Investigation into the suitability of wheat for ethanol production in the Western Cape

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Declaration

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Abstract

This study aimed to investigate the suitability of spring wheat in the Western Cape as a potential feedstock for a future bio-ethanol industry as well as initiate a pre-breeding effort to develop bio-ethanol -directed improved lines.

Determined primarily on grain yield, disease resistance and, direct as well as indirect assaying of important parameters, material was selected from a base-population for use as male parents. These were crossed with female parents sourced from the Stellenbosch University Plant Breeding Laboratory (SU-PBL) male sterility -mediated marker-assisted recurrent selection (MS-MARS) programme. This programme is constituted by an agronomically and disease-resistance -improved population, containing a dominant male sterility gene (*Ms3*). The progeny of these crosses was used to initiate the production of doubled haploids in order to ultimately derive higher ethanol yielding lines.

Multi-location field trial (MLFT) data revealed that 00K60-16-3-3 was the best adapted and highest yielding (2160.95 litres ethanol per hectare) advanced breeding line (ABL). Its performance was not statistically significantly less than first-ranked 03H86-8-2 (2184.62 litres per hectare) and both ABLs significantly ($P \leq 0.05$) out-performed six controls in the study. ABL 00K60-16-3-3 was also the most adapted in terms of potential yield in litres per ton of grain. ABL 03H86-8-1 was second recommended for the Western Cape, performing above the expected mean for yield in litres per hectare. Further adaptation of specific ABLs to the two major sub-regions of the Western Cape i.e. the Swartland and Southern Cape including the Rûens was also elucidated. Napier was significantly the highest yielding trial site although none of the considered sites were both stable and high yielding. It was also determined that entry X locality interaction (GxE) was indeed significant across the whole production area regarding litres per hectare as well as its two sub-regions. This is expected considering the environmentally diverse nature of the region as a whole.

Using several entries as examples, relationships between starch, ethanol production in litres ethanol per hectare and litres per ton where grain yield is not taken into consideration were illustrated. Overall applicable relationships other than clear grouped entry differences could not be established. What was clearly demonstrated however, is that the maximization of grain yield is paramount. Highlighted thus, is the individuality of a specific genotype where MLFTs will always be required to quantify genotype potential.

Uittreksel

Die studie het ten doel gehad om die geskiktheid van lentekoring vir die produksie van bio-etanol in die Wes Kaap te evalueer. Ook het dit ‘n voortelingsprogram geinisieer vir die teel van lyne met verhoogde bio-etanol opbrengs.

Materiaal vir gebruik as manlike ouers in ‘n basis-populasie is geselekteer gegrond grootliks op graanopbrengs, siekteweerstand en direkte sowel as indirekte etanolopbrengs kenmerke. Die gekose materiaal is gekruis met vroulike ouers verkry vanaf Stellenbosch Universiteit se Planteteeltlaboratorium (SU-PTL) se manlike steriliteits gedrewe merker bemiddelde herhalende seleksieprogram. Die program is saamgestel uit ‘n verbeterde populasie ten opsigte van siekteweerstand en agronomiese eienskappe. Dit bevat ook ‘n dominante steriliteitsgeen. Die nageslag van die kruisings is aangewend vir die inisiasie van die produksie van verdubbelde haploied lyne vir die verkryging van lyne met verhoogde etanol opbrangs.

Die ontleding van data ten opsigte van die multi-lokaliteitsproewe (MLP) het aangetoon dat gevorderde teellyn (GTL) 00K60-16-3-3 die beste aangepas was en ook die hoogste opbrengs (2160.95 liters etanol per hektaar) gegee het. 00K60-16-3-3 was ook nie statisties betekenisvol swakker as die eerste geplaaste 03H86-8-2 (2184.62 liters etanol per hektaar) en beide GTLs was statisties betekenisvol beter ($P \leq 0.05$) as die ses kontroles in die studie. GTL 00K60-16-3-3 was ook die beste aangepaste in terme van etanol opbrengs in liters per ton graan. GTL 03H86-8-1 was tweede aanbevole vir die Wes-Kaap met ‘n prestasie bo die verwagte gemiddelde opbrengs in liters per hektaar. Verdere aanpassing van spesifieke GTLs vir die twee mega-omgewings in Wes-Kaap nl. Swartland en Suid-Kaap insluitend die Rûens was ook afgelei. Napier was betekenisvol beter, maar nie enige van die lokaliteite was beide stabiel en hoë opbrengs lokaliteite nie. Dit was ook bepaal dat die inskrywing by lokaliteits interaksie (GXE) betekenisvol was oor die hele produksiegebied ten opsigte van liters per hektaar asook in die twee mega-omgewings afsonderlik. Dit was egter te verwagte gegewe die diverse aard van die omgewings in die streek as geheel.

Deur gebruik te maak van verskeie inskrywings as voorbeelde is die verwantskap tussen stysel, etanol produksie in liters etanol per hektaar en liters etanol per ton graan geillustreer sonder om graanopbrengs in ag te neem. Oorhoofs toepaslike verwantskappe anders as duidelike gegroepeerde inskrywings verskille kon nie afgelei word nie. Wat wel duidelik gedemonstreer kon word is dat maksimum graanopbrengs uiters belangrik was. Dit is dus duidelik dat weens die wisselende aard van spesifieke genotipes MLPs altyd van kardinale belang sal wees vir die kwantifisering van ‘n genotipe se potensiaal.

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List of abbreviations

2n, 4n, 6n	diploid, tetraploid, hexaploid
A/A	amylose/amylopectin ratio
AACC	American Association of Cereal Chemists
AAQ	autoamylolytic quotient
ABL	advanced breeding line
ADP	adenosine diphosphate
AFLP	amplified fragment length polymorphism
AMMI	additive main effects and multiplicative interaction
ANOVA	analysis of variance
APR	adult plant resistance
ATP	adenosine triphosphate
BME	β-mercaptoethanol
bp (Mb)	base pairs (million bp)
CIMMYT	International Wheat and Maize Improvement Centre
CMS	cytoplasmic male sterility
CO_2	carbon dioxide
CP	crude protein
CTAB	cetyl trimethylammonium bromide
CV	coefficient of variation
DDGS	distillers' dry grains with solubles
DH	doubled haploid
DME	Department of Minerals and Energy
DNA	deoxyribonucleic acid
dHPLC	denaturing high performance liquid chromatography
d.w.b	dry weight basis
ERM	embryo rescue medium
EST	expressed sequence tag
FFV	flexible-fuel vehicle
FN	falling number
FS	fermentable sugars/substances
GA_3	gibberellic acid
GxE	genotype X environment (interaction)
GHG	greenhouse gas
GPC	green parthenocarpic caryopses
GRR	gene-rich region
HAMS	high-amylose maize starch
sdH_2O	(sterile distilled) water
HCl	hydrochloric acid
HGCA	Home-grown Cereals Authority
HLM	hectolitre mass
HRWYT	high rainfall wheat yield trials
IEA	International Energy Agency
IP	intellectual property
LSD	least significant difference
MS-MARS	male-sterility -mediated marker-assisted recurrent selection
MAS	marker-assisted selection
MLFT	multi-location field trial
NaOAc	sodium oxaloacetate

NIRS	near-infrared reflectance spectroscopy
NSP	non-starch polysaccharide
PCA	principle component analysis (axis)
PCR	polymerase chain reaction
p.a.	per annum
pH	hydrogen potential
PLS-R	partial least squares analysis regression
QTL (eQTL)	quantitative trait loci (expressed QTL)
R&D (RD&D)	research and development (and deployment)
REN21	Renewable Energy Policy Network for the 21st Century
RMS	regular maize starch
RSH	raw starch hydrolyzing
SABA	South African Biofuels Association
SADC	Southern African Development Community
SSF	simultaneous saccharification and fermentation
SU	Stellenbosch University
SU-PBL	Stellenbosch University – Plant Breeding Laboratory
TILLING	target induced local lesions in genomes
VHG	very high gravity
WDGS	wet distillers' grains with solubles
WSSD	World Summit on Sustainable Development
YPD	yeast-extract -peptone-dextrose

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Chapter 1: Introduction

In 2007, scientists from various collaborating nations finally confirmed the causal relationship between increasing greenhouse gas (GHG) emissions and ongoing climate change leading to global warming (Bernstein *et al.*, 2007). With this consensus reached, together with the fact that fossil fuels are a finite resource for which demand is predicted to increase by more than 50% by 2025 (Ragauskas, *et al.*, 2006), action is required now. These global efforts were formalized in the Kyoto Protocol of 1997, a legally-binding commitment by countries to lower their GHG emission levels to an agreed level by 2012. Developing countries including South Africa, are not legally bound under the protocol to curb emissions (Van der Merwe, 2007), but instead have to report their GHG emission levels. South Africa is however ranked within the top 10 GHG offenders in the world (Van der Merwe, 2007) therefore there is pressure on the country to reduce its emissions. Governments around the world, including that of South Africa, have responded by promulgating policy as well as backing research in this area (Ruth, 2008). This is not the first time that fuel alternatives have received such attention. The energy crisis of the 1970s (Organization of the Petroleum Exporting Countries, OPEC oil embargo) forced countries to look elsewhere until the oil price subsequently dropped, followed by a tripling in global oil consumption in the years that followed (Lovins, *et al.*, 2004).

In 2002 and in the context of sharply increasing global interest in renewable fuels, the Department of Minerals and Energy (DME) in South Africa strengthened international relationships in the area of renewable energy during the World Summit on Sustainable Development (WSSD). Following this, the White Paper on Renewable Energy (DME, 2003) set a target of 10,000GWh of energy to be produced from renewable sources (mainly biomass, wind, solar and small-scale hydroelectric) by 2013. After Cabinet approval of the White Paper, the DME proceeded with the development of a renewable energy strategy for South Africa. The abovementioned target was confirmed to be economically viable inclusive of subsidies and carbon financing, as well as achievable mainly through the production of grid, off-grid and biofuel facilities. To put this target into perspective, this would be equivalent to electrifying approximately two million households having an annual electricity consumption of 5,000kWh or alternatively, is equivalent of about 5% of the, then electricity generation in South Africa. Later, in December 2005, Cabinet approved the development of an industrial strategy targeted at creating jobs in the energy crops and biofuels value-chain to help bridge the gap between the first and the second economy. Later still, in 2006 Cabinet authorized the establishment of a Biofuels Task Team (BTT) to develop this strategy which culminated in a draft national biofuels policy (DME, 2006) released later that same year. (DME, n.d.)

Since that time, a significant change (DME, 2007) to the original draft strategy was to adopt a short-term focus (five-year pilot plan) to achieve a 2% market penetration by biofuels, or 400 million l p.a. This target was revised down from 4.5% initially proposed in the 2006 draft

document. For the production of bio-ethanol, it was proposed that sugarcane (*Saccharum officinarum* L.) and sugar beet (*Beta vulgaris* L.) be used as feedstocks while for biodiesel production; sunflower (*Helianthus annuus* L.), canola (*Brassica napus* L.) and soya beans (*Glycine max* L.) were recommended. The exclusion of other crops, at least in the initial stages, is based mainly on food security concerns. In addition, drought-tolerant *Jatropha curcas* which is suitable for biodiesel production could compete for land (and to a lesser extent impact water resources) within the forestry sector (DME, 2007). It was concluded that certainty on the ability of underutilized land in South Africa to produce, has to be established first, together with the establishment of the necessary measures to guard against food inflation. In addition, it was suggested that the proposed 2% market penetration can be achieved without jeopardizing food security. The strategy targets both new as well as additional agricultural land and in total requires about 1.4% of South Africa's arable land area. A 100% fuel tax exemption was also proposed for bio-ethanol as it can also be used in markets other than vehicle fuel that carry no levies at all.

South Africa has the potential to significantly reduce its dependence on foreign-sourced oil via biofuel production. Factors influencing such an industry include not only the oil price, but the need for government backing (legislation and subsidies) as well as local economic viability inclusive of feedstock (raw material) development. To date, most research with respect to wheat (*Triticum aestivum* L.) for biofuel production (a genetically complex trait) has been carried out in the European context. Notwithstanding the results of an economic feasibility study concerning a possible biofuels plant in the Western Cape Province (Richardson *et al.*, 2007), current South African government policy excludes staple foods for this purpose. During the period in which the original draft strategy was being drawn up, Grain South Africa (GSA) launched an investigation into the technical viability of bio-ethanol production in the Western Cape, a major wheat producing region of South Africa.

This study aimed to investigate the suitability of spring wheat in the Western Cape as a potential feedstock for a bio-ethanol industry in the Province and initiate a pre-breeding programme to develop ethanol-directed improved breeding lines. Three conceptual objectives followed from this aim, the first of which was the establishment of multi-location field trials (MLFTs) of advanced wheat material over the entire Western Cape wheat production area, in which commercial cultivars were included as controls alongside Stellenbosch University – Plant Breeding Laboratory (SU-PBL) advanced breeding lines (ABLs). Secondly, was the optimization of analytical protocols applied to the MLFT material. These comprised the indirect starch-related, as well as direct assaying of important parameters relating to the project aim. Finally, a pre-breeding programme was initiated through combining the already-established SU-PBL rust resistance male sterility -mediated marker-assisted recurrent selection (MS-MARS) programme with doubled haploid (DH) technology.

Chapter 2: Literature review

2.1 Bio-ethanol production

2.1.1 Global industry context

In recent years and on a global scale, uncertain oil supply coupled with high price, climate change and the need to develop rural economies, have all contributed to increased interest in bio-energy production. Crude oil accounts for about 36% of world energy consumption (Cockerill and Martin, 2008), with the United States remaining the biggest consumer followed by China (Sanchez and Cardona, 2008). Of that figure, the world transport sector consumes approximately 60% according to International Energy Agency (IEA) statistics (IEA, 2008b). Also, according to a 2008 British Petroleum (BP) Statistical Review of World Energy, the world's total proven oil, natural gas and coal reserves are: 168.6 billion t; 177.4 trillion m^3 and; 847.5 billion t respectively, ending 2007 (British Petroleum, 2008). At current consumption rates, the proven reserves-to-production ratios are 41.6, 60.3 and 133 years respectively. Energy influences a country's social, economic, and environmental development including livelihoods, access to water, agricultural productivity, health, population levels, education and, gender-related issues (Ejigu, 2008). Oil output and consumption also influences that of products such as fertilizer and plastics, as well as a range of other consumer goods (NEPAD, 2001; Singh and Sooch, 2004; Ejigu, 2008).

The Renewable Energy policy Network for the 21st Century (REN21) manages the 'Action Programs' to advance renewable energy policy in both developed and developing economies, where such programs were born out of a series of high-level conferences launched in Bonn, Germany in 2004. The last such conference was the Washington International Renewable Energy Conference (WIREC) held in March 2008 that served to publicize renewable energy opportunities. According to REN21 (2009), the current renewable energy industry is considered by most analysts to be a guaranteed-growth sector as well as crisis-proof, an opinion founded on its underlying drivers. Its growth has also surpassed predictions of those in the industry itself. Many renewable energy sector indicators have shown massive gains since the REN21 Renewables Global Status Report was first launched in 2004, including investment which has increased four-fold to reach US$120-billion in 2008. During this four-year period, biodiesel production increased six-fold to 12 billion l p.a. while ethanol production doubled to 67 billion l p.a. (34% increase) (Figure 1). Bio-ethanol achieved a 4% market penetration of the total 1,300 billion l of gasoline consumed globally. Sugarcane is used for bio-ethanol production in Brazil, while the United States and Europe utilize the starch-yielding crops corn (*Zea mays* L.) and, wheat and barley (*Hordeum vulgare* L.) respectively (Linde *et al.*, 2008). In 2007, the five major bio-ethanol -producing countries of the world utilized approximately 2.2% (11.4 million ha) of available arable land to produce bio-ethanol feedstocks (Balat and Balat, 2009). In Brazil, ethanol production surged from 18 billion l in 2006 to 27 billion l in 2008, with more than 400 operational processing plants plus 60 biodiesel plants

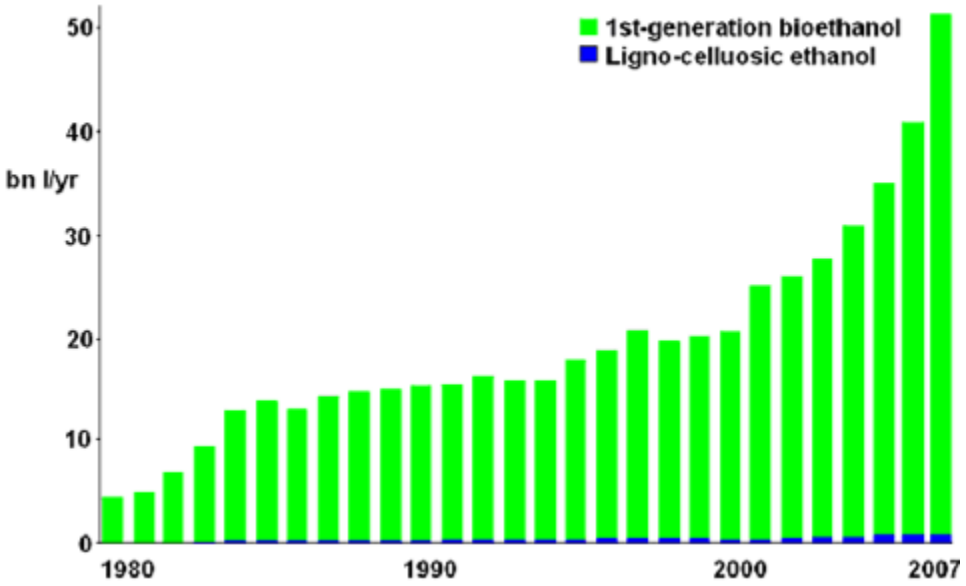

Figure 1. Global ethanol production derived from first- and second (lignocellulose) –generation biofuels.

operating by the end of 2008. About 15% of that country's ethanol production was exported during 2008. Balat and Balat (2009) illustrate the comparative factors underpinning Brazil's success, pointing out a long history of pro-active government support beginning with the National Alcohol Fuel Programme (ProAlcool) in 1975, to a current 80% share of the country's vehicle manufacture comprising flexible-fuel vehicles (FFVs) able to operate on anything from E-0 -100. The United States remains the world's leader in ethanol production in terms of output volume at 34 billion l in 2008, a year in which 31 new ethanol plants came online in that country and supported by about 1,900 E-85 refueling stations. Other notable ethanol producing countries include: Australia; Canada; China; Colombia; Costa Rica; Cuba; the Dominican Republic; France; Germany; India; Jamaica; Malawi; Poland; South Africa; Spain; Sweden; Thailand and; Zambia.

Similar to the Western Cape Province in South Africa in some years, European countries produce an annual surplus of wheat. In the United Kingdom, the BioResources Group of the Society of the Chemical Industry (SCI), held a conference in 2008 entitled Wheat for Biofuels, Bio-energy and High Value Bioproducts, concerning the possibility of using wheat as a feedstock (Society for the Chemical Industry, 2008). During this conference, it was concluded that wheat is the most widely grown, highest-yielding and often, most profitable crop in northwest Europe, from which higher-value chemicals could be extracted as part of the biofuel production process. Also mentioned, was the question of whether wheat is a sustainable feedstock, which is dependent on bioscience deliverables, economics and public perception. (Baylis, 2008)

2.1.2 Africa

Regarding ethanol production, sugarcane is considered to have the best energy balance and lowest production cost in most areas where it can be grown economically. Southern African

Development Community (SADC) sugar production is comparatively small and is led by Mauritius, South Africa and Zimbabwe, who together account for 65% of the region's total. Average South African sugar yields are low in contrast to Malawi, Zambia and Zimbabwe. Notably, Central Africa is the most suitable region for rain-fed (syn: *dryland*) sugarcane production while parts of West and East Africa namely, Tanzania and Uganda are considered marginally to moderately suitable. Rain-fed sugarcane production on the same scale as Brazil would be environmentally too costly. Nevertheless, it can be produced at smallholder-level together with other crops, making a smallholder out-grower based sugarcane bio-ethanol industry apparently viable on the continent. (Johnson and Matsika, 2006; Ejigu, 2008)

In 2007, the Mozambique government approved a US$510-million ethanol-from-sugarcane project backed by the British-based Central African Mining and Exploration Company (CAMEC) in Southern Gaza Province. In addition, Principle Energy, a renewable energy company, said that it will build a US$290-million bio-ethanol plant in that country in 2009. The country has also drafted a strategy for the production of biodiesel from the drought-tolerant non-food jatropha plant. (Reuters, 2009)

2.1.3 South Africa

The South African government has adopted a framework for climate policy, understanding that the future is a low-carbon economy. Winkler and Marquand (2009) reviewed the South African energy sector with emphasis on possible short-, medium- and long-term mitigation strategies that may be employed to move the country forward in this direction. It was concluded that to bring about the necessary transition, the current minerals and energy complex is so central to the economy that it is likely to take decades to change dramatically, requiring an industrial paradigm shift. The report proposes energy efficiency changes in the short-term, fuel-mix changes (biofuels) in the medium-term and, structural (industrial) changes in the long-term. The last-mentioned is the least well understood in terms of both cost and time-frame. Using 2007 data, it was also concluded that the energy (84% accounted for by large-scale synthetic fuel production) and industrial sectors on the supply side, as well as the industrial (66%) and transport (12%) sectors on the demand side, are the biggest contributors to GHG emissions (CO_2, CH_4 and N_2O) in South Africa. Only a few countries in the world have greater energy intensities (Hughes *et al.*, 2002) due mainly to: [1] the country's high dependence on coal, i.e. about 68% followed by crude oil at 19% primary energy supply according to the Department of Minerals and Energy (2006a) and; [2] a large proportion of energy-intensive industries. Both factors are strongly linked to historically low electricity costs, which in-turn is linked to the historically low price of coal in South Africa, i.e. around 40% of United States average prices for four decades. However, low-grade coal (about ⅔ of South Africa's production) is not significantly influenced by international energy prices, as it cannot be economically exported. Also, liquid fuels have historically been tightly regulated with recent renewables policy receiving widespread criticism, including from potential investors and the South

African Biofuels Association (SABA), an organization who's mandate it is to promote a sustainable biofuels industry in the country (Richardson *et al.*, 2007; Pringle, 2009). (Winkler and Marquand, 2009)

On February 23rd 2006, Ethanol Africa announced that it would construct eight maize (*Zea mays* L.) -fed bio-ethanol plants in South Africa at a value of R700-million each across three provinces. Prior to this, a free-fall in the maize price left producers overstocked and thus any profitable initiative to reduce stocks was welcome. Since then, government has excluded maize from its national biofuels strategy primarily over food security concerns, at least initially. The rationale at the time behind a proposed bio-ethanol plant in the Western Cape Province using wheat as a potential feedstock, included an ongoing harvest surplus of around 200,000 t. p.a. that was exported to other parts of the country at substantial cost (Lemmer, 2006). Other factors also contributed to the decreased economic viability of wheat production in the region. Richardson *et al.* (2007) quantified the economic viability of a proposed wheat-fed bio-ethanol plant in the Province using a Monte Carlo economic simulation model. It was concluded that huge government financial incentives would be required. Also noted, was the fact that South African agribusinesses generally consider the bio-ethanol industry to be a break-even industry. Investor concerns identified at the time included: uncertainty surrounding feedstock price and availability; lack of incentives; food-fuel price relationships and; a tendency to evaluate the industry's potential on point estimates, i.e. average, best- and worst-case scenarios. Notwithstanding the greater South African energy context described in the previous paragraph and recent events mentioned here, the business research group Frost and Sullivan estimated that the biofuels market could generate revenues for South Africa of more than US$750-million in 2010, climbing to over US$1-billion in 2013 (Bowker, 2008).

Focusing on future-orientated developments regarding biofuels, Stellenbosch University was awarded the Senior Chair of Energy Research (CoER) in March, 2007 (Biofuels and Other Alternative Clean Fuels) by the South African National Energy Research Institute (SANERI) (Le Roux, 2007). The CoER Biofuels is led by Prof. W.H. (Emile) van Zyl at the Department of Microbiology, together with core members. Their focus is on developing commercially viable value-adding chains for second-generation biofuel production in South Africa, as well as becoming a technology and service provider to other African biofuel producers who have better biomass potential. The PlantBio Trust has taken a lead in funding second-generation biofuels projects in South Africa (PlantBio, 2008). Candidate crop selection for funding by the Trust is primarily based on sugar content and the ability to grow on marginal land with low energy input, both of which are in-line with the national biofuels strategy.

2.1.4 What is bio-ethanol?

Ethanol (CH_3CH_2OH or C_2H_5OH) (syn.: *ethyl alcohol; grain alcohol; EtOH*) is a clear, colourless liquid. It has a high octane number, boils at 78°C within the distillation range of gasoline,

has a similar density and is fully miscible with it if in anhydrous form. If derived from agricultural sources (biomass) versus petrochemical (fossil) sources, this liquid fuel is referred to as *bio*-ethanol. Biofuels can be readily mixed (syn: *gasohol* and *diesohol*) with, or even substitute petroleum-based fuels. Theoretically, biofuels can be derived from any biological carbon source, although the most common and most abundant source is plant biomass. Biomass refers to all living organisms including their waste. As a whole, biomass from agricultural, industrial and municipal processes is under-utilized as an energy source. So-called 'energy crops' are grown specifically for commercial biomass production and comprise the feedstock for the industrial biomass-to-energy conversion process, also referred to as 'energy farming'. Suitable feedstocks are either high in sugar content such as sugarcane, sugar beet and sweet sorghum (*Sorghum bicolor* L.) or alternatively, high in starch such as maize or wheat grain. The abovementioned crops constitute first-generation bio-ethanol utilizing conventional technology in the conversion process. Second-generation biofuels are receiving attention to overcome technical (and thus economic) challenges to their production, such as the need for a whole suite of cellulases (enzymes) to break down lignocelluloses (Heinzelman *et al.*, 2009). By implication, non-food crops may also be utilized in second-generation processes or alternatively, inedible waste from food crops such as wheat straw, citrus peel or sawdust. More recent, is the advent of third-generation biofuels derived from algal/microbial sources. Some authors classify third-generation technology as feedstocks (food or non-food) with a genetically inbuilt (usually transgenic) ability to enhance the fuel production (conversion) process in some way, while referring to biofuels from microbial metabolic sources as fourth-generation technology. A fourth-generation process produces biofuels directly without the need for a fermentation step (Mannan, 2010). Currently, most biofuels are first-generation fuels while second-generation technology allows potential biomass availability to increase two-fold or more (Ragauskas, *et al.*, 2006).

Ethanol is currently the most widely adopted biofuel in the world and is usually blended with petroleum to between 10 (E-90) and 15% (E-85) as an oxygenate and octane extender to improve engine performance. Blending above E5 requires modification to refinery equipment. Therefore the South African Petroleum Industry Association (SAPIA) does not want an above-E5 mandate (Botes, W.C. Personal communication. 2009). Most existing gasoline engines can operate on E-15 without modification (Goldemberg, 2008). Favouring bio-ethanol, it has a higher octane number to reduce engine 'knock' or early ignition (Yoosin and Sorapipatana, 2007), broader flammability limits, greater flame speed and, higher vaporization heat; all allowing an engine to operate at greater thermal efficiency, i.e. run hotter by allowing an increased compression ratio (12:1 versus the standard 8:1). Shorter burn-time leads to theoretical efficiency advantages over gasoline (Balat, 2007). Bio-ethanol also contains 35% oxygen, thus significantly reducing its combustion emissions (Wang *et al.*, 1999; Demirbas, 2005; Malça and Freire, 2006), while petroleum essentially has none. (NREL, 2007; Demirbas, 2008; Balat and Balat, 2009)

A number of different biofuels can be produced from carbohydrates with the general formula (CH_2O). A case has been made for the adoption of all suitable alternatives to gasoline as well as for biodiesel over bio-ethanol (Jegannathan *et al.*, 2009), each with its own merits. Syngas can be derived from coal or natural gas, where both processes are already commercially well established. Syngas can also be produced from plant material as well as municipal waste, but still faces technical difficulties. Enzymatic and fermentation syngas processes in contrast to thermochemical processes, have characteristically been feedstock-specific to date in terms of efficiency. (Demirbas, 2007a, b; Mannan, 2010; Balat and Balat, 2009)

2.1.5 What are the concerns?

The social, economic and environmental impacts, as well as energy-balance of bio-ethanol production have all been questioned in numerous studies. There has however, been a lack of comprehensive assessments as the impacts of biofuel development go beyond the regional or national setting of domestic biofuels targets (Fischer *et al.*, 2009). Also, the precise economics are case-specific with feedstock type and financial incentives from government playing a major role (Amigun *et al.*, 2008; Balat and Balat, 2009).

Some of the key demerits of using bio-ethanol as a transport fuel are mentioned here. Due to its hygroscopic properties ethanol is potentially corrosive to fuel systems, although the context in which it can behave as a corrosive agent is restricted. For the same reason, fuel efficiency is reduced, engine starting is more difficult and intermittent operation (sputtering) is increased. Bio-ethanol also has a lower energy density than gasoline (66-67% of that of gasoline), lower flame luminosity, lower vapour pressure (making cold starts more difficult), is toxic to ecosystems (MacLean and Lave, 2003) and, has increased acetaldehyde exhaust emissions. However, since ethanol has a higher octane rating than gasoline, engine compression ratios can be increased, making ethanol-fueled engines 15% more efficient, thus partly compensating for its lower energy content (Hansen, 2004). The nett affect is that about 20% more ethanol versus gasoline is required per kilometer driven. (Balat and Balat, 2009)

In summary, current technology and agricultural outputs are not sufficient to replace fossil fuels entirely (Ruth, 2008). As the technology stands at present, in the case of maize ethanol for example and taking all factors into consideration, bio-ethanol production consumes about the same amount of fossil fuel as the ethanol itself replaces (Bourne, 2007). The use of food crops for bio-ethanol production is at the very least a contributing factor to recent upwardly-driven food prices for two main reasons namely, competition for: [1] the same crop yields and; [2] limited agricultural land area. The World Bank's World Development Report (WDR) of 2008, entitled Agriculture for Development is not entirely negative toward biofuels (World Bank, 2008). This report highlights the fact that there is not necessarily a linear relationship between energy security and the creation of a biofuels industry, and that current technologies can only marginally improve supply security. The report also cautions that the environmental benefits of reduced GHG emissions may be offset by

emissions released in growing, manufacturing and transporting such fuels. Also, some potential feedstocks have a higher environmental cost than others in a given location where comparative studies have been done. Similar sentiments are expressed by the Food and Agriculture Organization (FAO) annual report of 2008 entitled The State of Agriculture and Food, which points out that the removal of agricultural and biofuel subsidies and trade barriers creating artificial markets would benefit developing countries seeking a foothold in the sector (Food and Agriculture Organization, 2008). Biofuels are likely to play a significant role in a future total energy solution. Most studies have not ventured beyond energy and carbon balances and when compared, draw divergent conclusions (Blottnitz and Curran, 2007).

Brehmer and Sanders (2009a) challenge the idea that first-generation technology is as mature as is widely thought, pointing out that there is significant room for improvement on both the agricultural and processing sides. In the case of Brazil for example, it is not merely a clear jump to second-generation technology according to Brehmer and Sanders (2009a). It is also interesting to note that the Brazilian sugarcane-based bio-ethanol industry enjoys a huge positive fossil fuel nett energy value (NEV) in the order of eight, i.e. eight units of energy output for each unit input. In contrast, United States maize and European wheat struggle to achieve values above two (Bourne, 2007; Brehmer and Sanders, 2009b).

Not all findings are transferable to differing countries and regions and almost no published data is available relating to South Africa, which may be in part due to the perception of a financially unconducive environment for industry commercialization. However, an economic appraisal of biofuel industry potential in the country was undertaken by Meyer *et al.* (2008). This study highlighted (once again) the need for government financial support in the early stages. It was also pointed out that no country uses a staple food to produce biofuels. Grain sorghum (and maize) appear to be most promising where grain sorghum is more suited to drier and marginal areas, and has a starch fraction of around 75% (d.w.b.) (Stumpf, 2006). This contrasts with approximately 60% for wheat and triticale, although sugarcane has the highest ethanol yield and is also the cheapest to produce (Stumpf, 2006).

2.1.6 How is bio-ethanol made?

Three parts of the wheat plant have been studied as biofuel sources, namely: grain (primarily starch); straw (primarily lignocellulose) and; miller's by-products (mixed cellulose and starch) (Vidmantiene *et al.*, 2006; Das Neves *et al.*, 2007). Straw removal from the field should be balanced with soil carbon needs as well as erosion control (Johnson *et al.*, 2007). Biomass can provide thermal, electrical and mechanical energy services and in addition, higher conversion efficiencies are usually obtained by combining more than one such service by for example, combined heat and power plants (IEA BioEnergy, 2005).

