlorophyll a, chlorophyll b and carotenoids in the flag leaf

Chlorophyll a in (a) Hereward, (b) Alchemy and (c) Malacca, chlorophyll b in (d) Hereward, (e) Alchemy and (f) Malacca, and carotenoids in (g) Hereward, (h) Alchemy and (i) Malacca. Extracted from flag leaves at anthesis grown under control (C), 40% shade and 60% shade conditions. Pairwise comparison revealed no significant difference between NILs. N=5, error bars = S.E.

The chlorophyll a/b ratio was also analysed (Table 6.3), as this is has been reported to decrease under reduced light levels (Dong *et al.,* 2015; Li *et al.,* 2010; Valladares & Niinemets, 2008; Zhang *et al.,* 2011). Overall varieties there was no significant effect of shade on the chlorophyll a/b ratio (p=0.464), neither was there an effect of *lw1* (p=0.251) or variety (p=0.272). Furthermore there were no significant interactions between shade and *lw1* nor shade and variety. When analysed at the variety level for none of the three varieties was the effect of shade significant (p values in Table 6.3), and there was no interaction with *lw1*.

Overall, these pigment data show that for none of the three photosynthetic pigments assessed did shade significantly affect quantity in either the *Iw1+* or *iw1-* NILs, and the chlorophyll a/b ratio did not change.

Table 6. 3 The chlorophyll a/b ratio in flag leaves at anthesis grown under shade and control conditions

Values for Hereward, Alchemy and Malacca NILs. The overall p value for the effect of shade on the ratio is shown for each variety.

		Chlorophyll a/ b ratio Average ± S.E			
Variety	*Iw1*	No shade	40% shade	60% shade	p (shade)
Hereward	+	2.85 ± 0.03	2.79 ± 0.04	2.74 ± 0.04	0.220
	-	2.80 ± 0.02	2.76 ± 0.06	2.79 ± 0.05	
Alchemy	+	2.64 ± 0.03	2.69 ± 0.03	2.68 ± 0.11	0.965
	-	2.63± 0.04	2.62 ± 0.05	2.61 ± 0.05	
Malacca	+	2.75 ± 0.02	2.73 ± 0.07	2.72 ± 0.05	0.404
	-	2.74 ± 0.05	2.68 ± 0.04	2.70 ± 0.04	

6.4.2.3.2 Carbon assimilation

In order to further assess differences in acclimation to shade by the NILs, carbon assimilation at PAR levels between 0 and 1500 µmol m^{-2} s^{-1} was measured in flag leaves at anthesis for Hereward, Alchemy and Malacca under 60% shade and control conditions. The light curve parameters apparent quantum efficiency (AQE), light compensation point, light saturated carbon assimilation (A_{max}) and dark respiration (A_o) were calculated using the model described in methods section 4.3.6.1 (Table 6.4).

Overall there was no effect of shade on AQE (p=0.669). There was a significant difference between varieties (p<0.001), as Malacca NILs had a lower AQE that the other two varieties, but no effect of *Iw1* (p=0.254), nor any significant interaction between shade and *Iw1*. Pairwise comparison showed that for no NIL was there a significant difference between leaves from control or 60% shade. Similarly there was no overall effect of shade on A_{max} (p=0.090), light compensation point (p=0.992) or A_o (p=0.344). Additionally for no parameter was there an interaction between shade and *Iw1*, and pairwise comparison for each individual NIL showed no effect of shade on any parameter. In conclusion these data indicate that in this trial the efficiency of instantaneous carbon assimilation at various light levels was not affected by long-term shading, and there was no advantage attributed to NILs with *Iw1*.

Table 6. 4 Light curve parameters for field grown flag leaves from 60% shade and control conditions

AQE, light compensation point, A_{max} and A_o for NILs of Hereward, Alchemy and Malacca at anthesis from 60% shade and control. P values of pairwise comparison between control and shade leaves are shown. N=4.

AQE (mol CO_2 mol^{-1} PARi) Average ± S.E				
		Control	60% Shade	p
Hereward	*Iw1+*	0.042 ± 0.000	0.041 ± 0.001	0.43
	iw1-	0.038 ± 0.002	0.036 ± 0.001	0.949
Alchemy	*Iw1+*	0.044 ± 0.002	0.042 ± 0.001	0.532
	iw1-	0.045 ± 0.002	0.045 ± 0.003	0.988
Malacca	*Iw1+*	0.038 ± 0.002	0.040 ± 0.001	0.319
	iw1-	0.039 ± 0.002	0.040 ± 0.002	0.549

Light compensation (µmol^{-1} PARi) Average ± S.E				
		Control	60% Shade	p
Hereward	*Iw1+*	1.57 ± 0.47	2.25 ± 0.54	0.378
	iw1-	1.58 ± 0.93	1.13 ± 1.20	0.775
Alchemy	*Iw1+*	1.87 ± 0.50	1.79 ± 0.42	0.901
	iw1-	1.90 ± 0.38	0.68 ± 0.94	0.237
Malacca	*Iw1+*	1.21 ± 0.42	2.04 ± 0.40	0.199
	iw1-	1.40 ± 0.69	1.99 ± 0.59	0.834

A_{max} (µmol m^{-2} s^{-1}) Average ± S.E				
		Control	60% Shade	p
Hereward	*Iw1+*	22.30 ± 1.90	22.63 ± 0.96	0.881
	iw1-	18.68 ± 1.84	21.20 ± 3.49	0.756
Alchemy	*Iw1+*	27.50 ± 0.45	23.53 ± 1.25	0.024*
	iw1-	27.11 ± 1.39	23.67 ± 1.61	0.165
Malacca	*Iw1+*	22.76 ± 0.31	21.67 ± 0.57	0.145
	iw1-	22.59 ± 0.89	21.84 ± 2.32	0.731

A_o (µmol m^{-2} s^{-1}) Average ± S.E				
		Control	60% Shade	p
Hereward	*Iw1+*	-4.68 ± 0.27	-4.11 ± 0.56	0.383
	iw1-	-4.50 ± 0.58	-4.86 ± 1.59	0.575
Alchemy	*Iw1+*	-4.27 ± 0.21	-4.74 ± 0.47	0.400
	iw1-	-4.28 ± 0.34	-6.08 ± 0.88	0.086
Malacca	*Iw1+*	-4.36 ± 0.22	-3.56 ± 0.60	0.298
	iw1-	-4.43± 0.74	-4.16 ± 0.73	0.301

6.4.2.3.3 Chlorophyll fluorescence

Both dark and light adapted chlorophyll fluorescence parameters provide an indication of PSII function, and have been reported to alter in plants exposed to long term shade. Table 6.5 details the dark-adapted chlorophyll fluorescence parameters F_o, F_m and F_v/F_m for flag leaves of Hereward, Alchemy and Malacca NILs at anthesis grown under 60% shade and control. Overall, F_o was significantly reduced under 60% shade compared to control ($p<0.001$) and this effect can be seen in all NILs. There was no statistically significant effect of *Iw1* on F_o ($p=0.270$) nor interaction between *Iw1* and shade. However, when analysed by pairwise comparison the effect of shade on F_o was only significant for *Iw1+* NILs of Alchemy and Malacca. There was a significant effect of shade on F_m ($p<0.001$). In every NIL F_m was significantly increased in the shade, but again there was no significant effect of *Iw1* nor interaction between shade and *Iw1*. Similarly for F_v/F_m, flag leaves from 60% shade had overall significantly higher F_v/F_m than control leaves ($p<0.001$) and there was no difference between leaves with and without *Iw1* ($p=0.928$) and no significant interactions. This chlorophyll fluorescence data indicates that flag leaves from the shade have a more efficient PSII, and this effect appears to come more from an increase in F_m rather than reduction in F_o. However, the F_v/F_m values of control plants were still between 0.804-0.823, which is considered a healthy range indicating no severely detrimental effects on PSII.

Table 6. 5 Dark adapted chlorophyll fluorescence parameters for shade and control grown flag leaves at anthesis

F_o, F_m and F_v/F_m for flag leaves of Hereward, Alchemy and Malacca NILs grown under control and 60% shade conditions. P values for pairwise comparison between control and shade leaves are shown. N=40.

			F_o		F_m		F_v/F_m	
			Average ± S.E	p	Average ± S.E	p	Average ± S.E	p
Hereward	*Iw1+*	Control	466.10 ± 4.85	0.258	2585.44 ± 49.59	<0.001	0.817 ± 0.004	<0.001
		60% Shade	458.77 ± 4.26		2901.75 ± 35.74		0.841 ± 0.003	
	iw1-	Control	460.65 ± 5.01	0.758	2572.86 ± 38.39	<0.001	0.819 ± 0.003	<0.001
		60% Shade	458.54 ± 4.64		2877.39 ± 44.05		0.839 ± 0.003	
Alchemy	*Iw1+*	Control	468.63 ± 4.59	0.009	2650.18 ± 28.05	<0.001	0.822 ± 0.002	<0.001
		60% Shade	451.63 ± 4.31		2958.34 ± 22.53		0.848 ± 0.001	
	iw1-	Control	469.05 ± 4.10	0.118	2666.55 ± 32.22	<0.001	0.823 ± 0.003	<0.001
		60% Shade	460.70 ± 3.33		2969.75 ± 21.82		0.845 ± 0.001	
Malacca	*Iw1+*	Control	491.55 ± 4.58	0.050	2524.95 ± 36.14	<0.001	0.804 ± 0.004	<0.001
		50% Shade	471.40 ± 5.18		3021.30 ± 28.99		0.844 ± 0.001	
	iw1-	Control	493.79 ± 6.39	0.199	2618.72 ± 43.67	<0.001	0.809 ± 0.004	<0.001
		60% Shade	482.45 ± 4.90		3026.98 ± 29.37		0.839 ± 0.003	

The light adapted chlorophyll fluorescence parameters F_m' and F_s' were also measured in flag leaves under 60% shade and control conditions. ΦPSII and electron transport rate (ETR) were calculated from the data for Hereward, Alchemy and Malacca NILs (Figure 6.10). In Hereward there was a significant effect of shade on both ΦPSII (Figure 6.10a) and ETR (Figure 6.10b) at 1500, 1000 and 750 µmol m^{-2} s^{-1} ($p<0.001$). In both cases control leaves had significantly higher values. However,

although *Iw1+* leaves tended to have slightly higher ΦPSII and ETR than *iw1-* within both environments, this effect of *Iw1* was not significant at any light level. Furthermore the difference between NILs did not change significantly according to shade, indicated by the insignificant interactions between shade and *Iw1*. In neither Alchemy nor Malacca was there a significant effect of shade or *Iw1* on ΦPSII or ETR at any light level. However, *Iw1+* NILs in the shade did tend to have a (non-significantly) lower value of both ΦPSII and ETR than *iw1-* NILs grown in the shade and control leaves.

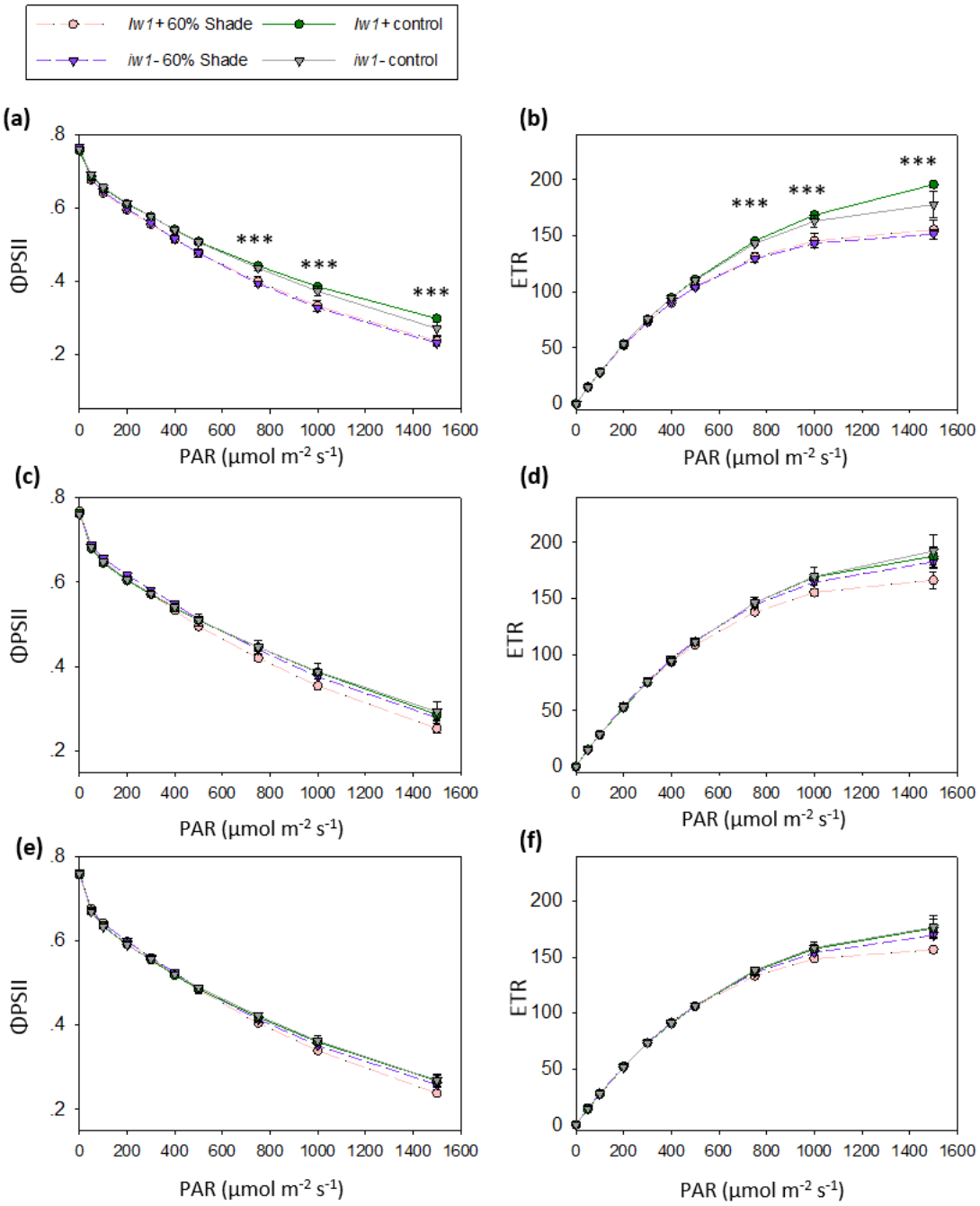

Figure 6. 10 Light adapted chlorophyll fluorescence parameters for flag leaves grown under control and 60% shade

ΦPSII for NILs of (a) Hereward (c) Alchemy and (d) Malacca. ETR under 60% shade and control conditions for (b) Hereward, (d) Alchemy, and (f) Malacca. Significance by pairwise comparison is indicated at p<0.001 (***). N=4, error bars = S.E.

6.4.2.4 Effects on the cuticle

In addition to photosynthesis, another component of plant physiology that may change under shade conditions is the plant cuticle. Cuticular conductance, epicuticular waxes, stomatal density and stomatal conductance were assessed to understand acclimation to shade by the NILs, and any interaction between the effect of *Iw1* on the cuticle and light availability.

6.4.2.4.1 Cuticular conductance

Cuticular conductance was assessed in flag leaves of NILs grown under control, 40% shade and 60% shade for Alchemy and Malacca NILs (Figure 6.11). Overall the effect of shade on rate of water loss was significant ($p<0.001$), but there was no significant difference between varieties or NILs. When analysed for each individual NIL the effect of shade on AS- (Figure 6.11a) was borderline significant ($p=0.052$), and was significant ($P<0.05$) for AS+ (Figure 6.11b), MS- (Figure 6.11c) and MS+ (Figure 6.11d). Although all three varieties showed the same trend, the exact relationship between the three environments did differ. Post hoc Tukey testing showed that for AS+ 40% shade leaves had significantly faster water loss than both control and 60% shade, for MS- 60% shade significantly differed from the other two environments, and for MS+ 40% and 60% shade were significantly different from one another, but neither differed from control.

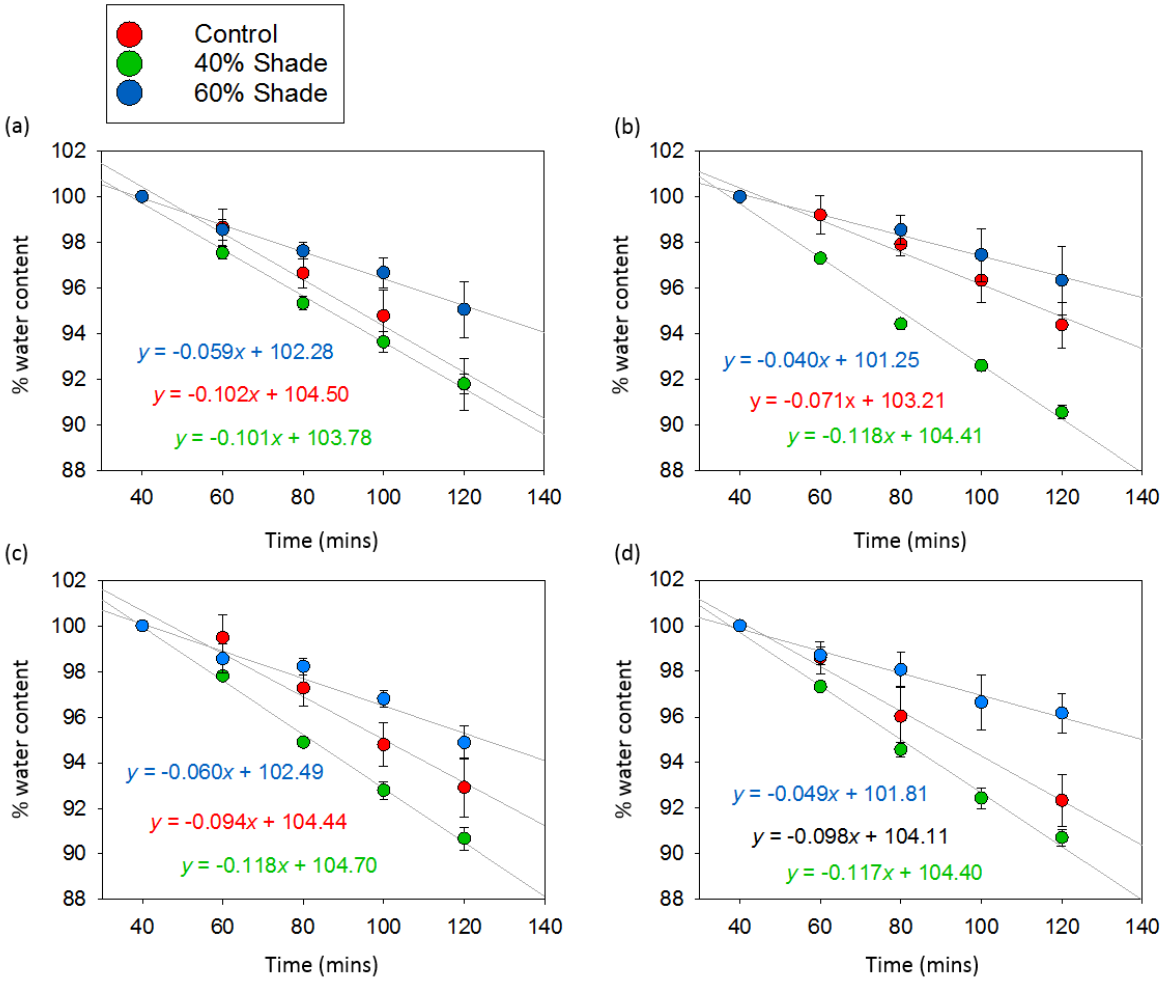

Figure 6. 11 Cuticular conductance of flag leaves grown under control, 40% shade and 60% shade

Water loss over an 80 minute period for (a) AS- (b) AS+ (c) MS- (d) MS+ for flag leaves from control, 30% shade and 60% shade conditions. Regression lines are shown, and the equation of each line is displayed in black for control, green for 40% shade and blue for 60% shade. N=5, error bars = S.E.

