Breeding for biofuel production

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ABSTRACT

The Stellenbosch University Plant breeding laboratory (SU-PBL) is currently conducting a study into the feasibility of wheat for bio-ethanol production in the Western Cape as an additional market for struggling producers. Since 2006 multi-location field trials were conducted, and material and field observations obtained used in order to develop and optimize analytical protocols for determining grain starch content and structure, fermentable sugar levels and ethanol yield. Correlations between grain ethanol yield and agronomic characteristics established, and used in order to quantify genetic variability of available germplasm. This data was also employed in order to establish a pre-breeding effort aimed at bio-ethanol yield advancement that will complement existing breeding activities. We have finished measuring the fermentable sugars, bio-ethanol yield and AAQ for the 2006 material and have observed that the current commercially available cultivars gave some of the best results. Especially SST 57 (63.86% starch, 11.88% protein and 692.99mM ethanol), SST 88 (64.83% starch, 11.97% protein and 635.72mM ethanol) and SST 015 (59.52% starch, 11.8% protein and 659.11mM ethanol) performed very well. Among the SU-PBL breeding lines 03H86-8 (62.51% starch, 12.73% protein and 851.60mM ethanol) and 97K1-15 (61.65% starch, 12.53% protein and 801.96mM ethanol) yielded the highest in terms of ethanol. During 2007 a pre-breeding program was initiated based on the recurrent mass selection (RMS) technique developed by the SU-PBL. Starting material was screened using rust inoculations and molecular markers. Male parents were selected based on their starch yields and presence of *Sr2*, and *Sr26*. Female lines were selected which carry *Lr24*/*Sr24*, *Lr37*/*Sr38*/*Yr17* and *Sr31*/*Lr26*/*Yr9*, 11 out of 180 screened. These lines are currently undergoing a third round of RMS and parallel to this material sourced is used for the creation of double haploid lines for field screening during the 2008/2009 season.

INTRODUCTION

Plant biomass is the most abundant renewable source of carbon on earth. The production of bio-ethanol forms part of the bio-energy industry, a fast-growing sector of the global biotechnology industry (Cezanne, 2004; LoGerfo, 2005). Biofuel production, which includes bio-ethanol and biodiesel, has been driven by the U.S.A. (maize) and Brazil (sugarcane). South Africa has the potential to meet 10% of its petrol and diesel needs through biofuels (Le Roux, 2005). Worldwide high crude oil prices and associated uncertain supply together with strong government backing are stimulating exponential interest. Local factors influence local economic viability and include available feedstock and their associated conversion efficiency in terms of overall energy balance. Currently, there exists little information on bio-ethanol production from wheat under South African conditions.

The aims of this (ongoing) project were to investigate the suitability of locally produced wheat (and triticale – not reported here) for ethanol production in the Western Cape province of South Africa, and to establish a pre-breeding effort aimed at bio-ethanol yield advancement. In order to achieve this we identified several objectives. These included the development and optimization of analytical protocols for determining grain starch content and structure, fermentable sugar levels, and ethanol yield. The establishment of correlations between grain ethanol yield and agronomic characteristics, and to use these in order to quantify genetic variability of available germplasm, both local and international. Another objective included exploring genetic markers for use in marker assisted selection (MAS). Subsequently a pre-breeding program based on marker assisted recurrent selection (MARS) were to be established.

MATERIALS AND METHODS

Material was sourced from the 2006 multi-location field trials (MLFT) of the Stellenbosch University Plant breeding laboratory (SU-PBL). It consisted of material from Welgevallen, Roodebloem, Tygerhoek, Vredenburg, Langgewens and Napier. When producing bio-ethanol from wheat, starch is usually converted to sugars, and the sugars are fermented to yield ethanol. The first step in quantifying potential ethanol yield is therefore to measure the starch content and structure. The MEGAZYME (Megazyme International Pty (Ltd), Ireland) total starch assay kit (K-TSTA) was utilized to determine starch content (AACC, American Association of Cereal Chemists - method 76-13) and the ratio of the starch components amylose and amylopectin (A/A) measured with the MEGAZYME amylose/amylopectin assay kit (K-AMYL), a modification of the Con-A method developed by Matheson and Yun (1990).

Near-Infrared reflectance spectroscopy (NIRS) was also used in order to measure starch content. Since every organic compound carries its own unique spectral 'fingerprint' determined by its molecular composition, which can be read by NIRS it allows rapid, non-contact, non-destructive, cost-effective analysis allowing multiple sample analyses to be performed simultaneously. Reflectance spectra were measured on a BÜCHI NIRLab N-200 NIR spectrophotometer on both whole grain and flour.

When quantifying the potential ethanol yield from wheat it is important to quantify the amount of fermentable substances (FS). The FS in grain is usually defined as the sum of the glucose and maltose content and were determined by denaturing high-performance liquid chromatography (dHPLC). We also calculated the autoamylolytic quotient (AAQ), a widely used measurement of ethanol yield potential. A small scale fermentation of the material was also attempted (modified protocol as described by Pieper and Senn (2000)).

During 2007 a pre-breeding program was initiated based on the recurrent mass selection (RMS) technique developed by the SU-PBL (Marais et al., 2000). One hundred and eighty plants of a population segregating for the *Ms3* male sterility gene, and for the resistant gene complexes *Lr24/Sr24, Lr37/Sr38/Yr17* and *Sr31/Lr26/Yr9* were obtained from the SU-PBL wheat RMS program and screened (to serve as female parents).