A conceptual outline of the first-generation bio-ethanol production process is given in Figure 2 below in which seven sub-systems can be identified and, where constituent steps in the

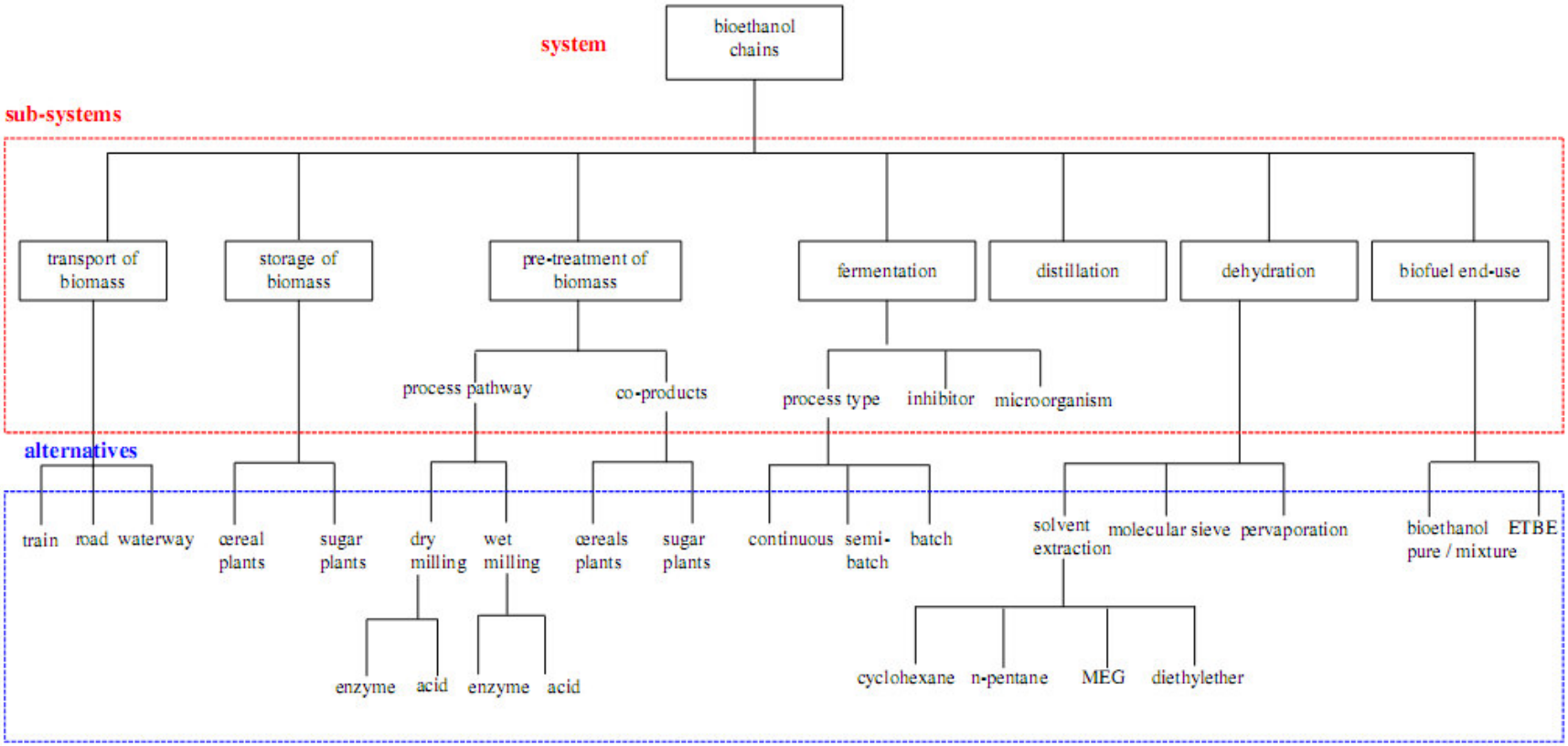

Figure 2. Hierarchal structure for first-generation bio-ethanol production pathways (Riviere and Marlair, 2009).

process vary according to feedstock type. In the case of starch grains such as wheat, the pre-treatment sub-system involves hydrolysis to convert the starch to fermentable sugars/substances (FS). This is carried out in either a dry-grind (Figure 3) or wet-mill process. Dry grinding is more prevalent due to lower capital requirements and operational costs (Nichols and Bothast, 2008) and is also applicable to small grains. The term 'dry-mill' refers to the same process but in the context of the food industry. The fermentation sub-system is either batch, fed-batch or continuous, with the last two being commercially dominant. The distillation and dehydration sub-systems are concerned with fuel purification. Distillation is characteristically 'energy-parasitic' (Figure 3), while dehydration can take varying forms. Simplistically, starch is hydrolyzed to glucose that in-turn is fermented to ethanol.

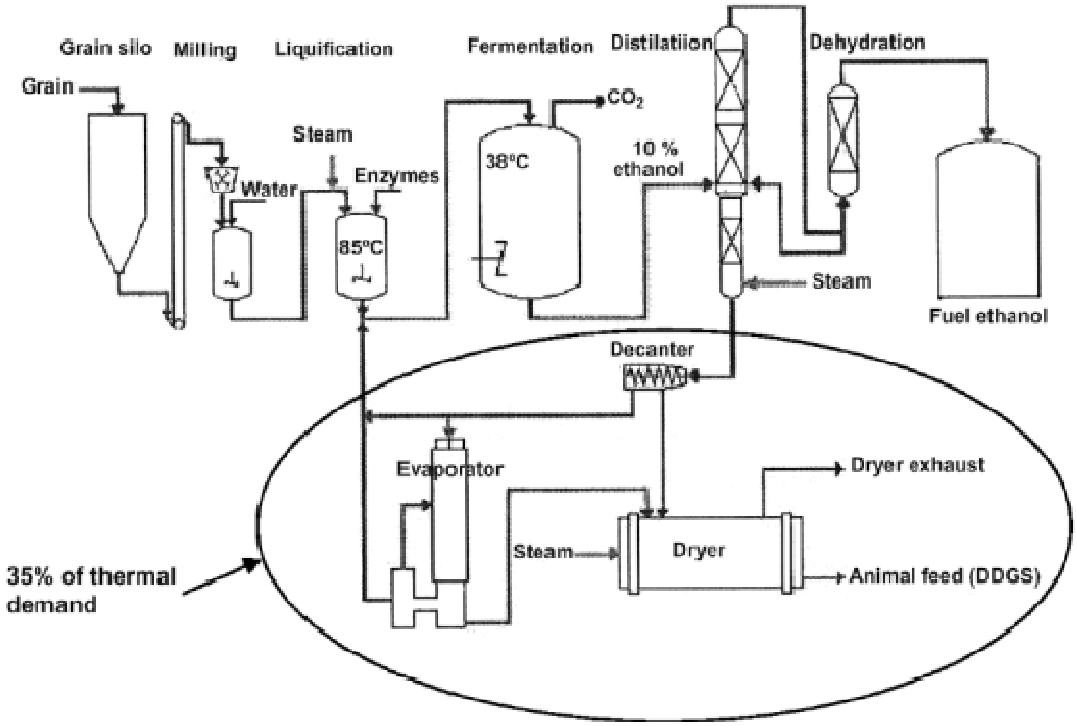

Figure 3. The dry-grind process of producing ethanol from wheat (Murphy and Power, 2008) Marlair, 2009).

The wet-mill process employs soaking (syn.: *steeping*) and fractionation to isolate a relatively pure starch stream from the germ, gluten protein and fibre; yielding a range of end-products in addition to ethanol (Johnson and May, 2003). Dry grinding (Figure 3) on the other hand, yields two end-products i.e. ethanol which is recovered through distillation and dehydration and, fermentation residuals known as distillers' dry grains with solubles (DDGS) which is sold as animal feed (Kelsall and Lyons, 2003). Alternatively, DDGS may be combined with some of the remaining thin stillage and sold as wet distillers' grains with solubles (WDGS). Three conceptual processes are common to both systems, where starch is [1] hydrated in aqueous suspension (syn.: *gelatinization* or *slurry preparation*), resulting in a viscous solution known as 'mash'. The starch-containing gel is then subjected to [2] liquefaction, reducing its viscosity and thus enhancing its

mechanical handling properties and decreasing energy input needs (Meredith, 2003). This is done by partially hydrolyzing the starch to dextrins using alpha-amylase and heating followed by cooling. The mash is then subjected to [3] saccharification, in which it is cooled further and the pH reduced. During saccharification, dextrins are hydrolyzed to the simple sugars glucose and maltose, by the addition of glucoamylase. Enzymes other than amylases may also be employed to enhance hydrolysis. (Nichols and Bothast, 2008)

In the dry-grind system, the fermentation yeast *Saccharomyces cerevisiae* (syn.: *brewer's yeast* or *baker's yeast*) is added with the glucoamylase in a process known as simultaneous saccharification and fermentation (SSF), where glucose is converted to ethanol as quickly as it is released by glucoamylase. This is not carried out in the wet-mill process. One molecule of glucose $(C_6H_{10}O_5)_n$ is converted to two ethanol ($2CH_2H_5OH$) and two carbon dioxide ($2CO_2$) molecules through a glucose ($C_6H_{12}O_6$) intermediate, giving a theoretical ethanol yield of 0.51g ethanol per g glucose consumed (Smith *et al.*, 2006). In addition to this, 0.49g CO_2 is also produced. About 90-93% (or more) of this theoretical yield is commercially realized, as some glucose is used in the production of yeast cells (Demirbas, 2005), glycerol and other fermentation by-products (Ingledew, 1999; Bai *et al.*, 2008). Typical of chemical processes in a complex background, unwanted side-reactions can also occur. Lactic acid -producing bacteria in particular, can inhibit fermentation through both chemical inhibitor production and substrate competition (Narendranath *et al.*, 1997, 2001; Connelly, 1999; Bayrock and Ingledew, 2004). (Nichols and Bothast, 2008)

Fermentation typically yields an ethanol concentration of 12% (w/v) or more, and is distilled from the mash to near-azeotropic (96%) form, where no more water can be removed. Although a too-high concentration will kill the yeast. Typically, distillation accounts for 46% of the parasitic energy of the entire process (IEA, 2008a). Dehydration using molecular sieves allowing water molecules to pass through while inhibiting larger ethanol molecules, yields a near-anhydrous (99.5%) ethanol product (Bibb Swain, 2003). Stillage drying to produce DDGS can account for 35% of the total parasitic energy of the whole process (IEA, 2008a). (Nichols and Bothast, 2008)

Despite bio-ethanol production from grain starch (and sugar) being a relatively technologically mature industry, scope for further improvement still exists. Notwithstanding better process engineering, improvements in first-generation technology by Bai *et al.* (2008) include: pervaporation to reduce product (ethanol) inhibition; yeast immobilization to increase ethanol productivity and decrease fermentor volume and; the use of self-flocculating yeast eliminating the need for yeast immobilization materials altogether. Very high gravity (VHG) systems utilize yeast strains tolerating higher ethanol concentrations where mash contains $250g.l^{-1}$ to yield an ethanol concentration of over 15% (v/v) (Bayrock and Ingledew, 2001; Lin *et al.*, 2002; Bai *et al.*, 2004a, b; Devantier *et al.*, 2005). High concentrations equal energy savings downstream with respect to distillation and stillage treatment. A recent development has been the development of raw (granular) starch hydrolyzing (RSH) enzymes such as Stargen™ 002 (Genencor) converting starch into dextrins at less than 48°C, and hydrolyzing those dextrins into sugars for subsequent

fermentation during SSF (Shetty *et al.*, 2005; Lewis, 2006; Robertson *et al.*, 2006; Williams, 2006). Thus, the need for high temperature jet-cooking is eliminated with concomitant energy savings. Much work has also been done to improve various aspects of the fermentation capabilities of *S. cerevisiae* (Nichols and Bothast, 2008), yet it still lacks commercially efficient pentose sugar (e.g. D-xylose and L-arabinose) fermentation capability. Near-infrared reflectance spectroscopy (NIRS) is also suited to substrates in a complex chemical matrix whose concentration is subject to significant batch-to-batch variation, such as mash glucose concentration and stillage ethanol concentration (Liebmann *et al.*, 2008). Perhaps most significant, is research into amalgamating the benefits of the wet-mill and dry-grind processes. This means: lower capital and operating costs with reduced energy and water usage; co-product generation; greater fermentation rates (and ethanol concentration) and; greater processing capacity as non-fermentables are excluded (Singh *et al.*, 2005; Rausch and Belyea, 2006). This is referred to as the 'biorefinery' concept and is in large-part an attempt to emulate what already happens in the petrochemical industry, only using biomass as a feedstock. Not unlike petroleum, biomass is also of a complex chemical composition. The biorefinery is a concept representing the integrated production of food, feed, chemicals and fuels (NRC, 2000). Koutinas *et al.* (2004), theoretically screened wheat as a biorefinery feedstock in terms of both efficiency and economics, and confirmed its suitability for this purpose. (Kamm and Kamm, 2008; Nichols and Bothast, 2008)

2.2 The wheat plant

Wheat (*Triticum aestivum* L.) belongs to the tribe Triticeae of the family Poaceae. Wheat is an annual self-pollinating grass that is cultivated from sea-level to over 3000m. It grows best in well-drained, clay-loam soil in temperate, arid or, semi-arid climates. The plant may grow to over 2m with roots penetrating just as deep. Most modern cultivars are about half this height or less and referred to as 'semi-dwarf.' Common or bread wheat (*T. aestivum*), durum (*T. durum*), and club wheat (*T. compactum*), account for approximately 90% of the crop across the globe. (Wiese, 1977; Poehlman and Sleper, 1995)

Bread wheat is classified according to: milling properties (hard or soft); dough rheology (strong or weak); bran colour (red or white) and; vernalization (cold-treatment) requirement (spring or winter) (Davies and Gooding, 1997). These classifications are continuous (versus categorical) across genotypes and thus not always distinct. Therefore, so-called facultative or intermediate wheat types also exist. Spring wheat is grown both north and south of the main winter wheat production areas where, with reference to the northern hemisphere for example, such areas are too cold and too warm respectively for winter wheat. Spring wheat grown at lower latitudes includes intermediate cultivars that are insensitive to the photoperiod stimulus and can therefore be planted at any time of the year in the subtropics. (Poehlman and Sleper, 1995)

2.2.1 Description of the wheat genome and its domestication

The process of wheat domestication was associated with artificial selection beginning with the loss of spike shattering at maturity thus reducing seed loss at harvest. Secondly, selection was applied to free-threshing or hull-less forms in which the glumes do not tightly adhere to the grain. Domestication together with an increase in ploidy level has altered the crop's morphological and physiological characteristics. The tillering phase has been shortened resulting in fewer flowering culms per plant. In addition, the harvest index, i.e. the proportion of above-ground plant mass in grain, as well as the grain-filling rate with respect to starch (endosperm), have also increased. (Poehlman and Sleper, 1995; Jantasuriyarat *et al.*, 2004; Nalam *et al.*, 2006; Simons *et al.*, 2006; Dubkovsky and Dvorak, 2007; Shewry, 2009)

In 1918, it was recognized that the wheat genus comprised di-, tetra- and hexaploid groups containing one, two and three genomes of seven, 14 and 21 chromosome pairs respectively (Sakamura, 1918). Hexaploid (syn.: *common* or *bread*) wheat has a large genome of about 16,000Mb (16 billion bp or base-pairs) distributed over 21 chromosomes. The chromosomes are arranged in seven homoeologous groups, such that each group comprises three related chromosomes (n=21) from each of three ancestral sub-genomes designated A, B, and D. The diploid (2n) progenitors of each of the sub-genomes have all been identified, although uncertainty still remains over the progenitor of the B genome (Gill *et al.*, 2004; Salse *et al.*, 2008, Kilian *et al.*, 2009). The current wheat genome is 5-times that of the human genome (Slade *et al.*, 2005). It is a product of both allopolyploidy and extensive evolutionary chromasomal duplications resulting in 80-83% of the genome comprising repetitive deoxyribonucleic acid (DNA). The majority of wheat genes are clustered within small highly recombinant genomic regions. (Gupta *et al.*, 1999; 2008)

The nomenclature convention followed by Zohary and Hopf (2000) and used by Kilian *et al.* (2009) is applied here as well. Only two species of wheat are of commercial significance namely, the allohexaploid (6n) *T. aestivum* and the allotetraploid (4n) *T. durum*. It is generally agreed that the first cultivation of wheat occurred about 10,000 years ago, of which the earliest cultivated forms were essentially landraces comprising 2n 'einkorn' *T. monococcum* (genome A^bA^b) and 4n 'emmer' *T. dicoccum* (A^uA^uBB, also known as *T. turgidum)*. Genetic relationships among the different forms of wheat suggest that it originated from Southeastern Turkey (Heun *et al.*, 1997; Nesbitt, 1998; Dubcovsky and Dvorak, 2007; Kilian *et al.*; 2009). Feldman (2001) describes the spread of wheat from there to the rest of the world. *T. monococcum* is the cultivated (domesticated) form of the wild einkorn *T. baeoticum* (thus also A^bA^b), while *T. dicoccum* is the cultivated form of the wild emmer *T. dicococcoides* (A^uA^uBB). *T. aestivum* (A^uA^uBBDD) is derived from the allopolyploidization (natural hybrid and likely occurring several times independently) of *T. dicoccum* and, the D-genome progenior *Aegliops tauschii* (DD) (syn.: *Ae. squarosa*). Little divergence has occurred within the D genome found in 6n and 2n species, suggesting a relatively early introgression into bread wheat. *T. dicoccoides* is derived from the 2n A-genome progenitor *T. urartu* (A^uA^u) and a yet undetermined 2n B-genome progenitor, where evidence suggests a wild

out-crossing of *Ae. speltoides* (SS or similar genotype) as the female parent with *T. urartu* as the male parent (Dvorak and Zhang, 1990; Huang *et al.*, 2002). *T. urartu* is in fact also an einkorn, but not a cultivated form. Thus, *T. monococcum* and *T. urartu* are both diploid for the A genome as their superscripts indicate. Also, two tetraploids exist, i.e. the above-discussed *T. dicoccum* and *T. araraticum* (A^uA^uGG) also known as *T. timopheevii*, which is sometimes cultivated. Both the B and G genomes occurring in these two tetraploids are widely considered to be modified S genomes derived from a common ancestor, yet a progenitor for the S genome is still uncertain (Gupta *et al.*, 2008; Kilian *et al.*, 2009). However, Salse *et al.* (2008) reported that the (genomic) relationship between the B genome found in wheat and the S genome of *Ae. speltoides* is not as close as was previously thought. *T. durum* (A^uA^uBB) also has its origins in *T. dicoccum* (Damania, 1998) and likely arose independently (Salamini *et al.*, 2002; Ozkan *et al.*, 2005), but is also uncertain (Haudry *et al.*, 2007). (Kilian *et al.*, 2009; Shewry, 2009)

The three chromosomes within the ABD homeologous groups often contain loci in common for a specific trait, suggesting a common ancestor. Genes located on the D genome influence the much-valued baking qualities of bread wheat. The B genome is partially homologous to many 2n species in the genus *Aegilops* while the D genome is completely homologous with *T. tauschii*. In summary, the A, B and D genomes are thus only partially homologous with each other. Despite this, the 4n and 6n wheats reproduce in the same manner as diploids (2n = 4x = 28 tetraploids, 2n = 4x = 42 hexaploids) on account of the *Ph1* and *Ph2* alleles on chromosome 5B. The nett effect of these (*Ph*) alleles is that each chromosome will only pair with its homolog from the same genome A, B or D, i.e. the same homeologous group. In the absence of functioning *Ph1* and *Ph2* alleles, chromosomes have the opportunity to pair with a homeologous chromosome from a differing genome. (Poehlman and Sleper, 1995; Gupta *et al.*, 2008; Shewry, 2009)

2.2.2 Genomic resources

The large size of the wheat genome, its polyploid nature and limited funding have slowed progress in functional genomics research, such that it has lagged well behind maize and rice (*Oryza sativa* L. Moench) (Varshney *et al.*, 2006; Gupta *et al.*, 2008). On the other hand, extensive cytogenetic stock availability (including aneuploids), relatively good knowledge of ancestry and the usefulness of the *Ph* gene system, have facilitated research. Due to the fact that wheat behaves like a diploid and yet its 6n genome tolerates (otherwise lethal) aneuploidy through the presence of triplicate alleles, a large number of aneuploids are available for genomic research (Gupta *et al.*, 2008). In addition to the above, the International Triticeae Mapping Initiative (ITMI) initiated whole-genome sequencing (WGS) in wheat and subsequently launched the International Genome Research on Wheat (IGROW), a multinational collaborative programme which later became the International Wheat Genome Sequencing Consortium (IWGSC) (Moolhuijzen *et al.*, 2007).

Molecular tools include the development of substantial expressed sequence tag (EST) collections, relatively dense genetic and physical maps (including ancestral progenitors), some full-

length complementary DNA (cDNA) genomic sequences and, gene-targeting systems. Maps have been used for: the development of functional markers; identification of gene-rich regions (GRRs); major gene and recombination 'hot-spot' identification and; the identification of quantitative trait loci (QTLs) and expressed QTLs (eQTLs) (Gill, 2004; Crossa *et al.*, 2007; Jordan *et al.*, 2007; Singh *et al.*, 2007; Gupta *et al.*, 2008). Composite maps of different molecular marker types have been constructed for: ESTs; amplified fragment length polymorphisms (AFLPs); single-nucleotide polymorphisms (SNPs) and; microarray-based (Doleˇzel *et al.*, 2005; Coram *et al.*, 2008) high throughput diversity array technology (DArT) markers (Akbari *et al.*, 2006; Semagn *et al.*, 2006; Gupta *et al.*, 2008). These marker types are being used to identify genes associated with specific traits, thus facilitating marker-assisted selection (MAS) and include: random DNA markers (RDMs); gene-targeted markers (GTMs) and; functional markers (FMs) (Bagge *et al.*; 2007; Jordan *et al.*; 2007; Gupta *et al.*, 2008).

In Australia, using a computer simulation model involving restricted back-crossing and DH technology, the application of MAS to the BC_1F_1 stage as well as haploids derived from their pollen led to a reduction in the cost of MAS of up to 40% (Kuchel *et al.*, 2005). The simulation model was later applied to and validated in a practical wheat breeding programme directed at quality and disease (rust) resistance improvement (Kuchel *et al.*, 2007). More difficult traits have been approached using regeneration and transformation protocols. Target induced local lesions in genomes (TILLING), ribonucleic acid interference (RNAi) and epigenetics, have also found application in functional wheat genomics including comparative genomics between related crop species (Devos, 2005).

2.3 Global wheat significance and the South African wheat industry

2.3.1 Global context

Wheat is counted amongst the world's 'big three' cereal crops with a global annual harvest exceeding 600 million t (FAO, 2009). Despite production steadily increasing over the last four decades, a decrease in output has ocurred in the last few years (FAO, 2009; Shewry, 2009). Wheat occupies approximately 17% of all crop acreage worldwide, feeding about 40% of the world's population and providing an estimated 20% of total food calories (Shewry, 2009). Uniquely, wheat is unrivalled in its cultivation range from 67°N to 45°S, including tropical and subtropical regions of higher altitude (Feldman *et al.*, 1995). Bread wheat currently accounts for about 95% of worldwide production. Wheat yields can also exceed 10t.ha^{-1} yet the global average is in the region of 2.8t.ha^{-1} (FAO, 2009; Shewry, 2009). Wheat can also be harvested mechanically using combine harvesters or alternatively, by hand as well as stored indefinitely, provided that the moisture content is less than 15% (d.w.b.) and pests are controlled (Shewry, 2009).

Wheat has diverse uses in industry from the traditional making of bread, confectionery, pasta and other products, to starch-based adhesives. Wheat also has important nutritive value despite lacking some essential amino acids, particularly lysine (Sarath *et al.*, 2008). The value of wheat to the food industry lies in the elastic characteristics of its dough i.e. rheological (mixing/handling) properties imparted by the gluten (gliadin and glutenin proteins) fraction which stretches and traps carbon dioxide as the fermenting dough expands (Bailey, 1941). (Poehlman and Sleper, 1995; Brandt, 2005; Sarath *et al.*, 2008; Shewry, 2009)

2.3.2 Introduction to the South African wheat industry

Wheat is the second most important cereal in South Africa in terms of monetary value, contributing approximately 15% to the gross national value of field crops for the 2008/9 season. A very small portion of this is durum wheat with the balance accounted for by bread wheat, most of which is utilized for human consumption while the remainder is used for animal feed or seed. Roughly 60% of flour and meal produced in South Africa is used for bread-making (Department of Agriculture, Forestry and Fisheries, 2006; 2009).

Wheat was introduced into South Africa in the Western Cape Province by early Dutch settlers with later expansion into inland summer rainfall areas (Jordaan, 1995). The cultivar 'Scheepers' played a pivotal role in the establishment of winter wheat production in the Free State Province. 'Inia 66' from the International Wheat and Maize Improvement Centre (CIMMYT, i.e. Centro Internacional de Mejoramiento de Maíz y Trigo) was significant with regards to spring wheat improvement in South Africa. Currently, two major wheat-producing regions exist in the country, namely: [1] the summer rainfall interior and; [2] the winter rainfall Southwestern and Southern Cape coastal areas. The summer rainfall rain-fed production area comprises four sub-regions namely, the Western, Central and Eastern Free State as well as parts of Mpumalanga Province. In these areas, mostly winter and intermediate-type wheat is produced.

There are two main winter rainfall production areas namely, the Swartland (adjacent to the Sandveld in the West) and Rûens (adjacent to the Southern Cape coast in the South). Temperatures in summer can reach 40°C but are usually between 15 and 27°C (Lambrechts, 1998; Van Niekerk, 2008). Some low-lying areas do experience mild frost in winter. Rainfall variation is influenced by altitude and in addition, the southern parts of the Swartland and eastern parts of the Rûens (towards the Boland) receive more rain (Lambrechts, 1998; Van der Walt, 2008). The region is environmentally diverse and hence cultivar choice is critical. Soils in both production regions of the Western Cape are variable but of the Bokkeveld group with low phosphorous and high sodium levels (Van Niekerk, 2008). In contrast to the Swartland (duplex soil structure), most soils in the Rûens are relatively shallow with a greater fraction of coarse fragments (Van Niekerk, 2008). Soil depth in this area and thus water retention capacity, is limited to a maximum depth of about 600mm or less versus 1200mm in the Swartland (Van Niekerk, 2008). Within the Rûens, the Middle-Rûens sub-region is the most suited to small grain production with an

annual rainfall of between 370 and 400mm p.a. (Van Niekerk, 2008). The Swartland receives between 400 and 700mm p.a depending on altitude (Lambrechts, 1998). Due mostly to the factors mentioned above including slopes greater than a 5 percent gradient, the Rûens is considered a high risk grain production area versus other parts of the world (Van der Walt, 2008). (Department Agriculture, Forestry and Fisheries: Western Cape, 1998; Jordaan, 1995; Barnard *et al.*, 2005)

In South Africa, planting is undertaken from mid-April to mid-June in the winter rainfall region and mid-May to the end of July in the summer rainfall areas. Winter wheat is planted early to meet its vernalization requirements while cultivars with a shorter growing season (spring types) are favoured under irrigation. The most significant regions in terms of production output are the Free State Province and the Western Cape, which together account for about 70% of national output. The Northern Cape Province leads the rest of the country in terms of output. Considering total land area planted to wheat, approximately 80% is grown under rain-fed conditions (Department of Agriculture, Forestry and Fisheries, 2008). Northern Cape production (irrigated) has been fairly stable over time in comparison to the country's two major production areas. Wheat production in South Africa and especially dryland production is characterized by low average yields in comparison with most major wheat producing countries (USDA-FAS, 2005; FAO, 2009). Nationally, there has been a decrease in output since 2003/04 as reported by the South African Grain Information Service (SAGIS), resulting in lower stock levels continuing into 2009 (Department of Agriculture, Forestry and Fisheries, 2009). Although the Western Cape has accounted for a little under half (about 350,000ha) of the total land area planted to wheat in South Africa (about 750,000ha), the production output differential compared to the Free State is due to a greater percentage of higher-yielding irrigation schemes in that Province. (Department of Agriculture, Forestry and Fisheries, 2008)

Alongside decreasing production, domestic consumption has steadily increased, creating the need for imports. Over the four seasons to 2006/07, the country produced 60-70% of its own wheat needs. Following a decent production year in terms of volume and quality, the majority of imports are 'filler' wheat types to be blended with local production and are mostly sourced from Argentina, while higher quality wheat is usually imported from the United States and Australia. (Department of Agriculture, Forestry and Fisheries, 2008; 2009)

2.4 Production constraints

Kosina *et al.* (2007) discusses all major constraints with respect to global wheat production, compiled from a survey conducted prior to the 2006 International Symposium on Increasing Wheat Yield Potential held in Ciudad Obregon, Mexico. The report identified heat stress, competition from weeds and disease as the most significant constraints. Yield losses caused by disease varied between 14 and 27.1% per surveyed region. The most serious diseases cited were: leaf and stripe

rusts (*Puccinia* spp.); Fusarium head blight (FHB) (*Fusarium* spp.); Septoria blotch (*Septoria tritici*); powdery mildew (*Erysiphe graminis*); tan spot (*Pyrenophora tritici* repentis); spot blotch (*Bipolaris sorokiniana*); bunts (*Tilletia* spp.) and; eyespot (*Cercosporella herpotrichoides*). Insect pests were usually reported as less damaging with estimated yield losses of 12.2-22%. The most often cited pests included: aphids; sunn pest (*Eurygaster* spp.); Hessian fly (*Mayetiola destructor*); weevils; termites; rodents and; birds.

2.4.1 Overview of diseases affecting wheat

Wheat is subject to an array of diseases particularly fungal. Wiese (1977) describes over 40 fungal diseases attacking all parts of the plant. Diseases may target the leaves and stem, roots and crown as well as the flowering culm. The rusts (*Puccinia* spp.) are considered the most widespread and economically significant worldwide. The total number of diseases afflicting wheat is unknown although almost 200 have been described, about 50 of which are routinely important from an economic point of view. Wiese (1977) points out that the type of pathogen, its population size, degree of virulence and, the presence of vectors are important influences.

In South Africa, the summer rainfall areas are less plagued by disease. Apart from the rusts, other important wheat diseases in South Africa include: FHB; karnal bunt (*Tilletia indica*) and; take-all (*Gaeumannomyces graminis*).

Irrigation wheat production in South Africa is impaired by the regular occurrence of FHB on an epidemic scale. Greater disease pressure is associated with increased conservation tillage as practiced in the Western Cape, as well as maize production in double-cropping systems with wheat. Producers are often challenged by a lack of summer rotation crops as well as high input costs, making a case for reduced tillage. Most South African wheat cultivars display low FHB tolerance levels and in addition, the disease has not been successfully controlled using fungicides (Kriel and Pretorius, 2008). However, removing maize residue before planting wheat has reduced the disease's severity. Climatic conditions during flowering are unpredictable although excess rainfall (including irrigation), lower night temperatures and thus high humidity are key to epidemiology of the disease. Gosman *et al.* (2009) demonstrated that the choice of semi-dwarfing genes (*Rht-B1* and *Rht-D1*) used in breeding programmes influences FHB resistance significantly. Research is currently focused on: resistance; soil cultivation; climatic triggers and; pathogen population genetics (Buerstmayr *et al.*, 2009). (Kriel and Pretorius, 2008)

2.4.2 The rusts

Three rust diseases affect wheat, namely: stem (black); leaf (brown) and; stripe (yellow) rust. These occur almost everywhere in the world where wheat is grown. As a disease group, all aerial plant parts are susceptible and under optimal conditions, stem rust for example, is not only restricted to the stems. The term 'rust' is derived from the characteristics of the sori or pustules that erupt to release spores through the plant's epidermis. Three highly specialized fungi are involved,

all belonging to the *Puccinia* genus. The fungi are biotrophic i.e. obligate parasites, requiring live plant tissue to survive, with highly complex multi-stage life cycles. Stem rust is caused by *Puccinia graminis* Pers f. sp. *tritici* Eriks; leaf rust by *P. triticina* Eriks and; stripe rust by *P. striiformis* West f. sp. *tritici* Eriks.

Each of these pathogens has innumerable pathotypes, differentiated/resolved by their pattern of virulence on a differential series of hosts (genotypes) in a process called 'infection typing' (McNeal *et al.*, 1971; Roelfs, 1992), as host genotypes influence physical sori appearance. The field frequency and thus economic significance of a particular race is more important than the actual number of races. Race prevalence and virulence towards specific host genotype resistance genes is dynamic and changes over seasons as well as geographic location.

There has been a swing toward durable polygenic (R-gene and APR) resistance in recent years. Historically, monogenic resistance has been easier and less costly to evaluate particularly with respect to seedlings. Pretorius *et al.* (2007) reviewed the history and challenges posed by rust diseases on small grains in South Africa with emphasis on wheat. The authors of this study concluded a general lack of capacity and fragmented efforts in combating wheat rusts in South Africa. Costly and environmentally questionable protective or eradicant fungicides are usually used as a control measure, although rust diseases are best controlled by cultivar resistance which in reality, usually means a higher degree of disease tolerance. Resistance can either be narrow (vertical) or broad (horizontal) -based, or a combination of both as discussed below with reference to each of the three rust types. (Singh and Rajaram, 1995; Feuillet *et al.*, 2005; Boyd *et al.*, 2006)

Leaf rust

Leaf rust is the most common rust type. *P. triticina* is heteroecious, requiring a telial/uredinial host (usually wheat) and an alternative (pycnial/aecial) host (*Thalictrum speciosissimum* or *Isopyrum fumaroides*) to complete its life cycle. *P. triticina* has often been historically overlooked as an important disease of wheat due to the fact that it has had less of an impact on grain quality compared to stem rust for example (Goswami and Kistler, 2004; Leonard and Szabo, 2005). Leaf rust usually decreases the number of kernels harvested per spike as well as lowering average kernel weight. It is highly probable that the sexual cycle does not epidemiologically contribute to the spread of this disease, and is thus an insignificant source of genetic variation in North America and most other wheat production areas. Together with molecular genotyping data for *P. triticina*, it was determined by Kolmer *et al.* (2007) that *Thalictrum* and *Isopyrum* species native to North America are relatively resistant to basidiospore infection (Jackson and Mains, 1921; Saari *et al.*, 1968). By 2007, 60 leaf rust resistance (*Lr*) genes had been designated in wheat (McIntosh *et al.*, 2007), of which most confer resistance in a race-specific gene-for-gene manner (Flor 1971). Characteristically, the effectiveness of such resistance is only transient when resistance (R) genes are deployed individually. Selection pressure is applied upon the pathogen to avoid recognition by its host through either mutation or the deletion of its

host-recognized effector molecule. Virulent rust types may also arise through the selection of sexual progeny that do not contain host-recognized effectors. Although the role of complementary pathogen effectors in wheat rust manifestation specifically is not known, other pathogen effectors are known to suppress basal host defense mechanisms in other plants (Huak *et al.*, 2003; DebRoy *et al.*, 2004; Kim *et al.*, 2005; Nomura *et al.,* 2005, 2006; Fu, 2007). Also, the cultivation of large areas of susceptible cultivars allows a large pathogen population to proliferate, effectively creating a reservoir for mutations and selection pressure upon them (Kolmer, 2005). *P. triticina* is thus characterized by a high degree of virulence variation and adaptation to diverse climatic conditions (Roelfs *et al.*, 1992; Kolmer, 2005).

According to Pretorius *et al.* (2007), the winter rainfall Western Cape wheat production region of South Africa as well as areas of the Free State with warm and moist spring conditions, are most susceptible to leaf rust infection. Neighbouring Lesotho is assumed to be an over-summering zone for wheat rust inoculum as in that country wheat is usually planted toward the end of the South African growing season (Terefe *et al.*, 2009). The most frequently occurring leaf rust pathotype in South Africa during the 2007 season was determined to be 3SA133[a] (76.8%) followed by 3SA126 (11.0%). In that year, the observed virulence profiles were similar to those of previous seasons and no new pathotypes were observed (Terefe *et al.*, 2009).