6.4.2.4.2 Epicuticular wax

To understand the effect of changing the light environment on epicuticular wax development and composition, flag leaf epicuticular wax biochemistry was analysed at anthesis, around a month after shade was introduced. Each compound class is present in the wax in a variety of chain lengths. To more clearly present overall trends, the various chain lengths have been grouped together to present one value per compound class in Figure 6.12. Detailed data for individual chain lengths are available in Appendix A6. Where there was a discrepancy between chain lengths in the response to shade within a compound class this has been highlighted in the text.

An overall analysis of total wax load (Figure 6.12a) inclusive of all three light environments and varieties indicated that there was no significant difference between NILs with or without *Iw1* ($p=0.352$), no effect of shading ($p=0.763$) and no statistical interaction between shade and *Iw1*. Analysis of each individual NIL shows that for no NIL was there a significant difference between the three environments in terms of total wax load. Repeating the same analysis for fatty acids (FA), primary alcohols (POH), and methylalkylresorcinols (MARs) showed there was no significant effect of *Iw1* ($p>0.1$) or shade ($p>0.1$) on quantity of any of these compound classes and no significant interaction between shade and *Iw1*. This can be seen from the bar charts, whereby there are no consistent shade dependent trends across NILs (Figures 6.12 b, d and e). *n*-Alkanes were significantly affected by presence of *Iw1* ($p=0.002$), with *Iw1+* NILs consistently having more *n*-alkanes in the epicuticular waxes than *iw1-* NILs (Figure 6.12c). This trend was present across all three shade environments, and there was no significant effect of shade on *n*-alkanes ($p=0.511$) and no statistically significant interaction between *Iw1* and shade ($p=0.655$). Although the three way interaction between *Iw1*, variety and shade was not significant statistically ($p=0.697$), there do appear to be some non-significant variety specific effects on n-alkane quantity. For example, in Alchemy the differences in *n*-alkane quantity between *Iw1+* and *iw1-* NILs in the control environment ($p=0.096$) were greater than the differences between NILs in 40% shade ($p=0.425$) and 60% shade ($p=0.786$).

β-diketones (β-DK) were the only wax component for which shade had a significant effect ($p=0.011$). In both Malacca and Hereward (Figure 6.12f) β-DK quantity appeared to decrease as light levels were reduced, although for neither variety was this effect significant when analysed at the variety level (Hereward, $p=0.110$; Malacca, $p=0.383$). However, there was a significant statistical interaction between shade and variety ($p=0.001$), as Alchemy *iw1-* NILs appeared to respond slightly differently. The effect of shading on Alchemy was statistically significant ($p=0.030$), with a negative relationship between light level and β-DK quantity (Figure 6.12f). However there was large standard error associated with the mean for 40% shade. Two of the samples this mean was based

on had a high value for β-DK and two were low. Therefore no data point could be excluded as an outlier. Unfortunately more samples were not available for this NIL to check these values.

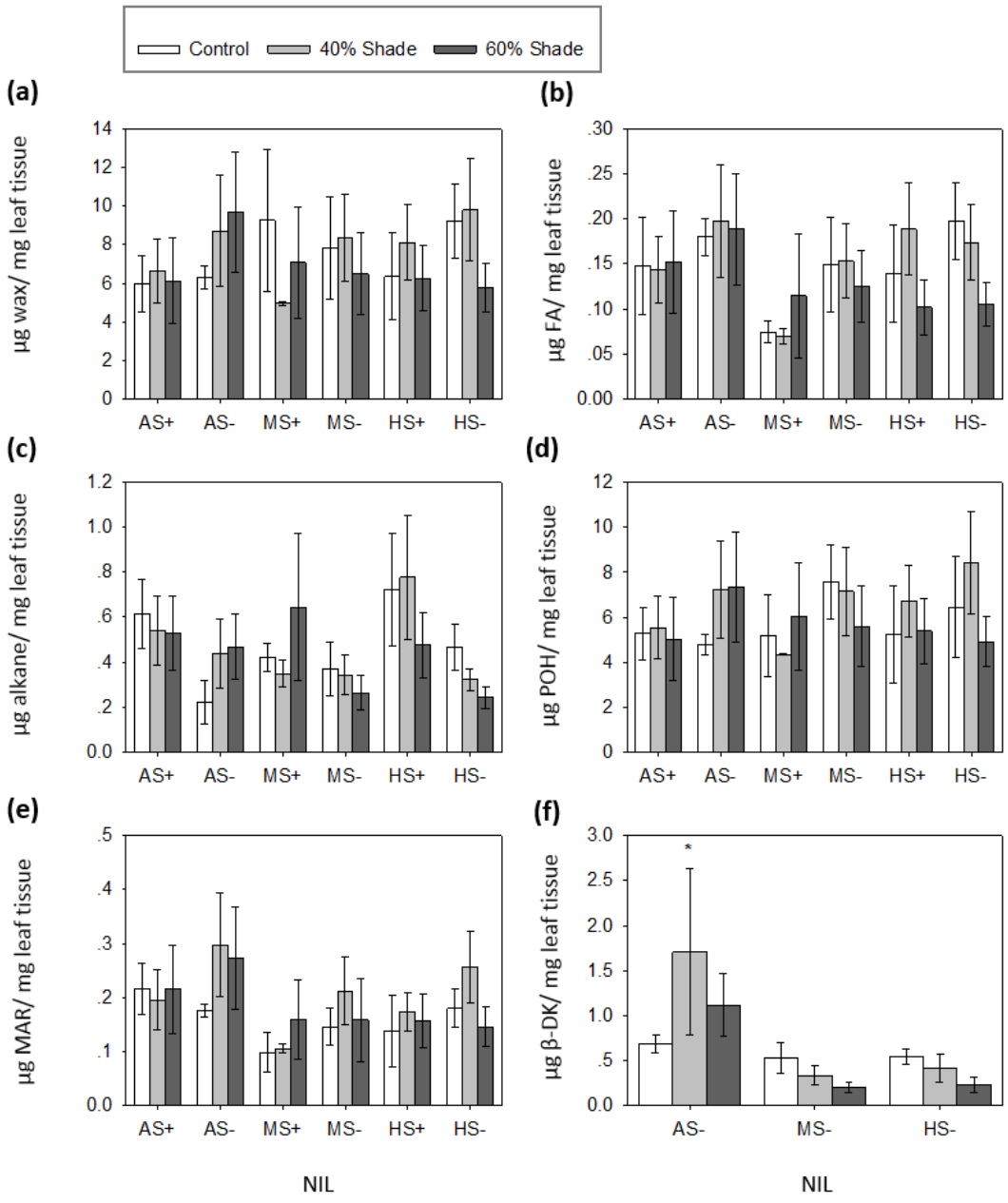

Figure 6. 12 Epicuticular wax composition at anthesis for flag leaves grown under control and shaded conditions

Charts showing (a) total wax load calculated from GCMS data (b) Fatty Acids (c) *n*-alkanes (d) primary alcohols (e) MARs and (f) β-diketones. Quantity of all chain lengths of each compound class have been grouped to give one overall value for each compound class, and then the average of five flag leaves taken. Significance is indicated at the p<0.05(*) level. N= 5, error bars = S.E.

Epicuticular wax composition was also assessed at 40 DPA which was around 10 days prior to full plant senescence. At this point the shading had been erected over the trial for around 2 months. Overall at 40 DPA there was a significant effect of shade on total wax load (p=008). This can be seen

in Figure 6.13a, where flag leaves from 40% shade plants control tend to have lower wax load than those from the control environment or 60% shade (with the exception of Alchemy *Iw1+* NILs). However, the response to shade did not significantly differ between NILs. There was no significant effect of *Iw1* on total wax load (p=0.106), and no significant interaction between *Iw1* and shade (p=0.429). Analysis of total wax load within each individual NIL showed that there was a significant effect on total wax load for *iw1-* NILs of Alchemy (p=0.001), with post hoc testing showing that this effect came from 40% shade which was significantly lower than the other two environments. There was also a significant effect in *Iw1+* NILs of Hereward (p=0.043), with a significant difference between 40 and 60% shade. The same pattern was seen in all other NILs, but the effect was not significant at this level.

Overall there was a significant effect of shade (p=0.002) and variety (p=0.013) on FA quantity (Figure 6.13b). However there was no significant effect of *Iw1*. Analysis at the level of individual NILs indicated that there was an effect of shade on FA quantity in all NILs with the exception of AS+. However, the overall trends were less consistent across NILs as with total wax load, although the interaction between shade and *Iw1* was not significant. In all cases control plants had the highest quantity of FAs. Post hoc testing showed that in AS- and HS+ leaves from 40% shade had significantly reduced levels in comparison to both control and 60% shade leaves. In HS- and MS- there was no significant difference between the two shade environments, but control leaves had significantly higher quantities of FAs. Looking at individual FAs the same overall trends were present in all NILs for C22, C24 and C28 FAs. However, there was no significant effect of shade on C32 FAs (p=0.162). The C32 FAs were present only in very small quantities, so this did not affect the overall trends seen in for the combined FA data.

At 40 DPA *Iw1* still had a significant effect on *n*-alkane quantity (p<0.001), with *Iw1+* NILs of all varieties having greater quantities of *n*-alkanes than *iw1-*. However, this effect of *Iw1* on *n*-alkanes was not dependant on light level, with no significant interaction between *Iw1* and shade. Independent of *Iw1,* shade did have a significant effect on quantity of *n*-alkanes (p<0.001), with a positive relationship between light level and *n*-alkane quantity in most cases. This effect of downregulation of in the shade was coming from the C27, C29 and C31 chain lengths, with levels of all three reduced in shade leaves compared to those from the control trial.

There was a significant effect of shade on POH quantity (p=0.011), although this effect was quite subtle in comparison to FAs and *n*-alkanes (Figure 6.13d). Across all six NILs leaves from 40% shade tended to have less POHs than those grown in control or 60% shade. However when analysed by NILs this was only significant for *iw1-* NILs of Alchemy (p=0.008). Overall *Iw1* did not affect POH

quantity, nor was there any interaction between *Iw1* and shade. Again, the effect of shade was significant across all the POH chain lengths and consistent with the combined data. The amounts of C24 and C26 alcohols were significantly higher in control leaves compared to 40 and 60% shade, whilst C28 and C30 had a significant reduction in 40% but no significant difference between control and 60% shade leaves.

There was an overall significant effect of shade on MARs ($p=0.001$). However, across the NILs the direction of this effect was inconsistent (Figure 6.13e). There was a trend in some NILs for quantity of MARs to be increased in shade leaves but this was not present in NILs of all varieties, supported by a significant interaction between *Iw1* and shade ($p=0.009$). At the compound specific level, as with the general data, MARs were less consistent than the other compound classes. For example in C19 MARs there was only a borderline significant effect of shade ($p=0.045$), and no interactions with any other factor, whereas in C21 MARs there was a significant effect of shade ($p=0.014$), variety ($p<0.001$) and *Iw1* ($p=0.002$), and a significant interaction between both shade and variety ($p=0.01$) and shade and *Iw1* ($p=0.02$). Notably MARs are present in very small quantities in comparison to the other compound classes present in the epicuticular wax.

Overall there was a significant effect of shade on β-DKs ($p=0.003$), with a positive relationship between light level and β-DK quantity present across all three varieties (Figure 6.13f). Post hoc testing showed that overall this effect was coming from the control leaves, which had significantly higher quantities of β-DKs than leaves from either shade environment (Tukey). Overall there was no significant interaction between shade and variety ($p=0.968$), although analysis at the variety level showed that the effect of shade was only significant for Hereward ($p=0.001$) with control being significantly different from the other two.

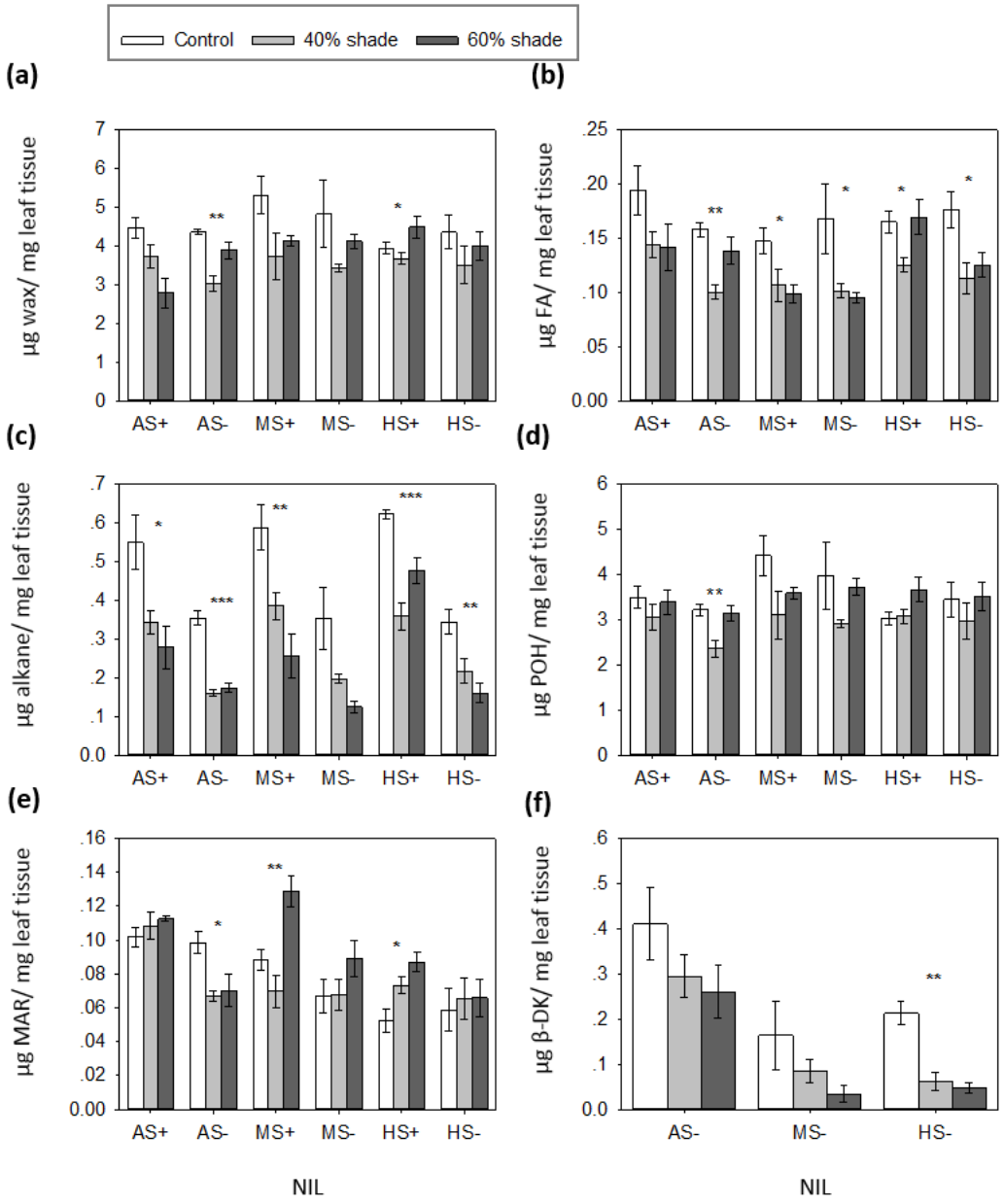

Figure 6. 13 Epicuticular wax composition at 40 DPA for flag leaves grown under shade and controlled conditions

Charts showing (a) total wax load calculated from GCMS data (b) Fatty Acids (c) *n*-alkanes (d) primary alcohols (e) MARs and (f) β-diketones. Quantity of all chain lengths of each compound class have been grouped to give one overall value for each compound class, and then the average of five flag leaves taken. A significant effect of shade for each NIL is indicated at level $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). N= 5, error bars = S.E.

In conclusion, although there appears to be no consistent effect of shade on the epicuticular waxes at anthesis, at 40 DPA two general trends are clear from the epicuticular waxes. For some compound classes there was a positive relationship between light level and quantity, whilst for others, including total wax load, leaves from 40% shade had reduced quantity of epicuticular wax compounds in comparison to leaves from the control and 60% shade groups. However, the effect of *lw1* on the epicuticular waxes did not change across the three shade environments.

6.4.2.4.3 Stomatal density

Stomatal density was measured for flag leaves from both shade and control conditions (Figure 6.14).

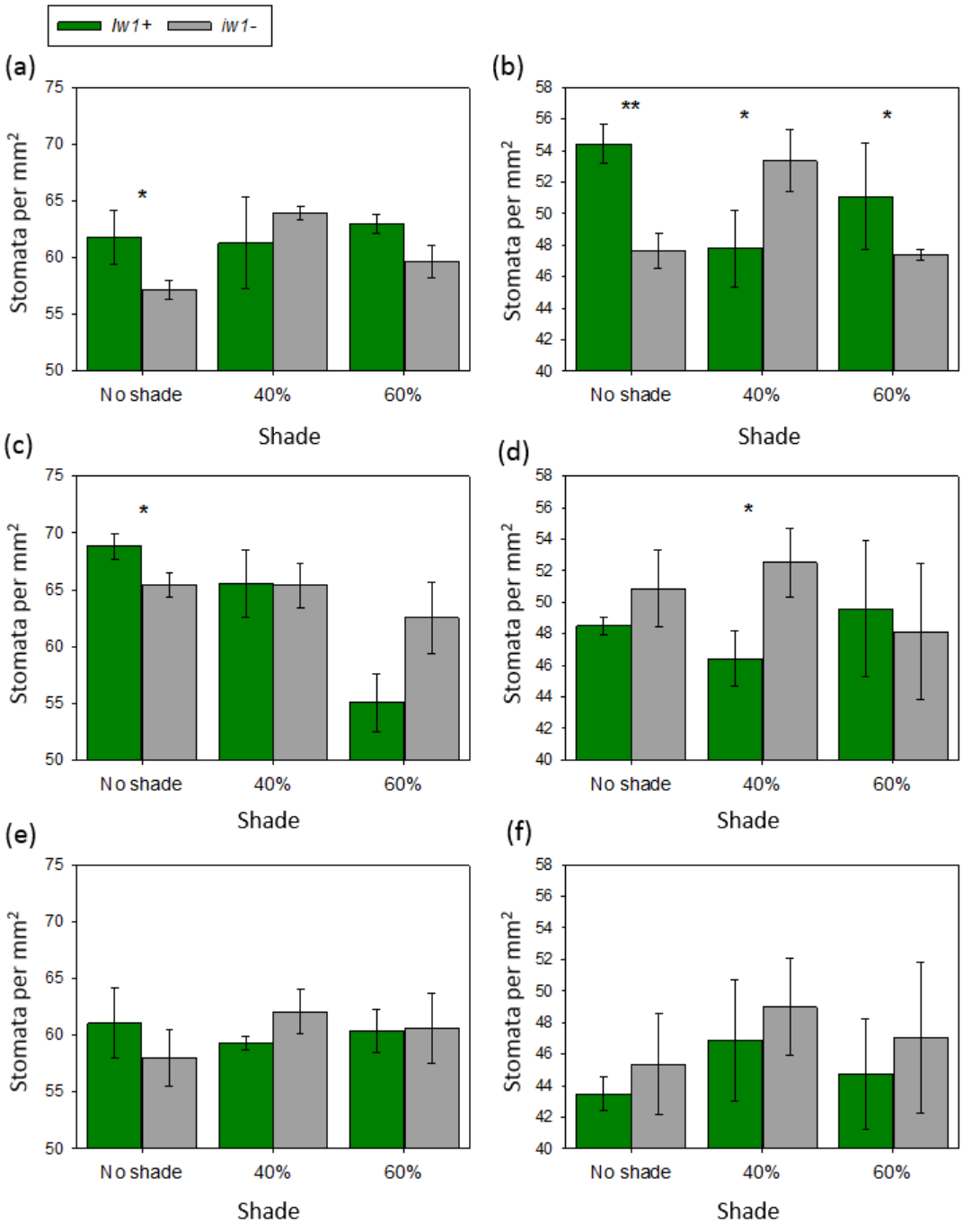

Figure 6. 14 Flag leaf stomatal density for NILs grown under controlled and shade conditions

NILs of Hereward on the (a) adaxial and (b) abaxial surface, Alchemy on the (c) adaxial and (d) abaxial surface and Malacca on the (e) adaxial and (f) abaxial surface. Significance between NILs by pairwise comparison is indicated on the chart $p<0.05$ (*). N=3, error bars = S.E.