Material which were obtained from the national germplasm bank at the ARC-SGI, Bethlehem, and postulated to carry *Sr2* and *Sr26* were rust tested, and also screened with markers for *Sr2* and *Sr26* (to serve as male parents). The male parents were additionally screened for seedling resistance to three of the most prominent stem rust pathotypes in the Western Cape, namely 2SA88, 2SA100 & 2 SA102.

Plant DNA extraction was done according to an adapted protocol from Doyle and Doyle (1990). The quality and quantity of extracted DNA was established with a NanoDrop® ND-1000 Spectrophotometer. Except for the *Lr24/Sr24* and *Lr37/Sr38/Yr17* complexes, each of the markers was amplified individually.

RESULTS AND DISCUSSION

NIR analysis revealed that total starch determined by wet chemistry showed less than satisfactory correlations (r = 0.66) with the whole grain and flour NIRS procedure. Moisture content did however show excellent correlation (r = 0.88). Initial determination of the A/A ratio yielded values in the region of 20%, and a significant correlation with ethanol yield could not be established. Since all materials examined were intended as bread wheat this was to be expected. From literature it is clear that high amylose lines may hold an advantage in ethanol yield.

Agronomic traits were generally poorly correlated with measured traits (data not shown). This was not entirely unexpected, but confirms the literature, i.e. although a trait like hectolitre mass (HM) is regarded as a good measure of starch yield, this starch yield is usually not directly correlated with a high ethanol yield.

The data from the small scale fermentations were however encouraging. Current commercially available cultivars gave some of the best results. Especially SST 57 (63.86% starch, 11.88% protein and 692.99 mM ethanol), SST 88 (64.83% starch, 11.97% protein and 635.72mM ethanol) and SST 015 (59.52% starch, 11.8% protein and 659.11mM ethanol) performed very well. Among the SU-PBL breeding lines 03H86-8 (62.51% starch, 12.73% protein and 851.60mM ethanol) and 97K1-15 (61.65% starch, 12.53% protein and 801.96mM ethanol) yielded the highest in terms of ethanol.

The markers used for screening the complexes *Sr24/Lr24, Lr37/Sr38/Yr17* and *Sr31/Lr26/Yr9* in the proposed female lines worked well in identifying those lines which carry more than one of the complexes in their genome. In total only 6% of the lines carried all three complexes, and were male sterile.

During seedling screening of the eleven potential male parent lines Eagle, Palmiet, Songlen and Steenbras showed low infection types (≤2c) against all three pathotypes. According to Pretorius et al. (2007), the most prominent stem rust pathotypes in the winter cereal production areas are however 2SA88 and 2SA102. The lines Cook and Avocet showed low infection types (≤2c) against both these pathotypes.

In seedling tests, *Sr2* gives a susceptible seedling reaction. When the seedling reactions of the male parent's material were compared with the marker data, it was found that Songlen and Steenbras gave very good resistance to all three stem rust pathotypes while Kite, Timgalen and Zaragoza gave susceptible reactions. This can be explained by the presence of other major genes in Songlen and Steenbras. *Sr26* was also screened for in the male parents and were found in Avocet and Eagle. *Sr26* is getting more and more attention for its effective use against the aggressive stem rust pathotype Ug99 (Singh et al., 2006).

The female and male parents selected for the target genes are currently in the process of undergoing a third round of RMS and parallel to this material sourced is used for the creation of double haploid lines for field screening during the 2008/2009 season. These doubled haploid lines will ultimately be used to compile a pre-breeding nursery which will have both excellent rust resistance and bio-ethanol quality in order for it to be useful as crossing parents i.e. a conventional pedigree breeding program.

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Table 1. Bio-ethanol related trait data.

Line	Starch (%)	Protein (%)	FS (mM)	Ethanol (mM)	AAQ (%)	A/A (%)
03H86-8	62.51	12.73	3.397	851.603	19.17	18.98
97K1-15	61.65	12.53	3.232	801.958	24.84	18.51
98K120-5-p17	67.09	14.13	3.772	794.652	18.42	19.68
97K1-15-2	61.08	12.39	3.529	792.379	11.20	19.20
01H9-5	59.92	10.93	3.381	766.300	14.88	19.00
03H18-4	62.02	12.28	4.313	753.107	22.31	20.24
00H11B-4	59.14	12.54	3.850	698.246	21.30	17.96
SST57	63.86	11.88	6.344	692.999	13.87	20.77
03H5-3	62.65	13.63	3.805	672.366	36.46	12.85
SST015	59.52	11.80	3.112	659.107	15.73	20.48
00H43D-1	61.69	9.53	3.763	646.317	19.55	17.39
SST88	64.83	11.97	4.088	635.724	23.76	20.02
97K1-15-4	64.47	12.62	3.904	622.278	18.88	21.06
03H5-4	60.03	12.80	2.317	617.362	27.39	13.10
97K1-4-8	59.47	11.67	3.497	598.463	24.70	20.94
98K120-5-p16	62.37	13.28	3.776	580.345	30.43	18.12
97K1-15-5	60.45	12.32	3.733	553.977	35.68	19.77
00K30-7-3	61.60	10.75	3.615	540.541	30.56	20.29
SST65	60.36	11.65	3.819	532.398	23.30	20.22
00K180-1	61.28	12.00	3.974	510.747	26.52	19.84