Stem rust

As a group, wheat rust has impacted the course of civilization through its destruction of an important food source. Many rust epidemics have been described over the last 150 years in regions such as the Near and Far East, Europe and the America's. Obligate biotrophic fungi, being entirely dependent on living host tissue for their reproduction, produce vast numbers of wind-dispersed spores (Brown and Hovmøller, 2002, Singh, 2006). Global disease monitoring is aimed at preventing a repeat of history or at least minimizing its impact. However, over the last 10-15 years reduced public funding is threatening preventative capacity (Singh *et al.*, 2006). Consequentially, rust diseases are on the increase worldwide. Stem rust has been effectively controlled for over 30 years until recently. Pathotype Ug99 (known as TTKS in northern America) was first recorded in Uganda in 1999 (Pretorius *et al.*, 2000) and has followed its own path of destruction through Africa and was recently reported in Yemen and the Arabian Peninsula including Iran (Paul, 2009). Jin *et al.* (2008) reported on virulence variation observed within the TTKS pathotype group. CIMMYT germplasm in Uganda succumbed to the pathotype which exhibited virulence to several resistance genes including *Sr31*, long since known for its durability (Pretorius *et al.*, 2000), as well as *Sr38* (Pretorius *et al.*, 2000). Several sources of resistance to stem rust exist, although not all equally effective (Singh *et al.*, 2006). Researchers suspect that Ug99 is making its way toward south Asia (Singh *et al.*, 2006; Stokstad, 2007). *Sr31* has been incorporated into CIMMYT spring wheat germplasm at a high frequency and is found in several

[a] Agricultural Research Council (ARC) nomenclature.

winter wheat cultivars grown throughout the world. Twenty five percent (50 million ha) of the world's wheat crop is believed to be at risk including more than 90% of cultivars in the suspected migration route (Reynolds and Borlaug, 2006; Singh *et al.*, 2006). A mutant of Ug99 was identified in Kenya in 2006 (Stokstad, 2007) that has also overcome *Sr24*. A number of *Sr* genes have been introgressed into wheat from wild relatives or other cereals (Singh *et al.*, 2006) yet cytogenetic manipulation and extensive breeding have often been required to maintain background germplasm quality. Multiple R-genes have had to be incorporated into cultivars, necessitating continual disease resistance maintenance. To facilitate this pyramiding/stacking approach to wheat disease resistance, robust (in terms of repeatability) molecular markers are currently available for a number of *Sr* and other genes. Gene pyramiding requires all breeders to adopt the same strategy in order for it to be effective or else resistance genes will be overcome in other geographic areas. Taking into account the spore dispersal capacity of rust, this translates into a global breeding strategy. Adult plant resistance (APR) genes are distinct from R-genes and present an alternative approach to disease-resistance breeding that may be deployed alongside R-gene pyramiding (Caldwell, 1968). APR genes behave quantitatively (versus the qualitative behaviour of R-genes) and usually impart resistance in adult plants only. Some developmentally regulated R-genes may also impart resistance in mature plants, but are mechanistically dissimilar. The most successful APR stem rust resistance gene to date has been *Sr2*, which has imparted partial resistance to all races of stem rust since the 1920s (McFadden, 1930; McIntosh, 1995). By itself, *Sr2* is not effective enough against Ug99 unless combined with other genes in what has been termed the (yet uncharacterized) *Sr2* gene complex (McIntosh, 1995; Singh *et al.*, 2006; McIntosh, 2008). Other wheat rust APR genes currently subject to positional cloning efforts include *Lr46*, which co-segregates with powdery mildew and stripe rust resistance. Significantly, molecular markers were recently developed for the *Lr34/Yr18/Pm38* (cloned) resistance gene complex by Lagudah *et al.* (2009). (Singh *et al.*, 2004; Borlaug and Reynolds, 2006; Ayliffe *et al.*, 2008)

Visser *et al.* (2009) compared the genetic structure of selected South African wheat stem rust races with that of Ug99. The local race UVPgt55 (syn: *TTKSF*, an isolate of 2SA88) grouped with Ug99 with a 100% similarity using SSR markers which divided the surveyed race population into two groups with 24.5% similarity. Including AFLP data in the results increased the similarity between the two groups to 66.7%. Hence, it was concluded that UVPgt55 was likely an exotic introduction into South Africa in contrast to the remaining races having locally evolved. It was also pointed out that sexual recombination has never been reported for wheat stem rust in South Africa (Pretorius *et al.*, 2007). UVPgt55 was detected for the first time in South Africa in 2000 (Boshoff *et al.*, 2002b) and was the first documented South African race to display virulence towards *Sr8b* and *Sr38* (Visser *et al.*, 2009). In this study, UVPgt55 also exhibited a similar avirulence-virulence profile to Ug99 (Pretorius *et al.*, 2000), but with the notable exception of avirulence towards *Sr31* (Pretorius *et al.*, 2007) in contrast with *Sr24*.

Yellow rust

A *Yr9*-virulent race of stripe rust was first reported in East Africa and subsequently spread to South Asia via the Middle East over approximately 10 years (Singh *et al.*, 2004). *P. striiformis* is a dikaryotic basidiomycete and unlike other wheat rusts, is not known to complete a sexual life cycle at all, as no alternative hosts have ever been identified (Stubbs, 1985; Moldenhauer *et al.*, 2008). In addition, no hyphal recombination has been described under natural conditions (Hovmøller *et al.*, 2002; Enjalbert *et al.*, 2005). Both nationally and regionally, genetic variability at the DNA level is often low in contrast to its virulence phenotype diversity (Steele *et al.*, 2001; Hovmøller *et al.*, 2002; Enjalbert *et al.*; 2005). Stripe rust epidemics have recently arisen in previously unaffected regions such as: South Africa in 1996 (Boshoff *et al.*, 2002a); the Eastern United States in 2000 (Chen 2005; Milus *et al.*, 2006) and; the state of Western Australia in 2002 (Wellings *et al.*, 2003). Using a rigorous sampling and AFLP- as well as virulence- based phenotype assessment strategy, Hovmøller *et al.* (2008) investigated *P. striiformis* diversity on a global scale to test the hypothesis of recent intercontinental spread in contrast to hypothesized local evolution. The resulting phylogeographical pattern provided the basis for a proposed sequence of dispersal events as well as their underlying causal mechanisms.

In South Africa, the bread wheat cultivar 'Kariega' displays complete APR to stripe rust. In contrast to documented concerns that the accumulation of partial and adult plant forms of stripe rust resistance may negate high yields (Brown, 2002), Kariega is still relatively high-yielding and is also a standard for baking quality and is thus of particular interest to breeders (Moldenhauer *et al.*, 2008).

2.5 Wheat quality and improvement

Wheat breeding has predominantly been public in the industrialized world with parts of Western Europe being an exception (Heisey *et al.*, 2002). A strong private wheat breeding presence has existed in the Southern Cone region of South America, especially Argentina, as well as South Africa. Also, the public sector has experienced a strong increase in pre-breeding focus (Botes, W.C. Personal communication. 2009). Wheat has traditionally been improved by crossing (hybridizing) phenotypically-identified high-yielding genotypes, with selection for transgressive segregates applied to subsequent segregating (filial) generations via either the pedigree, bulk-population or, single-seed descent methods. The crossing of autogamous (self-pollinating) wheat in large volume has also been facilitated by the discovery of cytoplasmic (genetic) male sterility (CMS) and associated fertility-restoring (*Rf*) genes (Marais and Botes, 2009). In addition to progress in agronomy, historical wheat improvement methods discussed by Reynolds *et al.* (2009) have been underpinned by the main elements of the 'international wheat improvement system' undertaken by CIMMYT. These elements have included: [1] 'shuttle breeding' at two

geographically contrasting locations in Mexico; [2] wide adaptation; [3] durable rust and *Septoria* resistance; [4] international multi-location testing and; [4] the deployment of genetic variation. (Poehlman and Sleper, 1995; Smale *et al*., 2001; Heisey *et al*., 2002; Ivanov and Dymshits, 2006; Ortiz *et al*., 2007; Pelletier and Budar, 2007)

Breeders need genetic variation sources as well as tools for its manipulation and validation. Haploid production finds application in developmental and genetic research as well as biotechnology and plant breeding. The main advantage of DH technology for plant breeders is the rapid advancement of selected heterozygous (usually F_1 or F_2) material to complete homozygosity in a single generation (Wu, 1986; Snape, 1989), versus what is possible with selfing or back-crossing. In addition, the process yields homozygous (true-breeding) progeny in which the number of possible gene combinations for traits of interest is reduced to more manageable proportions (Konzak *et al*., 1987), i.e. a smaller population size is required (Pienaar *et al*., 1997). Low efficiency is arguably still the greatest challenge to DH technology (Liu *et al*., 2002; Guzy-Wrobelska and Szarejko, 2003; Forster *et al*., 2007). Two routes to wheat DHs have emerged as commercially viable options, namely: [1] androgenesis via anther- (Barnabas *et al*., 2001) or alternatively, isolated microspore -culture (Zheng *et al*., 2001) and; [2] wide crossing using maize as the pollen donor (Suenaga, 1994). The steps in haploid production, namely: emasculation; pollination and; embryo culture (rescue), are also already familiar to breeders. According to Pienaar *et al.* (1997), most South African wheat genotypes respond poorly to the maize wide-crossing system. Pienaar *et al.* (1997) developed a wide-cross protocol at SU-PBL after evaluating 250 wheat 'haploidizer' (WH) media i.e. differing hormone treatments, as well as 50 embryo rescue media (ERM). In this study, the ERM was the same MS (Murashige-Skoog) barley anther culture medium of Olsen (1987) and modified by Daniel (1990) and Pienaar *et al.* (1997) but containing only 10% ammonium nitrate (NH_4NO_3). In wheat X maize crosses, normal fertilization occurs in most instances to produce a hybrid diploid (2n) zygote and endosperm. However the imbalance between the maternal and paternal genomes impedes endosperm development (Lin, 1984; Haig and Westoby, 1991) thus inhibiting embryonic growth (Zhang *et al*., 1996). Chromosomes from the pollen donor are eliminated from both zygote and endosperm during the first cell divisions (Zhang *et al*., 1996). The embryo therefore returns to the haploid (n) state (Mochida *et al*., 2004), but the endosperm usually aborts early thus necessitating *in vitro* embryo rescue in the absence of a normal endosperm (Zhang *et al*., 1996). (Bakos *et al*., 2005; Forster *et al*., 2007)

2.5.1 Improvement of wheat for bio-ethanol production

Until recently, little published data has been available concerning bio-ethanol production from wheat and other small grains. Consequentially, parameters influencing bio-ethanol yield are still generally poorly defined as they do not have the long breeding history associated with wheat developed for the food industry (Sarath *et al*., 2008). Kucerova (2007) lists the most significant quality parameters influencing bio-ethanol production from grain. Quality parameters for alcohol

processing from wheat are also reviewed in the Home-grown Cereals Authority (HGCA) Green Grain project, i.e. Research Review 61 (Smith *et al.*, 2006) (Figure 4). In addition, ethanol processing factors relative to sorghum have been identified by Wu *et al.* (2007). Work to identify the most important parameters is ongoing and non-starch polysaccharides (NSPs) are strongly implicated. NSPs displace grain starch in the endosperm and increase its viscosity (syn: *gelatinization*) during processing (Smith *et al.*, 2006; Kucerova, 2007). In conclusion and from a United Kingdom perspective at least, there has been too little time for wheat breeding to adapt to a biofuel market, despite scope for progress (Sylvester-Bradley and Kindred, 2008).

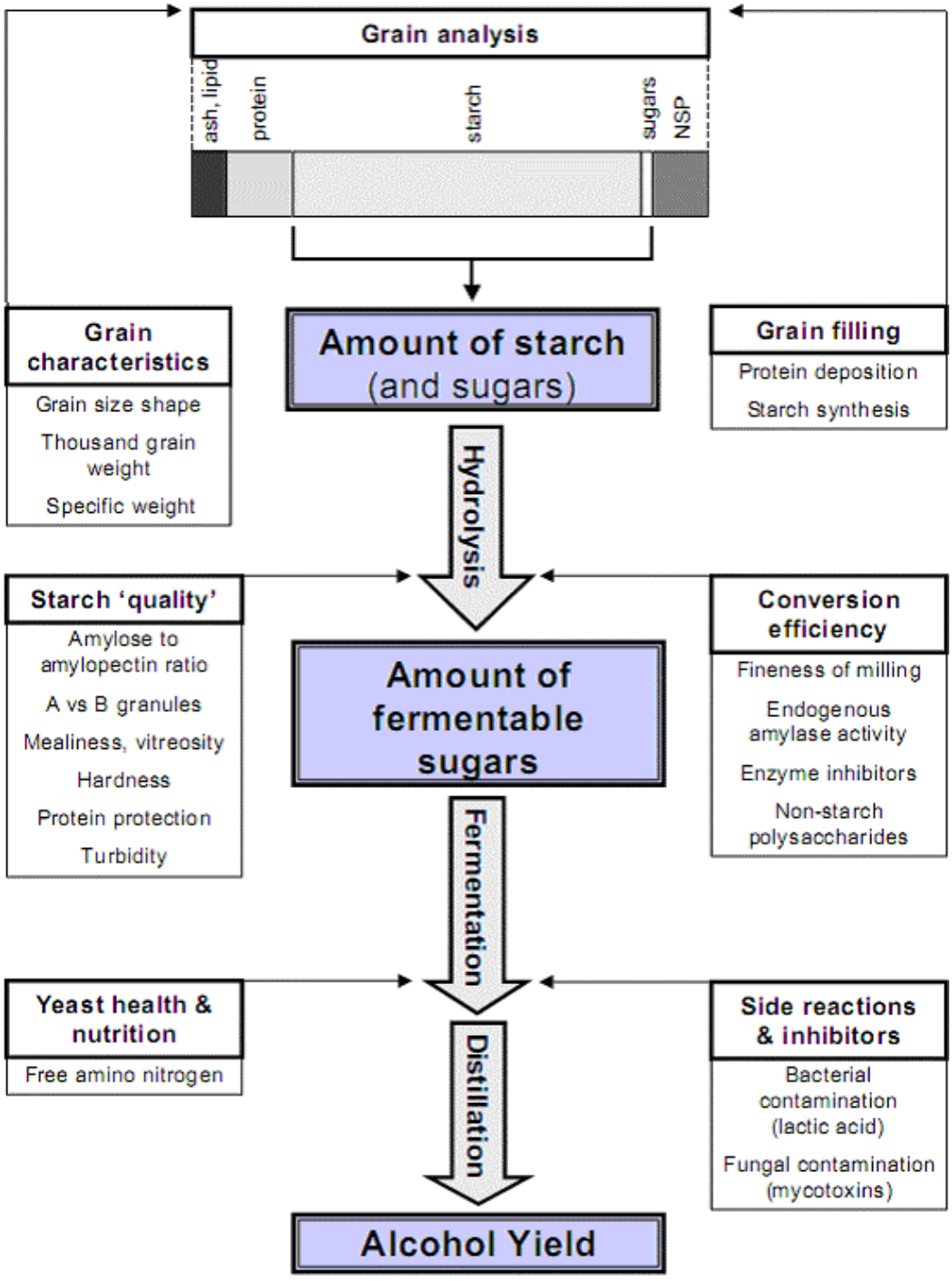

Figure 4. **Illustration of quality parameters and influences on ethanol yield** (Smith *et al.*, 2006).

As discussed by Manley *et al.* (2009), besides the obvious influence of grain yield on ethanol production, hectoliter-mass (HLM, syn.: *specific-, bushel-, test- or, hectolitre-weight*) measures grain bulk density (random-packing efficiency) as weight per unit volume (Donelson *et al.*, 2002). This is a crude measure of grain plumpness/soundness (Davies and Gooding, 1997) and thus flour yield (Dexter *et al.*, 1987). HLM is defined as the mass of a standard/specific volume of grain where 1hl = 100l. Flour yield coupled with other factors, influences bio-ethanol production from a ton of wheat where ethanol is derived from grain alone. Factors decreasing HLM and thus increasing proportional grain bran fraction include deformed, shriveled, damaged or weathered kernels as well as foreign material and high moisture content (above 12%) (Heyne, 1987). Transport and storage costs are also influenced by HLM. In addition, its relationship with moisture content is genotype-specific according to McLean (1987) and low HLM values do not necessarily equate with low flour yield (Heyne, 1987). Current wheat grading relies heavily on HLM as an indication of grain quality (Du Pisani, 2009) and thus realized price per ton, unless other factors such as protein fraction in Australia and South Africa for example (Manley *et al.*, 2009), falling number, weather or insect damage further negate the allocated grade. In South Africa, HLM is determined using a chondrometer (Davies and Gooding, 1997). The expected range of HLM values for sound wheat is 70-85kg.hl^{-1} (Troccoli and Di Fonzo, 1999).

Total starch content is a key parameter in bio-ethanol production (Smith *et al.*, 2006) and is well researched in terms of increasing its fraction in grain. Starch occurs as crystalline granules bound to protein in the grain endosperm. These granules have a hydrophobic core with pores extending to the granule surface (Nichols *et al.*, 2008). The insoluble starch biopolymer is comprised of two D-glucose -based polymers namely, amylose and amylopectin (Power, 2003; Pongsawatmanit *et al.*, 2007). The glucose units of amylose are linearly linked by α-1-4 bonds, while amylopectin is more branched with about 5% of its glucose units being linked by α-1-6 bonds causing branching. Plant biomass comprises 35-50% cellulose, 20-35% hemicellulose and 10-25% lignin (Mannan, 2010). Cellulose is a linear polymer of glucose whilst hemicelllulose is a branched polymer of xylose, a five-carbon sugar. Lignin is an aromatic polymer. Hartmann and Jacobi (2005) as well as Dahlberg (2007) illustrated that high ethanol yield is not necessarily strongly positively correlated with high total starch, but is an important starting point toward maximizing realized ethanol yield. Rosenberger (2005) and Labuschagne *et al.* (2007) also demonstrated that increased starch content is variably negatively correlated with protein content in terms of correlation strength (R^2). Realized ethanol yield is influenced by starch bio-availability which varies between genotypes (Moorthy, 2002).

The methodology for assaying total starch in grains as reviewed by Anon. (1987) and Smith *et al.* (2006) can broadly be categorized into acid hydrolysis as well as enzyme-based procedures. Both of these constitute a biochemical (syn.: *wet chemistry*) approach where the hydrolysis end-product (glucose) is quantified either polarimetrically or spectroscopically (syn: *colourimetric* approach). Alternative glucose determination methods also exist. Acid hydrolysis only

applies to pure starch samples while enzyme-based procedures differ from each other with respect to sample pre-treatment (Karkalis, 1985); starch solubilization, liquefaction and dextrin hydrolysis as well as glucose determination. (Megazyme International Ireland Limited ™, 2006b; Smith *et al.*, 2006; Huang *et al.*, 2008)

Every organic compound has its own unique spectral 'fingerprint' as determined by its molecular composition which can be revealed by NIRS. This approach to starch quantification allows for rapid, non-destructive (without milling) and cost-effective sample assaying. The amount of near-infrared light absorbed (i.e. not reflected or transmitted) by a sample is proportional to the amount of a particular component therein and illumination (scanning) at different wavelengths allows those components to be quantified (Smith *et al.*, 2006). However, a calibration using an already-quantified approach as a reference has to be established first as the relationship between the amount of light reflected (or transmitted) and the amount of a particular substance is not predictable (Smith *et al.*, 2006). (Huang *et al.*, 2008; Jirsa *et al.*, 2008; Jacobs, 2009)

The ratio of the starch components amylose and amylopectin also influences ethanol yield (Smith *et al.*, 2006). The amylose/amylopectin (A/A) ratio influences the physical and physico-chemical characteristics of starch including its gelatinization and recrystallization (Shelton and Lee, 2000; Tester *et al.*, 2004; Sarath *et al.*, 2008). Loaf volume is also influenced by amylose content (Lee *et al.*, 2001). Wild-type wheat starch contains about 75% amylopectin and 25% amylose (Sarath *et al.*, 2008). Amylose-free wheats are termed 'waxy' and research has shown that these are more efficient substrates in ethanol production, where the flour has a greater water-binding capacity and lower temperatures (energy inputs) are required to gelatinize waxy wheat (Wu *et al.*, 2006; Dowell *et al.*, 2009). According to Smith *et al.* (2006), high-amylose starch will not gelatinize below 100°C versus 60-70°C for standard starches. Bean *et al.* (2006) concluded that a lower amylose fraction is positively correlated with ethanol conversion efficiency in maize, especially where the starting amylose content was more than 35%. In conclusion, lower input energy requirements and quicker fermentations could benefit processing economics (Sarath *et al.*, 2008).

The amylose fraction of cereals is usually determined using a potentiometric (Bates *et al.*, 1943), amperometric (Williams *et al.*, 1958) or alternatively, colourimetric measurement of the iodine binding capacity of amylose (Matheson, 1971; Morrison and Laignet, 1983; Knutson, 1986; Chrastil, 1987; ISO, 1987). However, these three approaches are subject to experimental error in terms of repeatability as well as other problems described by Gibson *et al.* (1996). In addition, most alternative methodologies are highly laborious and have their own advantages and limitations. Using starches derived from several sources and with varying amylose contents i.e. amylose standards, Zhu *et al.* (2008) evaluated differing amylose-determination methodologies including that used in this investigation. To overcome the difficulties associated with the methodologies mentioned above, the lectin concanavalin-A (Con-A) forms a chemical complex with amylopectin, precipitating it out of solution (Matheson and Welsh, 1988; Yun and Matheson, 1990) and leaving amylose behind as a solute. The Con-A lectin complexes with branched polysaccharides

containing α-D-glucopyranosyl or alternatively, α-D-mannopyranosyl carbohydrate units at non-reducing end-groups under specifically defined biochemical (solution) conditions. In this way, amylopectin is precipitated out of solution leaving behind the linear amylose polymer (Megazyme International Ireland Limited ™, 2006a). This approach is applicable to all pure starches including cereal flours (Megazyme International Ireland Limited ™, 2006a). Although NIRS has not yet found wide application in the determination of the A/A ratio, Dowell *et al.* (2009) discusses this approach in terms of its ability to discriminate waxy starch from partially waxy (wild-type) wheat kernels.

Greater endogenous α-amylase activity, a viscosity index relating to polymer (starch) digestion, in the form of lower 'falling-numbers' (FN) is also desirable (Smith *et al.*, 2006). The Hagberg-FN assesses the α-amylase activity of grain and is in fact an indirect measure of gelatinized starch (Vaidyanathan, 1987).

In order to reach an almost total saccharification of starch to FS, two main groups of amylolytic enzymes (amylases) are required. Wheat and other cereals have their own endogenous amylolytic enzyme system. The so-called auto-amylolytic quotient (AAQ) is used to describe the entire endogenous auto-amylolytic hydrolytic enzyme system. This is determined by carrying out two fermentation tests on the same raw sample. The first includes the addition of technical enzymes, i.e. commercial amylases (+E) to determine the maximum ethanol yield obtainable, while the second is carried out without (-E) the addition of such enzymes (syn.: *malt*), i.e. ethanol yield obtained under AAQ conditions alone. The AAQ is defined as the ratio of ethanol yield (-E) to ethanol yield (+E). Under -E conditions, a high AAQ is required to be economically feasible (Ande *et al.*, 1998). The contribution of AAQ to realized ethanol yield is also influenced by genotypic and environmental effects. The contribution AAQ to ethanol yield is considered negligible in wheat and besides, technical enzymes are almost always added in industrial processes anyway (Smith *et al.*, 2006). AAQ is assessed using denaturing high performance liquid chromatography (dHPLC) preceded by fermentation to measure ethanol yield as described above. (Senn and Pieper, 2000; Miedl *et al.*, 2007)

Total FS in grain is usually defined as the sum of the glucose, maltose, maltotriose (a three-part glucose sugar) and, fructose residual sugar contents of the feedstock and can also be determined by dHPLC. A high starting value is desirable to maximize ethanol yield as during processing, starch is eventually completely hydrolyzed to simple-sugar substrates which are then fermented to ethanol (Smith *et al.*, 2006).

Higher grain (or straw in second-generation technology) moisture content is associated with lower ethanol yields. Moisture content is also inversely related to starch and protein content and too much is more common than too little. In addition to storage-related diseases and pre-sprouting in the field, grain respiration increases with increasing moisture content, leading to dry matter loss. Moisture content also influences the stability of biological and physico-chemical systems such as their susceptibility to chemical, enzymatic and microbial activity. Methodology to determine moisture content aims to exploit these physical or chemical system properties and

choice is dependent on a variety of factors including sample type. Moisture content can be determined by a number of methods. Samples may be weighed before and after drying. This approach is simple to conduct, has a low capital requirement and short analysis time and, is well established in industry. However, this method does not distinguish the loss of sample mass during drying caused by the vapourization of water versus that caused by the loss of other volatile compounds such as oils, alcohols and organic solvents. Also, a too-high drying temperature may result in sample solids decomposition which may also be confounded with moisture loss (Ohaus Corporation, n.d.). Other less time consuming methods are also available including NIRS (Williams, 1975; Smith *et al.*, 2006) and nuclear magnetic resonance (NMR) (Stenning and Channa, 1987). (Smith *et al.*, 2006)

It has been demonstrated that high crude protein (CP) content in cereals is negatively correlated with ethanol yield (Aufhammer *et al.*, 1996; Smith *et al.*, 2006). It is also known that CP is negatively correlated with total starch fraction as mentioned (Labuschagne *et al.*, 2007). However and in consideration of DDGS, CP still needs to be held at optimum levels. Assaying the nitrogen content of grains gives a rough indication of CP, as gluten is a nitrogenous compound. Besides NIRS (Osborne and Fearn, 1983; Smith *et al.*, 2006), two methods are commonly used for determining protein in wheat grain namely, the Kjeldahl method and Dumas method. Both of these measure grain CP (Smith *et al.*, 2006). In the Kjeldahl method, the sample is first digested with acid and the nitrogen converted to nitrate which is subsequently distilled and titrated.

Other quality parameters significant to bio-ethanol production include: starch gelatinization; saccharification temperature (Kucerova, 2007); flour fineness; endosperm hardness and; a desired low level of mycotoxins which otherwise might interfere with fermentation as well as DDGS quality (Smith *et al.*, 2006).

Sylvester-Bradley and Kindred (2008) are of the opinion that the focus of research as applied to food crops used as biofuel feedstocks, should be in areas exclusive of transgenic approaches due to both the cost of such technology and time considerations. According to the authors, this is because of such crop's already-established market value as a food source against a backdrop of emerging second-generation technology, as food crops are not optimized for the purposes of energy production (Heaton *et al.*, 2008; Sarath *et al.*, 2008). Fortunately though, these methodologies still include some exciting prospects for increasing plant starch fraction and its ability to be processed. Despite the emergence of second-generation technologies, the need for improved understanding of both starch and sugar metabolism will likely increase, as will knowledge of the energy balance in using cellulose (straw) versus starch (grain) as a feedstock. Very little is known about the relationships between carbon assimilation, its storage (carbon-sinks) and plant growth, notwithstanding insight gained from research on *Arabidopsis thaliana* (Smith and Stitt, 2007). Sucrose accumulation is even less well understood, but research on sugarcane suggests

that the total sugar fraction can be increased by sucrose conversion into a non-metabolizable isomer. (Smith, 2008; Sylvester-Bradley and Kindred, 2008)

To illustrate the total nett maximum theoretical energy conversion efficiency, Heaton *et al.* (2008) demonstrated that the theoretical conversion efficiency of whole spectrum solar energy into biomass is 4.6-6% dependent on plant type and, that the best year-long efficiencies are approximately 3%. According to this study, the best photo-voltaic (PV) solar cells are about as effective as the average leaf although in photosynthesis, most lost energy dissipated in the form of heat during the synthesis of biomass supports the construction and maintenance of the whole system. However and according to Shewry (2009), of greater significance to plant breeders and any grain-utilizing industry, is the year-to-year variability in growth conditions, which are predicted to increase in future due to global warming (Porter and Semenov, 2005).

Using existing knowledge, a specification (quality) standard for wheat intended as a biofuel feedstock has been recommended for the United Kingdom, inclusive of its agronomic production and industrial processing (Smith *et al.*, 2006). The underlying principles are similar to those applicable to the feed or distilling (potable alcohol) markets, yet there are clear differences arising from environmental costs and the less exacting protocols for the production of fuel alcohol. The development of accreditation standards is a high priority, considering that the British government intends that biofuel feedstock rewards should relate to GHG savings post-2010. For this purpose, the HGCA developed a GHG Calculator (Woods *et al.*, 2005). Meeting accreditation standards ought to be realistically attainable for producers, but 'ideal' accreditation needs to validate grain and straw yield, grain drying, nitrogen-use and starch fraction (potential alcohol yield) at various stages of each grain lot, from harvest to processing. However, grain is normally mixed and remixed at most stages of this process. NIRS can be employed to predict alcohol yield (Uthayakumaran *et al.*, 2005). It is known that low-yielding wheat concentrates its nitrogen reserves in the grain. Therefore financially rewarding low grain-nitrogen will by implication, encourage yield maximization and simultaneous GHG savings. Feedstock production cost and ethanol yield are both dependent upon the previous rotational crop, soil, and the environment (Rosenberger *et al.*, 2002; Nichols and Bothast, 2008). (Smith *et al.*, 2008; Sylvester-Bradley and Kindred, 2008)

In competition to wheat as a potential feedstock, triticale is currently mostly used for animal feed and has a higher grain yield, specific weight, heat and moisture stress tolerance, low soil pH tolerance and, lower pest and disease susceptibility (Oettler, 2005), as well as nitrogen requirement (Aufhammer *et al.*, 1996). In addition, triticale has greater endogenous amylolytic activity i.e. specifically α-amylase (Apar and Özbek, 2004; Mojović, 2006; Vucurovic and Pejin, 2007; Pejin *et al.*, 2009), thus potentially reducing the consumption of technical enzymes by up to 50% (Šimůnek, 1996) under the currently prevalent 'cold' processing technique (Vucurovic and Pejin, 2007; Pejin *et al.*, 2009). Davis-Knight and Weightman (2008) compared 13 winter triticale cultivars with a good distilling winter wheat cultivar at the same protein levels in the United

Kingdom in terms of both alcohol yield and GHG savings, using the HGCA biofuels GHG Calculator introduced in the preceding paragraph. In this study, comparable ethanol yields were obtained and better GHG savings were calculated for triticale, in large part due to the crop's lower agronomic nitrogen requirements. Regarding the concept of what may be considered the ideal biofuel, Sylvester-Bradley and Kindred (2008) summarize the most desirable feedstock characteristics as being: [1] high-yielding, thus reducing land requirements; [2] having a low nitrogen fertilizer requirement; [3] minimal cultivation to establish the crop; [4] does not need post-harvest drying; [5] large straw yield, either sequestering carbon back into the soil or being burned as fuel; [6] high FS, starch and sugar fractions, while conversely having low protein, NSPs, oil and ash and finally; [7] is easy to accredit with respect to as many of its key attributes as possible.

2.5.2 Examples of wheat improvement for bio-ethanol production

The United Kingdom HGCA Green Grain project explored the possibility of breeding wheat with low nitrogen requirements and maximum alcohol processing potential, focusing on nitrogen utilization rather than capture. The use of biofuel-directed wheat in the European Union when future prices will be governed by GHG savings through feedstock accreditation (Defra, 2007) will depend on cultivars yielding well with low nitrogen inputs. According to Sylvester-Bradley and Kindred (2008), nitrogen use efficiency has yet to be sufficiently addressed by breeders. There are two factors in efficient nitrogen use (nitrogen response) namely, its capture (nitrogen uptake in kg.soil-available-nitrogen^{-1}) and utilization (grain formed in kg.nitrogen-uptake^{-1}). In terms of capture, the average fertilizer nitrogen recovery by wheat is about 60% (Bloom *et al.*, 1988). This has historically been improved by yield-directed breeding (Foulkes *et al.*, 1998). Two recent innovations may further improve this trait. The first is introgressing the capacity to produce root exudates inhibiting the conversion of soil ammonium to nitrate (Subbarao *et al.*, 2007). Secondly, is the up-regulation of alanine-aminotransferase (Good *et al.*, 2004; Lea and Azevedo, 2007), already achieved in oilseed rape where fertilizer nitrogen requirements were halved (Good *et al.*, 2007). Citing three independent trials, Sylvester-Bradley and Kindred (2008) highlighted the following consistencies in wheat nitrogen use efficiency, namely: [1] that there is no significant increase in yield without applied nitrogen either in grain yield or nitrogen off-take (kg.ha^{-1}) (Figure 5); [2] breeding has not improved soil-derived nitrogen capture versus the capture of applied nitrogen held in the topsoil; [3] the yield-justified increase in nitrogen requirement is less than if there had been no improvement in nitrogen capture at all and; [4] breeding has not significantly improved nitrogen utilization. Sylvester-Bradley and Kindred (2008) also point out that unlike testing cultivars with no fungicides or plant growth regulators (PGRs), low nitrogen testing is more urgent because the traits influencing nitrogen response are less obvious. Despite little variation (about 2% d.w.b.) in grain CP in bread and feed wheat cultivars in the United Kingdom grown under the same conditions including nitrogen application (Snape *et al.*, 1993) with about 67% environmental control over CP (Vogel *et al.*, 1978), gliadins constitute approximately 40% of grain protein. Gliadins are also the most responsive to nitrogen supply (Kindred *et al.*, 2008). A low gliadin fraction does not

reduce seed germination but does enhance alcohol yield. Sylvester-Bradley and Kindred (2008) further point out that sufficient variation exists in United Kingdom distilling wheats for direct gliadin selection.

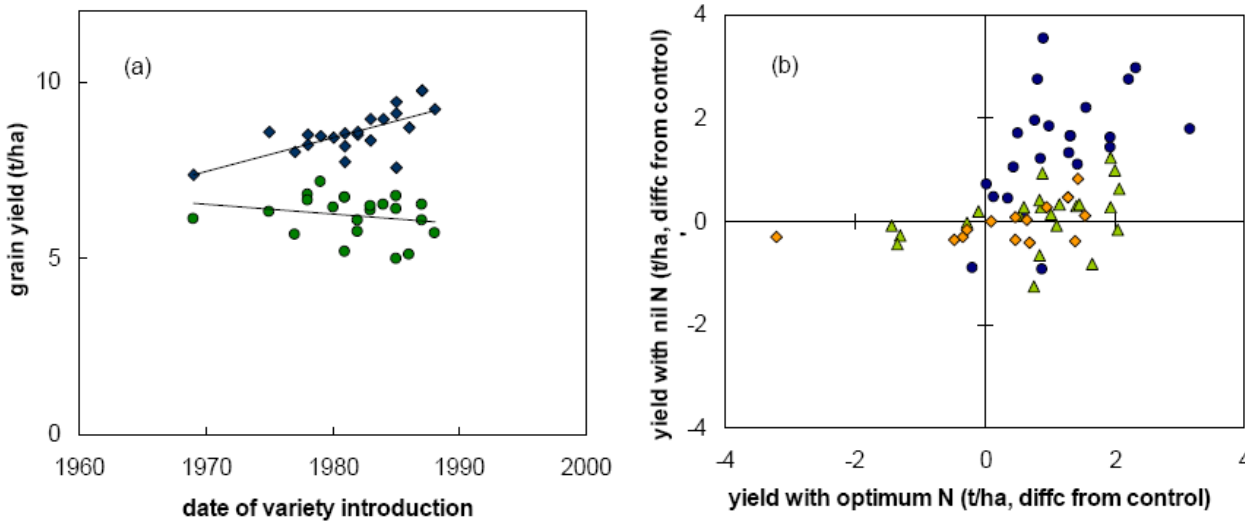

Figure 5. **(a) For 22 varieties introduced from 1969-1988, trends in grain yield with optimum nitrogen (diamonds) and nil nitrogen (circles) *Data from Foulkes et al. (1998*). (b) For 24 comparisons of 2 old (1st on RL 1977-1987) and 2 new (1999-2005) varieties conducted in 2005-2007, yield differences with optimum nitrogen and with nil nitrogen. *Data source: HGCA Project 3084*** (Sylvester-Bradley and Kindred, 2008).