An overall analysis inclusive of all three varieties and shade environments indicated that there was no significant effect of *Iw1* (p=.162) or shade (p=0.269) on stomatal density of the adaxial surface (Figure 6.14a, c and e). On the abaxial surface (Figure 6.14b, d, f), although there was no significant effect of shade on stomatal density (p=0.372), there was a significant effect of *Iw1* (p=0.012), a significant interaction between *Iw1* and shade (p=0.001), and borderline significant interaction between *Iw1*, shade and variety (p=0.056).Further analysis at the variety level shows these interactions in more detail.

On the abaxial surface of Hereward (Figure 6.14b) although shade had no significant effect on stomatal density overall (p=0.696), there was a significant interaction between shade and *Iw1* (p<0.001). Under both control and 60% shade conditions *Iw1+* flag leaves had significantly increased stomatal density (p<0.05), whilst this effect was reversed under 40% shade. In Alchemy (Figure 6.14d) under 40% shade *iw1-* flag leaves also had significantly higher stomatal density on the abaxial surface (p<0.05), although unlike Hereward this effect was also present, but not significant, under control conditions. In Malacca there was no effect of shade on stomatal density on either the adaxial (Figure 6.14e) or abaxial (Figure 6.14f) surface.

6.4.2.4.4 Stomatal conductance

In addition to stomatal density, stomatal conductance at varying levels of PAR between 0 and 1500 µmol m^{-2} s^{-1} was recorded to investigate stomatal function under optimum conditions. Figure 6.15 shows stomatal conductance in relation to carbon assimilation across this range of PAR. Across NILs of the three varieties there was no consistent or significant effect of *Iw1* or shade on stomatal conductance in relation to carbon assimilation. These measurements were taken in a leaf chamber under optimum conditions, so indicate no change in stomatal function or development between shade environments.

This data, together with information on stomatal density, indicates that the change in environmental conditions had no consistent effect on stomatal development and function across varieties, and there was no significant difference in the response of NILs with and without *Iw1*.

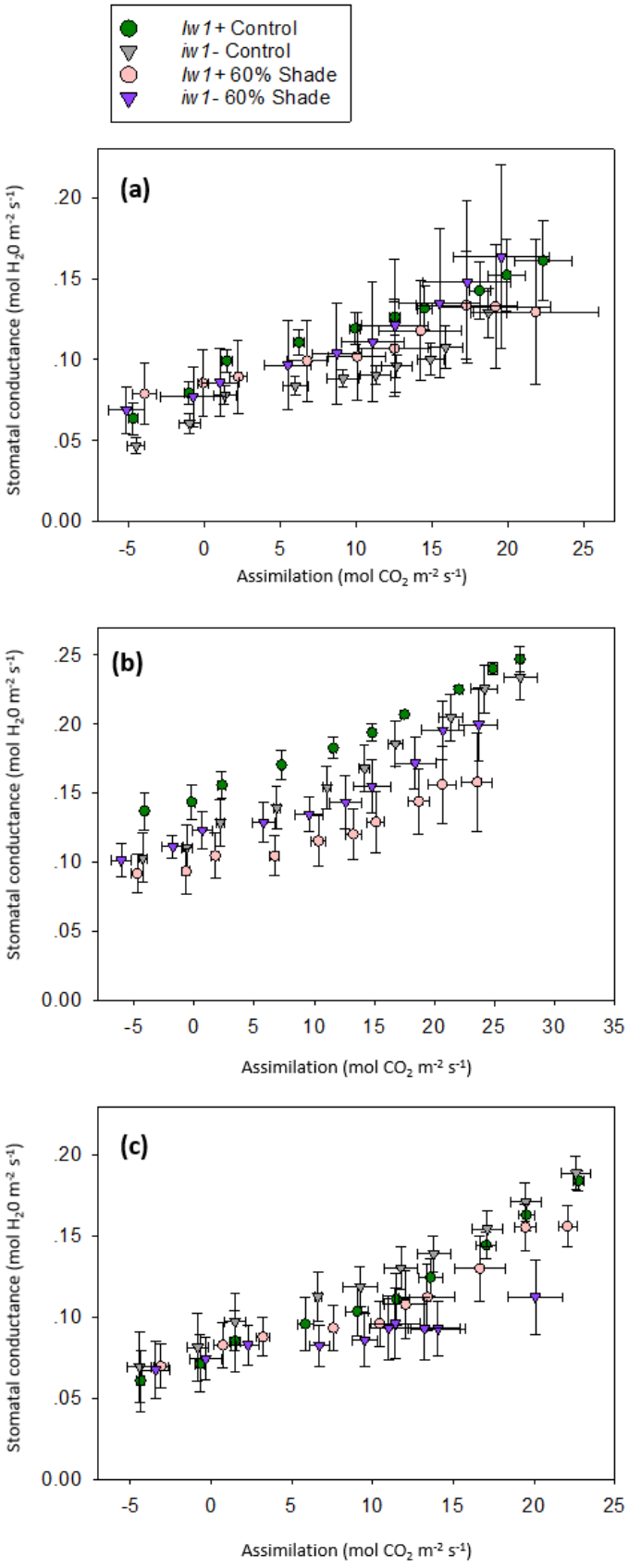

Figure 6. 15 Stomatal conductance relative to carbon assimilation at various levels of PAR for flag leaves grown under control and 60% shade

Charts show (a) Hereward, (b) Alchemy and (c) Malacca. For no variety was there a significant effect of shade or *Iw1* on the ratio of stomatal conductance to carbon assimilation at any level of PAR measured. N=4, error bars = S.E.

6.4.2.5 Water use efficiency

Photosynthesis and properties of the cuticle can change in a plant acclimated to lower light levels. Both of these factors can affect WUE. WUE will also be affected by temperature and RH, both of which were altered under the varying levels of shade. Overall WUE of NILs in the shade trial was assessed through measurement of instantaneous gas exchange at anthesis for flag leaves Hereward, Alchemy and Malacca NILs from control and 60% shade (Figure 6.16). Carbon isotope discrimination ($\Delta^{13}C$) of flag leaves at anthesis and 40 DPA was also measured for NILs of the three varieties under control, 40% shade and 60% shade (Figure 6.17). These data provide an integrated measure of water use across the growing season.

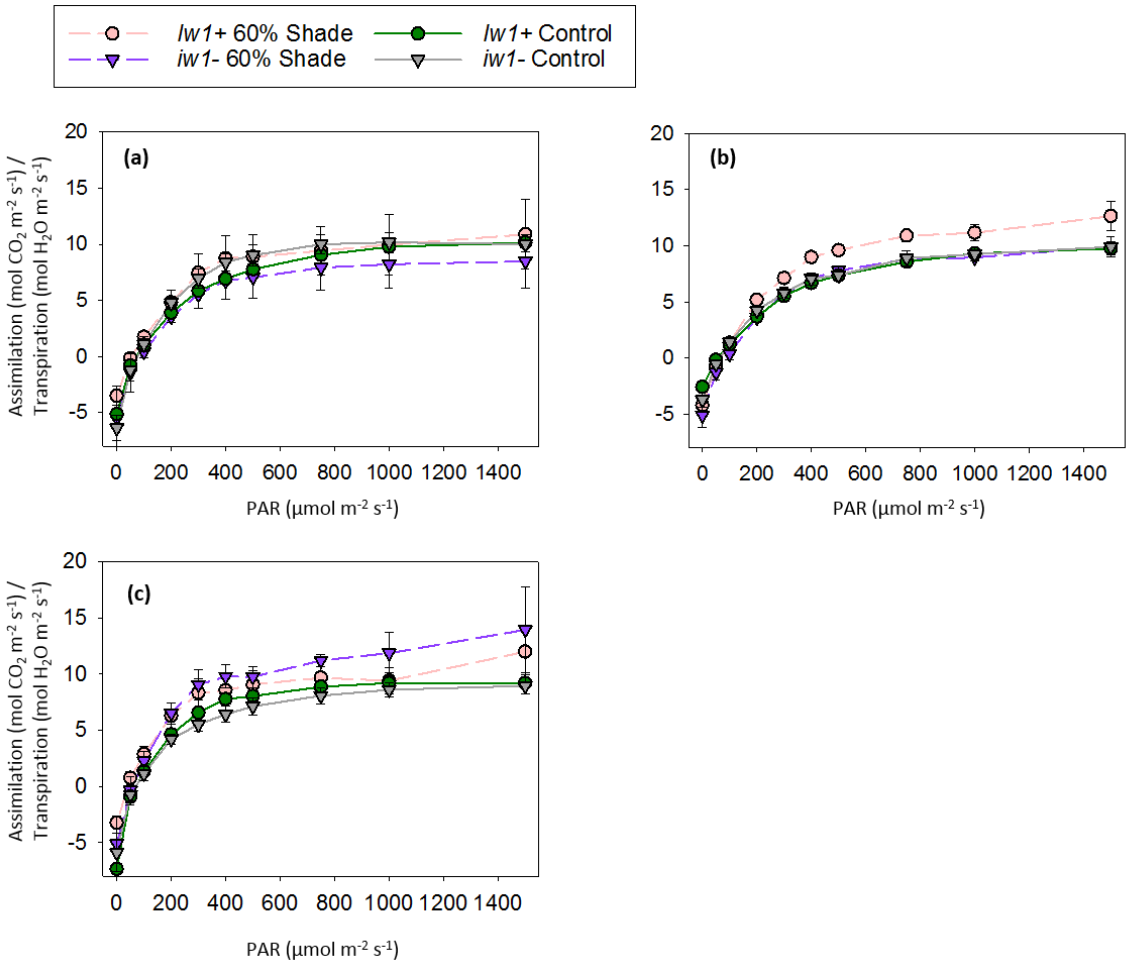

Figure 6. 16 Instantaneous water use efficiency for flag leaves grown under control and 60% shade

NILs of (a) Hereward, (b) Alchemy and (c) Malacca at anthesis from control and 60% shade. WUE was calculated from carbon assimilation and transpiration data measured at levels of PAR between 0 and 1500 µmol. There was no significant effect of shade or Iw1 for any variety. N=4, error bars = S.E.

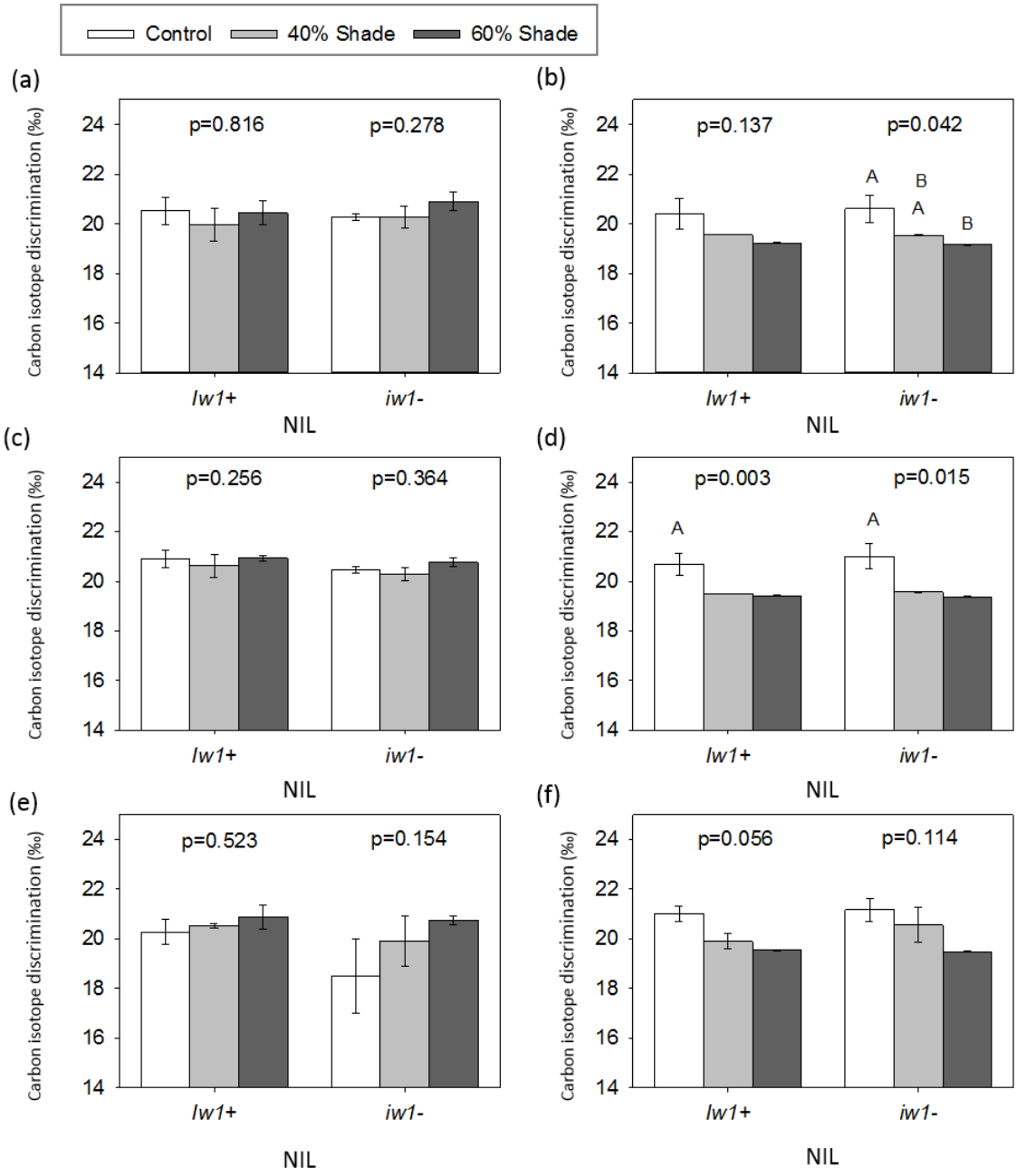

Figure 6. 17 Carbon isotope discrimination of flag leaf tissue grown under control and shade conditions

Hereward NILs at (a) anthesis and (b) 40 DPA, Alchemy NILs at (c) anthesis and (d) 40 DPA and Malacca NILs at (e) anthesis and (f) 40 DPA. P values show the difference between the three shade environments for each NIL. N=3, error bars = S.E.

The instantaneous WUE in the field calculated from gas exchange measurements of transpiration and assimilation indicated that for no variety was there a significant effect of either shade or *Iw1* on WUE at anthesis (Figure 6.16). Additionally there was no consistent trend across the three varieties. For example, in Alchemy (Figure 6.16b) *Iw1+* NILs within the 60% shade appear to have higher WUE than *iw1-* under shade and both NILs in the control environment, whereas in Hereward

(Figure 6.16c) *iw1*- NILs under 60% shade tended to have the lowest WUE. However, the $\Delta^{13}C$ data (Figure 6.17), which integrates the plant's WUE over the entire growing season provides a slightly different conclusion.

Overall at anthesis (Figure 6.17a, c and e) there was no significant effect of shade on $\Delta^{13}C$ ($p=0.138$). Neither was there a significant difference between NILs ($p=0.924$), or varieties ($p=0.325$). At 40 DPA however, the effect of shade was significant ($p<0.001$), with an overall positive relationship between $\Delta^{13}C$ and light availability. Post hoc testing (Tukey) showed that there was an overall trend for leaves grown under control conditions to have a significantly higher $\Delta^{13}C$ than leaves grown under 40% and 60% shade, whilst there was no significant difference between the two shade environments. This indicates that plants grown in the shade were more WUE than those from the control environment; shade leaves were losing less water for every mole of carbon that they assimilated. There was no effect of *Iw1* overall ($p=0.402$), nor a significant difference between NILs in any of the three environments (control, 40% shade or 60% shade) confirming previous findings that *Iw1* does not affect plant WUE (Chapter 5).

6.4.2.6 Field trial in 2015

This shade trial, with the same experimental design, was repeated in the 2015 harvest year. Yield and cuticular conductance were both measured. However, there were some technical issues with the netting during the trial, which could be seen from the RH and temperature data from 2015; there were periods of time whereby there was no difference between the shade blocks and control for these parameters. However, because it was not possible to measure incoming PAR across the growing season in the three blocks, the light conditions that the plants were experiencing during this time were not certain. Therefore data have not been included for interpretation within this chapter. However, although less significant, decreases in yield were observed between the three blocks (yield was reduced by around 15% within the 40% shade block and by around 30% under 60% shade) and there was still no difference between the responses of *Iw1* NILs of any variety. Cuticular conductance was also measured. Whilst a trend similar to 2014 was seen in Malacca and Alchemy, the differences between the three environments were heavily reduced and not significant. Hereward NILs were also measured in 2015 and no difference in cuticular conductance between the three blocks was recorded.

6.5 Discussion

6.5.1 Glaucousness conferred by β- and OH-β-diketones did not provide protection from high light levels

No difference was found between *Iw1+* and *iw1-* flag leaves of any variety in terms of ability to withstand a three hour period of high light stress. This indicates that epicuticular waxes with increased reflectance caused by presence of β- and OH-β-diketones provide no added protection from excessive PAR. Furthermore, there was no difference between NILs in terms of their ability to recover from high light stress. This lack of photoprotection provided by the glaucous epicuticular waxes could be explained by the integrating sphere work carried out in Chapter 4. The total PAR absorbed by single flag leaves did not differ between *Iw1+* and *iw1-* leaves, despite an increase in reflectance of 15-40% associated with glaucousness. Total PAR absorption should be investigated in other species where increased reflectance has been associated with glaucousness (Close *et al.,* 2007; Holmes & Keiller, 2002; Johnson *et al.,* 1983), as this could differ depending on epicuticular wax biochemistry. For example, the major difference between *Iw1+* and *iw1-* NILs is the presence of β- and OH-β-diketones. Any other differences between NILs in terms of epicuticular wax load and biochemistry are limited. It could be that significant increases in wax quantity, or upregulation of different compound classes within the wax, could provide more protection from high light. Other forms of glaucousness, such as that of sorghum or maize where visual wax appearance is determined by biochemical changes other than β- and OH-β-diketones could confer more photoprotective properties (Beattie & Marcell, 2002; Jenks *et al.,* 2000).

Alternatively, the lack of difference between *Iw1* NILs found in the present work could be a function of the methodology. The use of detached leaves to investigate characteristics such as resilience to high light stress offers a simple, practical approach that can be carried out with minimal space and resources. The methods employed here have been used in numerous previous studies to investigate photoinhibition in winter wheat and other species (Chen *et al.,* 2011; Gould *et al.,* 2010; Li *et al.,* 2010; Wang *et al.,* 2000; Yang *et al.,* 2006). However, the use of detached leaves may result in very different responses to stress than those that might occur with intact plants in the field. Sharma *et al.,* (2014) used 41 spring wheat cultivars known to differ in heat tolerance to test the difference in response of detached and intact flag leaves to heat stress. Using detached leaves significant differences in heat tolerance were found between cultivars, and response of each particular cultivar was consistent across two separate experiments. However, when the same experiment was carried out using intact plants results were different. Cultivar response did not correlate with the results in detached leaves. This work suggests that the genetic and physiological processes that determine severity of stress response are different in leaves that have been

detached from the plant and those that are still intact. Furthermore, varieties selected for their tolerance to a stress using a detached leaf method may not be the most stress tolerant in the field.

An additional issue with the use of detached leaves is that many responses to environmental stresses require feedback mechanisms from within the plant over the long term. For example plants transferred into a high light environment have been shown to develop thicker leaves, curled leaf edges, higher mesophyll and epidermal thickness, alter their concentrations of chlorophyll and photo protective carotenoids as well as develop differences in their light saturated photosynthesis (Bailey *et al.,* 2001; Sims & Pearcy, 1992). The ability to acclimate in this way cannot be assessed using detached leaves. The same is true for avoidance mechanisms employed by plants such as changes to leaf angle (Bonos & Murphy, 1999). This raises the possibility that over a longer period of exposure to high light stress acclimation, and productivity could differ between NILs.

In the present work it was not possible to investigate intact plant response to high light stress in the *Iw1* NILs. Although there was no difference between flag leaves in terms of PAR absorbed, the effect in the field could be different. Glaucous *iw1-* canopies in the field reflected 12-20% more PAR than their non-glaucous counterparts (Chapter 4). Light dynamics within the canopy can be very different from the spectral properties of single leaves. Therefore increased reflectance could offer greater protection to the population in the field. A time course experiment to assess chlorophyll fluorescence parameters throughout the day could assess any difference between the NILs in terms of their mid-day depression of photosynthesis and subsequent recovery. This work would need to be carried out in an environment where light levels at noon are consistently above 1500 µmol m^{-2} s^{-1} for an extended period of time to fully understand response to high light levels. In the UK, although these conditions do occur, weather can be unpredictable and it would be difficult to do such an experiment over an extended period of time.

One component of high light stress not investigated in this PhD was the effect of glaucousness on response to UV. It was not possible to measure leaf reflectance and transmission within the UV spectrum, and the HPS light used in the high light experiment does not emit light at UV wavelengths. Work by Holmes & Keiller (2002) suggests that glaucousness significantly increases reflectance of light at UV wavelengths (measured at 330 nm). Whilst exposure to excessive UV can be highly damaging to plant tissue resulting in impaired growth and photosynthesis (Caldwell *et al.,* 1989), more recent work has found that continuous exposure to moderate UV-B does not damage the plant tissue, but could provide a photoprotective advantage. Many leaf adaptations to UV including increased leaf thickness (Bornman & Vogelmann, 1991) also provide protection under high levels of PAR (Wargent & Jordan, 2013). Under field conditions, where levels are PAR are more likely to

be damaging than UV, plants have had slightly greater UV exposure could be at an advantage. Whether or not glaucous *Iw1* NILs display increased UV reflectance, and the effect of this on plant physiology, should be investigated in the future.

6.5.2 Non-glaucousness conferred no advantage under low level irradiance

The second part of this work focussed on the response of *Iw1* NILs to long term reductions in solar irradiation. Grain yield provides an overall assessment of plant productivity over the course of the growing season. In general, across all six lines, yield was reduced by around 30% and 50% compared to control under 40% and 60% shade respectively. This is quite a substantial yield loss, but does suggest some acclimation to the new conditions to compensate for reduced light availability. Reductions in shade of this severity over a long term period have not been widely studied in the literature. However, a short term study assessed grain yield parameters when plants were subject to 50% shade between GS32 and GS55 (heading). Shading reduced the number of grains by 55%, number of spikes by 45%, and number of grains per spike by 30% (Slafer *et al.,* 1994). These yield losses are more severe than in the present long term study, suggesting limited acclimation of the plants to this short term shade. More comparable to the present work, a study in China reduced incoming light from one month prior to anthesis until maturity in winter wheat by 33%. In the two wheat cultivars studied, Yangmai 158 and Yangmai 11, yield was reduced by 18 and 25% respectively (Mu *et al.,* 2010). The proportionate difference between percentage yield loss and percentage light reduction is comparable to that of the *Iw1* NILs.

Although all three varieties of wheat assessed in this study demonstrated a significant decline in yield on application of shade, for no variety was there a significant difference between NILs in terms of the response to low level light. This indicates that the non-glaucous phenotype did not provide an advantage under low level irradiance. For Hereward and Alchemy there did seem to be a small, consistent effect, whereby the difference in yield between *Iw1+* and *iw1-* NILs was reduced under 40% shade compared to control and 60% shade; under 40% shade any yield benefit of the *Iw1* introgression was lost. The yield benefit in non-glaucous NILs of Hereward and Alchemy is likely coming not from *Iw1* itself but from a closely linked gene (Chapter 3). This loss of yield benefit does not correlate with light availability, so could be a function of environmental conditions other than light. The 40% shade environment was potentially the least stressful, with intermediate levels of light, temperature and RH. It could be that the gene(s) responsible for the yield advantage offer no benefit under this specific combination of conditions. Alternatively, application of 60% shade in the present study could mask any subtle differences in response between the *Iw1* NILs. For example, further work in China with Yangmai 158 and 11 indicates that these cultivars were able to more effectively acclimate to lower levels of shading, with yield losses of only 7-10% associated with a

22-23% reduction of incoming light, and only a 2.3% loss of yield with 15% shade (Li *et al.,* 2010; Mu *et al.,* 2010).

6.5.3 There was no change in any photosynthetic parameter under shade conditions

When exposed to a change in prevailing light conditions plants respond over both the short and long term with a number of mechanisms. For example, leaf angle can change immediately, increasing the angle of incidence under low light thus decreasing the light reflected from the leaf allowing more light to be absorbed (Mc Millen & Mc Clendon, 1979; Raven, 1994). Chloroplast position in the leaf can also change over a short time-scale, moving towards the periclinal walls under low light to maximise light absorbance (Kasahara *et al.,* 2002). Acclimation over a longer term can also take place to maximise the light available to photosynthesis. Leaves exposed to long term shade can be thinner than sun leaves, resulting in more efficient light absorption by chloroplasts (Lichtenthaler *et al.,* 2007; Terashima *et al.,* 2001). In addition shade leaves have been reported to have a lower chlorophyll a/b ratio (Lichtenthaler *et al.,* 2007; Valladares & Niinemets, 2008), exhibit higher amounts of photosynthetic enzymes and changes to the electron transport rate (Murchie & Niyogi, 2011; Valladares & Niinemets, 2008). Other characteristics of shade leaf photosynthesis include a lower light compensation point and dark respiration rate, and a higher quantum yield (Valladares & Niinemets, 2008). All of these adaptations allow shade plants to use limited light more efficiently and maximise productivity.

In the *Iw1* NILs neither chlorophyll content nor the chlorophyll a/b ratio was altered under shade conditions compared to control, or was there a difference in the response of NILs with or without *Iw1*. Other studies in wheat that applied shading to the crop for a comparable amount of time did observe changes in pigment content. For example, significant increases in chlorophyll a and b were found by Mu *et al.,* (2010) after long term shading of 22% and 33% and by Li *et al.,* (2010) under shading as low as 8%. Zheng *et al.,* (2011) studied more severe shading comparable to the present work. In their study Chinese winter wheat of the variety Yangmai 13 was subject to 60% shade and 40% shade. These plants exhibited increases of 30-37% in concentration of both chlorophyll a and b in the flag leaf at anthesis, and a decrease in the chlorophyll a/b ratio from 3.41 under control conditions, to 3.16 under 60% shade and 2.92 under 80% shade. Interestingly, the *Iw1* NILs in the present work had a chlorophyll a/b ratio of around 2.62 – 2.85 under both shade and control conditions, which is lower than the value under 80% shade in the Chinese germplasm.

No significant change in A_{max} was found in the *Iw1* NILs under shade conditions, although Alchemy did display a non- significant decrease in A_{max} of around 2 µmol m^{-2} s^{-1} under 60% shade in both *Iw1+* and *iw1-* NILs. This was a parameter also measured by Zheng *et al.,* (2011), who had A_{max} values of around 25 µmol m^{-2} s^{-1} under controlled conditions at anthesis comparable to that of the *Iw1* NILs if not slightly lower. However, under 60% shade this declined significantly by 5 µmol m^{-2} s^{-1}. The work of Mu *et al.,* (2010) also found significant changes to A_{max} at anthesis under less severe shade conditions. Under 33% shade a reduction of 3 µmol m^{-2} s^{-1} was reported in a shade tolerant wheat cultivar, whilst A_{max} of the less tolerant cultivar reduced by twice as much.

It appears that the *Iw1* NILs have not acclimated their photosynthesis to the reduced light levels. It is possible that shading was applied too late in development for plants to acclimate by anthesis. However, measurements in this work were carried out on the flag leaf, which had not yet emerged when shade was applied. Therefore the flag leaf developed fully under reduced light conditions. Furthermore, the three Chinese studies described above all applied shade around one month prior to anthesis, which is comparable to the time scale used in the present work. The discrepancy between the result of the Chinese studies and the *Iw1* NILs could be due to germplasm; all of the studies listed were carried out in Chinese varieties. These varieties may be better able to adapt to new conditions. Alternatively the UK varieties used in the present work could already be adapted for growth under low light levels, as exemplified by the low chlorophyll a/b ratio. The result of this may be that there is limited room for further acclimation.

One parameter that did change according to shade (although not according to presence of *Iw1*) was dark adapted chlorophyll fluorescence. F_v/F_m was significantly increased in flag leaves from 60% shade compared to control conditions. This appears to stem more from an increase in F_m rather than a decrease in F_o. These data indicate that plants from the control plots had slightly reduced function of their PSII compared to shade leaves, likely due to photoinhibition from high light. Supporting this conclusion, levels of carotenoids were found to be positively correlated with light availability. These pigments are associated with protection against oxidative processes under high light, and can dissipate excess light energy (Bartley & Scolnik, 1995; Young, 1991), indicating more light stress within the control plots. However, differences between control and 60% shade leaves both in terms of carotenoids and F_v/F_m were subtle and do not indicate severe photoinhibition in the control plot. These changes in F_v/F_m are consistent with results previously reported in long term shade trials with wheat (Li *et al.,* 2010; Zheng *et al.,* 2011).

Although neither Alchemy nor Malacca NILs displayed any change in ETR or ΦPSII under shade, both *Iw1+* and *iw1-* NILs of Hereward demonstrated a significant decline in both ETR and ΦPSII

under 60% shade, an effect that has also been reported by Zheng *et al.,* (2011). This decline in light adapted fluorescence parameters corresponding with an increase in dark adapted F_v/F_m could indicate that although PSII has a higher potential activity in the shade, more of the excited energy may be dissipated in non-photochemical processes (Roháĉek, 2002). This could have been further explored had it been possible to calculate non-photochemical quenching (NPQ) in the *Iw1* germplasm.

In general there was no difference in the response of NILs with and without *Iw1* in terms of change in parameters related to photosynthesis, but there was potentially a small effect in terms of chlorophyll content. *Iw1+* NILs had significantly lower levels of chlorophyll a under control conditions than *iw1-* (Chapter4). This effect appeared to be amplified under 40% shade in Hereward and Alchemy, indicating that under 40% shade *iw1-* NILs had to further increase levels of chlorophyll a and were at greater disadvantage. However, the effect was not significant statistically and was not present in Malacca NILs. Conclusions drawn from this would be opposing to the yield data, whereby the any advantage conferred by *Iw1* tended to be reduced under 40% shade.

6.5.4 Total wax load was reduced under shade conditions

Many studies have shown that sun leaves have higher quantities of epicuticular wax than shade leaves of the same species (Osborn & Taylor, 1990; Pandey & Nagar, 2002; Sousa Paiva *et al.,* 2003). Analysis of epicuticular wax load of flag leaves at 40 DPA confirms that this is the case in the *Iw1* NILs. In general, flag leaves grown under control conditions had a higher wax load and higher quantity of individual wax components than leaves grown in the shade. However, this may not be an effect solely of the change in light level. In this field trial, as level of shade was increased, temperature decreased and RH increased. Separating out the effects of these three factors in the field is often difficult as they rarely occur in isolation. For example, in wheat and barley, epicuticular wax load has been shown to increase under drought stress (González & Ayerbe, 2010; Haley *et al.*, 1993), but these studies were carried out in the field where an increase in temperature often accompanies terminal drought. However, studies in glasshouse grown plants have shown that epicuticular wax load does increase in wheat (Uddin & Marshall, 1988) and sorghum (Premachandra *et al.,* 1994) when temperature is held constant and plants are exposed to water stress. Furthermore, a study in *Brassica oleracea* found epicuticular wax load to be negatively related with RH when all plants were kept at 21 ^{o}C. Interestingly, in this study when RH was held constant and temperature was varied between 15 and 35 ^{o}C, there appeared to be a negative relationship between temperature and epicuticular wax load (E. A. Baker, 1974). It is likely that the reduced wax load under shade conditions recorded in the *Iw1* NILs is a combination of both lower light levels and higher RH compared to control conditions. Notably, in some instances, wax load, and quantity of wax components is lowest under 40% shade, rather than 60% shade. This is possibly

because 40% shade presents the least stressful of the three environments and plants down-regulate wax production accordingly. Notably for no wax component (excluding β-diketones) was there a significant difference in the response to shade of *Iw1+* and *iw1*- NILs of any variety. From this it can be concluded that in no environment were the *Iw1+* (or *iw1*-) NILs at an advantage, and both glaucous and non-glaucous plants were experiencing equal levels of abiotic stress.

Despite these marked differences in epicuticular wax load at 40 DPA, there were no significant differences between the three environments at anthesis (GS61-69). It is possible that plants had not had time to acclimate to the new conditions at this stage. However, epicuticular wax synthesis is a dynamic process, and at this sampling point shading had been in place for around a month. A study in *Prunus laurocerasus* found that waxes had re-appeared on the plant only 2 days after complete removal of the epicuticular waxes, and normal wax development had resumed within 5 days (Jetter & Schäffer, 2001). This ability to regenerate and adapt the epicuticular waxes does seem to be heavily dependent on species and developmental stage (Neinhuis *et al.,* 2001). For example, a study on 16 species found that in leaves where epicuticular waxes were well established and no longer developing, wax removal only resulted in re-generation in 6 of the 16 species, whereas in developing leaves 13 out of 16 species exhibited wax regeneration, some starting to synthesise new waxes immediately after wax removal (Koch *et al.,* 2009). A time-course experiment in *Iw1* NILs of Malacca and Alchemy indicated that epicuticular wax composition was dynamic throughout plant development (Adamski *et al.,* 2013). Furthermore, the flag leaf had not yet emerged at the point when shading was applied, so the leaf and its waxes would have fully developed under shade conditions. From this I conclude that either the signals determining flag leaf epicuticular wax were already in place prior to shading and could not be altered, or altering the epicuticular wax composition would not have been beneficial to plant function at this stage.

6.5.5 *Iw1+* NILs had significantly higher cuticular conductance under 40% shade

Although there was no change in epicuticular waxes at anthesis, there did appear to be some effect of shade on cuticular conductance at this point, and a difference in response between NILs. Leaves from 40% shade had significantly faster rates of water loss through the cuticle than those from 60% shade or control. These measurements were taken in the laboratory using excised leaves, indicating an effect from some morphological change in the leaves themselves rather than altered evaporative demand. Although no consistent differences were found at anthesis in terms of total wax load or other wax component, epicuticular wax is not the only cuticular component sensitive to environmental changes. For example, increases in leaf thickness have frequently been reported under high light, including increased deposition of all cuticular components (Osborn & Taylor, 1990; Sousa Paiva *et al.,* 2003). Leaf thickness has also been reported to increase under low humidity (Torre *et al.,* 2003; Wright & Westoby, 2002) and high temperatures (Djanaguiraman *et al.,* 2011).

Neither leaf nor cuticle thickness were measured in the present study, and could have resulted in changes to cuticular conductance. For example leaves of Baroness roses (*Rosa x hybrid*) grown in 90% humidity had a poorly developed epidermis, more intercellular airspaces, and less palisade and spongy mesophyll parenchyma cells than plants grown under 70% humidity. This lead to significantly higher rates of water loss after being detached from the plant (Torre *et al.,* 2003). However, in the *Iw1* NILs, flag leaves with the fastest rate of water loss were recorded in the intermediate environment. This suggests a complex interaction between light, humidity and temperature. This is something that should be investigated under controlled conditions to understand precisely where the effect is coming from.

6.5.6 Stomatal function was not affected in either *Iw1+* or *iw1-* NILs

Stomatal density is heavily influenced by environmental factors. Studies in a number of species have found stomatal density to be significantly reduced in shade leaves compared to sun leaves (Gay & Hurd, 1975; Poole *et al.,* 1996), and a negative relationship between stomatal density and temperature (Beerling & Chaloner, 1993; Ciha & Brun, 1975; Luomala *et al.,* 2005) as well as humidity (Torre *et al.,* 2003). However, in the present work stomatal density was not significantly affected by the change in environmental conditions, and there was no consistent difference in response between *Iw1+* and *iw1-* NILs indicating no interaction between these environmental conditions and the action of *Iw1* on stomatal development. Other parameters known to be important in stomatal function such as patterning and anatomy (Lawson & Blatt, 2014) were not assessed in the present trial. However, instantaneous measurement of stomatal conductance under optimal conditions across various levels of PAR found no consistent difference between leaves from control and 60% shade conditions. This indicated no change in stomatal function under the shade.

6.5.7 Water use efficiency was negatively related to light availability

Overall at anthesis, instantaneous gas exchange measurement of transpiration indicated no difference between shade environments, and combined with the carbon assimilation data no difference in WUE was found between shade environments. Additionally there was no effect of *Iw1* on WUE in any environment. However, conclusions drawn from the carbon isotope discrimination data are slightly different. At anthesis there is no difference in flag leaf $\Delta^{13}C$ between the three environments which indicates no difference in WUE. However, at 40 DPA WUE appears to be negatively related to light availability. Under the shade, plants were exposed to lower temperatures and higher relative humidity than control. These conditions are conducive to low evapotranspiration. A reduction in evapotranspiration less than the corresponding reduction to photosynthesis under low light conditions would increase WUE. The discrepancy between the conclusions drawn from the $\Delta^{13}C$ at anthesis compared to 40 DPA could be due to the nature of

these data. $\Delta^{13}C$ is an integrated measure of plant activity over a period of time. At anthesis the plant had only been photosynthesising and transpiring under the shade for one month. At 40 DPA, over a month later, changes to the C^{12}/C^{13} ratio would have had more time to accumulate within the plant tissue.

6.5.8 Conclusions

In the low level irradiance trial, plants grown in the shade under low level irradiance did down-regulate quantity of epicuticular waxes. Although this was likely to be an effect not only of light, this result does indicate that epicuticular wax may play a role in protection from high light levels. However, in the water bath experiment there was no difference between the *Iw1+* and *iw1-* NILs of any variety in terms of their response to high light. This suggests that although β- and OH-β-diketones increase PAR reflection of the epicuticular waxes, this does not alter the plant interaction with the environment in terms of PAR made available to the photosynthetic tissues.