Following improved knowledge of starch biosynthesis (James *et al.*, 2003), starch can now be synthesized for specific end-uses (Jobling, 2005; Morell and Myers, 2005). Efforts to increase total starch fraction by manipulating ADP-glucose pyrophosphorylase for example, have only been marginally successful. More successful however, has been the genetic manipulation of specific starch synthases, increasing adenosine triphosphate (ATP) availability for starch synthesis and, the down-regulation of plastidial adenylate kinase as well as starch-degrading enzymes (Perry *et al.*, 2003). The introduction of starch synthesis into the cell cytosol is also a possibility. Selection for high-amylose fraction is more difficult in 6n (hexaploid) wheat in contrast to diploids, as it may be necessary to induce mutations within the whole homoeologous chromosome group (ABD) to gain a significant phenotypic effect (Yamamori *et al.*, 2000). Nakamura *et al.* (1995) developed wheat lines with non-functional granule-bound starch synthase (GBSS) and thus zero amylose fraction. A further example of starch manipulation is the application of RNAi technology to down-regulate starch-branching enzyme IIa (Regina *et al.*, 2006), resulting in lines with up to 80% amylose. (Torney *et al.*, 2007; Sarath *et al.*, 2008; Smith, 2008; Sylvester-Bradley and Kindred, 2008; Shewry, 2009)

Using current examples from differing feedstocks across the processing technology spectrum from first to fourth -generation ethanol production technologies, Mannan (2010) summarizes the biofuel-related intellectual property (IP) landscape, inclusive of feedstock improvement. During the period 2001-2007, 2796 biofuel-related patents were published in the

United States alone (Kamis and Mandar, 2008). The organization Public Intellectual Property Resource for Agriculture (PIPRA), comprising 45 institutional members across 14 countries, is currently mapping the biofuel technology IP landscape including germplasm development. Considering second-generation technology and beyond, there are two important influencing factors namely, the maximization of total biomass per unit land area (yield maximization) and, the ease at which feedstock can be processed. However, currently more is known about cellulose biosynthesis (Cosgrove, 2005) versus its deconstruction outside of the cell using either biological or chemical methods.

Illustrating biofuel-directed breeding outputs, maize hybrids have been developed specifically for biofuel processing and yield 2-5% more ethanol than non-specific hybrids (Bothast and Schlicher, 2005). High extractable starch (HES) and high total fermentables (HTF) hybrids have been developed for the wet- and dry-milling processes respectively, parameters which can both be measured using NIRS (Bothast and Schlicher, 2005). Other hybrids have been bred with an increased oil fraction as well as high free lysine for introduction into a modified dry-mill ethanol production process (Jessen, 2006). Further hybrids with inbuilt post-harvest starch-hydrolyzing ability were also being developed in 2004 (Craig *et al.*, 2004). (Torney *et al.*, 2007; Nichols and Bothast, 2008)

Chapter 3: Materials and methods

In order to achieve the aim of this investigation in evaluating the amenability of spring wheat to ethanol production in the Western Cape and making ethanol-improved ABLs available to industry, the following three objectives were pursued (Figure 6). Rain-fed advanced material MLFTs were conducted over the Western Cape commercial wheat production region to relate ethanol yield to differing production environments. These included commercial cultivars as controls that were part of the National Cultivar Evaluation Trials at the time. The second objective was to optimize the analytical protocols applied to the grain obtained from the MLFTs in determining ethanol-related traits. This included indirect starch-related as well as direct (fermentation) assaying of important parameters. Thirdly, a pre-breeding programme was initiated using the ethanol-directed MLFT material as parents together with material from the university's MS-MARS programme (Marais and Botes, 2009). Plants were selected from the bulked population for use as male (homozygous) parents (Figure 6). This was done on the basis of MLFT data for: [1] grain yield; [2] agrotype; [3] field (APR) rust resistance together with MAS (before and after bulking) and; [4] direct as well as indirect assaying of important parameters. Female (segregating) parents (Figure 6) were selected from the MS-MARS population containing the dominant male sterility (*Ms3*) gene, based on seedling rust-screening. These parents were randomly crossed and the wide-cross DH method employing maize as pollen donor was applied. The resulting homozygous progeny were the ethanol-directed improved lines incorporating available rust resistance genes set out in the aim of this project.

As part of optimizing analytical protocols applied to the male parents, it was also intended to establish a calibration (statistical prediction model) for total starch fraction, particularly for application to whole grain. Using NIRS data, a calibration was developed by Prof. Martin Kidd at the SU Centre for Statistical Consultation (CSC) using the 'R' statistical programming language. Partial least squares regression (PLS-R) using cross validation was used to determine the optimum number of principle components (PCs), i.e. the dimensionality of the prediction model.

To analyze MLFT data, Agrobase Generation II ™ (Agronomix, version 13.8.1) software was used with further data representation using Microsoft Excel™. Nearest neighbour analysis (NNA) was conducted on individual localities to remove any field trends in the ranking of entries. Genotype X environment (GxE) analysis of variance (ANOVA) was used to report the multiplicative (interaction) effects between locality and entry. Additive main effects and multiplicative interaction (AMMI; Gauch, 1988; Gauch and Zobel, 1988) as well as rank differences (Nassar and Huehn., 1987) were also employed as nonparametric (distribution-free) approaches to yield stability determination. Error was thus controlled by the application of NNA and AMMI, as they are both applied to orthogonal sources of variation i.e. error, genotypic and interaction variance (Crossa *et al.*, 1990).

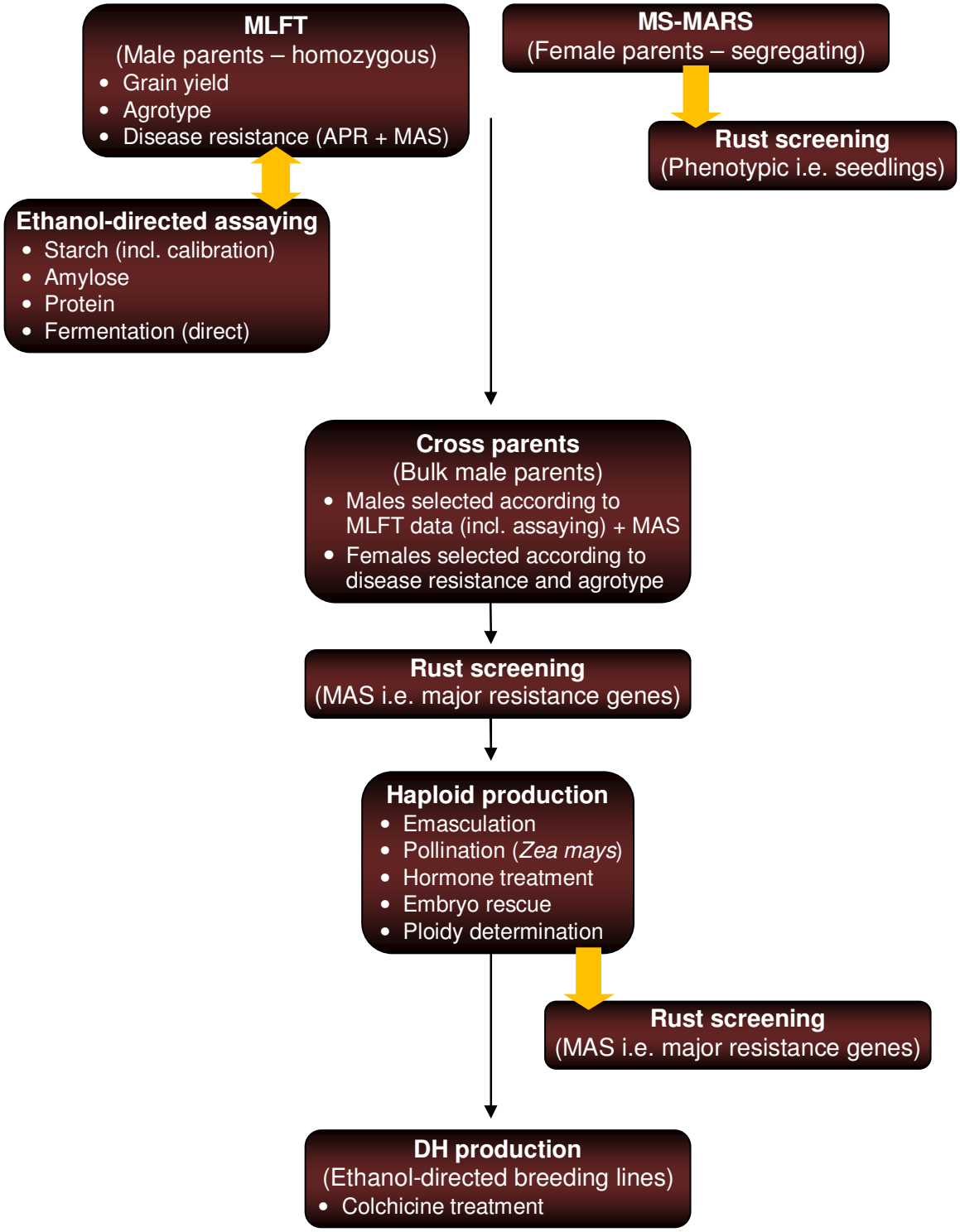

Figure 6. Conceptual outline of methodologies applied to this investigation.

3.1 Multi-location Field Trials

3.1.1 Planning, planting and harvesting

Advanced MLFT material was available from five localities (trial sites) across the Province during the 2006 season, namely: Langgewens; Roodebloem; Tygerhoek; Vredenburg and; Welgevallen (Figure 7). This season's material was primarily used for the optimization of analytical

Figure 7. **Western Cape Province depicting the ten SU-PBL trial sites across this commercial wheat growing region: See in association with Table 11 for the two component regions** (Both Welgevallen and Mariendahl are situated at Stellenbosch).

protocols that were to be applied to the 2007 harvest. A core of 16 entries common to all five localities were identified (Table 1). Material from Napier had to be foregone and there were not necessarily four replications of each entry from each site as originally planted. Thus the total number of experimental units for 2006 was 213. The 2006 MLFT design included four controls of which three plus four ABLs overlapped with the 2007 season (Table 1). For the 2007 season and for better environmental representation, 10 localities were chosen in the following three regions of the Western Cape Province: [1] Boland, with one location at Welgevallen (GPS coordinates: S33 50.836 E18 50.232); [2] Swartland, with four localities at Klipheuwel (S33 41.910 E18 42.060), Langgewens (S33 16.37 E18 42.575), Piketberg (S32 48.954 E18 50.984) and Vredenburg (S32 56.458 E17 56.066) and; [3] Southwestern Cape including the Rûens, with five localities at Albertinia (S34 12.289 E21 35.113), Napier (S34 28.311 E19 54.319), Riversdal (S34 05.715 E21 15.283), Roodebloem (S34 14.305 E19 25.778) and, Tygerhoek (S34 08.975 E19 54.871) (Figure 7). Each site included three replications of 20 entries each (Table 1) planted in a randomized complete block design (RCBD) for a total of 600 experimental units. Ten included controls were sourced from cultivars developed by Sensako, Pannar and the Agricultural Research Council (ARC). Of the remaining 10 entries, nine comprised ABLs whist ‘12th HRWYT 40-4’, was sourced

from CIMMYT as part of their high-rainfall wheat yield trials (HRWYT) in the region and selected from that nursery based on above average yield.

Table 1. Summary information for the entries involved in the 2006 and 2007 MLFT elite wheat trials.

Year	Count	Entry #	Genotype	Source	Intended use
2006 elite	1	1	**SST57**	Sensako	Bread flour
	2	3	**SST88**	Sensako	Bread flour
	3	4	**SST015**	Sensako	Bread flour
	4	2	**SST65**	Sensako	Bread flour
	5	37	03H86-8-1	US-PBL	Undetermined
	6	38	03H86-8-2	US-PBL	Undetermined
	7	7	97K1-15-5	US-PBL	Bread flour
	8	9	01H9-5	US-PBL	Bread flour
	9	5	00K180-2	US-PBL	Bread flour
	10	6	97K1-4-8	US-PBL	Bread flour
	11	8	00H43D-1	US-PBL	Bread flour
	12	19	97K1-15-2	US-PBL	Bread flour
	13	22	97K1-15-4	US-PBL	Bread flour
	14	34	03H5-3-1	US-PBL	Bread flour
	15	35	03H5-3-2	US-PBL	Bread flour
	16	36	03H18-4	US-PBL	Bread flour
2007 elite	1	1	**SST 57**	Sensako	Bread flour
	2	2	**SST 88**	Sensako	Bread flour
	3	3	**SST 015**	Sensako	Bread flour
	4	4	**SST 027**	Sensako	Bread flour
	5	5	**Kariega**	ARC	Bread flour
	6	6	**Biedou**	ARC	Bread flour
	7	7	**Baviaans**	ARC	Bread flour
	8	8	**W03/21**	ARC	Bread flour
	9	9	**W04/01**	ARC	Bread flour
	10	10	**PAN 3492**	Pannar	Bread flour
	11	12	03H86-8-1	US-PBL	Undetermined
	12	13	03H86-8-2	US-PBL	Undetermined
	13	11	97K1-15-5	US-PBL	Bread flour
	14	18	01H9-5	US-PBL	Bread flour
	15	14	03H380-5	US-PBL	Undetermined
	16	15	97K1-4-2	US-PBL	Bread flour
	17	16	00K60-16-2	US-PBL	Bread flour
	18	17	00K60-16-2-2	US-PBL	Bread flour
	19	19	00K60-16-3-3	US-PBL	Bread flour
	20	20	12th HRWYT 40-4	CIMMYT	Undetermined

Bold type controls i.e. commercial cultivar's (2006 = 4; 2007 = 10)
controls i.e. commercial cultivar's common to both years (3)
breeding lines common to both years (4)
international germplasm (CIMMYT)

Planting of the 6m^2 plots (experimental units) was undertaken using an Oyord (Wintersteiger) seed drill at a density of 300 seeds.m^{-2}. The trial sites measured 0.18ha.

Fertilizer was applied at planting as well as 35-40 days afterwards. On both occasions, Turbo 31 and 46N were applied to all sites excluding Albertinia, Piketberg and Napier. Ureum,

mono-ammonium phosphate (MAP) and copper oxychloride (CuOCl) were applied pre-emergence to the three sites mentioned above, while Ureum and 46N/20P/40K were applied post-emergence. All fertilizer was applied by hand at rates according to individual site soil analysis.

Herbicides were applied both pre- and post-emergence. Application was undertaken by hand using a knapsack sprayer. Logran^{TM} (30g.ha^{-1}, active ingredient: triasulfuron) was applied for the selective pre-emergence control of broadleaf weeds and suppression of some grasses, as well as post-emergence broadleaf weed control. Cossack^{TM} (300g.ha^{-1}, sulfonylurea and safener) and Buctril DS ^{TM} (375ml.ha^{-1}, bromoxynil) were applied in conjunction with the surfactant Ballista^{TM} (500ml.ha^{-1}) and insecticide Mospilan^{TM} (50g.ha^{-1}, acetamiprid). Fungicides were not applied at all with the purpose of emulating commercial growing conditions in terms of disease pressure. The sites were regularly visited during the growing season and disease notes recorded.

After sufficient drying of the grain in the field and in accordance with prevailing weather conditions, harvesting commenced in late October and proceeded for about six weeks. Using a NurseryMaster Elite (Wintersteiger) field plot combine at Welgevallen for example, each plot was harvested individually.

3.1.2 Grain yield determination

Harvested seed was transported to dry storage conditions at SU-PBL Welgevallen. The bags were then weighed and thereafter, the seed was cleaned of debris and chaff using a Hub-o-Mat Type SC800 (K. Huber Engineering) gravity-sieve -type seed-cleaner. The mass of each replicated entry was recorded as the official grain yield value.

HLM was also determined for both the 2006 and 2007 material using a chondrometer (Davies and Gooding, 1997) while thousand-kernel weight (TKW) was determined for the 2007 season only, using a numerical seed counter (Tripette and Renaud). The bags were then re-sealed and stored in cool, dry conditions.

Samples were prepared for later quality parameter assaying over the coming months by weighing approximately 145g of whole grain from each replicated entry bag. The samples were placed in labeled airtight (screw-lid) transparent 375ml plastic jars. The jars were stored in a cool (about 23°C), dry and dark location.

3.1.3 Total starch determination

In this investigation, total starch was estimated by first using a NIRS approach applied to the whole grain samples. The samples were milled and subjected to enzyme-based colourimetric biochemical starch determination and then NIRS applied once more to the milled flour equivalents. NIRS was applied to all experimental units for both seasons, i.e. 213 and 600 for 2006 and 2007 respectively. Biochemical assaying was applied to the 16 identified core-entries of 2006 (Table 1) and replication two of 2007 for a total of 120 and 200 samples for each respective year. NIRS reflectance spectra were determined using a NIRLab N-200 (BÜCHI) NIR spectrophotometer.

Milling was undertaken using a Cyclotec 1093 (Tecator) sample-mill fitted with a 0.5mm screen. Milled samples were stored in the same manner and under the same conditions as whole grain.

A thermogravimetric approach was used to determine moisture content in which the sample mass was recorded before heating and again when the mass reaches a so-called 'steady state'. Moisture content was determined on the day of wet chemical starch assaying. An IR35 (Denver Instrument) moisture analyzer was used for this purpose, employing infrared radiation drying technology. The selected drying profile conditions were: a 3g flour sample; a target temperature of 100°C and; automatic heat shut-off. Drying time under the selected drying profile conditions applicable to wheat flour was approximately 5min 30s per sample.

The MEGAZYME (Megazyme International Ireland Limited ^{TM}) Total Starch Assay Procedure (K-TSTA, 05/06 – kit form) was the enzyme-based wet chemistry approach used to approximate total starch in this investigation. This method is based on the thermostable α-amylase- amyloglucosidase. (AA/AMG) enzyme-based procedure of McCleary *et al*. (1997) and, concurs with: [1] the American Association of Cereal Chemists (AACC) Method 76-13; [2] Association of Analytical Communities (AOAC) International, Method 996.11 and; [3] the International Association for Cereal Science and Technology (ICC) Standard Method No. 168. This procedure can be applied to most cereal products, both natural and processed.

The MEGAZYME Total Starch Controls Kit (K-TSCK, 08/02), was utilized to validate the above K-TSTA procedure. This was carried out prior to assaying the 2006 and 2007 material. Validation was performed using five starch standards as supplied in the kits contents. For the 2006 material, these standards were repeated twice together with four 100µl D-glucose (1.0mg.ml^{-1} in 0.2% w/v benzoic acid, utilized as supplied) standards. For the 2007 material, a four-times starch standard replication was used.

The calculation of total starch fraction was determined as set out in Box 1 below and can be found and verified in the instruction leaflet enclosed with the K-TSTA kit. Alternatively, these calculations can also be performed in Microsoft Excel^{TM} format using the downloadable Megazyme Mega-Calc^{TM} form available from the MEGAZYME website.

Box 1. Calculation of total starch fraction.

$$\text{Starch (\% w/w d.w.b.)} = \text{starch (\% w/w 'as is')} \times \frac{100}{100 - \text{moisture content (\% w/w)}}$$

$$\text{Starch (\% w/w 'as is')} = \Delta A \times F \times 1000 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} = \Delta A \times \frac{F}{W} \times 90 \text{ where:}$$

ΔA = absorbance (reaction) read against the reagent blank

W = mass of flour in mg ('as is')

1000 = volume correction (0.1ml aliquoted from 100ml)

$F = \frac{100}{\Delta A}$ (conversion from ΔA to µg)

$\frac{1}{1000}$ = conversion from µg to mg

$\frac{100}{W}$ = factor to express starch as a % of flour mass

$\frac{162}{180}$ = adjustment from free D– glucose to anhydro D– glucose

3.1.4 Amylose/amylopectin ratio determination

After bulking the same MLFT material used for starch assaying, the A/A ratio was approximated using the MEGAZYME Amylose/Amylopectin Assay Procedure (K-AMYL, 04/06 – kit form). The protocol is based upon a modification of the Con-A -based enzyme procedure developed by Yun and Matheson (1990) where, an ethanol pretreatment step removing lipids from the initially-dispersed starch solution is incorporated as the modification (Morrison and Laignelet, 1983). Following precipitation of the amylopectin fraction by the Con-A lectin, an aliquot of the amylose-containing supernatant was hydrolyzed to D-glucose by the addition of AMG as was done for total starch determination. This solution was then colourimetrically quantified using the same GOPOD D-glucose -reacting determination reagent. A separate aliquot of the acetate solution was also removed for total starch estimation, hydrolyzed to D-glucose and quantified in the same manner. The amylose fraction was then estimated as the ratio of GOPOD absorbance at λ=510nm of the Con-A -precipitated supernatant, to that of the simultaneously prepared total starch aliquot. (Megazyme International Ireland Limited ™, 2006a)

The above K-AMYL procedure was validated using the enclosed HAMS (64±1.5% w/w d.w.b.) amylose standard supplied with the kit. Validation was carried out prior to the assaying of both the 2006 and 2007 material. For both seasons, the standard was repeated three times together with two 100µl D-glucose (1.0mg.ml^{-1} in 0.2% (w/v) benzoic acid, utilized as supplied) standards.

The calculation of the A/A ratio was determined as set out in Box 2 below and can be found and verified in the instruction leaflet enclosed with the K-AMYL kit.

Box 2. Calculation of amylose/amylopectin ratio.

$$\text{Amylose (\% w/w)} = \frac{\Delta A_a}{\Delta A_b} \text{ x } 66.8 \left(\text{i.e.} \frac{6.15}{9.2} \text{x } 100\right) \text{ where:}$$

ΔA_a = amylose (Con–A) absorbance

ΔA_b = total starch absorbance

6.15 = dilution factor for the amylose (Con–A) extract

9.2 = dilution factor for the total starch extract

3.1.5 Total protein determination

The Micro-Kjeldahl method (AACC Method 46-13) was employed in this investigation, using a wheat-specific conversion factor of 5.87 (Jones, 1931; Draper and Stewart, 1979). This procedure determines total nitrogen content in very small samples of flour, and was performed by the Department of Animal Sciences at SU.

3.1.6 Ethanol determination

A simplified and scaled-down (volume) version of the cold processing SSF technique as employed in newer first-generation bio-ethanol plants was developed by SU-PBL. The procedure was based on that described by Senn and Pieper (2000). The RSH enzyme Stargen™ 002 (Genencor), a blend of endo-acting α- and exo-acting gluco- amylase enzymes which convert (hydrolyze) raw (granular) starch into dextrins and, hydrolyze those dextrins into sugars at only 30℃ in the presence of yeast during SSF, was used. Stargen™ 002 was specifically developed for the hydrolysis of starch from small grains in contrast to maize-specific Stargen™ 001. The addition of Stargen™ 002 also allows for the use of a low-pH citrate buffer (50mM; pH3.6), making the need for antibiotics to control contamination during fermentation obsolete. The α-amylase -active *Saccharomyces cerevisiae* yeast strain D5-α sourced from the Department of Microbiology at SU, was used as the fermentation agent. This strain has the desirable characteristics of high fermentation capacity, high ethanol yield, high ethanol tolerance and, generally regarded as safe (GRAS) status.

The procedure comprises the addition of citrate buffer to the milled wheat samples followed by the mash viscosity-reducing (technical) enzyme Optimash™ BG (Genencor), a β-glucanase/xylanase enzyme complex hydrolyzing cellulose and hemi-cellulose. The mash is then incubated for 2h in an oven at a moderate temperature, i.e. higher than that required for optimum RSH enzyme activity. This initiates the fermentation process and releases some of the sugar substrate for subsequent yeast activity. Once cooled to at least 30℃, the D5-α yeast and RSH enzyme (Stargen™ 002) are added. The samples are then incubated at a temperature optimum for RSH enzyme activity for 72h, and hence complete fermentation of the mash.

The described protocol was optimized and validated by SU-PBL in 2006 prior to its implementation in 2007. Influenced by commercial availability at the time, Stargen™ 001 was used and no Optimash™ BG was included. Optimization involved quantifying the rate of fermentation in terms of ethanol yield. FS and AAQ were also determined via dHPLC carried out by the Central Analytical Facility (CAF) at SU. The aim was to achieve a complete-as-possible hydrolysis of starch to glucose (FS) and subsequent fermentation of that glucose to ethanol in the shortest possible time period. The AAQ is also a measurement of ethanol yield potential, but was not directly taken into consideration in the optimization of the procedure. With respect to wheat at least, technical enzymes are added in industrial fermentation processes anyway.

S. cerevisiae yeast cultures were prepared in advance and placed in temporal storage at 4℃ until required for fermentation shortly thereafter. On the day that the yeast culture was to be used, sample assaying proceeded as set out hereon. Under the sterile conditions of a laminar-flow hood, a 20g flour sample from each experimental unit was weighed out directly (without using a weigh-boat) into labeled and sterile 250ml Schott bottles. Bottle caps were replaced and sealed immediately to avoid the introduction of contaminants, specifically wild yeast strains. Before closing, a magnetic stirrer surface-sterilized in 100% (v/v) ethanol was included with each flour sample.

Working within the laminar-flow hood, 100ml sodium citrate buffer was pipetted into each bottle in-turn using a 5-30ml EM (Hirshmann Laborgerate) bench dispenser. Immediately thereafter, 1ml of Optimash™ BG technical enzyme was added using a sterile 5ml syringe. The bottles were then recapped, sealed and shaken vigorously five times using the included magnetic stirrer as an agitator to homogenize the mixture (mash).

The samples were then incubated for 2h at 53℃ in a pre-warmed ProLab PL001 oven to initialize/activate the fermentation process (hydrolysis). The bottles were shaken briefly by hand at half-hour intervals. While incubating, replacement caps housing a rubber grommet for the later insertion of a S-bend stopper as used in the wine industry (Kinear and Associates), were surface-sterilized in a beaker containing 100% (v/v) ethanol and allowed to dry in the laminar-flow hood. Upon removal of the samples from the oven, the bottles were allowed to cool to 30℃ or less, as required for the next step. One of the 200ml bottles of earlier-prepared yeast culture was removed from storage (4℃) and the yeast cells gently re-suspended by shaking.

Working sequentially with each sample in-turn, ten millilitres of the *S. cerevisiae* D5-α yeast culture was added to the mash using a sterile 5ml syringe. Immediately following this, 1ml Stargen™ 002 RSH technical enzyme was added to each bottle using a separate syringe. Following these two additions, the caps were removed (discarded) and replaced with the (now dry) ethanol-sterilized caps housing the rubber grommet with an inserted S-bend stopper. Immediately prior to inserting the stopper through the grommet, the bottom end of the stopper i.e. that part to be accommodated inside the sample bottle, was also surface-sterilized in 100% (v/v) ethanol. After resealing the bottles with the S-bend stoppers in place, each stopper was filled with sdH_2O

followed by partial unscrewing of the bottle cap (about ½-turn) to allow the water level in the stopper to equilibrate. Following this, the bottle caps were sealed to ensure an air-tight fermentation system. Working with each batch in-turn, the mass of all samples was recorded, i.e. the mass of the whole fermentation system 'as is' with water-filled stoppers in place.

The samples were then transferred to the oven and positioned on a 15-place (VELP Scientifica) multi-stirrer for an incubation (fermentation) period of 72h at 30°C. At the end of this period, the mass of each fermentation system was recorded once more.

From the chemical equation for starch hydrolysis (Smith *et al.*, 2006), the reaction starting products (starch plus water) equal the intermediary (glucose) and end-products (ethanol and CO_2). From this equation and taking grain yield as well as starch percentage into account in the determination of theoretical yield, the calculation of ethanol proceeded as illustrated in Box 3 below.

Box 3. Calculation of theoretical and actual realized ethanol yields.

Theoretical value:

[1] $1\text{g starch} \times \frac{1.11\text{g Glc}}{1\text{g starch}} \times \frac{0.511\text{g EtOH}}{1\text{g Glc}} = 0.567\text{g EtOH}$ or,

[2] $1.11\text{g EP} \times 0.511 = 0.567\text{g EtOH}$ or,

[3] $1.11\text{g EP} \times \frac{92}{180} = 0.567\text{g EtOH}$ and,

$$1\text{g EtOH} \times \frac{1\text{L}}{0.789\text{kg}} = \frac{1.267\text{L EtOH}}{\text{g EtOH}} \times \frac{1}{1000} = \frac{\text{L EtOH}}{\text{t grain}} \times \frac{\text{t grain}}{\text{ha}} = \frac{\text{L EtOH}}{\text{ha}}$$ where:

$\text{EP} = \text{end product}$

$\frac{1\text{L}}{0.789\text{kg}} = \text{density of ethanol}$

$\frac{1}{1000} = \text{conversion factor to t}^{-1}$

In summary: $\text{g starch} \times 1.11 \times 0.511 \times 1.267 \times 0.001 = \text{l.t}^{-1} \times \text{t.ha}^{-1} = \text{l.ha}^{-1}$

Actual value (with l.ha^{-1} derived as above):

$\text{g EtOH} = \text{g sample} \times 1.11 - \text{g } CO_2$ where:

$\text{g } CO_2 = \text{g}_{\text{start}} - \text{g}_{\text{end}}$ (i.e. g CO_2 ~ mass of the fermentation system)

$1.11 =$ conversion factor to theoretical EPs (through glucose intermediary product)

3.2 Establishing a pre-breeding programme

3.2.1 Bulking the male parents

Using the MLFT-derived advanced wheat material of the 2006 and 2007 SU-PBL rust resistance nurseries and now evaluated for ethanol yield potential as described thus far, a bulked population was established at SU-PBL (Welgevallen) in 2008 (Figure 6). This population was established for the purpose of random crossing with the MS-MARS -derived female parents (Figure 6). The entries in these two nurseries totaled 100 and 204 from the 2006 and 2007 MLFT seasons respectively. Entry selection was based on grain yield, agrotype, rust resistance (field APR and MAS) as well as ethanol-directed screening. MAS for disease (rust) resistance was also conducted after bulking (Figure 6). The genetic constitution of the included material with respect to height was mostly *Rht-D1b* and to a lesser extent *Rht-B1b*. These were scored via genotypic molecular markers obtained from Ellis *et al.* (2002) and performed by SU-PBL.

Prior to establishment, soil was prepared by disking followed by a short fallow period in which weeds were allowed to develop. The weeds were then sprayed with Roundup^{TM} (active ingredient: glyphosphate). After irrigation (out-of-season summer planting), seeds were sown by hand in pre-drilled single 1m rows at a density of 3g per row with rows spaced 30cm apart. After planting, overhead irrigation was established as summers in the Western Cape are characteristically hot, dry and windy. Also after sowing, nitrogen was applied by hand following soil analysis. Just post-anthesis, individual rust 'spreader' plants were interspersed at every 25th row. These spreader plants comprised the rust-susceptible cultivar 'Marocco'.

3.2.2 Disease screening: MAS

Rust screening using molecular marker -based MAS was applied to the bulked male parent population (Figure 6) with reference to the genes in Table 2 below. MAS was also applied to the MLFT (Welgevallen) material prior to bulking as well as to later DH production (Figure 6). It was known that certain genes were already present in the bulked population as this advanced (MLFT) material was in fact already derived through the MS-MARS programme. Genes (and associated gene complexes) of interest to this investigation (Table 2) comprised: *Lr19; Lr24/Sr24; Lr37/Sr38/Yr17; Sr2; Sr26 and; Sr31/Lr26/Yr9*. The scoring of *Lr19, Sr2, Sr26* and *Sr31* was undertaken by SU-PBL. The preparation of the *Lr24* and *Lr37* PCR reaction mix/volume and conditions associated with the reaction are summarized in Table 3.

Genomic DNA (gDNA) was extracted from adult plants using an adaptation of the procedure described by Doyle and Doyle (1990), excluding a RNase and phenol treatment step. PCR was conducted using Kapa Biosystems ^{TM} PCR products including the use of KapaTaq Readymix^{TM} DNA Polymerase (2X, 0.05U.µl^{-1} with Mg^{2+}, 0.4mM of each dNTP plus loading dye) or, individual reaction mix components from either Kapa Biosystems ^{TM} or Bioline^{TM}. As a band size marker, Bioline HyperLadder^{TM} II producing 15 bands ranging in size from 50 to 2000bp was used

Table 2. Summary information relative to genes/complexes scored by marker-assisted selection (MAS) (*Lr34* was included in later DH production).

Gene/ complex	Primer	Sequence	Aneal -ing (°C)	Frag- ment (bp)	Literature reference
Sr2	Xgwm533-F Xgwm533-R	AAG GCG AAT CAA ACG GAA TA GTT GCT TTA GGG GAA AAG CC	62	120	Hayden *et al.* (2004)
Sr26	SR26-43-F SR26-43-R	AAT CGT CCA CAT TGG CTT CT CGC AAC AAA ATC ATG CAC TA	58	207	Mago *et al.* (2005)
Sr31/Lr26/ Yr9	lag95-F lag95-R	CTC TGT GGA TAG TTA CTT GAT CGA CCT AGA ACA TGC ATG GCT GTT ACA	52	1000	Mago *et al.* (2002)
Lr19	12c-F 12c-R	CAT CCT TGG GGA CCT C CCA GCT CGC ATA CAT CCA	60[b]	130	Prins *et al.* (2001)
Lr24/Sr24	$SCS73_{719}$-F $SCS73_{719}$-R	TCG TCC AGA TCA GAA TGT G CTC GTC GAT TAG GAG TGA G	60[c]	719	Cherukuri *et al.* (2003)
Lr34/Yr18/ Pm38	L32DINT9-F L34PLUS-R	TTG ATG AAA CCA GTT TTT TTT CTA GCC ATT TAA CAT AAT CAT GAT GGA	58	517	Lagudah *et al.* (2009)
Lr37/Sr38/ Yr17	VENTRIUP LN2	AGG GGC TAC TGA CCA AGG CT TGC AGC TAC AGC AGT ATG TAC ACA AAA	65	259	Helguera *et al.* (2003)

Table 3. MAS PCR multiplex reaction and conditions for *Lr24* and *37*.

Components	[Final]	X1 Reaction	PCR conditions		
H_2O		3.5µl	94°C	4min	
Readymix™ (2X)		7.5µl	94°C	1min	X30
$SCS73_{719}$-F (10µM) $SCS73_{719}$-R (10µM)	0.5µM	0.75µl ea.	60°C	1min	
VENTRIUP (10µM) LN2 (10µM)	0.5µM	0.75µl ea.	72°C	1min	
DNA (~200ng.µl^{-1})	~100ng.µl^{-1}	1.0µl	72°C	7min	
Reaction volume		**15µl**	4°C	∞	

during electrophoresis. Three microliters of ladder was loaded together with either 10 or 12µl of PCR reaction mix (Table 3). Electrophoresis was carried out using standard horizontal electrophoretic equipment and agarose gels (1.5-2%) in association with a one-times Tris -boric-acid -EDTA (TBE) buffer. All gels were run at 100V although the runtime varied between 45 and 90min depending on gel size. Gel imaging was performed on a UVIpro Silver (Version 12.5, Uvitec) gel imaging system.

3.2.3 Deriving the female parents and crossing with the male parents

Male-sterile (segregating) parents sourced from the MS-MARS programme (Marais and Botes, 2009), an already-improved population from an agronomic and disease resistance point of

[b] 58°C according to Honing *et al.* (unpublished)
[c] 55°C according to Honing *et al.* (unpublished)

view and containing the dominant male sterility (*Ms3*) gene, were selected on the basis of seedling rust resistance and crossed with the male-fertile (homozygous) parents of the bulked population (Figure 6).

The F_1 female parents were planted in the glasshouse during the winter (growing season) as an integral part of the MS-MARS programme at SU-PBL. This material was inoculated 10-14 days after emergence with the stem rust race UVpgt55 and leaf rust races UVprt8 and UVprt13. Two to three weeks after inoculation and depending on the extent of rust development, this material was phenotypically scored according to the subjective scale of Roelfs *et al.* (1992). One motivation for the application of genotypic markers linked to resistance genes in this investigation is that the pyramiding of resistance genes through phenotypic-based infection type assaying is complicated if differing R-genes yield similar infection types (Sharp *et al.*, 2001; Babu *et al.*, 2004). Those individuals allocated a '1' or '2' categorization corresponding with 'resistant' and 'moderately resistant' phenotypes were selected. Male parents selected from the bulked population on the basis of disease resistance as determined by MAS (Figure 6), were planted in the same greenhouse. The parents were later randomly crossed, losing their identity (pedigree) as selection thereafter was based on the genotypic marker constitution of the cross progeny in terms of disease resistance. Seed harvested from these crosses was planted and grown in a cooled (+ and -5°C ambient temperature in winter and summer respectively) greenhouse for later DH production.

3.2.4 Doubled haploid production

In this investigation, an adaptation of the protocol described by Pienaar *et al.* (1997) was applied to the F_1 progeny of the random parental cross described earlier. MAS with respect to the genes listed in Table 2, was applied to both the F_1 cross progeny as well as haploids derived therefrom. Specific PCR reactions and conditions are presented in Table 4, 5 and 6. The haploid

Table 4. MAS PCR multiplex reaction and conditions for *Lr19, 24* and *37*.

Components[d]	[Final]	X1 Reaction	PCR conditions		
H_2O		0.7µl	94°C	5min	
Readymix™ (2X)		7.5µl	94°C	1min	X30
12c-F & R (10µM ea.) $SCS73_{719}$-F & R (10µM ea.)	0.53µM	0.85µl ea.	55°C[e]	1min	
Sr24-12-F & R (10µM ea.)[f] VENTRIUP & LN2 (10µM ea.)	0.47µM	0.75µl ea.	72°C	1min	
DNA (~100ng.µl^{-1})	~150ng.µl^{-1}	1.5µl	72°C	7min	
Reaction volume		**16.1µl**	4°C	∞	

[d] This multiplex reaction was optimized by SU-PBL.
[e] Not 60°C as reported by Honing *et al.* (unpublished) in which case *Lr19* is not visible on the gel.
f Included from Mago *et al.* (2005).

procedure was tested in 2007 using the CIMMYT-sourced entry '12th HRWYT 40-4' included in the 2007 MLFT (Table 1), as well as the sweet corn cultivar 'Bonita' (Agricol) as pollen donor.

Table 5. MAS PCR multiplex reaction and conditions for *Lr34, Sr2* and *26*.

Components[g]	[Final]	X1 Reaction	PCR conditions		
H_2O		1.9µl	94°C	5min	
$MgCl_2$ (25mM)		0.2µl	94°C	1min	X30
Readymix™ (2X)		7.5µl	55°C	1min	
L32DINT9F & L34PLUSR (10µM ea.) Sr26#43F & R (10µM ea.)	0.4µM	0.6µl ea.	72°C	1min	
Xgwm533F & R (10µM ea.)	0.5µM	0.75µl ea.	72°C	7min	
DNA (~100ng.µl^{-1})	~150ng.µl^{-1}	1.5µl	4°C	∞	
Reaction volume		**15µl**			

Table 6. MAS PCR reaction and conditions for *Sr31*.

Component	[Final]	X1 Reaction	PCR conditions		
H_2O		5.0µl	95°C	3min	
Readymix™ (2X)		7.5µl	95°C	30s	X35
lag95-F (10µM)	0.5µM	0.75µl	52°C	30s	
lag95-R (10µM)	0.5µM	0.75µl	72°C	50s	
DNA (~100ng.µl^{-1})	~100ng.µl^{-1}	1.0µl	72°C	10min	
Reaction volume		**15µl**	4°C	∞	

At an appropriate stage of development, wheat spikes were emasculated with the aid of sterile blunt-ended tweezers (forceps). The emasculated spikes were then covered with a glycine paper bag. All records concerning emasculation (date), pollination and hormone application were recorded on a tag attached to the glycine bag.

Between one and three days later depending on the anticipated anthesis date, the emasculated florets were cut back to just above the stigma to ensure that the later-applied maize (c.v. 'Bonita', Agricol) pollen made good contact with the stigma surface. With the aid of a 100ml glass beaker and a sheet of paper, maize pollen was collected on the day it was to be used thus ensuring it was fresh. The prepared wheat florets were fertilized within half an hour of pollen collection using a small artist's paint brush approximately 5mm in diameter. The glycine bags were then replaced and the date and time of pollination recorded on the attached tags.

[g] This multiplex reaction was optimized by SU-PBL.

At 30h after pollination the floral cups were filed with a freshly made hormone solution (50mg.l^{-1} 2,4-D plus 10mg.l^{-1} GA_3) using a 1ml syringe fitted with a 21G-1½" size needle. Spikes were then re-bagged and the date and time recorded.

Seeds were harvested within a period of 14 (no later than 15) days following hormone application. Using the fingers, the seeds (i.e. GPCs or green parthenocarpic caryopses) were removed from the glumes. The GPCs were then transferred to 10ml sterile plastic screw-capped vials with spike tags (records) attached to corresponding vials.

Working in a laminar-flow hood, GPCs were then surface-sterilized according to the following regime: [1] 70% (v/v) ethanol (30s); [2] 30% (v/v) sodium hypochlorite with six drops of Tween 20 (surfactant) in 200ml of the said solution (8min) followed by; [3] four rinses with sdH_2O (3min each). The embryos were then immediately excised under a stereoscope with the aid of sterile narrow-ended forceps and a stylet. The white to opaque embryo was lifted out of the internal matrix on the tip of the stylet and carefully placed on the surface of 20-25ml semi-solid ERM in the tissue culture jars. Four to five embryos were cultured per jar with labels placed on the underside of the jar.

The tissue culture jars were then incubated in the dark (21°C) for 10-12 days. When regenerated shoots were about 1cm long, the jars were transferred to a growth chamber (24°C and 14h light cycles, otherwise 18°C) until the plantlets had grown large enough to transplant, i.e. approximately three to four weeks later. At this stage, the plants were transplanted into pots containing moistened african violet (*Saintpaulia* spp.) medium. The pots were covered with a transparent plastic bag secured around the base of the pot with an elastic band to prevent desiccation of the young plantlets. The pots were then moved to a controlled environment (18°C and 14:10h day:night regime, incandescent and sodium-vapour lights) for a period of hardening-off. During this hardening-off period and after about seven days, the plastic bags covering the pots were cut across the corner to hasten the hardening-off process. The plant material took a further two to three weeks to properly establish and reach a sufficient size for ploidy determination.

Ploidy was determined according to the method described by Darlington and La Cour (1960) with minor modifications where for example, solutions were replaced with suitable alternatives.

Chapter 4: Results and discussion

4.1 Multi-location field trials

Table 7 below summarizes the coefficient of variation (CV) and heritability values (H^2, broad sense) for all ten localities in the Western Cape over the 2007 season. NNA did not reveal strong trends regarding the three summarized traits (Table 7) as the reported NNA weight did

Table 7. Coefficient of variation (CV) and heritability (H^2) values for grain and ethanol yield: 2007.

Location	Grain yield (t.ha^{-1})		Ethanol yield (l.t^{-1})		Ethanol yield (l.ha^{-1})	
	CV	H^2	CV	H^2	CV	H^2
ALB	9.97	0.57	5.78	0.66	5.81	0.83
KLI	22.81	0.59	4.92	0.66	4.95	0.97
LAN	17.13	0.63	7.53	0.38	7.16	0.91
NAP	8.20	0.77	4.53	0.77	4.77	0.90
PIK	22.62	0.55	4.85	0.29	4.79	0.96
RIV	45.59	0.67	3.49	0.88	4.04	1.00
ROO	16.95	0.38	4.61	0.70	4.61	0.91
TYG	15.44	0.64	3.72	0.73	3.79	0.95
VRE	*18.16	*0.16	4.41	0.72	4.22	0.97
WEL	16.29	0.74	*5.21	*0.23	5.45	0.97

* Unadjusted (no trends)

not exceed 0.39 over any of the locations for either 2006 or 2007. From this Table, Klipheuwel, Piketberg and Riversdal were excluded from further analysis due to grain yield CV values exceeding 20% (Taylor *et al.*, 1999; Bowman, 2001; Shah *et al.*, 2009) as highlighted. These were coupled with only moderate H^2 values (Falconer and Mackay, 1996) (Table 7). In addition, mean square errors (MSEs) are usually not homogeneous in multi-environment trials as they are influenced by circumstance, with lower values (and thus higher CVs) from low yielding environments (Bowman and Watson, 1997). Welgevallen was also excluded from the analysis of the Western Cape as a whole, as the Boland sub-region is not a major wheat production area in the Province. Also and where repeatable interaction exists, the best breeding material for less favourable environments cannot be identified by selecting for yield in favorable environments such as experiment stations, particularly in developing countries (Ceccarelli, 1996). However, this site is considered apart from the rest later on. Similarly, Vredenburg and Welgevallen were excluded from the 2006 data set for the same reasons. This left only three remaining trial sites for that year and in common with the 2007 MLFT. This is borne in mind when comparing entry and location performance over both seasons. Bird damage in both years contributed to the higher than expected grain yield CV values at certain trial locations (Table 7).

4.1.1 Grain yield

The grain yield of each entry (t.ha^{-1}) averaged across all trial locations is presented in Figure 8 and 9 for the two respective seasons. Controls are highlighted (using a red square/box) apart from the ABLs with the 5% Fisher's least significant difference (LSD, P≤0.05) also indicated as a basis for determining statistically significant differences. A summary interpretation of the MLFT grain yield data for both seasons is also given in Table 8.

From Table 8 and Figure 8 it is evident that 03H86-8-2 was the best ABL performer and entry overall in the Western Cape in terms of grain yield in 2006. In 2007, its performance was bettered in the Province by the inclusion of 00K60-16-3-3 (Table 8 and Figure 9). The superior performance of 03H86-8-2 was statistically significant compared to two and one control for each respective season (Figure 8 and 9). 00K60-16-3-3 significantly outperformed five of the ten controls in 2007 (Figure 9). Also, 03H86-8-2 was not significantly better than any of the other 2007 ABLs or the mean for that year (Figure 9). In contrast, in 2006 it yielded statistically significantly above the mean and significantly better than eight of the remaining 11 ABLs (Figure 8). In 2007, 00K60-16-3-3 significantly bettered the lowest ranked ABL namely 97K1-15-5, but not the mean for that year (Figure 9). The overall lead of 03H86-8-2 in 2006 (Table 8 and Figure 8) was eclipsed by SST 027 in 2007 (Table 8 and Figure 9). As for 00K60-16-3-3, the overall superior performance of SST 027 in 2007 was significantly better than five controls but statistically indifferent to the mean

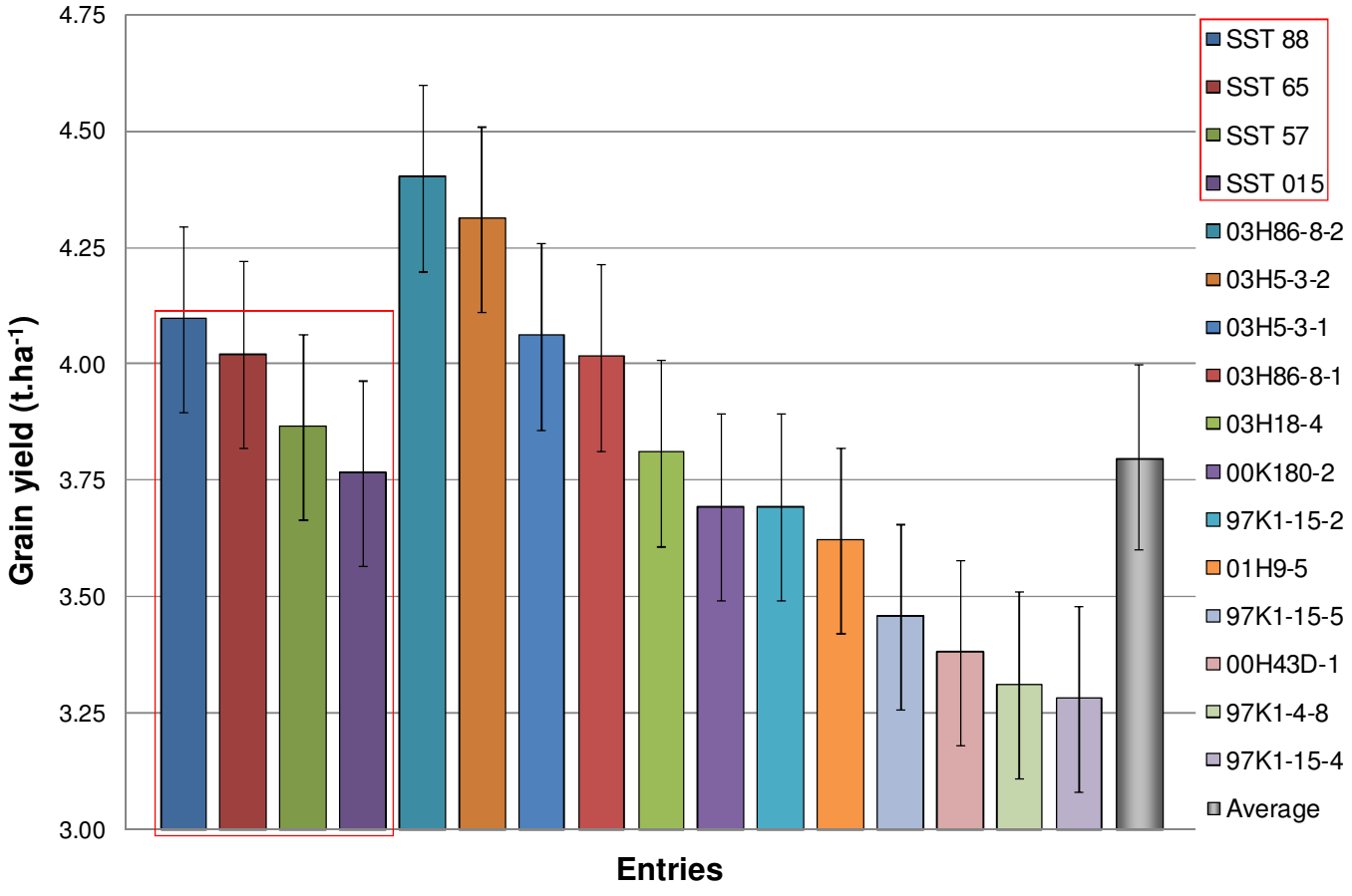

Figure 8. **Average grain yield (t.ha^{-1}) for 2006 entries across all localities** (5% LSD indicated with each).

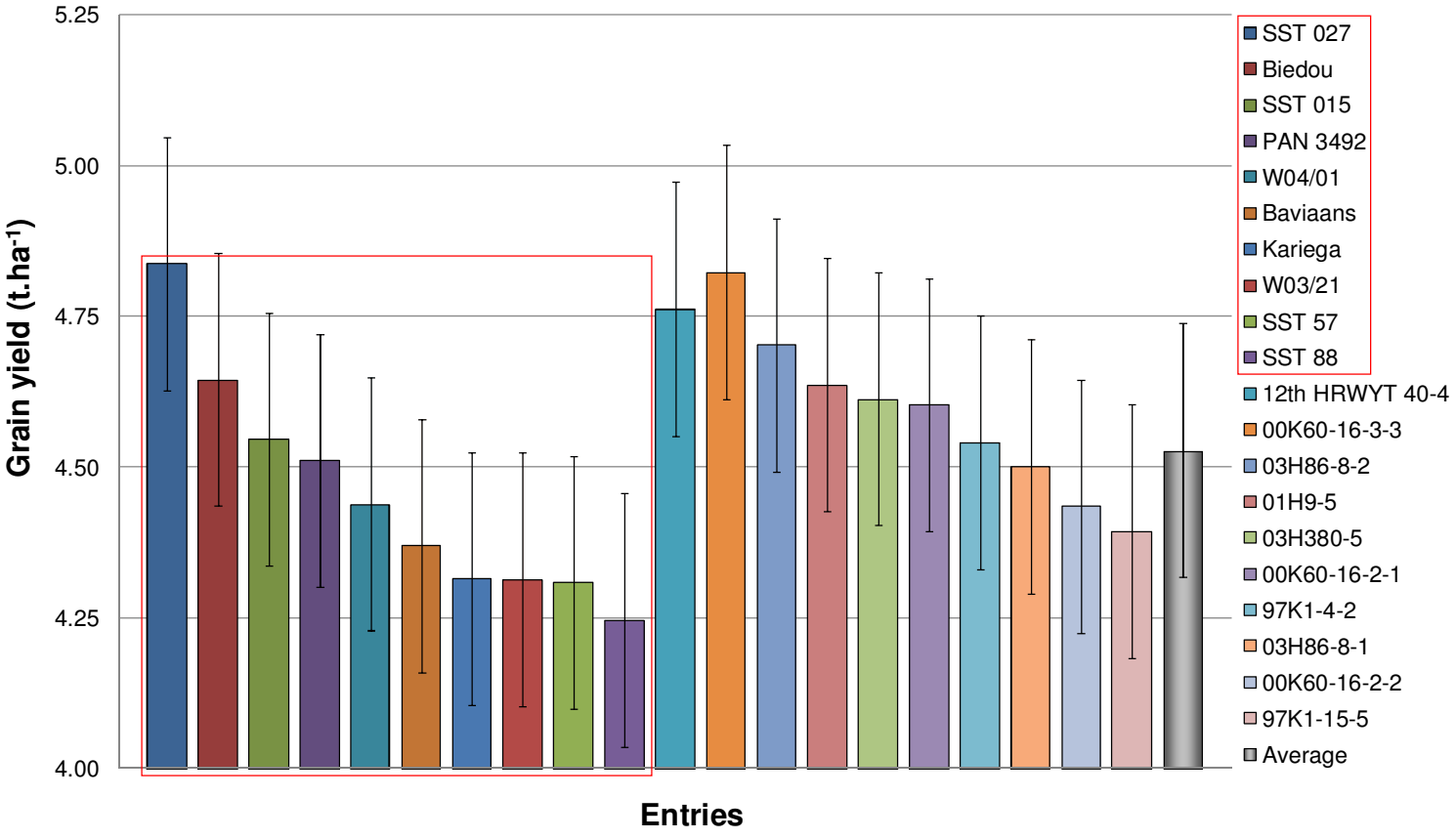

Figure 9. **Average grain yield (t.ha^{-1}) for 2007 entries across all localities** (5% LSD indicated with each).

(Figure 9). The lowest yielding ABLs for each respective season were 97K1-15-4 and 97K1-15-5 (Table 8; Figure 8 and 9). Their poor performance was statistically significantly worse than all four and one control respectively (Figure 8 and 9). 97K1-15-4 was also the lowest yielding entry overall in 2006 (Table 8 and Figure 8) whereas SST 88 was the poorest performing entry overall in 2007 (Table 8 and Figure 9). SST 88 also yielded significantly less than one control (Figure 9). No statistically significant differences were recorded between the leading three ABLs for either season (Figure 8 and 9). However in 2006 and as for 03H86-8-2 during that year, second-ranked 03H5-3-2 also significantly out-performed two controls and the mean for that year (Figure 8).

AMMI analysis was conducted on the 2007 MLFT data for the whole Western Cape. The results are summarized in the spatial bi-plot of Figure 10. From the AMMI ANOVA table (not shown), the interaction principle component analysis (IPCA1) axis explained 53.15% of the observed interaction variance. Only the first two axes were significant ($P \leq 0.05$, AMMI2 model) in determining the expected (adjusted for interaction effects) values. The CV and correlation coefficient (R^2) values for the model were 14.10% and 0.85 as reported in Table 8. AMMI analysis was also conducted on the 2006 grain yield data (bi-plot not shown). For that season, IPCA1 explained 75.68% of the observed interaction variance. As for 2007, only the first two axes were significant. Using singular vectors, meaningful inferences can be made with regards to specific entry X location combinations from the resultant bi-plot (Figure 10). Displacement along the

Table 8 . MLFT summary interpretation for grain yield (t.ha^{-1}): 2006 and 2007.

Year	Location	Mean (t.ha^{-1})	CV (%)	H^2	Best entry	Best 3 ABLs (ranked) 1	2	3	Worst entry	Worst ABL
2006	LAN	4.27±0.047	8.82	0.78	03H86-8-2	03H86-8-2	03H5-3-2	97K1-15-2	00H43D-1	00H43D-1
	ROO	4.33±0.056	10.38	0.87	SST 88	03H86-8-2	03H86-8-1	03H18-4	03H5-3-2	03H5-3-2
	TYG	3.12±0.078	20.13	0.82	03H5-3-1	03H5-3-2	03H5-3-1	03H86-8-2	00H43D-1	00H43D-1
	GxE (W Cape)[a]	3.90	12.70		03H86-8-2	03H86-8-2	03H5-3-2	03H5-3-1	97K1-15-4	97K1-15-4
2007	LAN	4.44±0.098	17.13	0.63	12th HRWYT 40-4	01H9-5	00K60-16-2-3	00K60-16-3-2	97K1-15-5	97K1-15-5
	VRE	2.57±0.060	[e]18.16	0.16	Biedou	01H9-5	00K60-16-3-3	03H86-8-1	PAN 3492	03H86-8-2
	GxE (Swartland)[b]	3.50	18.00		12th HRWYT 40-4	01H9-5	00K60-16-3-3	97K1-4-2	W04/01	97K1-15-5
	NAP	5.60±0.059	8.20	0.79	00K60-16-3-3	00K60-16-3-3	03H86-8-2	00K60-16-2-2	SST 88	03H86-8-1
	ALB	5.59±0.072	9.97	0.57	00K60-16-2-2	00K60-16-2-2	03H380-5	03H86-8-2	SST 88	97K1-15-5
	TYG	5.20±0.10	15.44	0.64	12th HRWYT 40-4	03H86-8-1	03H86-8-2	00K60-16-2-1	00K60-16-2-2	00K60-16-2-2
	ROO	3.77±0.082	16.95	0.38	00K60-16-3-3	00K60-16-3-3	00K60-16-2-1	01H9-5	W03/21	03H380-5
	GxE (S Cape & Rûens)[c]	5.04	12.74		03H86-8-2	03H86-8-2	00K60-16-3-3	00K60-16-2-1	SST 88	01H9-5
	GxE (W Cape)[d]	4.53	14.10		SST 027	00K60-16-3-3	03H86-8-2	01H9-5	SST 88	97K1-15-5

[a] R^2 = 0.79, entry X location F (P≤0.05) = 4.49 (0.0000)

[b] R^2 = 0.81, entry X location F (P≤0.05) = 1.44 (0.1334)

[c] R^2 = 0.75, entry X location F (P≤0.05) = 1.58 (0.0144)

[d] R^2 = 0.85, entry X location F (P≤0.05) = 1.69 (0.0008)

[e] Unadjusted (no trends)

Means for best grain-producing localities in each region

Blue Best lines within the best grain-producing localities and within all (GxE) localities, as well as across both seasons

Red As for blue type but with respect to the tail-performance grain-producing localities

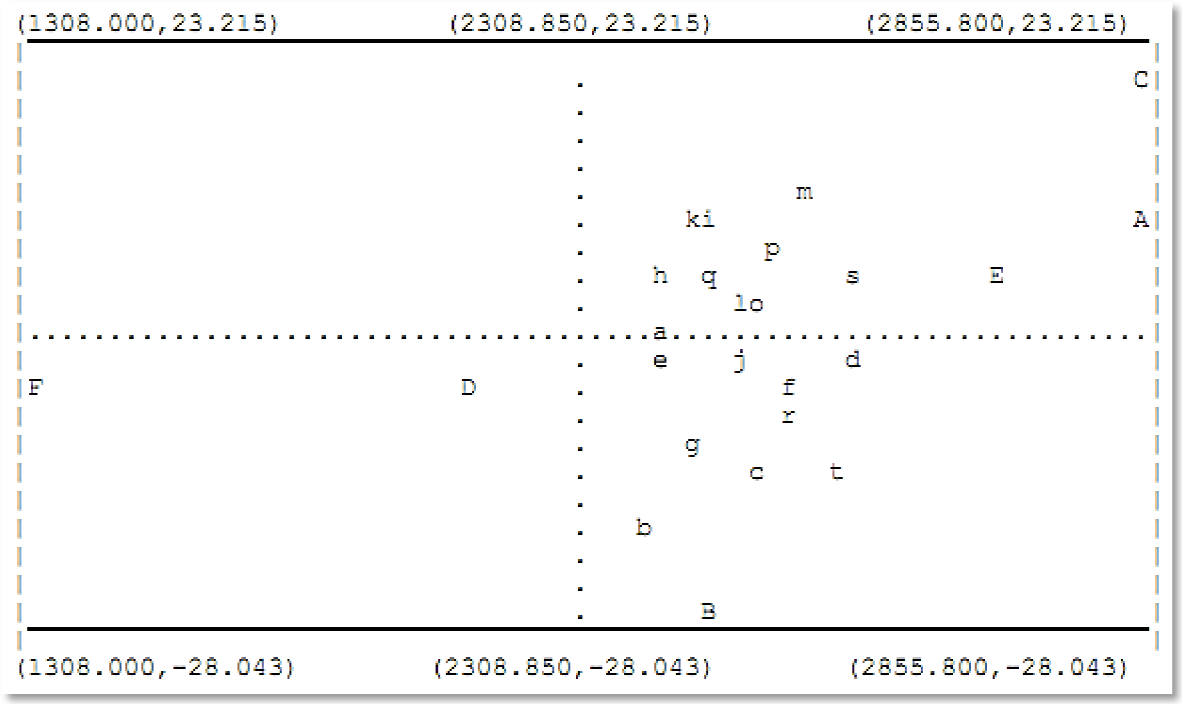

Figure 10. Additive main effects and multiplicative interaction (AMMI) analysis of the 2007 MLFT data for grain yield in the Western Cape (kg.ha^{-1}). Refer: text for explanation of symbols.

abscissa represents main effects differences (kg.ha^{-1}) between specific environments (capitalized letters) and entries (lower case letters) with a common adjusted mean. Displacement along the ordinate (IPCA1) represents interaction differences between the said main effects where values closer to zero represent less adjustment and thus increased stability/adaptation. The ranking of main effects with large absolute value IPCA1 scores is also less statistically reliable than those with scores nearer zero. In general, the abscissa represents the overall relative quality of locations and general breeding status for entries (Crossa *et al.*, 1990).

AMMI analysis for the 2006 grain yield data revealed a greater spread (increased diversity) of the entries around the abscissa mean in contrast to 2007. This indicates that all the 2007 entries as a group are expected to perform either better or worse than the mean for individual environments (Figure 10). The ranking of entries is also more likely to change at less stable locations such as Napier and Langgewens (Figure 10). Additionally, the expected (genotypic) value for all 20 entries in 2007 was higher than the expected mean (Figure 10). In 2006, 03H86-8-2 was also only marginally more adapted to multi-environments than in 2007 considering that only three versus six (2007) environments were analyzed in that year. Also, 03H5-3-1 and 03H5-3-2 were both poorly adapted in comparison to 03H86-8-2. The Nassar and Huehn (1987) ranking test revealed 03H86-8-2, 97K1-4-8 and SST 57 as the three respective and overall most adapted entries in 2006. AMMI analysis recommended SST 57, 97K1-15-4 and SST 015 in that order. Also, all three environments in 2006 were very unstable in terms of their differentiating genotypes. Common to both years, Langgewens and Roodebloem both displayed negative IPCA1 scores in

contrast to Tygerhoek, although with differing magnitudes in each season as expected for differing sets of entries in each year.

Spatial patterns in the 2007 grain yield data as revealed in Figure 10 confirm what is reported in Table 8. Controls are represented by a-j in the Figure while ABLs are represented by k-s. The entry 12th HRWYT 40-4 is located at position t. Locations A, C and F represent Albertinia, Napier and Vredenburg respectively and contrast the two highest versus the lowest yielding trial site (Table 8 and Figure 10). It is evident from Figure 10 that Tygerhoek (E) was more stable in terms of differentiating genotypes planted there, albeit lower yielding than Albertinia and Napier. Genotype X entry combinations in the top and bottom right quadrant respectively are especially adapted to each other. Opposite quadrants represent negative interaction. Both Napier and 03H86-2 (m) displayed large positive interaction in contrast to large negative interaction between Langgewens (B) amd 03H86-8-2 for example. Albertinia, Napier and Tygerhoek can also be grouped into a mega-environment. The top three ABLs in the Western Cape during 2007 i.e. 00K60-16-3-3 (s), 03H86-8-2 and 01H9-5 (r) (Table 8 and Figure 10), all reveal their genetic superiority in terms of grain yield (Figure 10). Also, 00K60-16-3-3 and 01H9-5 were equally and more adapted than 03H86-8-2. This accounts for the reported ranking of the best three ABLs in Table 8 relative to the Province in 2007. In addition, SST 027 (d) was the highest yielding entry overall and among the most environmentally adapted along with SST 57 (a), Kariega (e), PAN 3492 (j), 03H86-8-1 (l) and 97K1-4-2 (o) (Figure 10). According to the non-parametric ranking test of Nassar and Huehn (1987), the three most adapted entries overall in the Western Cape during 2007 were 97K1-4-2, SST 57 and W03/21 (h) respectively. This test lends equal weight to each environment and thus entries with fewer rank changes across environments are expected to be more stable (Nassar and Huehn, 1987). AMMI analysis recommended SST 57, PAN 3492 and SST 027 in that order according to absolute adjustment values if considering adaptation apart from genotypic yield values. Further AMMI analysis of adaptation and stability to and within the two regions of the Province was conducted with respect to ethanol yield discussed later on in this Chapter.

From Table 8, the ABL 01H9-5 was the highest yielding in the Swartland region where it significantly out-performed three controls during 2007. However, it did not perform statistically significantly better than the second- or third-ranked ABL for the region i.e. 00K60-16-3-3 and 97K1-4-2 respectively (Table 8). Additionally, 00K60-16-3-3 yielded significantly more than one of the included controls. The region's overall highest yielding entry (12th HRWYT 40-4) also performed significantly better than three controls. In common with the whole Western Cape in 2007 (Table 8, Figure 8 and 9), 97K1-15-5 was the worst performing ABL and performed significantly worse than five controls. The best performing ABL in the Province over both seasons i.e. 03H86-8-2 (Table 8; Figure 8, 9 and 10) was ranked as the second lowest ABL entry in the Swartland although not statistically significantly different from 97K1-15-5. The lowest yielding entry overall namely W04/01 (Table 8) yielded significantly worse than five of the ten included controls.

In contrast to the Swartland, 01H9-5 was the poorest yielding ABL in the Southern Cape and Rûens during 2007 (Table 8), although its poor performance was not statistically significantly worse than any of the ten included controls. Within this region however, both Roodebloem and Tygerhoek recorded better 01H9-5 performance in 2006 relative to the set of entries for that season. Further illustrating regional multi-environment adaptation, 03H86-8-2 was the highest yielding entry overall in the Southern Cape / Rûens during 2007 (Table 8) and significantly out-performed seven controls. This contrasts with its second lowest ABL ranking in the Swartland mentioned in the previous paragraph. The superior performance of 03H86-8-2 in the Southern Cape / Rûens was statistically indifferent to the second- and third-ranked ABL entries for that year, namely 00K60-16-3-3 and 00K60-16-2-1 respectively (Table 8) which significantly out-yielded three controls each. The region's worst performing entry i.e. SST 88 (Table 8) performed significantly worse than three controls.

4.1.2 Total starch

The motivation behind employing both NIRS and biochemical approaches to the quantification of total starch was to establish a calibration (statistical prediction model) that might prove useful in estimating the total starch fraction of especially whole grain samples, as their milling for quality trait determination is a laborious and costly task. The aim was to replace costly y-reference measurements (wet chemistry for example) with quick and inexpensive x-reference (spectroscopic for example) measurements. Also, if it could be established that there was a relatively large range (domain) of starch values from highest to lowest estimates, a more useful and widely applicable by implication prediction model would result. To the best knowledge at the time when this project was conceived, the above-discussed NIRS approach had never been attempted on spring wheat grain from a bio-ethanol point of view in a South African context.