Irrespective of *Iw1*, the germplasm used in this study appeared to compensate to some extent to the long term shade conditions, demonstrated by losses of yield under low light proportionately smaller than the levels of shading applied. However none of the physiological parameters measured were significantly altered in the shade environments compared to control. This indicates that other factors not measured may have been changing under the shade conditions to compensate for the reduced light. Additionally, yield losses could have been further reduced had these parameters acclimated to the shade conditions. Moving forwards it could be beneficial to identify UK wheat germplasm that can better acclimate to reduced light.

A recurring theme throughout the long term shade study was for the 40% shade environment to differ from both control and 60% shade both in terms of overall trends and, in some cases, interactions between *Iw1+* and *iw1-* NILs. Each of the three environments assessed within this study had a unique set of environmental conditions and any changes in physiology could not be attributed to any one parameter. The 40% shade environment could be considered the least stressful of the three environments, with lower temperatures and higher humidity than control, but higher light levels than 60% shade. It is possible that this unique set of conditions set it apart from control and 60% shade. Alternatively, 60% shade could have been so severe that any minor differences in acclimation between NILs were masked. Further investigation of more low level shading in the range of 10- 50% shade could provide more insight into the ability of UK germplasm to adapt to lower light levels, and any differences between the glaucous and non-glaucous phenotype.

This work has opened up a number of avenues for further exploration, and as only one year of data was available could be treated as a preliminary study to more in depth work. Further field trials

using UK germplasm in addition to experimentation under controlled conditions whereby the effects of light, temperature and RH can be distinguished would provide more insight.

Chapter 7: Conclusions and synthesis

There is currently limited variation in domesticated wheat germplasm for epicuticular wax type, with the majority of cultivated varieties displaying a glaucous, visible wax, phenotype. However, our understanding of the effect of glaucousness on the physiology of wheat and other cereal crops is based mainly on research carried out within a Mediterranean climate. This could therefore be of limited relevance for other environments. To contribute towards this gap in the scientific literature, this PhD aimed to answer the question: Does non-glaucousness, as conferred by *Iw1*, provide an advantage for yield and physiology of UK wheat?

This chapter will summarise the key findings of the PhD in relation to this overall aim, and identify any specific areas in which the research should be taken forward. Limitations to the research, and suggestions for how these issues might be overcome in future studies will then be explored. Finally, I consider the key lessons learnt during the PhD process in regards to the study of crop physiology, and provide some suggestions as to how these could be addressed in the future.

7.1 Key findings and future directions

7.1.1 Non-glaucousness conferred by *Iw1* offered no yield advantage

The majority of existing literature suggests that the glaucous phenotype confers a yield benefit under drought (Clarke & McCaig, 1982; Febrero *et al.,* 1998; Jefferson *et al.,* 1989; Johnson *et al.,* 1983; Merah *et al.,* 2000; Premachandra *et al.,* 1994; Richards *et al.,* 1986), whilst non-glaucousness may offer an advantage under optimum conditions (Merah *et al.,* 2000; Simmonds *et al.,* 2008). However, during this PhD project **the non-glaucous phenotype (*Iw1*) was found to offer no yield advantage in UK field trials**. Yield is a complex and variable trait. The outcomes of this PhD, and their opposition to existing literature, highlight the importance of studying yield over multiple years and locations in a number of genetic backgrounds prior to drawing conclusions.

Although no yield benefit was associated with *Iw1* itself, this work found that **one or a number of genes within the introgressed region on chromosome 2BS might confer a yield advantage.** Two of the six varieties tested, Hereward and Alchemy, did show a yield benefit associated with the *Iw1* introgression of around 3% and 5% respectively. Work with the recombinant lines showed that this yield effect was most likely coming from a closely linked gene rather than *Iw1* itself. Given that global wheat yields are currently only increasing by 0.9% per year (Ray *et al.,* 2013), potential yield improvements of the magnitude described above would be worth pursuing. The next steps will be

to identify exactly where this effect is mapping on the chromosome and identify any possible candidate genes. **Non-glaucous NILs of Hereward had significantly higher light saturated photosynthesis of the flag leaf than their glaucous counterparts**. Improved photosynthesis of the flag leaf has been linked to higher yields in wheat (Fischer *et al.,* 1998; Jiang *et al.,* 2003), suggesting a possible mechanism by which yield was improved in the *Iw1+* NILs. However, this same effect was not recorded in the Alchemy NILs, and further exploration with Hereward recombinant lines did not map this effect to the same place as yield. This indicates that yield is being improved via an alternative mechanism.

7.1.2 Non-glaucousness as conferred by *Iw1* had no effect on water use efficiency

Components of assimilation and transpiration, in addition to carbon isotope discrimination, were measured in order to understand WUE in the field. The conclusion was reached that **there was no effect of *Iw1* and non-glaucousness on WUE** confirming previous work in the UK using *Iw1* NILs (Adamski *et al.,* 2013). However, this is contrary to previous work carried out in Mediterranean environments, where both positive (Richards *et al.,* 1986) and negative (Febrero *et al.,* 1998; Merah *et al.,* 2000; Monneveux *et al.,* 2004) effects of glaucousness have been found on water use. In addition to environmental conditions, the effect of glaucousness on WUE could be determined by the specific biochemical make-up of the epicuticular waxes. This could differ between studies depending on the underlying genetics. Therefore, to understand this discrepancy, *Iw1* would need to be introgressed into various glaucous Mediterranean wheat varieties, and WUE assessed under drought stress. This would provide direct comparison with the present work.

Previously, concerns around WUE may have prevented the introgression of traits linked to non-glaucousness into breeding varieties. However, this work demonstrates that, at least where *Iw1* is concerned, this should not be factored into decisions regarding UK breeding. For example, the yield benefit in Hereward appears to be mapping very closely to *Iw1* in a way that the yield and non-glaucous trait cannot currently be separated by recombination. This region can now be further investigated on the understanding that there would be no negative effect on WUE.

7.1.3 Reduced reflectance of non-glaucous epicuticular waxes does not change PAR absorbance

Epicuticular wax morphology determines light scattering from the plant surface, and glaucousness has been widely reported to increase PAR reflectance (Febrero *et al.,* 1998; Jefferson *et al.,* 1989; Johnson *et al.,* 1983). Adding to this knowledge, this PhD conclusively showed that **it is the β-diketones and OH-β-diketones in the epicuticular waxes that increase PAR reflectance of the flag**

leaf. Waxes lacking these compounds did not alter flag leaf reflectance. However, this work also showed that even though non-glaucous *Iw1+* flag leaves reflect less PAR, **there was no overall increase in PAR absorbance of *Iw1+* flag leaves.** This conclusion was supported by data that showed no effect of glaucousness on flag leaf photosynthesis at varying light levels.

This PhD also showed that ***Iw1+* NILs had reduced PAR reflectance at the crop canopy level**, confirming findings of previous studies**.** However, measurement of light interception at various levels in the canopy proved inconclusive. Canopy dynamics can be quite different to the single leaf level, so it is possible that the reduced reflectance does change the light available further down the canopy. Light interception is an important component of radiation use efficiency, which itself is a target for yield improvement. Therefore, this will be an important parameter to measure in the future as better technologies become available. Also at the crop canopy level, the increased reflectance of glaucous canopies has previously been linked to significantly lower temperatures (Jefferson *et al.,* 1989; Richards *et al.,* 1986). However, in the *Iw1* NILs **there was no effect of glaucousness on canopy temperature**.

7.1.4 The increased reflectance of glaucous (*iw1*-) epicuticular waxes offers no additional photoprotection from high PAR, nor do they present a disadvantage under low light conditions

To further investigate the difference in spectral properties between NILs, plant response to high PAR intensity was measured in the laboratory setting. However, **the increased reflectance of glaucous *iw1*- epicuticular waxes offered no added photo-protection from high levels of PAR**. This could be because there was no difference in total PAR absorbance between NILs. Alternatively this could have been due to methodology. It was only possible to do this work in a laboratory setting using excised leaves. This may limit the relevance of the conclusions in terms of application in the field. Reflectance outside of the PAR spectrum, and response to other forms of radiation, such as excessive light in the UV spectrum or infrared was not explored in this PhD. Glaucous epicuticular waxes have been shown to increase reflectance outside of the visible light spectrum (Holmes & Keiller, 2002), so this could be an avenue for future exploration with respect to the *Iw1* NILs.

Although high light intensities could not be investigated over the long term in the field, it was possible to study the effect of low-level irradiance on plant physiology. The main conclusion of this work was that **the reduced reflectance of *Iw1+* waxes offered no advantage under low-level irradiance.** In a more general sense, changes that might be expected in certain physiological parameters on exposure to shade, such as increased pigmentation, were not observed in any of the three varieties. It is possible that UK germplasm is already adapted to low light levels, and cannot

improve further. Alternatively, factors other than those measured such as canopy structure and morphology were changing. Experiments of this kind within a UK setting are uncommon, and thus it is difficult to draw solid conclusions from this initial set of data. Further experiments of this kind will be important to answer these open questions and understand how wheat varieties can best be adapted to changing light conditions.

Overall, based on the data presented in this thesis, and the previous literature I have reached the conclusion that ***Iw1*** **offers no physiological advantage to wheat within the current UK environment.** This conclusion could perhaps be applied to other agricultural systems with similar climatic conditions. However, the UK climate is projected to change over the coming decades. In order to future-proof food production we need to understand how these environmental changes will manifest and adopt particular sets of traits accordingly.

7.2 Limitations

Specific limitations associated with the various methodologies employed in this research have been discussed within the relevant chapters. However, there were a number of limitations that persisted throughout the PhD. Ability to overcome these could have significantly improved progress and allowed a more in depth understanding of plant physiology.

7.2.1 Measurement at the canopy level was not always possible

Lack of equipment to take physiological measurements at the canopy level in the field proved to be a major limitation. For example, all gas exchange measurements were taken using an IRGA on single leaves. During these measurements, leaves are placed into a chamber and gas exchange measured under optimum conditions. However, the activity of the canopy as a whole could be quite different to that of single leaves. Therefore an understanding of gas exchange, not just of the flag leaf but the entire plant or canopy, would be beneficial. The use of carbon isotopes did provide an integrated measure of WUE over a period of time (but again, just in single organs), and measurement of yield indicated productivity over the entire growing season. However, within both of these surrogate measures, specific components contributing to the end result cannot be separated out.

Another aspect of this research where measurement at the canopy level would have been beneficial was the work on spectral properties. Although reflectance from the crop canopy was successfully measured, measurement of the amount of light penetrating the canopy was less conclusive. This is an area that is a challenge for crop physiology in general as the majority of cereal

crops are drilled in rows. This makes measurement of light interception difficult due to interference from large gaps in the canopy. Progress is being made in this area and new techniques are being developed. For example imaging technologies able to measure amount of shadow cast by forest canopies could have application in the crop sciences. However there is still progress to be made. Light interception is an important component of radiation use efficiency, which itself is a target for improving yield potential. Therefore this will be a valuable area to focus resources in the future.

7.2.2 Low-throughput phenotyping

The amount of data that could be collected during this PhD was limited by the low-throughput nature of many of the phenotyping techniques employed. For example the use of an IRGA system to take gas exchange measurements allowed only four to five flag leaves per day to be measured in the field. Another example was the integrating sphere used to measure leaf spectral properties. This took considerable time to take measurements on each leaf. These labour intensive, time-consuming processes, place limits on data generation and require careful prioritisation of germplasm for phenotyping. For example, I chose to focus on the flag leaf for the majority of measurements to gain a comprehensive picture of the physiology of this organ in glaucous and non-glaucous plants. However, measurement on multiple organs such as the inclusion of the spike and leaf sheath would have been more informative and provided a more complete over-view of plant physiology. Higher-throughput processes (or more time) would have made this possible.

The availability of instrumentation and techniques for high-throughput phenotyping is becoming more widespread. This is an area of research that has rapidly progressed over the past 3 years of this PhD project. For example, the use of drones and image analysis are becoming more commonplace in field phenotyping. It is now possible to measure components such as transpiration, canopy temperature and plant growth over large populations in the field in short periods of time. However, although this generates large quantities of data, there can be a trade-off with resolution. Although the low-throughput phenotyping methods employed in this PhD did not allow assessment of large amounts of plant material, they did provide detailed information on a small subset. Ideally both low and high throughput phenotyping techniques would be used in parallel in future work to provide both a general overview of the population, and detailed information about specific individuals.

7.3 Personal thoughts moving forwards

The research also highlighted a number of areas in which I think the crop physiology field as a whole should treat as priority in the future. A theme running throughout this PhD was the importance of

using well-defined germplasm to assess crop physiology. The *Iw1* NILs and recombinant lines used in this work had a clearly defined introgressed region into chromosome 2BS. The genetics of *Iw1* was understood as far as possible, and the use of molecular markers within the region allowed traits to be mapped to specific locations on the chromosome. Furthermore, the effect of *Iw1* on epicuticular wax biochemistry had been previously studied in depth in a variety of organs at a number of developmental stages (Adamski *et al.,* 2013). This genetic and biochemical understanding informed the conclusions drawn from the physiology work. This allowed any differences between the NILs to be attributed, or not, to *Iw1* and the non-glaucous trait. Past work regarding the effect of glaucousness on yield and physiology has not always used near isogenic lines or characterised the genetics and biochemistry underlying the wax phenotype. This can make comparison between studies difficult and does not allow clear understanding of any observed difference between glaucous and non-glaucous phenotypes.

Leading on from this, a second lesson learned from this research is the importance of integrating genetics and physiology. Without inter-disciplinary research combining these two areas, progress will be slower in terms of identifying complex traits that could contribute to yield. This is also vital for an understanding of how the benefits conferred by a trait may differ between environments. Developments in molecular biology have moved forwards very fast in recent years. For example, during the course of this PhD the draft genome for *T. aestivum* was published (Mayer *et al.,* 2014), allowing great progress in genetic marker selection for desirable traits and identification of candidate genes. These developments are resulting in very large amounts of genetic data being generated about crop plant species. However, it is very important that developments in physiology keep pace with this. Identification of novel candidate genes and QTL for yield improvement cannot be applied in the field without an accompanying understanding of plant physiology.

Finally, this work demonstrates the importance of studying a trait across multiple environments in a variety of germplasm. Taking account of the conclusions from this PhD, and from other studies into glaucousness in the literature, the same phenotype can lead to very different effects on yield and physiology dependent on environmental conditions. This will become increasingly important into the future as the climate changes. To maximise the efficiency of different agricultural systems, the cultivars and phenotypes used must be optimised for those particular environmental conditions. Knowledge of how a particular trait affects yield over a range of environments would aide informed decision making regarding which traits to prioritise during breeding. For example, regions projected to experience greater drought and heat might consider adopting the glaucous phenotype in their wheat varieties, whereas those projected to experience temperate conditions would not need to consider glaucousness as a priority when choosing germplasm characteristics.

Having clear recommendations connecting environmental conditions with a particular set of physiological traits would help select germplasm for growing regions both now and in the future.

Abbreviations

Δ13C	Carbon isotope discrimination
Amax	Light saturated assimilation
A0	Dark respiration point
AQE	Apparent quantum efficiency
BC2	Backcross 2
BC4	Backcross 4
DH	Doubled haploid
DNA	Deoxyribonucleic acid
DPA	Days post anthesis
ETR	Electron transport rate
GC-MS	Gas chromatography mass spectrometry
GS	Growth stage
IRGA	Infrared gas analyser
KASPar	Kbioscience competitive allele specific PCR genotyping system
MAR	Methylalkylresorcinols
NIL	Near isogenic line
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
POH	Primary alcohol
PSII	Photosystem II
QTL	Quantitative trait loci
RH	Relative humidity
RUE	Radiation use efficiency
SEM	Scanning electron microscope
WUE	Water use efficiency

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Appendices

A1 KASPar Markers for genotyping recombinant lines

SNP name	FAM primer	HEX primer	Common primer
BS00084668	CACCATAGTCGCCCTCAAATCCT	ACCATAGTCGCCCTCAAATCCC	GCTGAGGCCACTAAACCAACATTCAT
BS00009972	GATGCATATATTTTTGGCTCTACAGG	CATGATGCATATATTTTTGGCTCTACAGA	GTGCCAGAAATGAGACGGTTGAAGAT
BS00070900	AGGATGCGTTGTTTGCCAACTCTA	GGATGCGTTGTTTGCCAACTCTG	GCCATCCACCACCACCATAAGTTA
BS00010318	GACAAAGTTGGTGGTAAATTCTCTTAC	GGACAAAGTTGGTGGTAAATTCTCTTAT	GCAAGCATGCCTTCCACCACCAA
BS00045163	CCTCTGCAACGCCGCCGC	CCTCTGCAACGCCGCCGT	TATGCGGGTCGGCGATGACGTT
BS00010637	AGCCCCCAAGGTACTCGATC	AGCCCCCAAGGTACTCGATT	CATGAGTGACGATCCAAGTTTCAAAGATT
BS00073542	AGGATGCGTTGTTTGCCAACTCTA	GGATGCGTTGTTTGCCAACTCTG	GCCATCCACCACCACCATAAGTTA
BS00063694	ACTGCAATGATTCTTGTGCGAGCA	CTGCAATGATTCTTGTGCGAGCC	GATGATGGCATCAACCATGGTTTAAACAT
BS00006788	GTGTATCATTTCCTTTGTGCGGATTC	GTGTATCATTTCCTTTGTGCGGATTT	GTGCAGTTTCTACTTGAAGCGACAAATTT
BS00065040	AAGGTCAGTTCTTGGTTTGCCTCA	GGTCAGTTCTTGGTTTGCCTCC	AAGCAAAGCGCCAACTGCAACGTAA
Bra1190	TGTGGTGTATCATTTCCTTTGTGT	TGTGGTGTATCATTTCCTTTGTGC	CTTGAAGCGACAgATTTGAAGAATC
BS00009848	CACAAAGCGCGACCAAGATCATC	CACAAAGCGCGACCAAGATCATG	GGCTCGTGCTAAGGCTGCTGAT
BS00064156	CGCTGAGATGTTTGTTTGTTGCAG	CGCTGAGATGTTTGTTTGTTGCAC	CGTTTAGCATACTGCATACAGTAGTCATA
BS00022734	GGAAAAATCGATCTCACTGCT	CTGGAAAAATCGATCTCACTGCC	AATGAAGTGGCGCTGTCTTGAAATAGTTT
BS00022060	CGCGTCAGCACATCCTGCG	CCGCGTCAGCACATCCTGCA	GCTGGATGGTGCTCCTGGAACAA
BS00064155	ATATTCGTTTAGCATACTGCATACAGC	CATATTCGTTTAGCATACTGCATACAGT	GCTGAGATGTTTGTTTGTTGCACCATTAT
BS00003719	GCAGCAAAGAAGTTAGCAGAAATATAC	GCAGCAAAGAAGTTAGCAGAAATATAG	GAAGGGCGGTAGAAGAAGCAAACAT
BS00076982	AAATAGCGTTAAGATGTTTGGAGGAC	GAAATAGCGTTAAGATGTTTGGAGGAT	CAATATCCGCTATAGCAGGTCTTTTCATT
BS00071995	GTATGATCCATGATTAGCTGGCTTAC	AGTATGATCCATGATTAGCTGGCTTAT	CAAGTTAGCAATCCCTCTGGCCTAA
BS00064156	CGCTGAGATGTTTGTTTGTTGCAG	CGCTGAGATGTTTGTTTGTTGCAC	CGTTTAGCATACTGCATACAGTAGTCATA

A2 Hereward recombinant genotypes

Marker locations with the Shamrock allele are donated by B, and the Hereward allele as A.