4.1.2.1 NIRS calibration for starch yield

Table 9 below summarizes the R^2 correlation coefficients for the NIRS starch calibration. From this Table, it is evident that a useful prediction model likely exists with respect to the estimation of total starch fraction in contrast to moisture determination. The

Table 9. Summary of total starch calibration R^2-values determined by PLS-R.

	Wholegrain				Flour			
	Starch		Moisture		Starch		Moisture	
	Train	Test	Train	Test	Train	Test	Train	Test
Combined	0.801	0.820	0.498	0.420	0.858	0.830	0.491	0.417
2006	0.494	0.210	0.411	0.266	0.386	0.100	0.685	0.585
2007	0.809	0.908	0.601	0.602	0.844	0.807	0.614	0.569
				2007 only				
Raw	0.817	0.840	0.600	0.566	0.901	0.710	0.674	0.458
1st derivative	0.908	0.875	0.901	0.603	0.922	0.815	0.824	0.440
MSC-corrected	0.833	0.860	0.601	0.554	0.916	0.750	0.648	0.444

poor results obtained regarding moisture content are unexpected and in contradiction to the literature (Smith *et al.*, 2006). This may relate to the method chosen to quantify moisture content in this investigation and/or its execution. It was observed that the domain (spread of measurements) relative to the ordinate (NIR-predicted values) was relatively narrow. This contrasted with the domain along the abscissa (values from the moisture analyzer), accounting for the poor moisture prediction correlation values reported in Table 9. This was consistently observed in the analysis of both season's data individually as well as when combined.

Highlighted in Table 9, the analysis of combined season data is of particular interest from the point of view of model application. According to Prof. Martin Kidd (CSC, Stellenbosch University) (Personal communication. 2009), the inclusion of measurements from more than one season contributes to a more robust/rigorous prediction model. In addition, the transformation of the data to the first derivative resulted in a slightly improved model fit as highlighted (Table 9). The graphical model representations for whole grain are shown for the first derivative 2007 train and test data sets in Figure 11 and 12 below. It is also evident from Table 9 that the 2007 data set gave an improved starch prediction model in contrast to 2006. This is likely due to increased experience gained in handling the wet chemical assay procedure. In contrast though, the model determined for moisture content prediction remained statistically unreliable over both seasons.

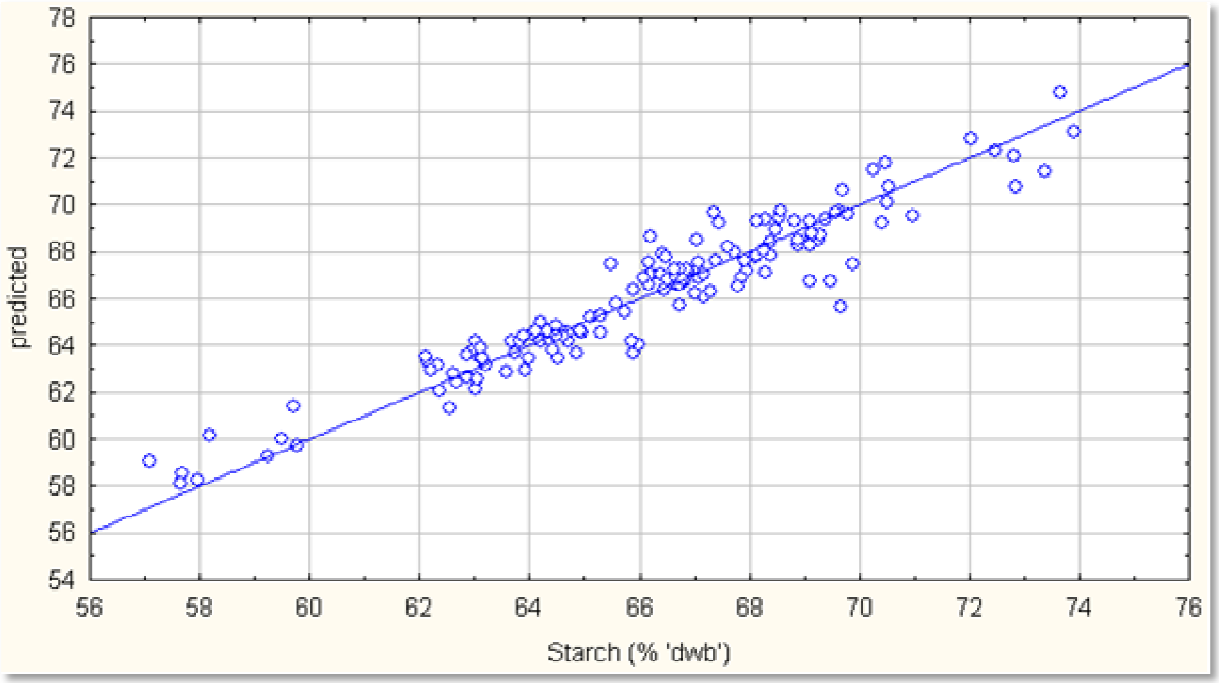

Figure 11. First derivative calibration regression for 2007 whole grain total starch content: 'train' data set.

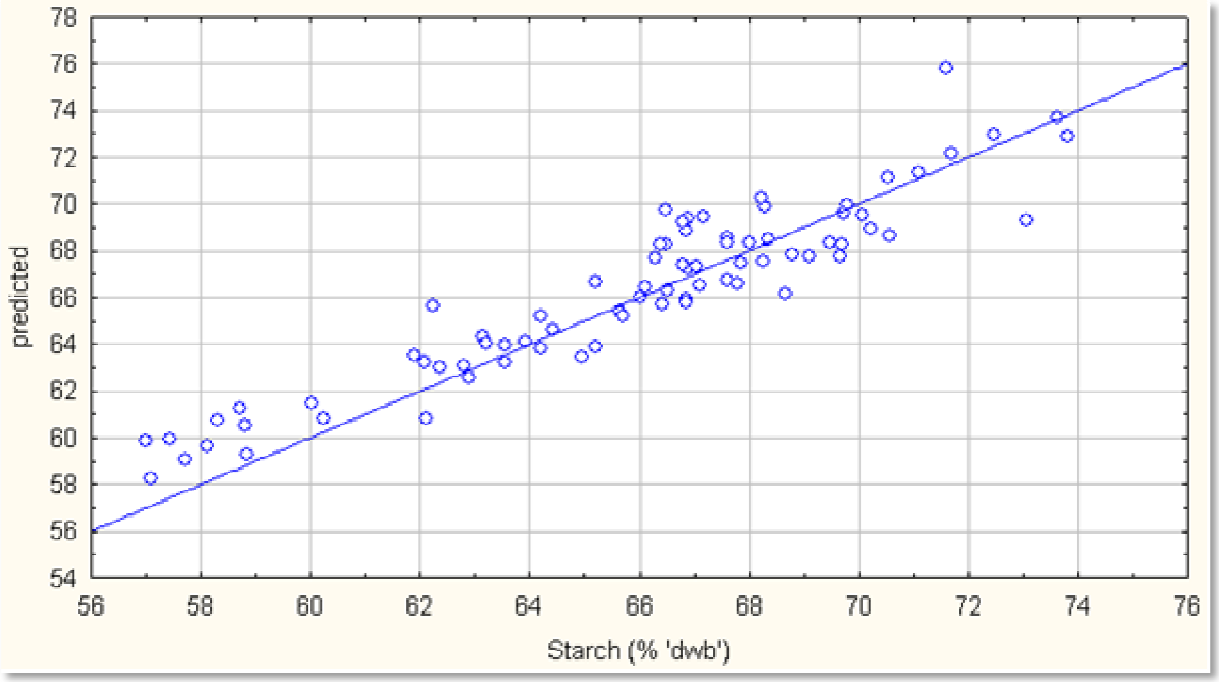

Figure 12. First derivative calibration regression for 2007 whole grain total starch content: 'test' data set.

4.1.2.2 Chemically determined starch yield

The timing of moisture content determination (immediately prior to wet chemistry), sample homogenization and, sample storage conditions (23℃ and relatively humid) are influential to the results of a moisture-dependent enzyme-based biochemical assay procedure. Also, drying profile optimization and the reproducibility of results are influenced by loaded sample volume (Ohaus Corporation, n.d.). It was observed that a temperature of 110℃ would begin to burn/discolour the loaded flour sample, confounding the loss of physical (solids) mass with moisture content. When weighing out flour on the moisture analyzer, the lid housing the heating element was kept fully open and away from the sample loading area. This consistently required the addition and/or removal of very small quantities of flour to reach the target 3g. The mass reported by the instrument was characteristically unstable and inclined to decrease slightly over time due to moisture vapourization before analysis had begun. This occurred despite following the manufacturer's instructions and a necessary high instrument operating temperature. Thermally unstable conditions are less than ideal for determining the mass of fine flour samples. However, with some practice this problem was well controlled but not completely eliminated. Allowing the instrument to cool down between every sample would also be impractical and itself may introduce error. An alternative approach might be to use a separate balance and weigh-boat, and then transfer the weighed sample to the moisture analyzer sample-pan. This is a laborious option that will nevertheless more accurately weigh out a 3g sample. A portion of that advantage may however be sacrificed in transferring the flour to the sample-pan.

The decision to analyze the centrally positioned replication two was chiefly motivated by principle as it relates to data collection/measurement from a field plant population. During sample loading and considering the more difficult handling properties of the included starch standard (adhering to the inside of the glass tubes) and, that only 200µl of liquid was to be added, any sample fraction that was not transferred to the bottom of the tube would be dissimilarly treated. Thus, smaller (shorter) tubes would be advantageous bearing in mind that the tubes only needed to hold a maximum volume of liquid of about 7.3ml during the entire procedure. Also, in character with the physico-chemical properties of water during the time that the bath lid was open during the starch cooking (100°C) step, the lost water temperature could not quickly be recovered. For this reason, sample vortexing was not performed at 2min (remaining) as prescribed by the protocol. The sequential vortexing of 21 tubes even if done briefly, took approximately 1min 15-30s to complete with the vortex running continuously at full velocity. This is a sizeable period relative to the 6min total incubation time.

The wet chemistry ranked percentage (d.w.b.) total starch fraction of all entries averaged across locations (as for grain yield) is graphically illustrated in Figure 13 and 14 for each respective season. Controls are highlighted apart from the ABLs and the 5% LSD (P≤0.05) value is indicated for each entry. In addition, starch data is summarized in Table 10.

Evident from Figure 13 and 14 is that the controls SST 015, SST 57 and SST 88 were of high total starch fractions relative to two largely different sets of entries in each season. The ABL

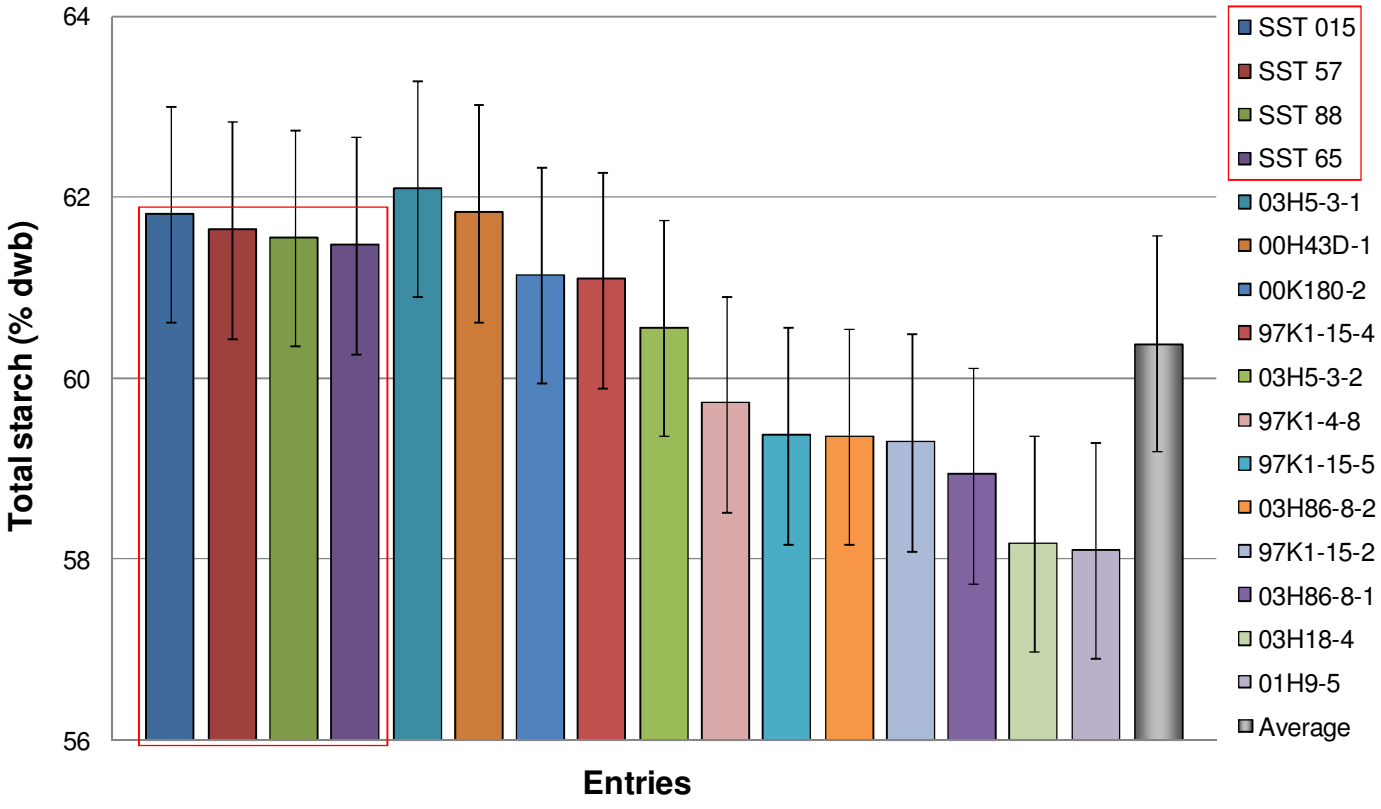

Figure 13. Average starch yield (% d.w.b.) for 2006 entries across all localities (5% LSD indicated with each).

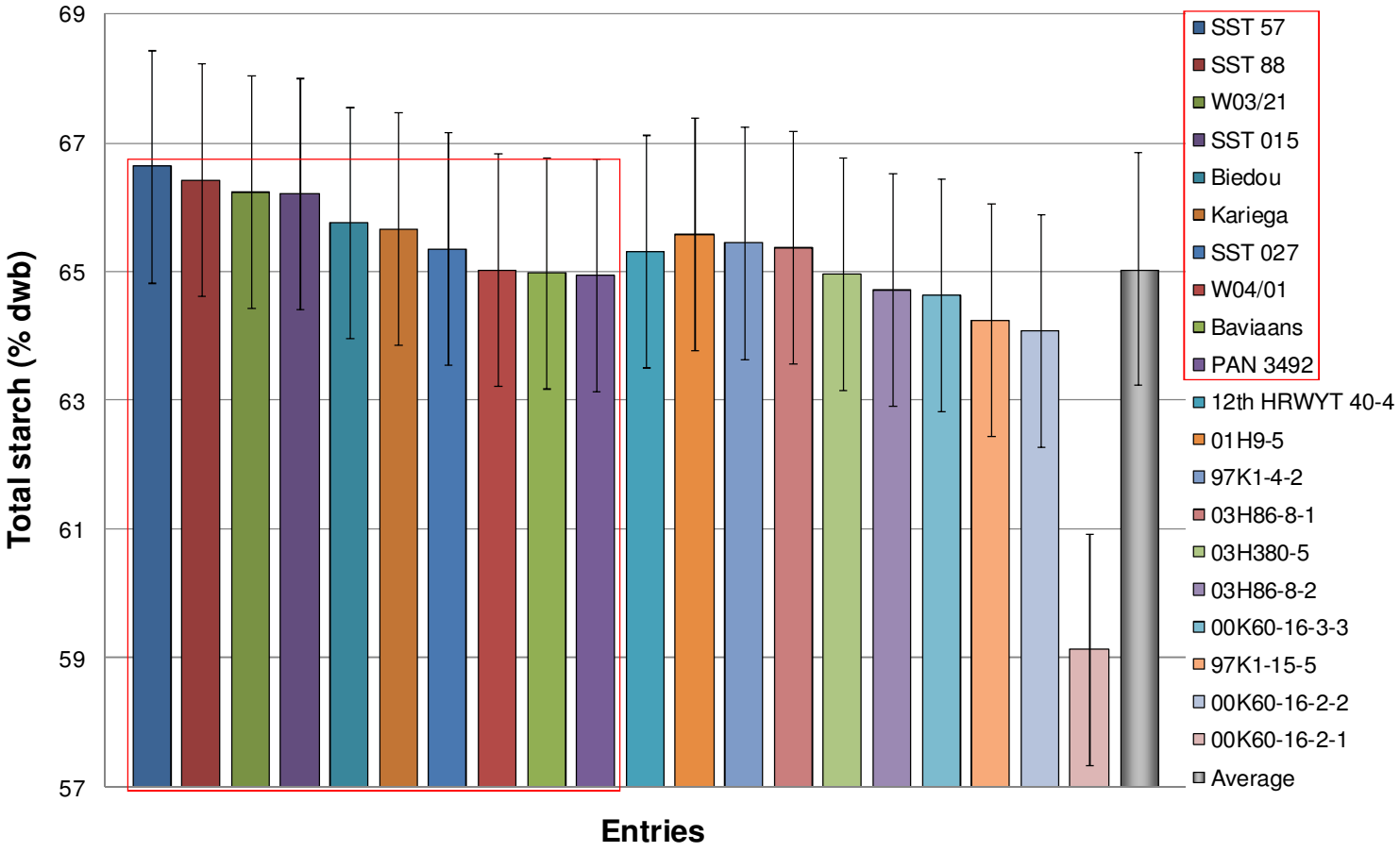

Figure 14. **Average starch yield (% d.w.b.) for 2007 entries across all localities** (5% LSD indicated with each).

Table 10. **MLFT summary interpretation for total starch (% d.w.b.): 2006 and 2007.**

Year	Location	Mean (%)	CV (%)	Best entry	Best 3 ABLs (ranked) 1	2	3	Worst entry	Worst ABL
2006	W Cape[a]	60.38	2.37	03H5-3-1	03H5-3-1	00H43D-1	00K180-2	01H9-5	03H18-4
2007	Swartland[b]	65.59	1.57	12th HRWYT 40-4	97K1-4-2	01H9-5	03H86-8-1	00K60-16-2-2	00K60-16-2-2
	S Cape & Rûens[c]	64.76	5.80	SST57	01H9-5	03H86-8-1	97K1-4-2	00K60-16-2-1	00K60-16-2-1
	W Cape[d]	65.04	4.84	SST57	01H9-5	97K1-4-2	03H86-8-1	00K60-16-2-1	00K60-16-2-1

[a] location F (P≤0.05) = 2.86 (0.0073)
[b] location F (P≤0.05) = 5.00 (0.0382)
[c] location F (P≤0.05) = 6.88 (0.0005)
[d] location F (P≤0.05) = 9.85 (0.0000)

01H9-5 reported contrasting results in each year for the same reason (Table 10; Figure 13 versus 14). 00K60-16-2-1 was statistically significantly lower than all ten controls and the mean during 2007 (Figure 14). Otherwise no significant differences existed between the entries for that year. In contrast, the three lowest ranked ABL entries in 2006 i.e. 03H86-8-1, 03H18-4 and 01H9-5 respectively, all had total starch values that were statistically significantly less than the four controls included in that season (Figure 13). Significant differences were also reported between the highest and lowest ranked ABLs (Figure 13). As explained earlier, statistically significant differences

between the genotypes were sought for the purposes of constructing a starch calibration model. Also evident from Figure 13 and 14, the controls as a group from both seasons displayed generally above-average starch fractions versus the included ABLs as a group. This trend was also evident in the Southern Cape / Rûens but not the Swartland during 2007 (data not shown). Additionally, the best overall (12th HRWYT 40-4) and best ABL (97K1-4-2) entries in the Swartland were statistically significantly higher than three and one control respectively. None of the highest starch fraction entries in the Southern Cape / Rûens (Table 10) were statistically significantly better relative to the included controls in 2007. Certain of the controls with relatively low grain yield such as SST 57 and W03/21 for example (Figure 9), were among the highest overall in terms of starch fraction (Figure 14). The third-ranked grain yield performer among the 2007 ABL entries namely 01H9-5 (Table 8; Figure 9 and 10), was the highest ranked ABL for total starch during that year (Table 10 and Figure 14). The best two 2007 grain yield performers of 00K60-16-3-3 and 03H86-8-2 respectively (Table 8; Figure 9 and 10), were mediocre with respect to total starch (Figure 14). 03H86-8-2 was also mid-ranked in 2006 regarding starch (Figure 13). SST 57 was the overall highest ranked entry in the Province and the Southern Cape / Rûens (Table 10 and Figure 14), although not statistically significantly better in either multi-environment. This entry ranked third overall in the Swartland but not significantly better than any of the included controls. Further; 97K1-4-2, 03H86-8-1 and 01H9-5 consistently ranked in the top three ABLs across all three multi-environments in 2007 (Table 10).

Regarding localities, in 2007 the highest mean was recorded at Piketberg and Welgevallen respectively, while Welgevallen recorded the highest mean in 2006. Statistically significant differences were also reported between the locations in 2006 ($P=0.0073$, $P\leq0.05$) and 2007 ($P=0.0000$, $P\leq0.05$) (Table 10).

4.1.3 Amylose/amylopectin ratio

In handling only ten samples per assay cycle it was not possible to analyze the sample solutions within 2h of Con-A solvent addition, as stipulated by the protocol. According to the protocol, amylose is inclined to retrograde and precipitate out of solution should the samples not be analyzed within the said time period, leading to underestimation (Megazyme International Ireland Limited ™, 2006a). This is despite a required and thus unavoidable 1h bench-incubation step to precipitate the amylopectin fraction out of solution by the Con-A enzyme preparation.

Problems were encountered with between-kit repeatability while assaying the 2007 MLFT bulked replication material. Based on the failure of the kits, later confirmed by the manufacturer (Megazyme International Ireland Limited ™), A/A data was excluded from this investigation.

4.1.4 Total protein

Total protein was determined using the Micro-Kjeldahl method carried out by the Department of Animal Sciences at SU. However, it was later established that results from 2006 and 2007 were inconsistent (Botes, W.C. Personal communication. 2009). Thus unfortunately,

protein data could also not be taken into consideration in this investigation. Due to a lack of available samples, protein and A/A assaying could not be repeated.

4.1.5 Ethanol

The fermentation procedure was initially optimized using 2006 MLFT material and Stargen™ 001 RSH enzyme. During 2007, the small grain -directed Stargen™ 002 and Optimash™ BG, a technical enzyme hydrolyzing cellulose and hemi-cellulose, became available. This led to further improvements in mean fermentation efficiency of 0.79 in 2006 versus 0.98 in 2007. Optimash™ BG also hydrolyzes the bran fraction of whole wheat flour to release further fermentable sugars, thus accounting for the observed fermentation efficiency values of over 1.0 evident in the 2007 data set (Botes, W.C. Personal communication. 2009). These factors are borne in mind when comparing the ethanol performance of entries across seasons in this Chapter, i.e. those entries common to both years (Table 1). Nevertheless, the relative entry performance within each season is also meaningful for comparison purposes. Both $l.t^{-1}$ and $l.ha^{-1}$ are in reference to 'actual' yield figures that do not take total starch percentage into account in contrast to 'theoretical' yield ($l.t^{-1}$), i.e. the maximum yield on which fermentation efficiency was determined. The reported actual ethanol yield in both units represents raw values as determined by fermentations converted from small-scale volume and mass to $l.t^{-1}$ and then $l.ha^{-1}$ after taking grain yield data into consideration. Thus, actual $l.t^{-1}$ represents an empirically determined breeder's 'potential' (maximum).

Fermentation data in terms of actual yield in both $l.t^{-1}$ and $l.ha^{-1}$ is illustrated in Figure 15 and 16 below for the 2006 (optimization) and 2007 (GxE data) seasons respectively. In these

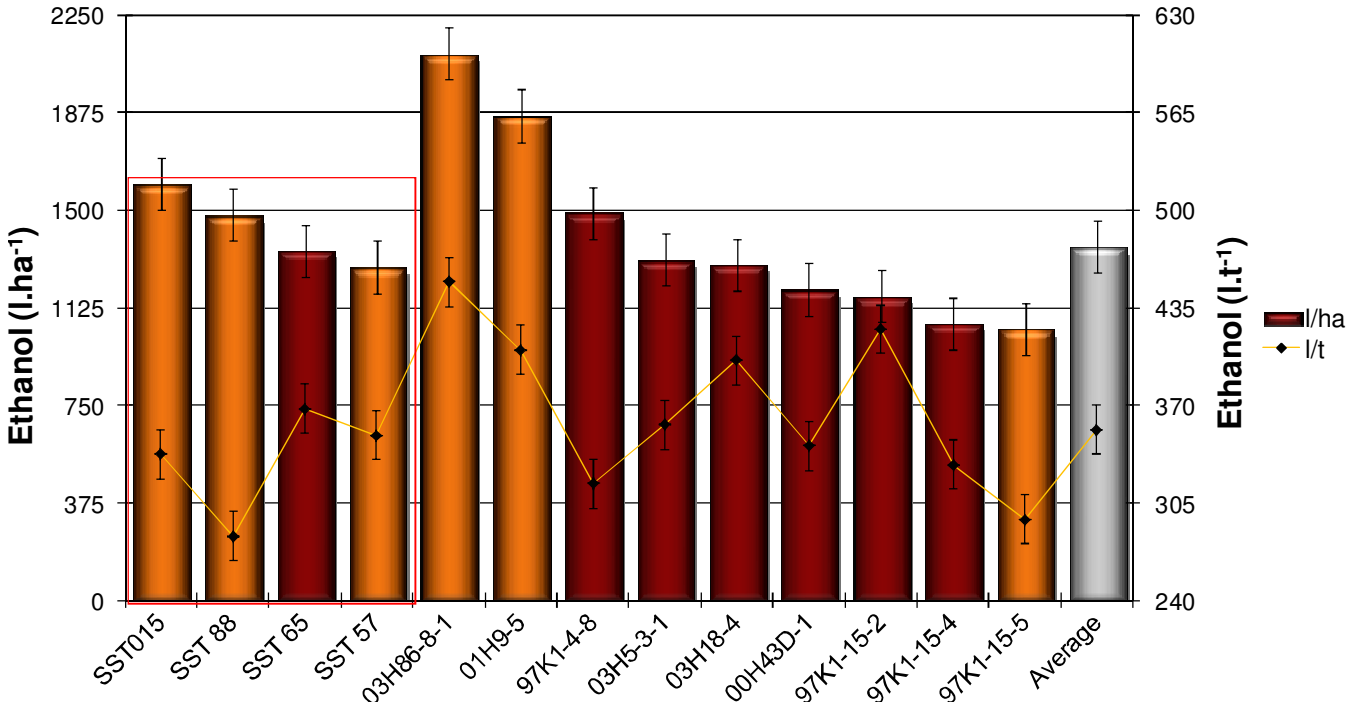

Figure 15. Average ethanol yield ($l.ha^{-1}$ and $l.t^{-1}$) for 2006 entries across all localities (5% LSD indicated with each).

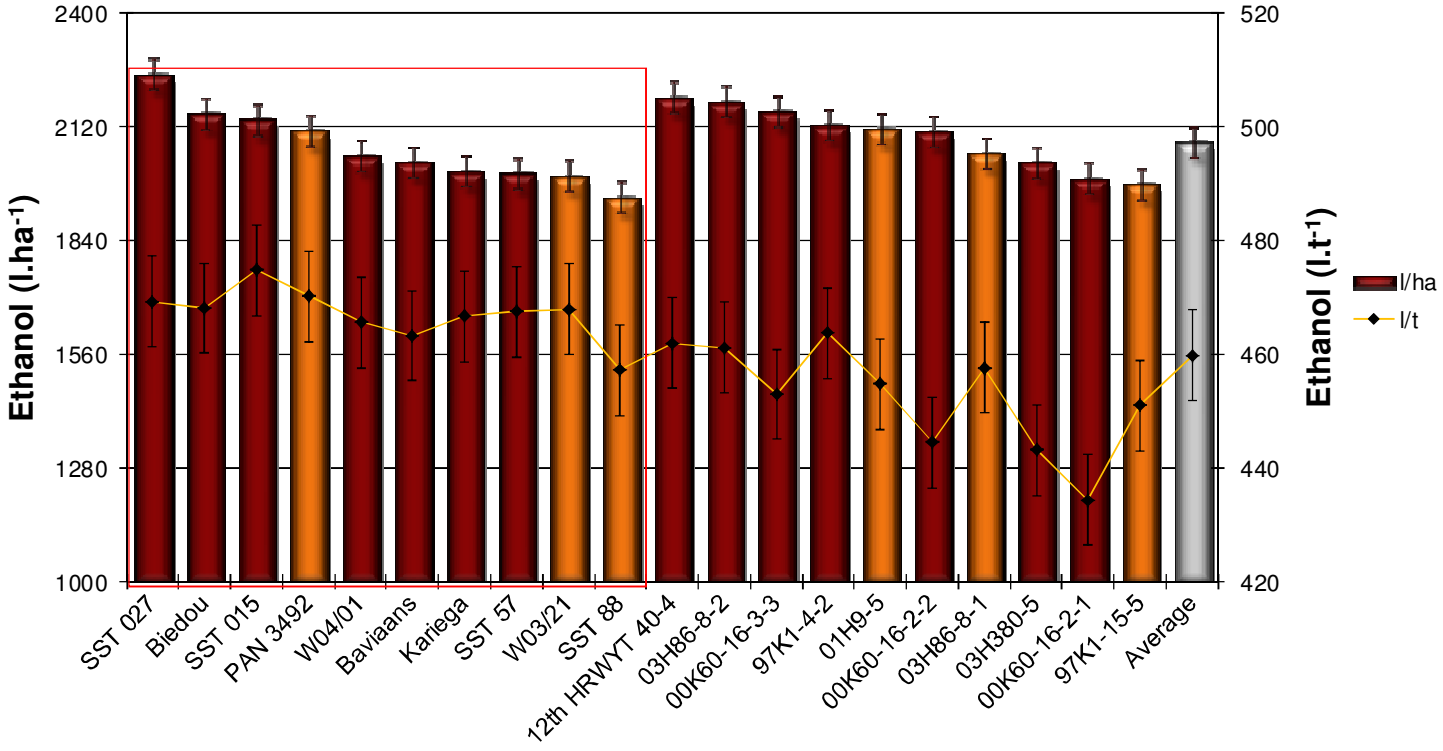

Figure 16. Average ethanol yield (l.ha^{-1} and l.t^{-1}) for 2007 entries across all localities (5% LSD indicated with each).

Figures, entry performance is ranked (bar graph) in terms of l.ha^{-1}, while l.t^{-1} values are given in the same co-ordinate plane (line plot). Both of these measures in 2007 (Figure 16) were calculated across the same localities as for grain yield (Figure 9). In interpreting these graphics, both performance measures are considered in isolation from one another as l.ha^{-1} takes grain yield into account in contrast to l.t^{-1}. Entries are thus interpreted relative to each other within each scale independently. As before, the controls are highlighted apart from the ABLs and the 5% LSD (P≤0.05) is indicated with each entry. In addition, a summary interpretation of the 2007 MLFT fermentation data illustrating best and worst entry performance in each locality as well as regional performance is presented in Table 11 (See in conjunction with Figure 16).

From Table 11 and Figure 16, the ABL 03H86-8-2 was the highest yielding in the Western Cape Province during 2007 in terms of ethanol yield in l.ha^{-1}. Its superior performance was statistically significantly better than six controls (Figure 16). The performance of this line was followed by 00K60-16-3-3 and 97K1-4-2 respectively (Table 11 and Figure 16) which also significantly out-yielded six controls each (Figure 16). No statistically significant differences were recorded between the three top-ranked ABL entries in the Province (Figure 16). As discussed earlier, SST 027 was the highest yielding entry overall in the Province in terms of grain yield (Table 8; Figure 9 and 10). This performance was mirrored with respect to ethanol yield in l.ha^{-1} (Table 11 and Figure 16) where it significantly out-yielded all remaining controls in the Province (Figure 16). In contrast, 97K1-15-5 was the lowest yielding and least adapted ABL entry for both traits in 2007 (Table 8 and 11; Figure 9, 10 and 16) and performed relatively poorly overall in terms of grain yield

Table 11. MLFT summary interpretation for ethanol yield in l.ha^{-1} (green rows) and l.t^{-1} (white rows): 2007.

Region	Location	Mean (l.ha^{-1} & l.t^{-1})	CV (%)	H^2	Best entry	Best 3 ABLs (ranked) 1	2	3	Worst entry	Worst ABL
Swartland	LAN	2092.76±19.36	7.16	0.91	12th HRWYT 40-4	01H9-5	00K60-16-3-3	00K60-16-2-2	97K1-15-5	97K1-15-5
		471.30±4.58	7.53	0.38	W04/01	03H86-8-1	03H86-8-2	97K1-4-2	00K60-16-2-1	00K60-16-2-1
	VRE	1183.33±6.45	4.22	0.97	Biedou	00K60-16-3-3	01H9-5	03H86-8-1	SST88	03H86-8-2
		461.53±2.63	4.41	0.72	SST015	00K60-16-3-3	00K60-16-2-2	97K1-15-5	Baviaans	97K1-4-2
	GxE[a]	1638.05	6.82		12th HRWYT 40-4	01H9-5	00K60-16-3-3	03H86-8-1	97K1-15-5	97K1-15-5
	GxE[b]	466.41	6.20		SST015	00K60-16-3-3	03H86-8-1	03H86-8-2	00K60-16-2-1	00K60-16-2-1
S Cape & Rûens	NAP	2643.54±16.26	4.77	0.90	SST027	00K60-16-3-3	03H86-8-2	00K60-16-2-2	SST88	03H86-8-1
		472.96±2.77	4.53	0.77	SST57	97K1-4-2	03H86-8-1	01H9-5	00K60-16-2-1	00K60-16-2-1
	ALB	2479.57±18.59	5.81	0.83	03H86-8-2	03H86-8-2	00K60-16-2-2	97K1-4-2	SST88	00K60-16-2-1
		444.02±3.31	5.78	0.66	Baviaans	03H86-8-2	01H9-5	97K1-15-5	00K60-16-3-3	00K60-16-3-3
	TYG	2364.05±11.57	3.79	0.95	12 HRWYT 40-4	03H86-8-1	03H86-8-2	97K1-4-2	SST88	01H9-5
		446.68±2.14	3.72	0.73	W03/21	97K1-4-2	03H86-8-2	97K1-15-5	00K60-16-2-1	00K60-16-2-1
	ROO	1742.62±10.36	4.61	0.91	00K60-16-3-3	00K60-16-3-3	03H86-8-2	01H9-5	03H380-5	03H380-5
		462.32±2.75	4.61	0.70	Biedou	97K1-4-2	03H86-8-2	00K60-16-3-3	00K60-16-2-2	00K60-16-2-2
	GxE[c]	2307.45	4.93		03H86-8-2	03H86-8-2	97K1-4-2	00K60-16-2-2	SST88	01H9-5
	GxE[d]	456.50	4.73		Biedou	97K1-4-2	03H86-8-2	01H9-5	00K60-16-2-1	00K60-16-2-1
	GxE[e]	2084.31	5.43		SST027	03H86-8-2	00K60-16-3-3	97K1-4-2	SST88	97K1-15-5
	GxE[f]	459.80	5.28		SST015	97K1-4-2	03H86-8-2	03H86-8-1	00K60-16-2-1	00K60-16-2-1

[a] R^2 = 0.97, entry X location F (P≤0.05) = 10.98 (0.0000)
[b] R^2 = 0.48, entry X location F (P≤0.05) = 1.15 (0.3218)
[c] R^2 = 0.95, entry X location F (P≤0.05) = 8.25 (0.0000)
[d] R^2 = 0.70, entry X location F (P≤0.05) = 1.68 (0.0066)
[e] R^2 = 0.97, entry X location F (P≤0.05) = 10.09 (0.0000)
[f] R^2 = 0.63, entry X location F (P≤0.05) = 1.45 (0.0136)

Means for best ethanol-producing localities in each region (Figure 32)
Blue Best lines within the best ethanol-producing localities and within all (GxE) localities, as well as relative to grain yield (Table 12)
Red As for blue type but with respect to the tail-performance ethanol-producing localities

in 2006 (Figure 8). In addition, it ranked last for ethanol yield in l.ha^{-1} during the optimization of the fermentation process in 2006 (Figure 15). The poor l.ha^{-1} performance of 97K1-15-5 in 2007 was statistically significant relative to four controls (Figure 16). The worst performing entry overall in the Province was SST 88 (Table 11 and Figure 16). Its poor performance in 2007 relative to regions and the Province as a whole regarding grain yield was discussed earlier. Also, its best grain yield performance in 2006 relative to the included controls (Figure 8), translated into better l.ha^{-1} performance during that same year (Figure 15).

The AMMI analysis bi-plot of ethanol yield in l.ha^{-1} from the Western Cape commercial wheat production area is presented in Figure 17 below. All five PCA axes were significant (P≤0.05, AMMI-F) according to the AMMI ANOVA (not shown). IPCA1 explained 48.09% of the observed interaction variance in determining the expected values. As in Figure 10 for grain yield, the genetically superior environments of Napier (C), Albertinia (A) and Tygerhoek (E) are contrasted with Vredenburg (F) and in addition, can be grouped into a mega-environment as before. Similar specific genotype X environment interaction groupings are also evident (Figure 10 versus 17). Roodebloem (D) and Vredenburg both performed below the adjusted locality mean for ethanol yield in l.ha^{-1} (abscissa). The superior ABL 03H86-8-2 (m) and respectively second- and third-ranked 00K60-16-3-3 (s) and 97K1-4-2 (o at r) are illustrated (Figure 17). Mentioned earlier, 12th HRWYT 40-4 (t) was ranked second overall in the Province for ethanol yield in l.ha^{-1} after SST 027 (d), although not statistically significantly different from it (Figure 16). The overall worst performer SST 88 (Table 11 and Figure 16), is located at position b and is not shown (Figure 17). Also apparent from Figure 17, the entries SST 57 (a), SST 027, Kariega (e), Biedou (f), Baviaans (g)

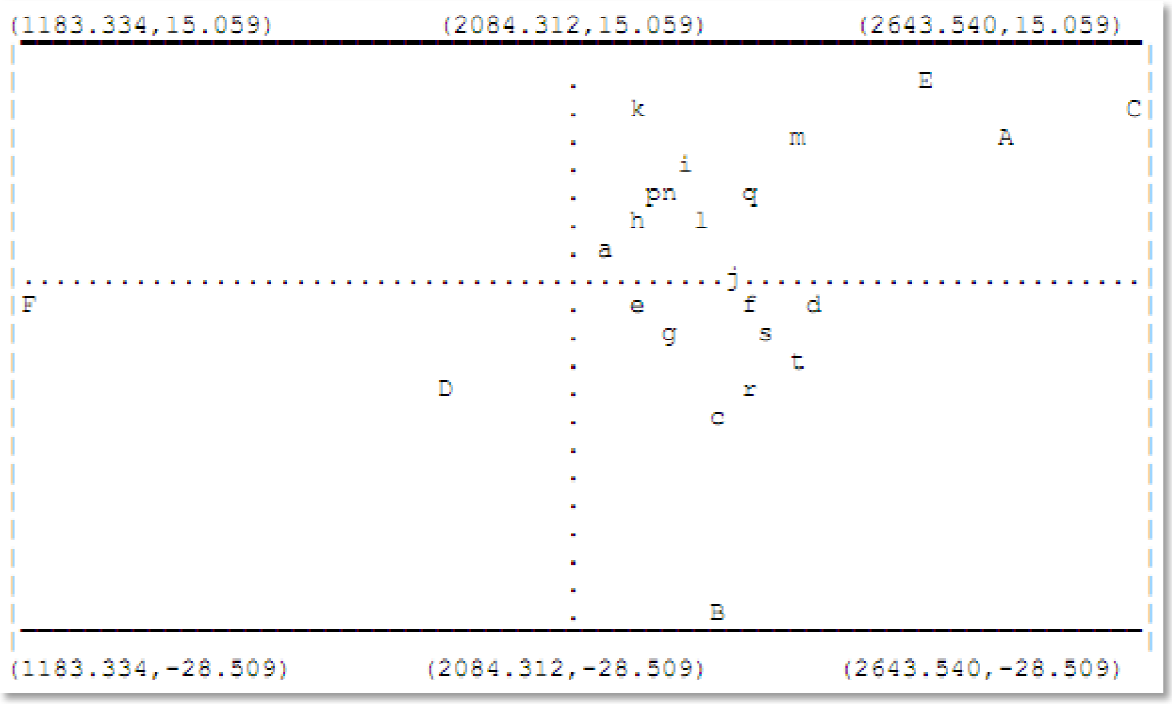

Figure 17. Additive main effects and multiplicative interaction (AMMI) analysis of the 2007 MLFT data for ethanol yield in the Western Cape (l.ha^{-1}).

and PAN 3492 (j) are the most adapted to multi-environments, some with a $l.ha^{-1}$ yield well above the expected mean. Also, none of these six entries are SU-PBL ABLs and none of the trial sites analyzed in 2007 were both stable in terms of their influence on genotypes and simultaneously high yielding (Figure 17). Further, all 2007 trial sites maintained the same top ranking in terms of both grain yield and ethanol yield in $l.ha^{-1}$ (Table 8 versus 11; Figure 10 versus 17). This is expected due to the influence of grain yield on ethanol yield in $l.ha^{-1}$ where ethanol is derived from the grain only. The Nassar-Huehn ranks test (Nassar and Huehn, 1987) ranked SST 015 (c) as the most adapted control and 03H86-8-1 (l), 01H9-5 (r) and 97K1-15-5 (k) as the most adapted SU-PBL ABL entries respectively. According to absolute adjustment values and not taking genotypic yields into consideration, AMMI analysis ranked the best three adapted ABL entries as 03H86-8-1, 00K60-16-3-3 and 00K60-16-2-1 (p) in that order. Similarly, PAN 3492 was ranked the most adapted control.

Considering ethanol yield in $l.t^{-1}$, SST 015 was the highest performing entry overall in the Western Cape (Table 11 and Figure 16). In addition, this was the best overall entry at Vredenburg as well but not in other localities (Table 11). Its performance was statistically significantly better than the control SST 88. SST 015 was also the highest ranked control entry in terms of total starch fraction in 2006 (Figure 13) and the fourth highest ranked in 2007 (Figure 14). Eight of the ten included controls occupied the first eight rankings for ethanol yield in $l.t^{-1}$ in the Province (Figure 16). As mentioned earlier in this Chapter, the controls as a group during 2007 (and 2006) reported higher total starch fractions than the ABLs as a group (Figure 14, also see Figure 13). ABL entries with the highest potential ($l.t^{-1}$) ethanol yield were 97K1-4-2, 03H86-8-2 and 03H86-8-1 respectively (Table 11 and Figure 16). Although not statistically significant (Figure 14), both 97K1-4-2 and 03H86-8-1 ranked second and third respectively with respect to starch in the Province in 2007 (Table 10). The total starch fraction of 03H86-8-2 was below the mean for 2007 (Figure 14) and especially 2006 (Figure 13), but also not statistically significantly better than the mean in either season. There were no significant differences in potential ethanol yield between these three lines and none significantly bettered any of the controls (Figure 16). As mentioned earlier, 03H86-8-1 was ranked first overall ($l.t^{-1}$ and $l.ha^{-1}$) in 2006 during the optimization of fermentation where it significantly out-performed all four controls in that year (Figure 15). This entry's potential for ethanol yield as measured in $l.t^{-1}$ is reflected in the 2007 season as well when compared to that year's set of entries (Figure 16), but not in comparison to the three controls common to both seasons (Figure 15 versus 16). In contrast to Vredenburg, 97K1-4-2 appeared in the top three ABL entries at four out of six individual localities during 2007 and was the best overall entry in terms of $l.t^{-1}$ in the Southern Cape and Rûens in addition to the Province (Table 11 and Figure 16). Similarly, 03H86-8-2 in addition to its second-ranked superior grain yield performance (Table 8; Figure 9 and 10), also appeared in the top three ranked ABL entries for potential ethanol yield at four localities and in both regions (Table 11). This is either in spite of or, possibly because of its mediocre total starch fraction (Figure 13 and 14). As for the Swartland and the Southern Cape /

Rûens, the worst performing ABL and entry overall in the Western Cape was 00K60-16-2-1 (Table 11 and Figure 16). Its $l.t^{-1}$ performance was statistically significantly worse than all ten controls (Figure 16), total starch included (Table 10 and Figure 14). As mentioned earlier, 97K1-15-5 was the overall lowest yielding ABL entry in terms of ethanol in $l.ha^{-1}$ (Table 11 and Figure 16). Its equally lowest ranked grain yield performance (Table 8 and Figure 9) countered its only fourth overall lowest potential ethanol yield in $l.t^{-1}$ (Figure 16). Also, from a starch point of view this entry ranked mediocre to poor in both seasons (Figure 13 and 14). Similarly, the $l.ha^{-1}$ performance of 00K60-16-2-1 (Figure 16) was probably explained by its lowest overall $l.t^{-1}$ rank (Table 11 and Figure 16), despite its only fifth lowest grain yield ranking (Figure 9). As mentioned above, it ranked lowest overall for total starch (Table 10 and Figure 14). A similar relationship was evident regarding the two best performing ABL entries in terms of ethanol yield in $l.ha^{-1}$ i.e. 03H86-8-2 and 00K60-16-3-3 respectively (Table 11 and Figure 16). Their $l.ha^{-1}$ ranking follows their $l.t^{-1}$ ranking (Figure 16) but is the reverse of their grain yield ranking (Figure 9). This relationship is further supported by the marginally higher total starch fraction of 03H86-8-2 versus 00K60-16-3-3 despite statistically insignificant differences between them (Figure 14). As a further example illustrating the relationship between grain yield, ethanol yield in $l.ha^{-1}$, potential ethanol yield in $l.t^{-1}$ and total starch fraction, 97K1-4-2 ranks fourth lowest among the ABLs for grain yield (Figure 9) but highest for potential ethanol yield (Table 11 and Figure 16). As mentioned above, it ranked second highest in the Province for total starch fraction (Figure 14). This probably explains its third highest ABL entry ranking in terms of ethanol yield in $l.ha^{-1}$ as reported in Table 11 and Figure 16. Conversely to the above discussed examples, despite SST 88 returning the lowest potential ethanol yield in $l.t^{-1}$ amongst the controls (in both years), no definite pattern is evident relating grain yield and ethanol yield in $l.t^{-1}$ or $l.ha^{-1}$, nor total starch amongst the four controls in 2006 (Figure 8, 13 and 15).

Similar to ethanol yield in $l.ha^{-1}$, the AMMI bi-plot for ethanol yield in $l.t^{-1}$ in the Province is illustrated in Figure 18 below. IPCA1 accounted for 100% of the observed interaction variance as only the first axis was significant ($P \leq 0.05$). Thus, only genotypic effects played a statistically significant role in entry ranking. The genetic superiority of the locations Langgewens (B) and Napier (C) are contrasted against Albertinia (A) and Tygerhoek (E) (Figure 18). Changes in locality rank are evident in Figure 18 as well as Table12 when comparing potential ethanol yield in $l.t^{-1}$ to yield in $l.ha^{-1}$ (Table 11 and Figure 17). However, Napier remained the highest yielding location in terms of both $l.t^{-1}$ and $l.ha^{-1}$ regarding realized (Table 11) and expected (Figure 17 and 18) values. In contrast to ethanol yield in $l.ha^{-1}$, both Roodebloem (D) and Vredenburg (F) yielded above the expected locality mean (Figure 17 versus 18). Similarly, the opposite is evident with respect to Albertinia and Tygerhoek. Langgewens was both relatively stable and high yielding (Figure 18) but not when considering yield in $l.ha^{-1}$ (Figure 17). Albertinia was the most stable environment concerning $l.ha^{-1}$ (Figure 17) and, the influence of grain yield (Figure 10) on locality stability (Figure 17) is also evident. Different interaction grouping is applicable here in comparison to ethanol yield in $l.ha^{-1}$ (Figure 17), with both Baviaans (g) and 03H86-8-2 (m) being well adapted to Roodebloem,

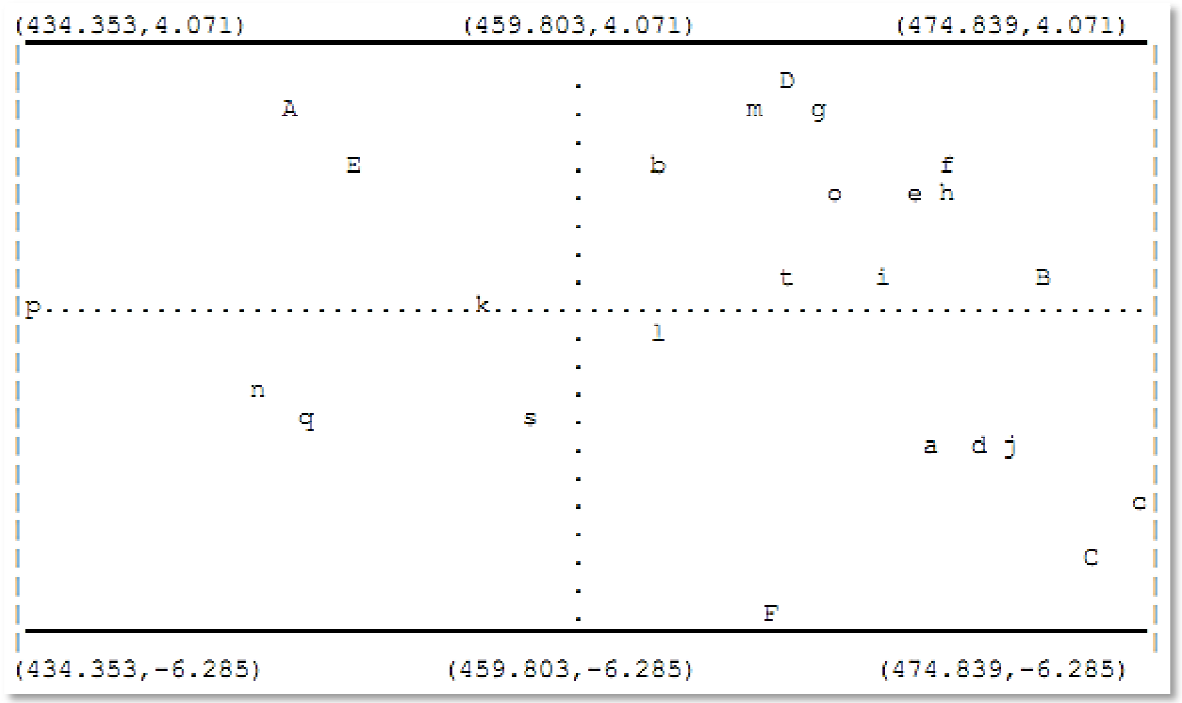

Figure 18. Additive main effects and multiplicative interaction (AMMI) analysis of the 2007 MLFT data for ethanol yield in the Western Cape (l.t^{-1}).

as is SST 015 (c) to Napier for example (Figure 18). As for differences in entry performance both within and across differing environments in terms of l.t^{-1} versus l.ha^{-1}, such differences are expected considering that ethanol yield in l.t^{-1} is influenced by *inter alia* total starch percentage (as is l.ha^{-1}) but not grain yield. Also apparent from Figure 18, is the spread of entries relative to the abscissa. This also reflects the fact that grain yield is not factored into the calculation of ethanol yield in l.t^{-1} (Figure 10 and 17 versus 18). As mentioned earlier, ethanol yield reported in l.t^{-1} represents a breeder's potential value. The highest yielding entry in the Western Cape in terms of yield in l.t^{-1} namely SST 015 (Table 11 and Figure 16) is illustrated in Figure 18. The three first-ranked ABL entries of 97K1-4-2 (o), 03H86-8-2 (m) and 03H86-8-1 (l) are also evident and account for the leading ABLs reported in Table 11 and Figure 16. Considering adaptation alone and according to the Nassar and Huehn (1987) ranks test, 97K1-4-2, 01H9-5 (r) and 03H86-8-2 were respectively the most adapted while SST 88 (b) was the most adapted control. AMMI adjustments revealed 97K1-4-2, 03H86-8-1 and 00K60-16-2-2 (q) as the respectively most adapted ABL entries for l.t^{-1} in the Province, with SST 57 (a) as the most adapted control. The worst performing entry overall in the Western Cape for l.t^{-1} (and starch) was 00K60-16-2-1 (p) (Table 10 and 11; Figure 14, 16 and 18). Eight of the ten controls (a-j) are positioned above the expected mean ethanol yield (Figure 18, see in conjunction with Figure 16). As discussed, this pattern was also observed regarding total starch (Figure 14) and similarly for 2006 (Figure 13). SST 027 (d), PAN 3492 (j) and 12th HRWYT 40-4 (t) were the best entries in terms of multi-environment adaptation and simultaneous above-average yield relative to both l.t^{-1} (Figure 18) and l.ha^{-1} (Figure 17). SST 57, W03/21 (h at a in Figure 17) and Kariega (e) were also adapted in terms of both ethanol measures

but lower yielding. As for ethanol yield in $l.ha^{-1}$, none of these most adapted and simultaneously high yielding entries include SU-PBL ABLs. Of the three leading ABL entries for ethanol yield in $l.ha^{-1}$ (Table 11 and Figure 16), 00K60-16-3-3 (s) was the most adapted in terms of both ethanol yield measures (Figure 17 and 18). However, 03H86-8-2 and 97K1-4-2 (o at r in Figure 17) both had better potential yield measured in $l.t^{-1}$ (Figure 17 versus 18).

In the Swartland, 01H9-5 significantly out-yielded six controls and was ranked the highest yielding ABL entry in this region in terms of $l.ha^{-1}$ (Table 11). Its performance was eclipsed by 12th HRWYT 40-4 as the highest yielding entry overall (Table 11) which significantly out-yielded seven controls. The second- and third-ranked ABL entries in the region were 00K60-16-3-3 and 03H86-8-1 respectively (Table 11). Each yielded statistically significantly better than four and two controls respectively. In addition, 01H9-5 yielded significantly better than 03H86-8-1. 00K60-16-3-3 yielded significantly better than 03H86-8-1 as well. As for the Province as a whole (Table 11; Figure 16 and 17), 97K1-15-5 was recorded as the worst performing ABL entry. In addition, it was the lowest yielding entry overall in the Swartland (Table 11) and yielded statistically significantly less than eight of the ten included controls.

The ABL 00K60-16-3-3 was the highest yielding in the Swartland with respect to $l.t^{-1}$ (Table 11). However, it ranked third lowest amongst the ABL entries in the region for total starch fraction although not statistically significantly worse than any of the included controls. The $l.t^{-1}$ performance of 00K60-16-3-3 in the region was also statistically insignificant. 03H86-8-1 was the second-ranked ABL regarding $l.t^{-1}$ yield (Table 11) and third-ranked for total starch in the region (Table 10). Third-ranked for ethanol yield was 03H86-8-2 (Table 11). Neither of these two runner-up entries was statistically significant regarding their superior $l.t^{-1}$ performance and no significant differences were recorded between the top three ABL entries. As for the Province as a whole, the best $l.t^{-1}$ ethanol performing entry overall was SST 015 (Table 11). It performed statistically significantly better than one control. In common with the Province, 00K60-16-2-1 was ranked as the lowest yielding ABL and entry overall (Table 11). Its poor performance was statistically significantly worse than four controls.

AMMI analysis was also conducted on ethanol yield data for the Swartland to assess stability and adaptation (bi-plot not shown). Considering that only two environments were involved after the culling of trial data following high grain yield CV values (Table 7), the one remaining IPCA axis was significant ($P \leq 0.05$) for both measures of ethanol yield. Of the three leading ABL entries for $l.ha^{-1}$ reported in Table 11 for this region, only second-ranked 00K60-16-3-3 performed above the expected mean for both ethanol measures. This line was also simultaneously relatively adapted compared to the first and third-ranked ABLs, i.e. 01H9-5 and 03H86-8-1 respectively (Table 11). 01H9-5 was however more adapted than 00K60-16-3-3 regarding both measures of ethanol yield but below the expected mean in terms of potential yield in $l.t^{-1}$. 03H86-8-1 performed below the expected mean for both measures and was not as adapted as 01H9-5. SST 015 was not only the highest yielding entry overall in this region, but also well adapted in terms of both $l.t^{-1}$ and

$l.ha^{-1}$. Neither Langgewens nor Vredenburg were stable in terms of their influence on genotypes planted there.

The second highest yielding ABL considering grain yield and the highest yielding considering ethanol yield in $l.ha^{-1}$ in the Western Cape (03H86-8-2), was also the best line in the Southern Cape and Rûens in terms of both grain yield and ethanol yield in $l.ha^{-1}$ (Table 11). Second- and third-ranked ABL entries in this region were 97K1-4-2 and 00K60-16-2-2 respectively (Table 11). These top-ranked lines performed statistically significantly better than nine, seven and seven controls respectively. 03H86-8-2 also significantly out-performed both ABL runners-up in this region. The most adapted ABL to the Swartland i.e. 01H9-5 was ranked last while the worst entry overall in the Southern Cape and Rûens was SST 88 (Table 11). The poor performance of these two entries was statistically significantly worse than three and nine controls respectively.

The best $l.t^{-1}$ ABL entry in the Western Cape i.e. 97K1-4-2 also performed best overall in the Southern Cape and Rûens (Table 11). However, it was statistically insignificant regarding better performance. Also, this line ranked third among the ABL entries for total starch in this region during 2007 (Table 10). Second- and third-ranked ABL ethanol performers were 03H86-8-2 and 01H9-5 respectively (Table 11). Their superior $l.t^{-1}$ yield was also statistically insignificant and no significant differences were recorded between the top-ranked three ABLs in this region. Also, the total starch fraction of 01H9-5 ranked first amongst the ABL entries for the region (Table 10). Although as mentioned earlier, it was not statistically significantly better than the included controls during 2007. In common with the Western Cape as well as the Swartland multi-environments, 00K60-16-2-1 was the lowest yielding line and entry overall (Table 11). It performed significantly worse than all ten controls.

The first AMMI analysis axis (IPCA1) explained 61.12% and 49.24% of the observed interaction variance for ethanol yield in $l.t^{-1}$ and $l.ha^{-1}$ respectively for the Southern Cape and Rûens. Also, three and two axes were significant ($P \leq 0.05$) for each respective ethanol measurement. The bi-plot (not shown) revealed a similar skewed spread of the entries relevant to the expected mean (abscissa) as observed for the Province as a whole, in terms of $l.t^{-1}$ (Figure 18) versus $l.ha^{-1}$ (Figure 17). This is expected with more trial sites from the Southern Cape / Rûens region representing the Province after data culling (Table 7). The Swartland however revealed a more similar $l.t^{-1}$ and $l.ha^{-1}$ entry spread. Firm conclusions cannot be made with so few sites representing the Swartland in comparison to the Southern Cape / Rûens. Among the locations representing the Southern Cape / Rûens, Napier was both stable and well above the expected mean for environments in terms of $l.ha^{-1}$ but the opposite regarding stability when considering ethanol yield in $l.t^{-1}$. It was still the highest ranked location in terms of potential ethanol yield in this region (Table 11). Of the three best $l.ha^{-1}$ ABL entries reported for the Southern Cape / Rûens (Table 11), 00K60-16-2-2 was the most adapted to multi-environments. Its potential ethanol yield in $l.t^{-1}$ was well below the expected mean for environments however, ranking third last overall. Both 03H86-8-2 and 97K1-4-2 performed above the expected mean for both ethanol measures with

97K1-4-2 being better adapted regarding $l.t^{-1}$ and $l.ha^{-1}$. Biedou followed by W04/01, was also well adapted to the region and simultaneously high yielding relative to the expected mean especially for ethanol yield in $l.ha^{-1}$.

In addition to Figure 18 and 17 illustrating the relative stability and expected performance in $l.t^{-1}$ and $l.ha^{-1}$ respectively of individual trial sites in relation to entries, Figure 19 below summarizes locality performance in the Province during 2007 where localities are colour-coded for each region. Welgevallen is also included for comparison. Napier and Langgewens were the two best $l.ha^{-1}$ performing trial sites in the Southern Cape / Rûens and Swartland regions respectively (Table 11; Figure 17 and 19). However, the performance of Langgewens was average for the Province while Welgevallen was the highest yielding trial site overall (Figure 19). In addition, most sites were significantly different from each other in respect of ethanol yield in $l.ha^{-1}$ versus fewer significant differences in $l.t^{-1}$ (Figure 19). This is expected considering that the Western Cape is environmentally diverse and the influence of those environmental differences on grain yield, not only at the genotype level but with regards to all entries at a specific location. Both Napier and Langgewens were also the best sites in terms of potential ethanol yield in $l.t^{-1}$ (Table 11; Figure 18 and 19). Although few sites were included overall after the culling of trial data for high grain yield CV values particularly in the Swartland (Table 7), a definite trend in locality performance between ethanol yield in $l.t^{-1}$ and $l.ha^{-1}$ was not evident (Figure 19). This was also true when taking locality-averaged starch data (not shown) into consideration, although such data was not replicated. Also, the situation regarding localities mirrors some individual entries, although not all as discussed earlier (Figure 13 versus 15 and 14 versus 16).

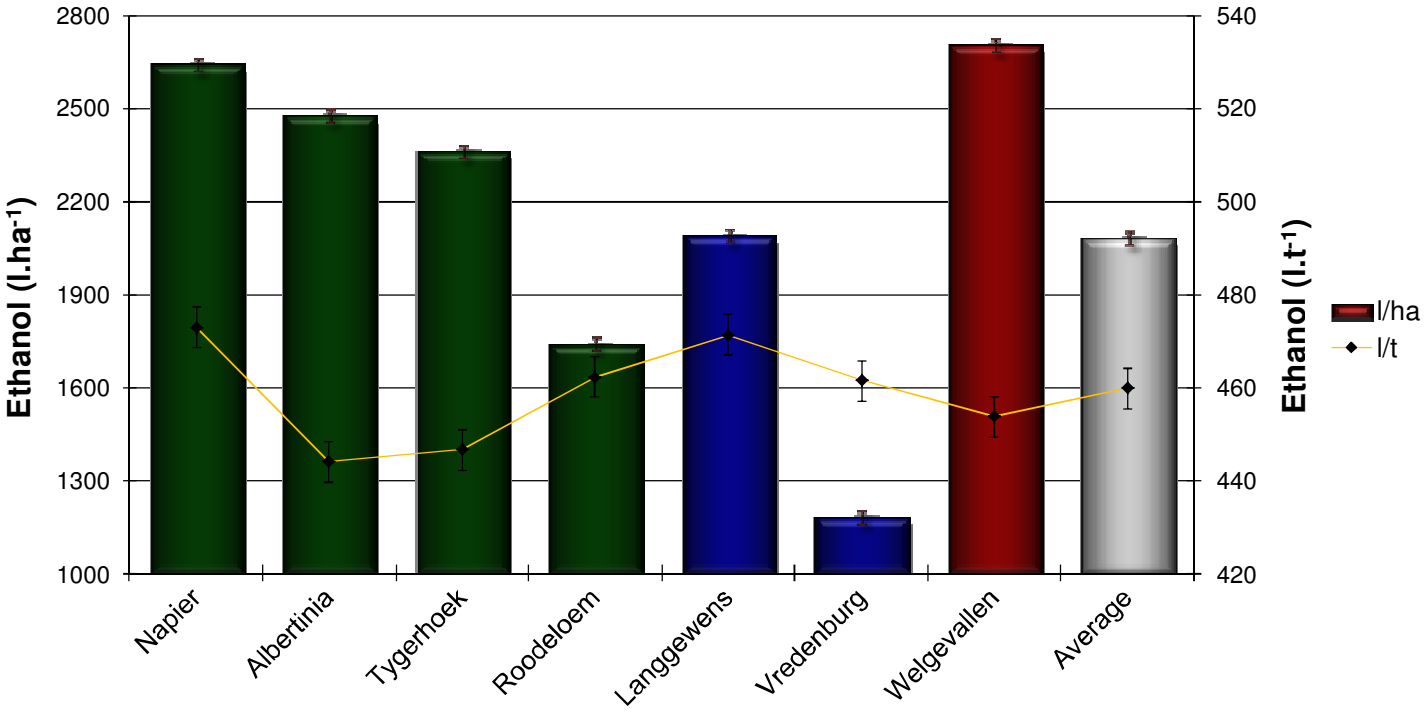

Figure 19. **Average ethanol yield ($l.ha^{-1}$ and $l.t^{-1}$) per locality for 2007** (5% LSD indicated with each).

4.1.6 MLFT summary interpretation and recommendations

Table 8 and 11 summarize interaction between entries and localities for grain yield and ethanol yield respectively. Within the Province, only the Swartland reported no statistically significant interaction (P=0.1334, P≤0.05) between the said main effects with regards to grain yield (Table 8). However, significant interaction (P=0.000, P≤0.05) was reported from the Swartland with respect to ethanol yield in $l.ha^{-1}$ (Table 11). In terms of $l.t^{-1}$, interaction was also statistically insignificant (P=0.3218, P≤0.05) in this region (Table 11). In all other multi-environments relating to grain yield and ethanol yield in both measures (Table 8 and 11), interaction was statistically significant (P≤0.05). In the Swartland and considering grain yield in 2007, localities were statistically significantly different from each other (P=0.0000) while entry differences were insignificant (P=0.1999). The main effects of entry and locality were both insignificantly different with regards to ethanol yield in $l.t^{-1}$ in the Swartland. The respective reported P-values (P≤0.05) were 0.4227 and 0.4738. Significant interaction is expected in the multi-environments considered, bearing in mind that the Western Cape is environmentally diverse even within its two sub-regions and in addition, the effect of environment on the traits measured. According to the heritability values reported in Table 7, genotypic variance had a strong influence on the observed differences between entries whether they were significant or not, particularly for ethanol yield measured in $l.ha^{-1}$. Had grain yield CV values not been as high as reported in Table 7, the inclusion of more trial sites in the Swartland may have given a different reflection of interaction in this region of the Province.

Despite the superior $l.ha^{-1}$ performance of 03H86-8-2 in the Province (Table 11; Figure 16 and 17), second-ranked 00K60-16-3-3 (Table 11) is recommended as the simultaneously best adapted and highest yielding SU-PBL ABL entry for ethanol yield in $l.ha^{-1}$ in the Western Cape (Figure 17). Its performance was not statistically significantly less than 03H86-8-2 and both of these lines significantly out-performed six controls each (Figure 16). As mentioned earlier, of the three leading ABL for $l.ha^{-1}$ yield (Table 11 and Figure 16), 00K60-16-3-3 was the most adapted in terms of both ethanol measures (Figure 17 and 18). However, 03H86-8-2 and 97K1-4-2 (o at r in Figure 17) both had better potential yield measured in $l.t^{-1}$ (Figure 17 versus 18). 97K-1-4-2 ranked third for $l.ha^{-1}$ (Table 11 and Figure 16) and also statistically significantly out-performed six controls and was not significantly different from 03H86-8-2 (Figure 16). 03H86-8-1 is the second recommended SU-PBL ABL for the Western Cape. It also performed above the expected mean for yield in $l.ha^{-1}$ (Figure 17). However, this line was also the second most adapted to the Province according to AMMI analysis and the most adapted ABL according to the Nassar and Huehn (1987) ranks test. In addition, it was also the second most adapted ABL entry according to AMMI adjustments considering potential yield in $l.t^{-1}$. This line also ranked first overall for both ethanol measures in 2006 during the optimization of fermentation (Figure 15). Also highlighted earlier, the controls SST 027, PAN 3492 and the CIMMYT-derived entry 12th HRWYT 40-4 were also best adapted and simultaneously high yielding regarding both measures of ethanol yield in the Province

(Figure 17 and 18). SST 027 statistically significantly out-performed all the remaining controls for ethanol yield in $l.ha^{-1}$ (Figure 16). Considering localities, Napier was the best performing location in the Province in terms of ethanol yield in both $l.t^{-1}$ and $l.ha^{-1}$ (Table 11 and Figure 19). Its genetic superiority is also reflected in Figure 17 and 18 regarding expected values but it was not as stable as Albertinia regarding yield in $l.ha^{-1}$ (Figure 17). As mentioned though, none of the six trial sites were both stable in terms of their influence on genotypes and simultaneously high yielding (Figure 17). In reference to Table 8 and 11 and in consideration of all entries included in both 2006 and 2007 (Table 1) as well as $l.t^{-1}$ data from the fermentations (Table 11; Figure 15 and 16), the 03H86-8 and 00K60-16 half-sib families produced the best ethanol performers generally speaking. 97K1-4 and 01H9-5 are also promising sources of selection.