A2.1 Hereward recombinants HS1 – HS17

2BS Distance (cM)	Marker	**HS1**	**HS1C**	**HS2**	**HS2C**	**HS3**	**HS4**	**HS5**	**HS5C**	**HS8**	**HS9**	**HS10**	**HS14**	**HS17**
0	BS00084668	-	A	B	A	-	B	A	B	A	B	A	B	A
1.15	BS00009972	-	A	-	A	B	B	A	B	A	B	A	B	A
1.77	***Iw1***	B	A	B	A	B	B	A	B	A	B	A	B	A
10.41	BS00070900	A	A	A	A	B	B	A	B	A	B	-	B	B
10.41	BS00010318	A	A	A	A	B	B	A	B	A	B	B	B	B
12.68	BS00045163	A	A	A	A	B	A	A	B	A	B	-	B	B
17.98	BS00010637	A	A	A	A	B	B	B	B	A	-	-	B	B
17.98	BS00065040	A	A	A	A	B	B	B	B	A	B	-	B	B
17.98	BS00063694	A	A	A	A	B	B	B	B	A	B	-	B	B
18.27	**Bra1190**	A	A	A	A	-	-	B	B	A	B	-	B	B
19.18	BS00009848	-	A		A	A	A	A	B	A	A	B	A	B
44.64	BS00064156	A	A	A	A	A	A	B	B	B	A	B	A	B
45.19	BS00022734	A	A	A	A	A	A	B	B	B	A	B	A	B
45.19	BS00022060	A	A	A	A	A	A	B	B	B	A	B	A	B
45.19	BS00064155	A	A	A	A	A	A	B	B	B	A	B	A	B

A2.2 Hereward recombinants HS18 – HS33

2BS Distance (cM)	Marker	**HS18**	**HS19**	**HS20**	**HS21**	**HS22**	**HS23**	**HS26**	**HS28**	**HS30**	**HS31**	**HS32**	**HS33**
0	BS00084668	B	A	B	A	B	A	B	A	B	A	B	A
1.15	BS00009972	B	A	B	A	B	A	B	A	B	A	B	A
1.77	***Iw1***	B	A	B	A	B	A	B	A	B	A	B	A
10.41	BS00070900	B	A	B	A	B	B	A	A	B	A	B	A
10.41	BS00010318	B	A	B	A	B	B	A	A	B	A	B	A
12.68	BS00045163	B	A	B	A	B	B	A	A	B	A	B	A
17.98	BS00010637	A	A	B	B	B	B	A	B	A	A	A	B
17.98	BS00065040	A	A	B	B	B	B	A	-	A	A	A	B
17.98	BS00063694	A	A	B	B	B	B	A	B	A	A	A	B
18.27	**Bra1190**	A	A	B	B	B	B	A	B	A	A	A	B
19.18	BS00009848	A	B	B	B	B	B	A	B	A	A	A	B
44.64	BS00064156	A	B	B	B	A	B	A	B	A	B	A	-
45.19	BS00022734	A	B	B	B	A	B	-	B	A	B	A	-
45.19	BS00022060	A	B	B	B	A	B	A	B	A	B	A	B
45.19	BS00064155	A	B	B	B	A	B	A	B	A	B	A	-

A3 Alchemy recombinant genotypes

Marker locations with the Shamrock allele are donated by B, and those with the Alchemy allele with an A.

A3.1 Alchemy recombinants AS1-AS7

2BS Distance (cM)	Marker	AS1	AS1C	AS2	AS3	AS4	AS4C	AS5	AS5C	AS6	AS7
0	BS00084668	B	A	B	B	A	A	A	A	A	A
1.15	BS00009972	B	A	B	B	A	A	A	A	A	A
1.15	***lw1***	B	A	B	B	A	A	A	A	A	A
9.26	BS00010318	B	A	A	B	A	A	A	A	A	B
9.26	BS00070900	B	A	A	B	A	A	A	A	A	B
11.53	BS00073542	B	A	A	B	A	A	A	A	A	B
16.88	BS00063694	B	A	A	A	A	A	B	A	A	B
16.88	BS00006788	B	A	A	A	A	A	B	A	A	B
17.00	Bra1190	B	A	A	A	A	A	B	A	A	B
18.03	BS00003719	B	A	A	A	A	A	B	A	A	B
18.61	BS00076982	A	A	A	A	A	B	B	A	B	B
32.68	BS00071995	B	A	A	A	A	B	A	A	A	A
43.49	BS00064156	B	A	A	-	A	B	A	A	A	A

A3.2 Alchemy recombinants AS7C – AS15

2BS location (cM)	Marker	AS7C	AS8	AS8C	AS9	AS9C	AS10	AS11	AS12	AS13	AS14	AS15
0	BS00084668	A	A	B	B	B	A	B	A	A	A	B
1.15	BS00009972	A	A	B	B	B	A	B	A	A	A	B
1.15	***lw1***	A	A	B	B	B	A	B	A	A	A	B
9.26	BS00010318	A	A	B	A	B	A	A	A	A	A	B
9.26	BS00070900	A	A	B	A	B	A	A	A	A	A/?	B
11.53	BS00073542	A	A	B	A	B	A	A	A	A	A/?	B
16.88	BS00063694	A	A	A	A	B	A	A	A	B	B	B
16.88	BS00006788	A	A	A	A	B	A	A	A	B	B	B
17.00	Bra1190	A	A	A	A	B	A	A	A	B	B	B
18.03	BS00003719	A	B	B	A	B	A	A	A	B	B	A
18.61	BS00076982	A	B	B	A	B	B	A	B	B	B	A
32.68	BS00071995	A	A	A	A	A	A	A	A	A	A	A
43.49	BS00064156	A	A	A	A	A	A	A	A	A	A	A

A4 Church Farm field plans

Within the field plans, NILs are indicated as described in Chapter 2 section 2.1.1: Malacca = MS+, MS-; Alchemy = AS+, AS-; Hereward = HS+, HS-; Xi19 = XS+, XS-; Einstein = ES+, ES-; Robigus = RS+, RS-. Parent varieties are also shown using the key Malacca = M, Alchemy = A, Hereward = H, Xi19 = X, Einstein = E, Robigus = R, Shamrock = S. Recombinant lines of all varieties are also shown using the nomenclature parent x parent followed by a number, for example Malacca recombinant 1 = MS1. Germplasm with no relevance to this PhD was removed from the plan and shown as a blank box. Each of the five blocks within each experiment is shown in a different colour.

A4.1 2013 field plans

In 2013 Alchemy, Robigus, Einstein and Hereward germplasm were grown in one trial, and Malacca and Xi19 in a second. Trials were next to each other in the same field, but are shown here on individual field plans.

Alchemy, Robigus, Einstein, Hereward:

10	9	8	7	6	5	4	3	2	1	
A		RS1		ES2	AS-	R	H		ES-	1
AS-		RS1C		ES3	AS-	RS-	HS-		ES+	2
AS+		RS2		ES3C	AS+	RS-	HS+		ES-	3
AS-		RS3		ES1	AS+	RS+	HS+		ES+	4
AS+		RS4		ES1C	A	RS+	HS-		ES1	5
AS2		RS5		E	AS4	RS7	HS2		ES1C	6
AS3		RS5C		ES-	AS4C	RS5	HS2C		ES2	7
AS1		RS6		ES+	AS1	RS5C	HS1		ES3	8
AS1C		RS7		ES-	AS1C	RS1	HS1C		ES3C	9
AS4C		RS8		ES+	AS2	RS1C			AS-	10
AS4		RS9		RS9	AS3	RS2			AS+	11
E		RS9C		RS9C	HS-	RS9			AS-	12
ES-		R		RS3	HS-	RS9C			AS+	13
ES+		RS-		RS5	HS+	RS6			AS1	14
ES-		RS+		RS5C	HS+	RS3			AS1C	15
ES-		RS-		RS6	HS1	RS4			AS2	16
ES3		RS+		RS7	HS1C	RS8			AS3	17
ES3C		AS2		RS8	HS2	ES-			AS4	18
ES1C		AS3		RS4	HS2C	ES+		E	AS4C	19
ES1	RS-	AS4	ES2	RS2	H	ES-		A	HS-	20
ES2	RS+	AS4C	ES1	RS-	RS1	ES+			HS+	21
HS2	RS-	AS1	ES1C	RS-	RS1C	E		R	HS-	22
HS2C	RS+	AS1C	ES3	RS+	R	ES1		H	HS+	23
HS1	RS1	A	ES3C	RS+		ES1C		RS2	HS1	24

HS1C	RS1C	AS-	ES+			ES3		RS3	HS1C	25
HS+	RS2	AS+	ES+			ES3C		RS4	HS2	26
HS-	RS3	AS-	ES-			ES2		RS5	HS2C	27
HS+	RS4	AS+	ES-			AS3		RS5C	RS-	28
HS-	RS9C	H	E			AS1	A	RS6	RS+	29
H	RS9	HS1	HS-			AS1C	AS-	RS7	RS-	30
R	RS7	HS1C	HS+			AS2	AS+	RS8	RS+	31
RS5C	RS8	HS2	HS-			AS4	AS-	RS9	RS1	32
RS5	RS6	HS2C	HS+			AS4C	AS+	RS9C	RS1C	33

Xi19 and Malacca:

10	9	8	7	6	5	4	3	2	1	
XS36-1	XS18-1	XS21-1	XS12-1	XS10-C4	XS13-2	XS50	XS11-1	XS14-1	MS-	1
XS19-1	XS7-1	XS15-1	XS12-2	XS10-2	XS13-C2	XS18-1	XS11-C5	XS14-2	MS+	2
XS53	XS5-1	XS16-1	XS12-C1	XS10-C2	XS13-4	XS49	XS11-C1	XS14-C1	MS-	3
XS30-1	XS39-1	XS8-1	XS12-C2	XS10-1	XS13-C1	XS30-1	XS11-2	XS14-C2	MS+	4
XS28-1	XS25-1	XS19-1	XS-	XS52	XS16-1	XS26-1	XS1-1	XS15-1	MS1	5
XS50	XS17-1	XS29-1	XS+	XS40-1	XS22-1	XS47-1	XS2-1	XS16-1	MS1C	6
XS48	XS14-1	XS32-1	XS-	XS9-1	XS49	XS27-1	XS35-1	XS17-1	MS2	7
XS45-1	XS14-2	XS33-1	XS+	XS9-C4	MS-	XS25-1	XS4-1	XS18-1	MS3	8
XS34-1	XS14-C1	XS4-1	XS34-1	XS9-C1	MS+	XS10-1	XS53	XS19-1	MS4	9
XS40-1	XS14-C2	XS23-1	XS27-1	XS9-4	MS+	XS10-C4	XS42-1	XS20-1	MS4C	10
XS22-1	XS38-1	XS30-1	XS37-1	XS47-1	MS-	XS10-C2	XS13-C2	XS21-1	M	11
XS15-1	XS9-4	XS17-1	XS11-1	XS35-1	MS3	XS10-2	XS13-2	XS22-1	S	12
XS33-1	XS9-C1	XS20-1	XS11-2	XS24-1	MS2	XS40-1	XS13-C1	XS23-1	X	13
XS46-1	XS9-1	XS22-1	XS11-C1	XS39-1	MS1	XS14-C1	XS13-4	XS24-1	XS-	14
XS23-1	XS9-C4	XS36-1	XS11-C5	XS21-1	MS1C	XS14-2	XS31-2	XS25-1	XS+	15
XS27-1	MS-	XS35-1	XS40-1	XS36-1	MS4	XS14-C2	XS3-1	XS26-1	XS-	16
XS3-1	MS+	XS24-1	XS38-1	XS18-1	MS3C	XS14-1	XS16-1	XS27-1	XS+	17
XS11-C1	MS-	XS39-1	XS52	XS23-1	M	XS9-1	XS19-1	XS28-1	XS1-1	18
XS11-1	MS+	XS47-1	XS10-2	XS28-1	S	XS9-C4	XS12-C2	XS29-1	XS2-1	19
XS11-2	MS3	XS14-1	XS10-1	XS32-1	X	XS9-C1	XS12-1	XS30-1	XS3-1	20

XS11-C5	MS1	XS14-C1	XS10-C2	XS30-1	XS+	XS9-4	XS12-2	XS31-2	XS4-1	21
XS35-1	MS1C	XS14-C2	XS10-C4	XS38-1	XS-	XS21-1	XS12-C1	XS32-1	XS5-1	22
XS32-1	MS3	XS14-2	XS13-4	XS25-1	XS-	XS8-1	XS39-1	XS33-1	XS6-1	23
XS47-1	MS4C	XS49	XS13-C1	XS19-1	XS+	XS33-1	XS15-1	XS34-1	XS7-1	24
XS31-2	MS4	XS48	XS13-C2	XS27-1	XS11-2	XS43-1	XS45-1	XS35-1	XS8-1	25
XS51	M	XS3-1	XS13-2	XS20-1	XS11-C5	XS46-1	XS48	XS36-1	XS9-1	26
XS16-1	S	XS6-1	MS2	XS33-1	XS11-C1	XS23-1	XS36-1	XS37-1	XS9-4	27
XS20-1	X	XS28-1	MS3	XS42-1	XS11-1	XS37-1	XS7-1	XS38-1	XS9-C1	28
XS37-1	XS-	XS2-1	MS1	XS5-1	XS48	XS24-1	XS5-1	XS39-1	XS9-C4	29
XS44-1	XS-	XS43-1	MS1C	XS31-2	XS12-1	XS29-1	XS22-1	XS40-1	XS10-1	30
XS13-4	XS+	XS45-1	MS4	XS44-1	XS12-C1	XS32-1	XS44-1	XS41-1	XS10-2	31
XS13-C1	XS+	XS44-1	MS4C	XS43-1	XS12-C2	X	XS38-1	XS42-1	XS10-C2	32
XS13-2	XS12-1	XS51	MS-	XS41-1	XS12-2	S	XS17-1	XS43-1	XS10-C4	33
XS13-C2	XS12-C1	XS7-1	MS+	XS45-1	XS50	M	XS28-1	XS44-1	XS11-1	34
XS41-1	XS12-C2	XS1-1	MS+	XS46-1	XS26-1	MS2	XS51	XS45-1	XS11-2	35
XS52	XS12-2	XS41-1	MS-	XS29-1	XS17-1	MS1	XS6-1	XS46-1	XS11-C1	36
XS2-1	XS8-1	XS50	M	XS6-1	XS51	MS1C	XS34-1	XS47-1	XS11-C5	37
XS29-1	XS49	XS31-2	S	XS8-1	XS15-1	MS4	XS52	XS48	XS12-1	38
XS26-1	XS6-1	XS18-1	X	XS37-1	XS53	MS4C	XS41-1	XS49	XS12-2	39
XS4-1	XS42-1	XS5-1	XS42-1	XS7-1	XS1-1	MS3	XS20-1	XS50	XS12-C1	40
XS43-1	XS10-C2	XS46-1	XS9-C1	XS2-1	XS14-C2	MS-	XS-	XS51	XS12-C2	41
XS21-1	XS10-1	XS53	XS9-1	XS4-1	XS14-1	MS+	XS+	XS52	XS13-2	42
XS24-1	XS10-2	XS26-1	XS9-C4	XS34-1	XS14-2	MS-	XS-	XS53	XS13-4	43
	XS25-1	XS9-4	XS3-1	XS14-C1	MS+	XS+	XS13-C2	XS13-C1		44

A4.2 2014 field plans

All six varieties were grown in the same trial in 2014. They have been separated by variety here for ease of interpretation.

Xi19:

XS45-0	XS16-1	XS47-1	XS3-1	XS13-2	XS41-1	XS1-1	XS72	XS63	1
XS45-1	S	XS20-1	XS5-1	XS46-1		XS14-1	XS25-1	XS11-1	2
XS68	X	XS19-1	XS9-1	XS12-1	XS58	XS10-1	XS48	XS17-1	3
XS-	XS-	XS+	XS+	XS30-1	XS60	XS51	XS50	XS15-1	4
XS10-1	XS9-1	XS60	XS1-1	XS15-1	XS19-1	XS25-1	XS51		5
XS58		XS14-1	XS50	XS72	XS47-1	XS12-1	XS46-1	XS16-1	6
XS45-1	XS30-1	XS48	S	XS+	XS-	XS63	XS13-2	XS5-1	7
XS20-1	XS3-1	XS11-1	X	XS-	XS+	XS17-1	XS68	XS41-1	8
XS25-1	XS68	XS9-1	XS16-1	XS1-1	XS51	XS72	XS48	XS13-2	9
XS12-1	XS19-1	XS5-1	XS45-1	XS20-1	XS10-1		XS17-1	XS46-1	10
XS50	XS+	XS+	XS41-1	XS58	XS15-1	XS30-1	XS11-1	X	11
	XS-	XS-	XS63	XS14-1	XS3-1	XS47-1	XS60	S	12
XS10-1	XS17-1	XS14-1	XS30-1	XS48	XS72	XS20-1	XS25-1		13
XS11-1	XS9-1	XS51	XS45-1		S	X	XS5-1	XS60	14
XS47-1	XS16-1	XS46-1	XS15-1	XS1-1	XS41-1	XS13-2	XS-	XS+	15
XS50	XS63	XS12-1	XS19-1	XS68	XS3-1	XS58	XS-	XS+	16
XS14-1	XS3-1	XS5-1	XS9-1	XS58	XS13-2	XS48	XS20-1	XS19-1	17
XS1-1	XS60	XS11-1	XS10-1	XS47-1	XS41-1	XS16-1	XS72	XS51	18
XS30-1	XS63	XS68	XS46-1	XS17-1	XS45-1	XS50	XS25-1	XS12-1	19
	S	X	XS-	XS+	XS+	XS-	XS15-1		20
1	2	3	4	5	6	7	8	9	

Malacca:

S	MS7C	MS8	MS12	MS1	MS5C	M	1
MS11	MS5	MS6	MS1C	MS23	MS10	MS14C	2
MS-	MS-	MS19	MS3	MS4	MS4C	MS2	3
MS+	MS+	MS18	MS15	MS7	MS20	MS14	4
MS8	MS20	MS11	MS12	MS6	MS18	MS10	5
MS23	MS14C	M	S	MS14	MS3	MS4C	6
MS15	MS7C	MS7	MS+	MS+	MS-	MS-	7
MS1	MS1C	MS2	MS4	MS19	MS5C	MS5	8
MS4	MS4C	MS14	MS8	MS6	MS3	MS1C	9
MS7C	MS7	MS14C	MS23	MS2	MS5	MS1	10
MS+	MS-	MS15	MS5C	MS18	MS19	M	11
MS-	MS+	MS20	MS11	MS10	MS12	S	12
MS20	MS10	MS8	MS6	MS7	MS7C	MS12	13

MS2	MS3	MS18	MS14	MS14C	MS23	MS19	14
MS15	MS1	MS1C	M	MS5	MS+	MS-	15
MS4	MS4C	MS11	S	MS5C	MS-	MS+	16
MS10	MS7C	MS7	MS4	MS4C	MS2	MS11	17
MS19	MS23	MS3	MS18	MS1C	MS1	MS8	18
MS14C	MS14	MS15	MS12	MS20	MS5	MS5C	19
S	M	MS-	MS+	MS-	MS+	MS6	20
10	11	12	13	14	15	16	

Robigus:

RS33	RS41	RS6	RS38	RS7	RS26	RS37		1
RS39	RS36	RS1	RS40	RS15	RS9	RS21	R	2
RS2	RS42	RS43	RS4	RS23	RS8	RS30	RS11	3
RS4	RS-	RS+	RS-	RS+	RS14	RS3	S	4
RS23	RS2	RS36	RS30	RS4	RS1	RS42	RS41	5
R	RS5	RS8	RS39	RS43	RS21	RS38	RS14	6
S	RS26		RS37	RS6	RS9	RS+	RS+	7
RS11	RS7	RS15	RS33	RS3	RS40	RS-	RS-	8
RS9	RS14	RS37	RS21	RS15	RS8	RS39	RS26	9
RS3	RS40	RS41	RS43	RS-	RS+	RS11	RS38	10
RS30	RS23	RS42	RS6	RS-	RS+	RS7	RS5	11
RS33	RS2	RS1	RS4	R	S		RS36	12
RS40	RS37	RS21	RS7	RS5	RS11	RS4	RS9	13
	RS1	RS23	RS26	RS38	RS15	RS41	RS2	14
RS39	RS43	RS33	RS8	RS42	RS-	RS+	R	15
RS14	RS3	RS36	RS6	RS30	RS-	RS+	S	16
RS7	RS41	RS30	RS1	RS43	RS4	RS37	RS23	17
RS-	RS+	RS39	RS11	RS15	RS2	RS26	RS21	18
RS-	RS+	RS3	RS40	RS36	RS9	RS6	RS14	19
	R	S	RS42	RS5	RS38	RS33	RS8	20
17	18	19	20	21	22	23	24	

Hereward:

HS3	HS31	HS2	HS10	HS28	HS+	HS+	HS33	1
HS18	HS22	HS2C	HS26	HS21	HS-	HS-	HS14	2
HS19	HS4	HS1C	HS1	HS5	HS32	HS17	HS8	3
	HS30	S	H	HS5C	HS20	HS23	HS9	4
HS17	HS21	HS30	HS8	HS31	HS3	HS32	HS1C	5
HS28	HS5	HS5C	HS4	HS23	HS26	HS2C	HS1	6
HS14	HS19	HS20	HS18	HS10	HS22	HS2	S	7
HS-	HS-	HS+	HS+	HS33	HS9	H		8
H	HS28	HS21	HS3	HS32	HS4	HS33	HS5	9
S	HS2C	HS10	HS17	HS26	HS19	HS31	HS5C	10
HS8	HS2	HS23	HS1	HS18	HS22	HS-	HS+	11
	HS9	HS20	HS1C	HS30	HS14	HS+	HS-	12
HS30	HS32	HS8	HS9	HS19	HS17	HS3	HS26	13
HS2C	HS2	HS10	HS31	HS23	HS28	HS22	HS20	14
HS-	HS+	HS33	HS14	H	HS21	HS1	HS1C	15
HS+	HS-	HS4	HS18	S	HS5C	HS5		16
HS31	HS10	HS5	HS5C	HS22	HS23	HS20	HS18	17
HS32	HS26	HS19	HS21	HS9	HS30	HS8	HS28	18
HS33	HS14	HS3	HS-	HS+	HS2	HS4	HS17	19
	HS1C	HS1	HS+	HS-	HS2C	H	S	20
25	26	27	28	29	30	31	32	

Alchemy:

S	AS8	AS9C	AS2		1
AS12	AS11	AS1C	AS+	AS-	2
AS4	AS3	AS14	AS-	AS+	3
AS4C	AS10	AS1	A	AS15	4
AS11	AS15	AS10	AS4C	AS1C	5
AS-	AS+	AS-	AS+	AS1	6
AS9C	AS12	AS2		A	7
AS3	AS8	AS14	S	AS4	8
	AS4C	AS10	AS-	AS+	9
AS8	AS11	AS3	AS+	AS-	10
AS14	AS1	A	S	AS2	11
AS15	AS9C	AS12	AS1C	AS4	12
AS9C	AS15	AS11	AS8	AS12	13
AS4	AS-	AS-	AS+	AS+	14
S	AS14	AS10	AS1	AS2	15
A	AS4C	AS3	AS1C		16
AS1	AS3	AS8	AS14	A	17
AS1C	AS2	AS4C	AS12	S	18

AS-	AS+	AS4	AS11	AS15	19
AS+	AS-	AS9C	AS10		20
33	34	35	36	37	

Einstein:

ES4	ES2	ES11	ES-	ES+	1
ES4C	ES9	ES7	ES+	ES-	2
ES6	ES3	ES7C	S	ES1	3
ES5	ES3C	ES5C	E	ES1C	4
S	ES+	ES9	ES4	ES7C	5
E	ES-	ES3	ES4C	ES3C	6
ES1C	ES-	ES1	ES7	ES6	7
ES11	ES+	ES5	ES5C	ES2	8
ES5	ES7C	ES+	ES+	ES1	9
ES2	ES5C	ES-	ES+	ES4C	10
ES11	ES1C	E	ES3	ES9	11
ES6	ES4	S	ES3C	ES7	12
ES9	ES5	ES4	ES4C	E	13
ES5C	ES6	ES2	ES7	S	14
ES1	ES3	ES1C	ES-	ES+	15
ES3C	ES11	ES7C	ES+	ES-	16
ES7C	ES7	ES6	ES9	ES11	17
ES4	ES4C	ES5C	ES5	ES3C	18
ES+	ES-	ES-	ES+	ES3	19
E	S	ES1	ES1C	ES2	20
38	39	40	41	42	

A4.3 2015 field plans

Only Hereward and Alchemy germplasm were grown in 2015 in two trials next to each other in the field.

Hereward:

	HS28	HS17	HS9	H	HS+	HS-	HS-	HS+	S	HS14	HS27		17
HS3C	HS8	HS3	HS32	HS25	HS18	HS5	HS8C	HS21	HS16	HS20	HS6		16
HS5C	HS7	HS15	HS33	HS12	HS23	HS24	HS4	HS1-	HS6C	HS30	HS1	HS13	15
HS1	HS5	HS31	HS21	HS13	HS17		HS31	HS19	HS2	HS11	HS22	HS26	14
HS23	HS15	HS27	HS10	HS25	HS14	HS32	HS12	HS3	HS7	HS22	HS19	HS156	13
HS33	HS8C	HS8	HS3C	HS6C	HS20	HS26	HS6	HS30	HS+	HS-	HS+	HS-	12
HS18	HS6	HS4	HS24	HS9		HS11	HS2	HS18	H	S	HS5C	HS28	11
HS16	HS30	HS9	HS20	HS2	HS22	HS7	HS10	HS8	HS25	HS12	HS28	HS15	10
HS5C	HS6C	HS3	HS17	HS24	HS23	HS21	H	S	HS31	HS3C	HS4	HS33	9
HS-	HS-	HS+	HS+	HS1	HS11	HS5		HS19	HS13	HS27	HS32	HS8C	8
HS20	HS25	HS2	HS28	HS11	HS18	HS27	HS26	HS16	HS15		HS14	HS26	7
HS9	HS10	HS22	HS31	HS17	HS30	HS7	HS32	HS3	HS29	HS19	HS6C	HS6	6
HS24	HS33	HS14	HS12	HS21	HS8C	HS13	HS1	HS3C	HS5C	HS23	HS4	HS8	5
HS28	HS29	HS30	HS31	HS32	HS33	HS-	HS+	HS+	HS-	H	S	HS5	4
HS15	HS16	HS17	HS18	HS19	HS20	HS21	HS22	HS23	HS24	HS25	HS26	HS27	3
HS5	HS5C	HS6	HS6C	HS7	HS8	HS8C	HS9	HS10	HS11	HS12	HS13	HS14	2
	H	S	HS+	HS-	HS-	HS+	HS1	HS2	HS3	HS3C	HS4		1
1	2	3	4	5	6	7	8	9	10	11	12	13	

Alchemy:

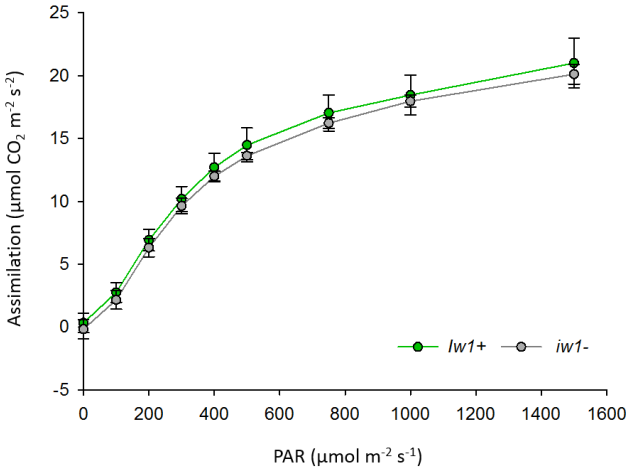
	AS+	AS+	AS-	AS-	S	A		17
AS10	AS4	AS6	AS1		AS15	AS9	AS8	16
AS14	AS12	AS5	AS13	AS9C	AS11	AS7	AS5C	15
AS9C	AS11	AS3			AS8C	AS3	AS2	14
S	AS6	AS5C	AS13	AS8	AS7C	AS8C	AS2	13
A	AS14	AS1	AS15	AS10	AS9	AS12	AS5	12
		AS7	AS4	AS-	AS+	AS-	AS+	11
AS11	AS15	AS12	AS1	AS9C	AS2	AS9		10
AS4	AS7C	AS+	AS+	AS-	AS-	AS5C	AS13	9
AS10	AS3	AS8C	A	S	AS5	AS14	AS6	8
AS+	AS-	AS4	AS10	AS1		AS8	AS7	7
AS-	AS+	AS8	AS8C	AS7C	AS14	AS5C	AS15	6
AS5	AS9	AS9C	AS7	AS12	AS6	A	S	5
AS13	AS14	AS15		AS2	AS13	AS11	AS3	4
AS7C	AS8	AS8C	AS9	AS9C	AS10	AS11	AS12	3
AS1	AS2	AS3	AS4	AS55	AS5C	AS6	AS7	2
	A	S	AS+	AS-	AS-	AS+		1
1	2	3	4	5	6	7	8	

A5 Robigus and Xi19 light curves

Light curves for NILs of Robigus and Xi19 were obtained using a LI-COR 6400XT during the harvest year of 2013.

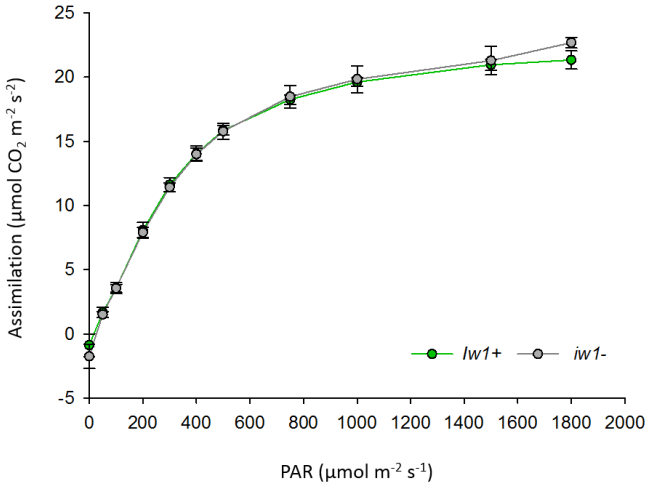
A5.1 Robigus

	Average ± S.E		p
	lw1+	*iw1-*	
AQE	0.045 ± 0.001	0.045 ± 0.001	0.774
Light Compensation	4.44± 0.87	3.86± 0.73	0.628
Amax	21.00 ± 1.97	20.12 ± 0.78	0.690
Ao	0.33 ± 0.77	-0.167 ± 0.75	0.660



A5.2 Xi19

	Average ± S.E		p
	Iw1+	*iw1-*	
AQE	0.045 ± 0.003	0.044 ± 0.002	0.979
Light compensation	3.64 ± 1.63	4.61 ± 0.51	0.589
Amax	21.24 ± 0.51	21.51 ± 1.21	0.844
Ao	0.50 ± 0.91	0.19 ± 0.71	0.451



A6 Shade trial wax profiles

FA = fatty acid, POH = primary alcohol, MAR = methylacylresorcinol

P values show the effect of shade when each NIL was analysed by ANOVA individually

T indicates trace amounts were present less than 0.0000 µg/mg plant tissue

- Indicates missing data

A6.1 Alchemy wax profile

	Wax quantity in µg/mg leaf tissue			
	Anthesis		Senescence	
C22 FA				
	AS+	AS-	AS +	AS-
Control	0.0098 ± 0.0031	0.0072 ± 0.0036	0.0101 ± 0.0009	0.0120 ± 0.0006
30% Shade	0.0106 ± 0.0023	0.0145 ± 0.0058	0.0087 ± 0.0063	0.0071 ± 0.0058*
50% Shade	0.0103 ± 0.0027	0.0173 ± 0.0055	0.0139 ± 0.0062	0.0093 ± 0.0005
p	0.976	0.397	0.308	p<0.001
C24 FA				
	AS +	AS -	AS +	AS -
Control	0.0297 ± 0.0122	0.0530 ± 0.0203	0.0234 ± 0.0045	0.0138 ± 0.0009*
30% Shade	0.0221 ± 0.0095	0.0182 ± 0.0067	0.0171 ± 0.0026	0.0084 ± 0.0007*
50% Shade	0.0321 ± 0.0153	0.0254 ± 0.0102	0.0281 ± 0.0092	0.0105 ± 0.0018
p	0.816	0.22	0.457	0.021
C28 FA				
	AS+	AS -	AS +	AS -
Control	0.1024 ± 0.0396	0.1138 ± 0.0088	0.1601 ± 0.0175	0.1310 ± 0.0051
30% Shade	0.1112 ± 0.0254	0.1358 ± 0.0409	0.1173 ± 0.0119	0.0829 ± 0.0062*
50% Shade	0.1087 ±	0.1445 ±	0.1995 ±	0.1165 ±

	0.0380	0.0461	0.0860	0.0121
p	0.982	0.845	0.107	0.004
C32 FA				
	AS+	AS -	AS +	AS -
Control	T	0.0009 ± 0.0006	0.0002 ± 0.0001	0.0010 ± 0.0001
30% Shade	T	-	0.0003 ± 0.0001	0.0018 ± 0.0004
50% Shade	T	0.0005 ± 0.0002	0.0003 ± 0.0001	0.0021 ± 0.0008
p	0.488	0.612	0.580	0.336
C24 POH				
	AS+	AS -	AS +	AS -
Control	0.7918 ± 0.1976	0.2195 ± 0.1255	0.5353 ± 0.0526	0.2751 ± 0.0030*
30% Shade	0.8043 ± 0.1951	0.5020 ± 0.1675	0.4481 ± 0.0344	0.1725 ± 0.0151*
50% Shade	0.7217 ± 0.2374	0.5840 ± 0.1973	0.7263 ± 0.2982	0.2290 ± 0.0277
p	0.959	0.341	0.345	0.007
C26 POH				
	AS+	AS -	AS +	AS -
Control	0.3607 ± 0.1882	0.3537 ± 0.1701	0.1666 ± 0.0128	0.1438 ± 0.0073*
30% Shade	0.4732 ± 0.1963	0.6094 ± 0.3023	0.1446 ± 0.0126	0.0922 ± 0.0081*
50% Shade	0.4886 ± 0.3422	0.9670 ± 0.5157	0.2222 ± 0.0791	0.1256 ± 0.0152
p	0.916	0.551	0.506	0.015
C28 POH				
	AS+	AS -	AS +	AS -
Control	3.6107 ± 0.7623	4.0812 ± 0.2190	2.6544 ± 0.2229	2.6679 ± 0.1120
30% Shade	4.0973 ± 0.9656	4.8944 ± 1.3427	2.3400 ± 0.2254	1.9910 ± 0.1664*
50% Shade	3.6926 ±	5.6025 ±	4.2516 ±	2.6577 ±

	1.2166	1.6773	1.5736	0.1349
p	0.927	0.728	0.498	0.01
C30 POH				
	AS+	AS -	AS +	AS -
Control	0.1124 ± 0.0269	0.1018 ± 0.0029	0.0882 ± 0.0073	0.0758 ± 0.0054
30% Shade	0.1425 ± 0.0365	0.1266 ± 0.0342	0.0780 ± 0.0078	0.0515 ± 0.0045*
50% Shade	0.1133 ± 0.0466	0.1462 ± 0.0472	0.1352 ± 0.0523	0.0764 ± 0.0064
p	0.796	0.697	0.643	0.01
C27 *n*-Alkane				
	AS+	AS -	AS +	AS -
Control	0.0870 ± 0.0310	0.0307 ± 0.0083	0.0757 ± 0.0159	0.0397 ± 0.0039*
30% Shade	0.0199 ± 0.0045	0.0229 ± 0.0078	0.0432 ± 0.0059	0.0133 ± 0.0036
50% Shade	0.0728 ± 0.0164	0.0488 ± 0.0128	0.0586 ± 0.0300	0.0203 ± 0.0025
p	0.716	0.617	0.051	0.001
C29 *n*-Alkane				
	AS+	AS -	AS +	AS -
Control	0.3269 ± 0.1043	0.0805 ± 0.0557	0.3069 ± 0.0422*	0.1611 ± 0.0084*
30% Shade	0.0177 ± 0.0094	0.0298 ± 0.0096	0.1957 ± 0.0156	0.0744 ± 0.0053
50% Shade	0.3391 ± 0.1059	0.2151 ± 0.0668	0.3023 ± 0.1547*	0.0892 ± 0.0068
p	0.925	0.316	0.018	<0.001
C31 *n*-Alkane				
	AS+	AS -	AS +	AS -
Control	0.1729 ± 0.0442	0.1110 ± 0.0362	0.1665 ± 0.0375	0.1534 ± 0.0142*
30% Shade	0.1870 ± 0.0963	0.1363 ± 0.0594	0.1034 ± 0.0309	0.0728 ± 0.0040
50% Shade	0.1170 ±	0.2034 ±	0.1420 ±	0.0649 ±

	0.0405	0.0657	0.0512	0.0128
p	0.771	0.51	0.66	<0.001
MAR19				
	AS+	AS -	AS +	AS -
Control	0.0270 ± 0.0061	0.0212 ± 0.0016	0.0126 ± 0.0006	0.0138 ± 0.0017*
30% Shade	0.0621 ± 0.0111	0.0446 ± 0.0169	0.0135 ± 0.0014	0.0084 ± 0.0008
50% Shade	0.1783 ± 0.0715	0.1990 ± 0.0668	0.0142 ± 0.0026	0.0070 ± 0.0013
p	0.967	0.616	0.564	0.01
MAR21				
	AS+	AS -	AS +	AS -
Control	0.0601 ± 0.0139	0.0524 ± 0.0038	0.0280 ± 0.0014	0.0270 ± 0.0018*
30% Shade	0.2908 ± 0.0550	0.1829 ± 0.0554	0.0289 ± 0.0021	0.0190 ± 0.0008*
50% Shade	0.0001 ± 0.0001	0.0759 ± 0.0429	0.0378 ± 0.0070	0.0198 ± 0.0030
p	0.946	0.642	0.584	0.032
MAR23				
	AS+	AS -	AS +	AS -
Control	0.0683 ± 0.0192	0.0600 ± 0.0051	0.0369 ± 0.0022	0.0327 ± 0.0023*
30% Shade	0.1870 ± 0.0963	0.1363 ± 0.0594	0.0383 ± 0.0027	0.0235 ± 0.0009*
50% Shade	0.0004 ± 0.0004	1.1186 ± 0.3523	0.0518 ± 0.0095	0.0256 ± 0.0029
p	0.989	0.712	0.354	0.024
MAR25				
	AS+	AS -	AS +	AS -
Control	0.0206 ± 0.0051	0.0205 ± 0.0015	0.0155 ± 0.0013	0.0145 ± 0.0013
30% Shade	T	0.1100 ± 0.0622	0.0166 ± 0.0025	0.0109 ± 0.0011
50% Shade	0.0295 ±	0.0348 ±	0.0214 ±	0.0104 ±