Although only two localities represented the Swartland region, second-ranked $l.ha^{-1}$ performer 00K60-16-3-3 (Table 11) yielded above the expected mean for both ethanol measures. This line was also simultaneously relatively adapted compared to the first and third-ranked 01H9-5 and 03H86-8-1 respectively (Table 11). 01H9-5 was however more adapted regarding both measures but below the expected mean in terms for yield in $l.t^{-1}$. SST 015 was the highest yielding entry overall and well adapted in terms of $l.t^{-1}$ and $l.ha^{-1}$. In spite of the superior genotypic quality of Langgewens (Figure 17 and 18), neither this location nor Vredenburg were stable in terms of their influence on genotypes planted there.

Napier was both stable and well above the expected mean for environments in the Southern Cape / Rûens in terms of $l.ha^{-1}$ but the opposite regarding stability when considering ethanol yield in $l.t^{-1}$. Third-ranked ($l.ha^{-1}$) 00K60-16-2-2 (Table 11) was the most adapted to multi-environments in this region and simultaneously high yielding. Its $l.t^{-1}$ performance was well below the expected mean for environments however. Second-ranked 97K1-4-2 (Table 11) performed above the expected mean for both ethanol measures and was better adapted regarding both measures than the best performing $l.ha^{-1}$ ABL entry i.e. 03H86-8-2 (Table 11). Biedou was also well adapted to the region and simultaneously high yielding especially for ethanol yield in $l.ha^{-1}$.

Ignoring statistically significant differences and only considering the ranked performance of all entries in 2007, MLFT data is summarized in Table 12. This Table also illustrates the skewness of entry ranking relative to the mean. It is evident that no trends exist in relating the measured traits that can be applied to all entries individually. As highlighted earlier though, the controls as a group had higher $l.t^{-1}$ ethanol (Figure 16) and starch (Figure 14, also see Figure 13) values compared to the ABLs as a group (Table 12). This relationship was also revealed when considering total starch fraction and ethanol in $l.t^{-1}$ calculated over all ten harvested localities for 2007 (not shown) as both of these parameters are independent of grain yield. The $l.t^{-1}$ performance of entries common to both seasons (Table 1; Figure 15 and 16) were also compared bearing in mind the discussed difference in RSH enzyme plus the addition of technical enzyme in 2007. Also, a largely different set of entries was compared between the two seasons as a reference point. As a result of differences in enzymes used mentioned earlier, the fermentation efficiencies of entries

Table 12. Ranked MLFT data (GxE) summary performance for 2007 entries.

Grain yield	Total starch	Ethanol (l.ha^{-1})	Ethanol (l.t^{-1})
SST 027	SST 57	SST 027	SST 015
00K60-16-3-3	SST 88	12th HRWYT 40-4	PAN 3492
12th HRWYT 40-4	W03/21	03H86-8-2	SST 027
03H86-8-2	SST 015	00K60-16-3-3	Biedou
Biedou	Biedou	Biedou	W03/21
01H9-5	Kariega	SST 015	SST 57
03H380-5	01H9-5	97K1-4-2	Kariega
00K60-16-2-1	97K1-4-2	01H9-5	W04/01
SST 015	03H86-8-1	PAN 3492	97K1-4-2
97K1-4-2	SST 027	00K60-16-2-2	Baviaans
PAN 3492	12th HRWYT 40-4	03H86-8-1	12th HRWYT 40-4
03H86-8-1	W04/01	W04/01	03H86-8-2
W04/01	Baviaans	Baviaans	03H86-8-1
00K60-16-2-2	03H380-5	03H380-5	SST 88
97K1-15-5	PAN 3492	Kariega	01H9-5
Baviaans	03H86-8-2	SST 57	00K60-16-3-3
Kariega	00K60-16-3-3	W03/21	97K1-15-5
W03/21	97K1-15-5	00K60-16-2-1	00K60-16-2-2
SST 57	00K60-16-2-2	97K1-15-5	03H380-5
SST 88	00K60-16-2-1	SST 88	00K60-16-2-1

Red	SU-PBL breeding lines
Blue	CIMMYT entry
Black	Controls
= border	Mean

relative to theoretical maximum yield in l.t^{-1} differed greatly between the seasons, thus confounding comparisons between the two years. What the data does consistently reveal though, is the clear influence that grain yield has on ethanol production in l.ha^{-1} where such production is derived from the grain only. This is illustrated in Figure 20, which shows that for a given genotype it is more meaningful to pursue increased ethanol yield through increasing (and securing) grain yield. It is evident from Table 8, 11 and 12 in conjunction with Figure 8, 9, 10, 13, 14, 15 16, 17 and 18 relating 2006 and 2007 MLFT data, that certain individual entries and the controls versus ABLs as a group cluster toward the top and bottom of the rankings for each considered trait. In theory, an ideal bio-ethanol -directed wheat genotype would aim to combine high grain yield, high total starch fraction and high potential ethanol yield in l.t^{-1}. As far as multi-environment adaptation is concerned, SST 027 comes closest to this ideal (Table 12). Such a suite of MLFT-proven characteristics, preferably empirically established/confirmed over two or more seasons, should produce top ethanol performers relative to competing genotypes. The influence of grain yield on realized ethanol yield in l.ha^{-1} is especially relevant in an environmentally diverse region such as the Western Cape. Thus, cross-over -type GxE where the ranking of genotypes changes with differing environments is expected as was observed in this investigation (Ceccarelli, 1996). Individual genotypes have differing ethanol-influencing parameter values relative to each other as

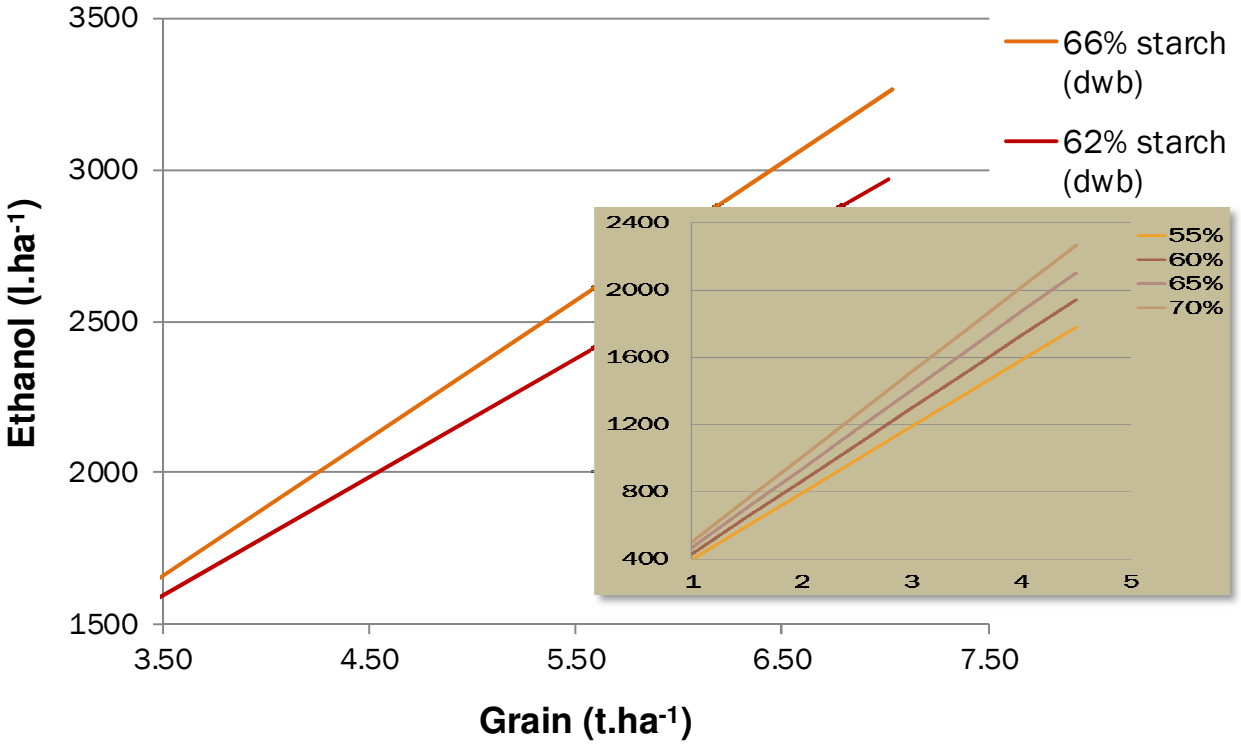

Figure 20. Average theoretical ethanol versus grain yield regression over localities for entries with differing total starch content (triticale determined by SU-PBL).

Wheat

discussed. No doubt, these differences extend to amylose fraction and total protein as well. Patterns in relating the measured traits were discussed and revealed concerning: 97K1-4-2; SST 015; 03H86-8-1, 03H86-8-2; 00K60-16-3-3; 00K60-16-2-1 and; 97K1-15-5 as examples. In other entries such as SST 88, no trends were evident. Wheat material at an advanced stage of selection is expected to demonstrate less statistically significant differences regarding certain traits. This makes the elucidation of trends in the data more difficult. However and especially in an environmentally diverse region, individual genotypes and locations will perform differently and thus have to be assessed for ethanol yield using MLFTs. In this scenario, both specific and multi-environment adaptation is expected as was reported in this investigation.

Triticale

4.2 Pre-breeding programme

4.2.1 Bulked male parent population

The frequencies of the genes of interest (Table 2) in the bulked male parent population are presented in Table 13 below. *Lr24* and *Lr37* were the most frequently occurring (63.0% and 2.9% respectively) inclusive of their mutual occurrence (13%). Feint banding for *Lr24* (719bp) is also evident in Figure 21 versus that for *Lr37* (259bp). This is likely due to primer interaction and/or non-specific binding under multiplex conditions.

Table 13. Frequencies of major rust resistance genes of interest as determined in the bulked male parent population.

Gene	*Lr24*	*Lr37*	*Sr26*	*Lr19*	*Sr31*	*Sr2*
Lr24	63.00					
Lr37	13.00	2.90				
Sr26	0.00	0.00	0.00			
Lr19	0.03	0.00	0.00	0.01		
Sr31	0.14	0.02	0.00	0.00	0.20	
Sr2	0.15	0.04	0.00	0.01	0.07	0.21

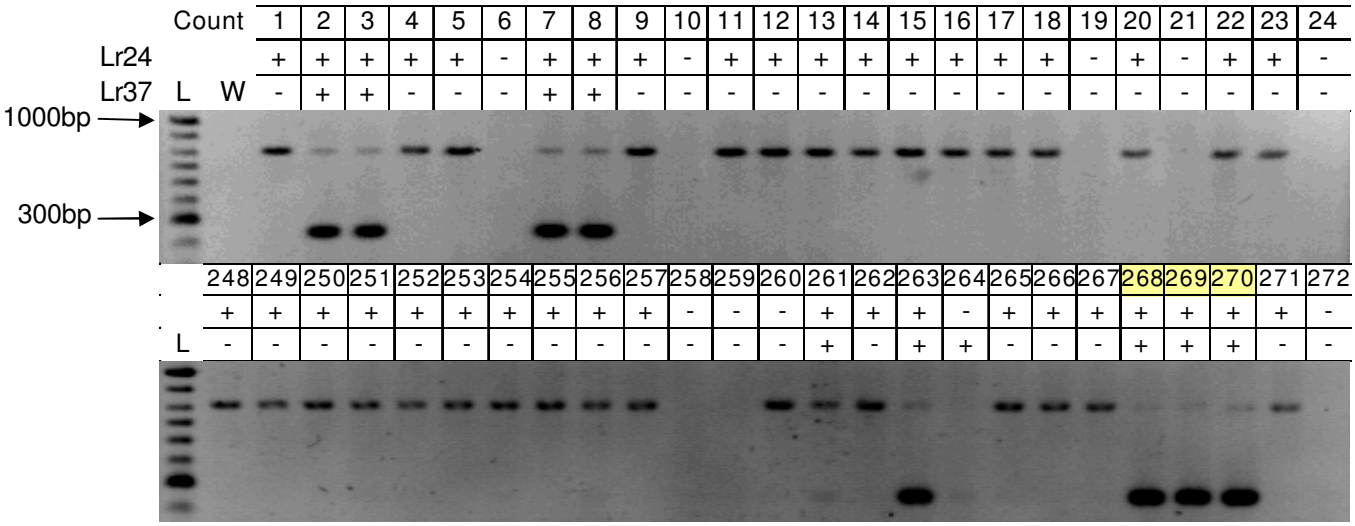

Figure 21. MAS applied to the bulked male parent population: *Lr24* and *Lr37* (part of the sample set with highlighted entries later confirmed on a separate gel).

4.2.2 Doubled haploids

Initially, the pollen donor only reached anthesis (growth chamber) a short while after the random cross progeny (cooled greenhouse). As a result, primary wheat tillers were cut back in anticipation of the revised anthesis date. Haploid plant production was therefore carried out on the secondary tillers only.

During ploidy determination, the root tips became very soft in terms of handling after their incubation in the fixing solution, regardless of how long this incubation period was. This especially affected the handling of the root tips during the later incubation in 60°C HCl solution in which repeated handling (transfers) was necessary. The potential difficulty lay in ensuring that all the root tips spent only 7½min in the acid solution, bearing in mind that each vial (corresponding with each individual plant) contained up to several root tips. In addition, the use of forceps limits the operator's ability to transfer a number of tips more or less simultaneously. It was noticed during validation of the procedure that root tips would readily break when handled with forceps especially when time pressure was applied to transfer a number of tips simultaneously during the HCl

incubation step. This severely limited maximum throughput. Before HCl treatment, the sdH_2O (30min rinsing step) was drained off using a 1ml pipette. Immediately, the vial of root tips (rinsing water drained off) was placed in a heating block for about 30s alongside heated HCl in a separate vial corresponding with each plant label. In this manner, the glass vial containing the tips was pre-warmed just briefly enough without harming the roots before decanting the corresponding vial of HCl into it. The vial of root tips plus heated HCl was immediately returned to the heating block for the required 7½min. In this manner, physical handling of the brittle tips was avoided. Also, the start point of the HCl treatment step was exactly the same for every tip from a given plant (vial). Using two timing devices simultaneously, the second vial of tips was pre-warmed in the heating block for about 30s and treated in the same manner. Immediately after the 7½min acid incubation, two sequential sdH_2O rinsing steps were required of one to two minutes each to halt the hydrolysis of the DNA by the acid. Using one of the two timing devices, the HCl solution was drained from the vial using a filter-tipped 1ml pipette, after which the rinsing sdH_2O was added immediately. It was therefore known that the start of the acid-treatment step was about 30s apart for each vial. This presented a 30s window period at the end of the 7½min incubation to drain off the acid and replace it with rinsing water for each vial in-turn that entered the incubation period at 30s intervals (after pre-warming). If the root tips spent a little longer in the rinsing water this was not considered critical unlike ensuring a precise and controlled acid-treatment step. The 7½min acid incubation period comfortably permitted 12-14 vials to be treated sequentially at 30s intervals in the manner just described while avoiding the physical handling of brittle root tips one-by-one and, ensuring a precise start and end-point to this influential step.

Molecular marker -based MAS was applied to the progeny of the random parent cross (Figure 6), in addition to the bulked male parent population. This was done to track gene occurrence for the purposes of selection in the 41 cross progeny as it was known that they carried at least two of the genes of interest (Table 2). Secondly, MAS was later applied to confirm/validate the rust resistance allele status of the haploid end-products themselves. Differing from the application of MAS to the bulked male parent population (Figure 6), *Lr34* was included in the screening of haploid plants. A suitably validated (by SU-PBL) protocol applicable to this gene complex was only published in the literature shortly before. The multiplexing of the genes of interest (Table 2) had also been established by SU-PBL.

In contrast to the bulked male parent population and using a separate primer set (Table 4 versus 2), differing positive controls for the *Lr24/Sr24* complex were included. Although still scorable, *Lr34* produced disappointing results when multiplexed (Table 5), despite prior optimization by SU-PBL at the time using the same primer set (Table 2), positive control and PCR components. Also, for reasons that could not be identified at the time that this stage of the project was reached, the PCR protocol for *Sr31* (Table 6) repeatedly failed in its application to both the 41 random cross progeny as well as the haploid end-products. This was separately confirmed by SU-

PBL. Before this, the protocol had produced good results where differing concentrations of the primer set (Table 2) were tested during validation.

The multiplexed MAS protocol for *Lr19*, *Lr24* and *Lr37* (Table 4) was applied to the 41 random cross progeny (Figure 22). In addition to the mentioned failure of the *Sr31* PCR protocol (Table 6), these plants were not scored for the remaining two *Sr* genes (Table 2) as a decision was taken that sufficient information was present for selection purposes. It was also decided to attempt haploid plant production from as many of the 41 individuals as possible due to a relatively low percentage of green plants regenerated during prior protocol testing. Also, secondary tillers were being used. It is known that fewer secondary tillers are produced in contrast to primary tillers.

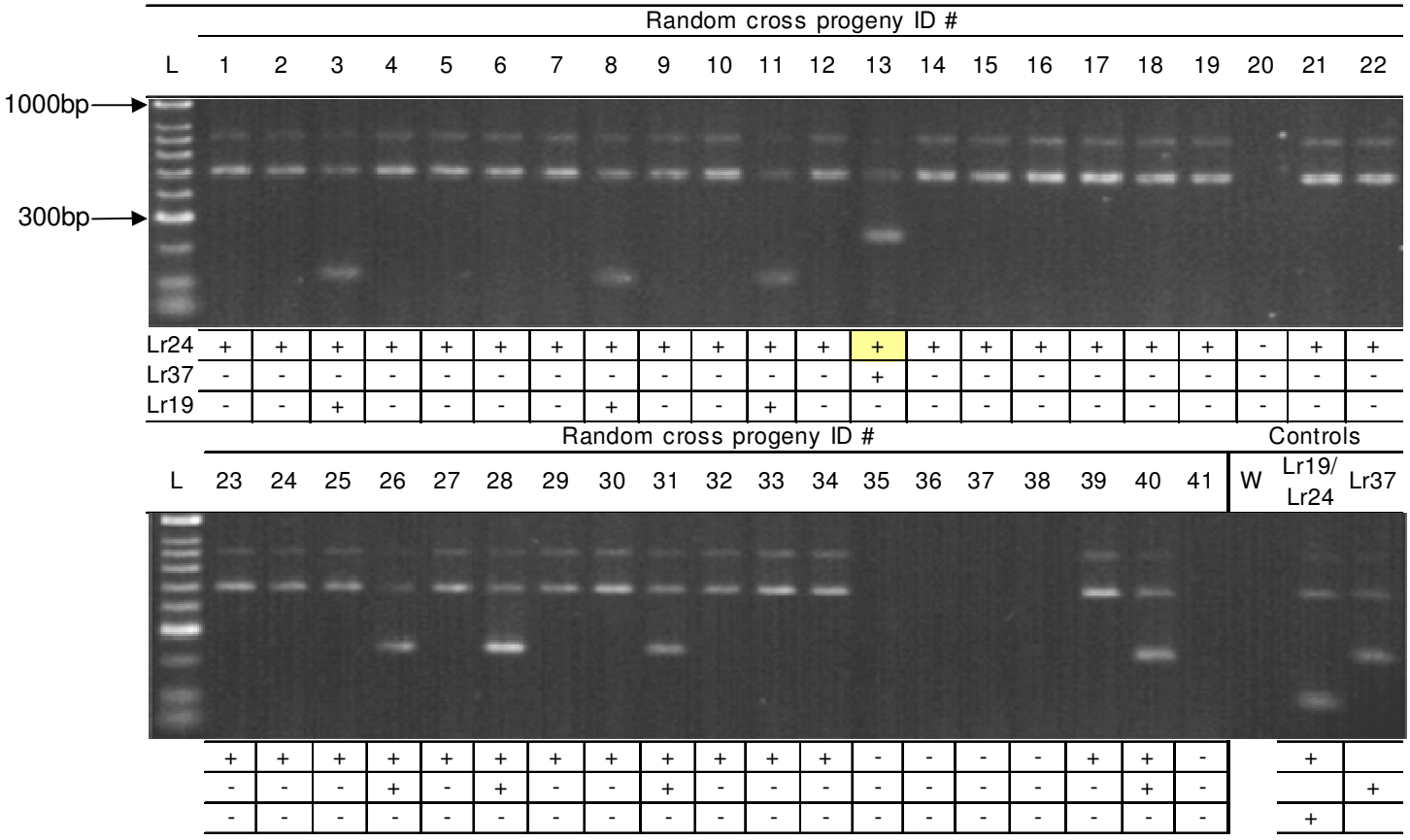

Figure 22. MAS applied to the parental cross progeny: *Lr19*, *Lr24* and *Lr37*.

Table 14 below provides a summary of the wide-cross DH results. These plants were not doubled with in chromosome number due to time constraints at the end of the project. Very little contamination was recorded i.e. within two tissue culture jars only, plus a third that was transferred to the growth chamber with regenerating embryos (green plants). Possible recalcitrant genotype issues aside, the results closely mirrored those from the prior testing of the procedure. This was in spite of minor procedural improvements gained through experience. The most significant similarity was the very low number of regenerated green plants i.e. only nine in total (Table 14). Although substantial in number, room also exists for further improvement in the number of GPCs recovered

Table 14. Summary of haploid data for the parental cross progeny.

Random cross progeny ID #	Spikes treated	Florets pollinated	Seeds set	GPCs recovered	Embryos rescued	Green plants regenerated	Remarks
1	3	54	49	22	1	0	
2	1	24	23	20	1	0	
3	1	27	26	13	2	0	
4	1	22	19	8	1	0	
5	3	76	65	32	3	0	all formed roots only
6	2	51	42	21	5	1	4 formed roots only
7	1	26	20	11	5	0	4 formed roots only
9	1	14	10	2	0	0	
10	4	88	73	39	17	2	6 formed roots only
11	3	65	59	20	5	0	all formed roots only
12	2	44	41	17	4	1	2 formed roots only
13	1	28	23	12	5	1	green plant died + 2 formed roots only
14	2	52	43	24	3	0	
15	1	28	26	19	6	0	1 jar of 4 embryo's contaminated
16	1	24	21	11	0	0	
17	1	23	23	8	0	0	
19	1	14	12	9	4	0	contamination
20	1	28	25	20	1	0	
21	3	74	70	39	2	0	
22	1	26	21	15	0	0	
23	1	20	15	11	0	0	
24	1	24	21	12	2	0	1 formed roots only
25	1	28	22	14	0	0	
26	3	85	72	38	5	0	1 formed roots only
27	2	50	43	25	11	1	8 formed roots only
28	1	26	8	6	2	0	all formed roots only
29	1	32	29	14	0	0	
30	1	26	25	11	0	0	
31	1	19	17	14	10	3	1 green plant died + 3 formed roots only
32	1	24	22	0	0	0	
36	1	26	21	12	0	0	
40	1	28	25	10	1	0	formed roots only
Totals:	**49**	**1176**	**1011**	**529**	**95**	**9**	

and embryos rescued therefrom (Table 14). After much practice, it was evident whether or not embryos were present in a GPC or not, so this was not considered a likely cause. Also, fresh pollen was always used and 30h hormone application timing was strictly adhered to. These results were howeverbased on secondary and not primary tillers as was much of the work done during protocol testing. Due to their morphology, some secondary tillers were practically unusable regarding emasculation. Also mirroring earlier protocol testing results (not shown), a large proportion of the embryos produced roots only, while albino shoot production was also evident (Table 14). Very few rescued embryos were likely dead (unviable) as the vast majority responded (grew) to the ERM medium in the given culture conditions, yet not in the form of green plant regeneration. According to Elsabet Wessels (SU-PBL) (Personal communication. 2009), several areas exist for improvement in the procedure, some minor and relating to protocol execution and others likely influential on the success rate measured in green plant regeneration. These include later (one day) emasculated floret pollination at a slightly more advanced stage of stigma development. The use of a different maize (sweetcorn) cultivar was also mentioned. Also and immediately after embryo rescue, jars should be held at 4℃ in the dark for three to four days and then transferred to the growth chamber for shoot development. In this work, jars were incubated in the dark at ambient (21℃) temperature for 10-12 days until shoots developed and only then transferred. Maternal parents (wheat) should also be held in a greenhouse at exactly 18℃ prior to emasculation up until the GPCs are removed. Hormone application can also occur at 28h versus 30h. Finally, smaller embryos (without flattened appendages characteristic of 'too old' embryos) i.e. those rescued at 13-14 days post- hormone application were also said to be more viable. It is also preferable to use tubes to culture single embryos versus four to five cultured in a jar. If contamination is encountered, only one potential green plant is lost. In addition, usually not all plantlets in a single jar are simultaneously large enough to plant over to pots, necessitating repeated sterile removal as the plants develop.

The results of MAS application to the nine regenerated individuals is presented in Figure 23 below. Three individuals were derived from a single embryo (Figure 23), the third of which lagged well behind the first two in terms of morphological development. Hence, less DNA could be extracted from this individual as is evident from the resultant band intensity (Figure 23). The haploid (*n=21*) status of eight of the nine regenerated individuals was unambiguously established (Figure 24) where 21 chromosomes are clearly visible. No suitable seminal root tips were available on the smallest plant (individual #12(3)) after cutting the roots back and allowing them to grow out. Ploidy determination had to be carried out under narrow time constraints and only a very small developing seminal root close to the base of this plantlet could be identified. In conjunction with Figure 23, a summary of the rust resistance gene status of eight of the nine regenerated and confirmed haploid status plants is depicted in Table 15.

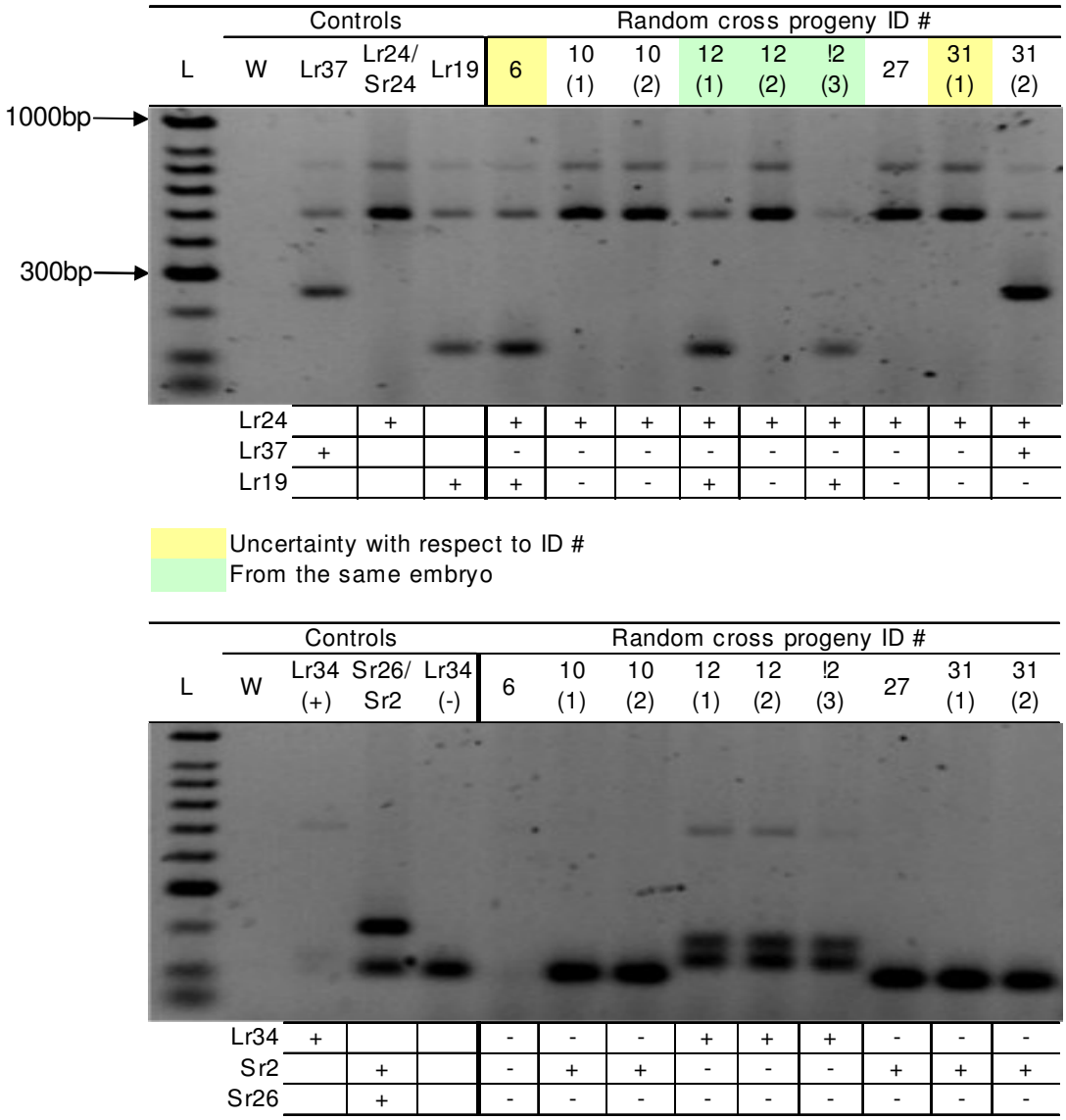

Figure 23. MAS applied to the haploid parental cross progeny: *Lr19*, *Lr24*, *Lr37*, *Sr2*, *Sr26* and *Lr34*.

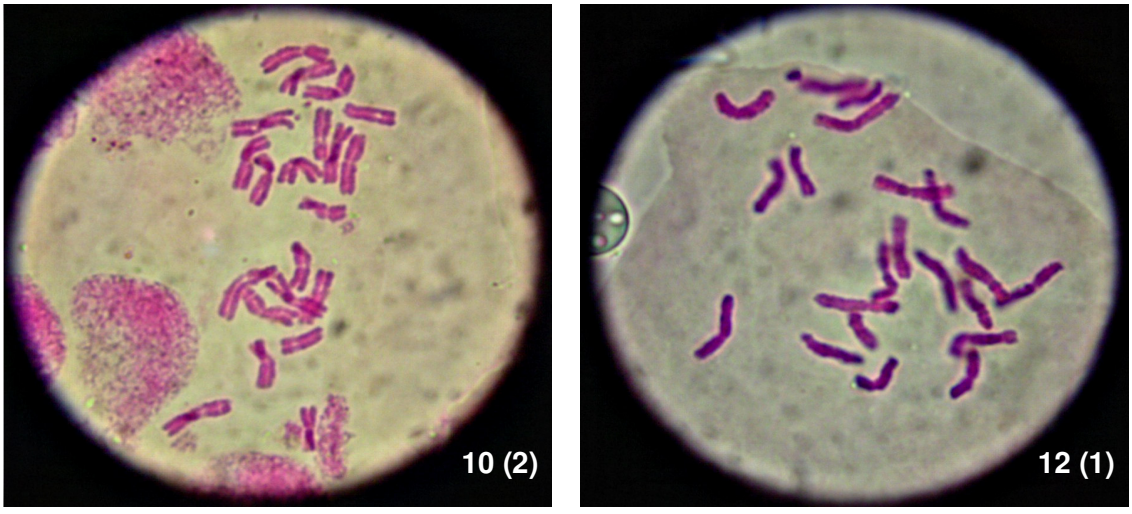

Figure 24. Ploidy determination of the parental cross progeny: #10 (1) and 12 (1).

Table 15. Summary of rust resistance genes within the confirmed haploid status progeny.

Gene	Individual							
	1	2	3	4	5	6	7	8
Lr19	X			X		X		
Lr24	X	X	X	X	X	X	X	X
Lr37								X
Lr34				X	X	X		
Sr2		X	X				X	X
Sr26								
Total:	2	2	2	3	2	3	2	3

Chapter 5: Conclusion

This investigation got underway against the back-drop of a possible bio-ethanol plant in the Western Cape. Since then, updated legislation by the South African government together with an economic feasibility study for the proposed conversion plant ruled wheat out as a candidate feedstock, at least for the immediate future. Industry viability factors inclusive of feedstock suitability, first need to be validated in the local context as not all knowledge is transferable across differing geographic regions.

In accordance with the objectives following from the aim of this project, the best spring wheat ethanol-producing germplasm was identified. This included commercial cultivars and SU-PBL ABLs. As expected in an environmentally diverse region, both specific- and multi-environment adaptation in the form of crossover GxE was reported. In addition, promising lines for further selection were also identified as well as the best localities for bio-ethanol production in the Western Cape amongst the locations considered. Potential ethanol yield in $l.t^{-1}$ was also related to entries, individual localities, the two sub-regions and the Province as a whole. In the process of advanced MLFT material assaying, protocols were optimized in accordance with the project's objectives. This included an increase in mean fermentation efficiency between the two seasons as influenced by enzyme availability. Disappointingly though, the A/A ratio and total protein data had to be excluded due to a lack of repeatability. To expidite homozygosity, haploid progeny incorporating available rust resistance genes at the time were also produced. Green plant regeneration was identified as the greatest obstacle, although a relatively large number of embryos were rescued and ploidy determination was very successful. Several possible influencing factors were identified and discussed that might lead to improved green plant regeneration.

In consideration of the differing sets of entries, the reduction of the number of sites in both years due to high grain yield CV values resulting in skewed regional representation and, differences in fermentation caused by enzyme availability, a more meaningful indication of entry performance was given by the 2007 data set. However, 2006 and 2007 data largely mirrored each other in terms of overall conclusions. This applies especially to the best and worst entry performers and demonstrates the value of ranking even where statistically significant differences may be few (Hühn, 1997).

In addition, rust resistance has a critical influence on securing grain yield and thus ethanol yield in $l.ha^{-1}$, the measure of ultimate interest to this investigation. In consideration of first-generation bio-ethanol production from wheat, the importance of grain yield as the most influential trait was clearly demonstrated. Highlighted, was the fact that grain yield is more influential than total starch fraction and thus the importance of maximizing and securing grain yield through rust resistance. No overall applicable relationship between potential ethanol yield in $l.t^{-1}$ and total starch fraction could be established. However, group differences were reported between the commercial cultivars and SU-PBL ABLs. Using several examples and in spite of few statistically significant

differences in total starch fraction between genotypes across environments, several entries demonstrated the influence of both starch and potential ethanol yield in $l.t^{-1}$ on ethanol yield measured in $l.ha^{-1}$, after taking grain yield into consideration.

Considering that the research emphasis has since switched to second-generation ethanol production from plant mass, future work on wheat grain (alone) as a potential feedstock for bio-ethanol production would only apply in a climate of viability. It was also intended to use A/A ratio data for the development of a calibration model outside the scope of this project. A reassessment of total starch in terms of data range may also be appropriate although both of these parameters are notoriously difficult to measure in terms of repeatability. However, for the purposes of selection (including calibration) and in fact this entire investigation so far as MLFT data is concerned, the ranking of genotypes is what is important from a breeding point of view.

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