	0.0102	0.0117	0.0048	0.0013
p	0.965	0.833	0.873	0.085
MAR27				
	AS+	AS -	AS +	AS -
Control	0.0201 ± 0.0040	0.0218 ± 0.0013	0.0088 ± 0.0025	0.0105 ± 0.0011*
30% Shade	0.0015 ± 0.0015	1.2784 ± 0.7799	0.0110 ± 0.0012	0.0050 ± 0.0013*
50% Shade	0.0684 ± 0.0250	0.0848 ± 0.0298	0.0136 ± 0.0028	0.0074 ± 0.0012
p	0.9	0.609	0.655	0.026

A6.2 Malacca wax profile

	Wax quantity in µg/mg leaf tissue			
	Anthesis		Senescence	
C22 FA				
	MS+	MS-	MS+	MS-
Control	0.0181 ± 0.0056	0.0149 ± 0.0050	0.0121 ± 0.0008*	0.0122 ± 0.0025
30% Shade	0.0078 ± 0.0020	0.0159 ± 0.0098	0.0090 ± 0.0018	0.0076 ± 0.0019
50% Shade	0.0126 ± 0.0084	0.0111 ± 0.0046	0.0071 ± 0.0007*	0.0087 ± 0.0007
p	**0.341**	**0.71**	**0.035**	**0.226**
C24 FA				
	MS+	MS-	MS+	MS-
Control	0.0460 ± 0.0156	0.0178 ± 0.0060	0.0151 ± 0.0023	0.0151 ± 0.0031
30% Shade	0.0070 ± 0.0021	0.0206 ± 0.0123	0.0141 ± 0.0018	0.0090 ± 0.0023
50% Shade	0.0193 ± 0.0173	0.0162 ± 0.0092	0.0138 ± 0.0034	0.0090 ± 0.0008
p	**0.246**	**0.883**	**0.929**	**0.13**
C28FA				
	MS+	MS-	MS+	MS-
Control	0.1171 ±	0.1169 ±	0.1200 ±	0.1403 ±

	0.0475	0.0420	0.0097*	0.0267*
30% Shade	0.0548 ± 0.0038	0.1165 ± 0.0719	0.0831 ± 0.0126	0.0823 ± 0.0042
50% Shade	0.0867 ± 0.0530	0.0975 ± 0.0265	0.0767 ± 0.0047	0.0757 ± 0.0034
p	**0.549**	**0.562**	**0.016**	**0.014**
C32 FA				
	MS+	MS-	MS+	MS-
Control	T	0.0002 ± 0.0001	T	0.0004 ± 0.0001
30% Shade	0.0001 ± 0.0001	0.0002 ± 0.0001	T	0.0025 ± 0.0019
50% Shade	-	0.0005 ± 0.0004	0.0008 ± 0.0004	0.0012 ± 0.0003
p	**0.623**	**0.863**	**0.66**	**0.468**
C24 POH				
	MS+	MS-	MS+	MS-
Control	1.4189 ± 0.4609	0.4881 ± 0.1631	0.6027 ± 0.0552*	0.3224 ± 0.0659
30% Shade	0.5484 ± 0.0980	0.5657 ± 0.3680	0.4347 ± 0.0609	0.2204 ± 0.0058
50% Shade	1.0859 ± 0.4901	0.4292 ± 0.1627	0.3310 ± 0.0667*	0.2041 ± 0.0190
p	**0.282**	**0.538**	**0.026**	**0.078**
C26 POH				
	MS+	MS-	MS+	MS-
Control	1.3026 ± 0.5366	0.7816 ± 0.4279	0.2279 ± 0.0168	0.1906 ± 0.0372
30% Shade	0.2043 ± 0.0234	0.7097 ± 0.5570	0.1692 ± 0.0316	0.1379 ± 0.0040
50% Shade	0.4862 ± 0.3061	0.3836 ± 0.2337	0.1599 ± 0.0104	0.1370 ± 0.0109
p	**0.276**	**0.767**	**0.091**	**0.147**
C28 POH				
	MS+	MS-	MS+	MS-
Control	6.7009 ±	5.0228 ±	3.4110 ±	3.2760 ±

	1.9203	1.5996	0.3795	0.6072
30% Shade	3.4620 ± 0.0857	5.6612 ± 3.3190	2.3667 ± 0.3988	2.4544 ± 0.0948
50% Shade	5.1414 ± 2.6087	4.6480 ± 1.3642	2.9512 ± 0.1554	3.2451 ± 0.1833
p	**0.405**	**0.637**	**0.123**	**0.16**
C30 POH				
	MS+	MS-	MS+	MS-
Control	0.1923 ± 0.0539	0.1311 ± 0.0414	0.1194 ± 0.0107	0.1146 ± 0.0260
30% Shade	0.1191 ± 0.0001	0.1606 ± 0.0990	0.0844 ± 0.0199	0.0662 ± 0.0033
50% Shade	0.1863 ± 0.1248	0.1142 ± 0.0372	0.0978 ± 0.0067	0.0896 ± 0.0039
p	**0.569**	**0.582**	**0.226**	**0.074**
C27 *n*-Alkane				
	MS+	MS-	MS+	MS-
Control	0.1625 ± 0.0492	0.0599 ± 0.0185	0.0705 ± 0.0116*	0.0524 ± 0.0152*
30% Shade	0.0118 ± 0.0006	0.0191 ± 0.0126	0.0420 ± 0.0050	0.0232 ± 0.0060
50% Shade	0.1255 ± 0.0545	0.0494 ± 0.0165	0.0267 ± 0.0068*	0.0166 ± 0.0010*
p	**0.123**	**0.547**	**0.009**	**0.03**
C29 *n*-Alkane				
	MS+	MS-	MS+	MS-
Control	0.5157 ± 0.2100	0.2337 ± 0.0773	0.3768 ± 0.0357*	0.2228 ± 0.0494*
30% Shade	0.0155 ± 0.0014	0.0226 ± 0.0160	0.2449 ± 0.0221	0.1274 ± 0.0112
50% Shade	0.5880 ± 0.2607	0.1683 ± 0.0480	0.1557 ± 0.0361	0.0727 ± 0.0097*
p	**0.541**	**0.554**	**0.001**	**0.006**
C31 *n*-Alkane				
	MS+	MS-	MS+	MS-
Control	0.1303 ±	0.0680 ±	0.1163 ±	0.0496 ±

	0.0489	0.0152	0.0027*	0.0026*
30% Shade	0.0924 ± 0.0116	0.0816 ± 0.0477	0.0776 ± 0.0058	0.0417 ± 0.0069
50% Shade	0.1676 ± 0.0739	0.0464 ± 0.0112	0.1095 ± 0.0029*	0.0338 ± 0.0049*
p	**0.382**	**0.553**	**0.004**	**0.013**
MAR19				
	MS+	MS-	MS+	MS-
Control	0.0262 ± 0.0080	0.0269 ± 0.0074	0.0151 ± 0.0032	0.0112 ± 0.0020
30% Shade	0.0376 ± 0.0084	0.0500 ± 0.0286	0.0075 ± 0.0010	0.0110 ± 0.0011
50% Shade	0.1300 ± 0.1102	0.1306 ± 0.0561	0.0123 ± 0.0013	0.0110 ± 0.0010
p	**0.529**	**0.617**	**0.065**	**0.995**
MAR21				
	MS+	MS-	MS+	MS-
Control	0.0580 ± 0.0168	0.0428 ± 0.0114	0.0221 ± 0.0020	0.0189 ± 0.0036
30% Shade	0.2202 ± 0.0414	0.2118 ± 0.1223	0.0185 ± 0.0031	0.0187 ± 0.0023
50% Shade	0.0001 ± 0.0001	0.0150 ± 0.0109	0.0341 ± 0.0035*	0.0232 ± 0.0032
p	**0.718**	**0.527**	**0.007**	**0.501**
MAR23				
	MS+	MS-	MS+	MS-
Control	0.0639 ± 0.0235	0.0480 ± 0.0087	0.0343 ± 0.0031	0.0237 ± 0.0046
30% Shade	0.0924 ± 0.0116	0.0816 ± 0.0477	0.0298 ± 0.0053	0.0233 ± 0.0028
50% Shade	T	0.1985 ± 0.0551	0.0507 ± 0.0038*	0.0319 ± 0.0041
p	**0.615**	**0.647**	**0.01**	**0.228**
MAR25				
	MS+	MS-	MS+	MS-
Control	0.0184 ±	0.0137 ±	0.0106 ±	0.0085 ±

	0.0063	0.0036	0.0015	0.0014
30% Shade	T	0.0277 ± 0.0266	0.0098 ± 0.0013	0.0094 ± 0.0026
50% Shade	0.0225 ± 0.0071	0.0276 ± 0.0129	0.0176 ± 0.0018*	0.0144 ± 0.0026
p	**0.78**	**0.695**	**0.007**	**0.214**
MAR27				
	MS+	MS-	MS+	MS-
Control	0.0180 ± 0.0039	0.0142 ± 0.0033	0.0062 ± 0.0016	0.0048 ± 0.0021
30% Shade	T	0.3342 ± 0.2363	0.0042 ± 0.0011	0.0052 ± 0.0015
50% Shade	0.0562 ± 0.0332	0.0467 ± 0.0238	0.0141 ± 0.0013*	0.0087 ± 0.0014
p	**0.938**	**0.525**	**<0.001**	**0.227**

A6.3 Hereward wax profile

	Wax quantity in µg/mg leaf tissue			
	Anthesis		Senescence	
C22 FA				
	HS+	HS-	HS+	HS-
Control	0.0103 ± 0.0031	0.0200 ± 0.0043	0.0091 ± 0.0005	0.0126 ± 0.0014
30% Shade	0.0156 ± 0.0026	0.0165 ± 0.0035	0.0065 ± 0.0002*	0.0089 ± 0.0010
50% Shade	0.0096 ± 0.0029	0.0116 ± 0.0022	0.0096 ± 0.0005	0.0086 ± 0.0007
p	**0.583**	**0.367**	**0.001**	**0.099**
C24 FA				
	HS+	HS-	HS+	HS-
Control	0.0175 ± 0.0106	0.0277 ± 0.0070	0.0132 ± 0.0029	0.0142 ± 0.0015
30% Shade	0.0351	0.0204	0.0087	0.0093

	± 0.0109	± 0.0047	± 0.0016	± 0.0013
50% Shade	0.0239 ± 0.0092	0.0169 ± 0.0051	0.0146 ± 0.0041	0.0092 ± 0.0008
p	**0.442**	**0.534**	**0.301**	**0.37**
C28 FA				
	HS+	HS-	HS+	HS-
Control	0.1171 ± 0.0475	0.1169 ± 0.0420	0.1424 ± 0.0082	0.1491 ± 0.0136
30% Shade	0.0548 ± 0.0038	0.1165 ± 0.0719	0.1094 ± 0.0061*	0.0941 ± 0.0123
50% Shade	0.0867 ± 0.0530	0.0975 ± 0.0265	0.1448 ± 0.0115	0.1067 ± 0.0102
p	**0.747**	**0.515**	**0.018**	**0.069**
C32 FA				
	HS+	HS-	HS+	HS-
Control	T	T	T	0.0002 ± 0.0001
30% Shade	0.0001 ± 0.0001	0.0002 ± 0.0001	0.0004 ± 0.0001	0.0004 ± 0.0001
50% Shade	0.0001 ± 0.0001	0.0003 ± 0.0001	0.0003* ± 0.0001	0.0006 ± 0.0002
p	**0.376**	**0.325**	**0.033**	**0.468**
C24 POH				
	HS+	HS-	HS+	HS-
Control	0.9447 ± 0.2733	0.6976 ± 0.1499	0.6250 ± 0.0304	0.3431 ± 0.0582
30% Shade	1.1036 ± 0.3757	0.6751 ± 0.1552	0.4653 ± 0.0386*	0.2502 ± 0.0286
50% Shade	0.6723 ± 0.1687	0.3535 ± 0.0709	0.6241 ± 0.0456	0.2650 ± 0.0308
p	**0.931**	**0.462**	**0.02**	**0.259**
C26 POH				
	HS+	HS-	HS+	HS-
Control	0.4346 ± 0.2716	0.8415 ± 0.2453	0.1569 ± 0.0062	0.1797 ± 0.0232
30% Shade	0.6780 ±	0.7073 ±	0.1383 ±	0.1439 ±

	0.2657	0.2692	0.0066	0.0121
50% Shade	0.4544 ± 0.2394	0.4913 ± 0.2101	0.1898 ± 0.0067*	0.1289 ± 0.0133
p	**0.823**	**0.641**	**0.002**	**0.12**
C28 POH				
	HS+	HS-	HS+	HS-
Control	3.6513 ± 1.2132	5.8761 ± 1.2119	2.1140 ± 0.1179	2.8082 ± 0.3057
30% Shade	4.6963 ± 0.8932	6.8088 ± 1.7923	2.3199 ± 0.1419	2.4849 ± 0.3475
50% Shade	4.1135 ± 1.0422	3.9442 ± 0.8067	2.6579 ± 0.2103	2.9832 ± 0.2657
p	**0.836**	**0.619**	**0.117**	**0.831**
C30 POH				
	HS+	HS-	HS+	HS-
Control	0.1475 ± 0.0524	0.1361 ± 0.0296	0.0922 ± 0.0019	0.0671 ± 0.0060
30% Shade	0.2045 ± 0.0682	0.1820 ± 0.0527	0.0994 ± 0.0088	0.0606 ± 0.0090
50% Shade	0.1344 ± 0.0359	0.1062 ± 0.0244	0.1286 ± 0.0186	0.0898 ± 0.0079
p	**0.817**	**0.522**	**0.099**	**0.78**
C27 *n*-Alkane				
	HS+	HS-	HS+	HS-
Control	0.1110 ± 0.0381	0.1006 ± 0.0222	0.1018 ± 0.0032*	0.0766 ± 0.0099*
30% Shade	0.0164 ± 0.0026	0.0222 ± 0.0052	0.0459 ± 0.0073	0.0368 ± 0.0056
50% Shade	0.0544 ± 0.0166	0.0390 ± 0.0073	0.0584 ± 0.0114	0.0223 ± 0.0032
p	**0.981**	**0.159**	**0.002**	**<0.001**
C29 *n*-Alkane				
	HS+	HS-	HS+	HS-
Control	0.4821 ± 0.1635	0.3003 ± 0.0655	0.4039 ± 0.0078*	0.2178 ± 0.0200*
30% Shade	0.0257 ±	0.0358 ±	0.2348 ±	0.1393 ±

	0.0054	0.0093	0.0246	0.0210
50% Shade	0.3067 ± 0.0933	0.1507 ± 0.0316	0.3082 ± 0.0201	0.1033 ± 0.0172*
p	**0.933**	**0.255**	**0.001**	**0.005**
C31 *n*-Alkane				
	HS+	HS-	HS+	HS-
Control	0.1977 ± 0.0583	0.0765 ± 0.0254	0.1406 ± 0.0108	0.0782 ± 0.0157
30% Shade	0.1542 ± 0.0595	0.0628 ± 0.0121	0.0993 ± 0.0093	0.0464 ± 0.0031
50% Shade	0.1159 ± 0.0355	0.0535 ± 0.0111	0.0740 ± 0.0131	0.0357 ± 0.0044
p	**0.945**	**0.544**	**<0.001**	**0.1**
MAR19				
	HS+	HS-	HS+	HS-
Control	0.0183 ± 0.0072	0.0364 ± 0.0079	0.0094 ± 0.0020	0.0130 ± 0.0037
30% Shade	0.1178 ± 0.0322	0.0574 ± 0.0074	0.0116 ± 0.0018	0.0117 ± 0.0024
50% Shade	0.1195 ± 0.0306	0.1006 ± 0.0276	0.0090 ± 0.0013	0.0086 ± 0.0013
p	**0.98**	**0.633**	**0.563**	**0.567**
MAR21				
	HS+	HS-	HS+	HS-
Control	0.0414 ± 0.0164	0.0519 ± 0.0107	0.0125 ± 0.0017*	0.0154 ± 0.0030
30% Shade	0.5045 ± 0.1819	0.2031 ± 0.0278	0.0167 ± 0.0013	0.0192 ± 0.0037
50% Shade	T	0.0147 ± 0.0066	0.0211 ± 0.0004	0.0170 ± 0.0028
P	**0.07 ± 0.02**	**14.74 ± 6.58**	**21.05 ± 0.37***	**16.99 ± 2.78**
MAR23				
	HS+	HS-	HS+	HS-
Control	0.0429 ± 0.0244	0.0516 ± 0.0088	0.0196 ± 0.0027	0.0180 ± 0.0026
30% Shade	0.1542 ±	0.0628 ±	0.0257 ±	0.0221 ±

	0.0595	0.0121	0.0016	0.0044
50% Shade	0.0002 ± 0.0002	0.2314 ± 0.0804	0.0358 ± 0.0007*	0.0230 ± 0.0032
p	**0.827**	**0.46**	**0.002**	**0.715**
MAR25				
	HS+	HS-	HS+	HS-
Control	0.0144 ± 0.0069	0.0173 ± 0.0037	0.0054 ± 0.0009*	0.0071 ± 0.0011
30% Shade	0.0025 ± 0.0022	0.0295 ± 0.0142	0.0085 ± 0.0004*	0.0075 ± 0.0012
50% Shade	0.0176 ± 0.0053	0.0229 ± 0.0053	0.0124 ± 0.0004*	0.0084 ± 0.0013
p	**0.962**	**0.652**	**<0.001**	**0.449**
MAR27				
	HS+	HS-	HS+	HS-
Control	0.0216 ± 0.0104	0.0234 ± 0.0045	0.0054 ± 0.0021	0.0053 ± 0.0024
30% Shade	0.0653 ± 0.0646	0.4139 ± 0.1526	0.0107 ± 0.0008	0.0050 ± 0.0030
50% Shade	0.0460 ± 0.0138	0.0440 ± 0.0112	0.0088 ± 0.0044	0.0089 ± 0.0034
p	**0.953**	**0.4**	**0.267**	**0.431**

A7 Shade trial field plan

= Soissons

Block:	1	2	3	4	5
60% SHADE					
	AS+	MS+	HS+	AS-	MS-
	AS-	MS-	HS-	AS+	MS+
	HS+	AS-	MS-	HS-	AS+
	HS-	AS+	MS+	HS+	AS-
	MS-	HS-	AS+	MS+	HS+
	MS+	HS+	AS-	MS-	HS-
40% SHADE					
	AS+	MS+	HS+	AS-	MS-
	AS-	MS-	HS-	AS+	MS+
	HS+	AS-	MS-	HS-	AS+
	HS-	AS+	MS+	HS+	AS-
	MS-	HS-	AS+	MS+	HS+
	MS+	HS+	AS-	MS-	HS-
CONTROL					
	AS+	MS+	HS+	AS-	MS-
	AS-	MS-	HS-	AS+	MS+
	HS+	AS-	MS-	HS-	AS+
	HS-	AS+	MS+	HS+	AS-
	MS-	HS-	AS+	MS+	HS+
	MS+	HS+	AS-	MS-	HS-