Screening for *Sclerotinia sclerotiorum* resistance using detached leaf assays and simple sequence repeat markers in soybean cultivars

P.P. Mbedzi[a,b], L. van der Hoven[a], B.J. Vorster[a,b], J.E. van der Waals[a]

[a]Department of Plant and Soil Sciences, University of Pretoria, Private Bag X20 Hatfield, 0028, South Africa,

[b]Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

Highlights

- Detached leaf assays and SSR showed potentially *S. sclerotiorum* resistant soybeans.
- Most South African cultivars were genetically closer to W82 than to Maple Arrow.
- Cultivars closer to MA may have some genetic resistance to *S. sclerotiorum.*

Abstract

Sclerotinia sclerotiorum (Lib.) de Barry, causal agent of Sclerotinia stem rot of soybeans, is one of the pathogens that could have a potentially devastating impact on the growth of the soybean industry in South Africa. Several quantitative trait loci (QTLs) that play a role in soybean resistance to Sclerotinia stem rot have been identified and mapped on the soybean integrated genetic linkage map. No cultivars planted in South Africa have been screened for the presence of these QTLs and their underlying markers, and limited current information on the resistance of these cultivars is available. A detached leaf assay was used to assess resistance of 29 soybean cultivars that are commercially grown in South Africa at temperatures of 20^0 C and

25^0 C as well as under low and high relative humidity. These cultivars were further screened for resistance to Sclerotinia stem rot using simple sequence repeat (SSR) markers, that are linked to resistance traits associated with Sclerotinia stem rot in soybean. Detached leaf assays revealed a significant difference ($P<0.001$) in disease response across tested cultivars, while SSR markers revealed 10 cultivars that potentially have genetic-based resistance against Sclerotinia stem rot. Cultivars that showed a level of resistance to infection during the detached leaf assay were also more closely related to the Sclerotinia stem rot resistant cultivar Maple Arrow than to highly susceptible cultivar Williams 82; indicating the possible genetic resistance of these cultivars to Sclerotinia stem rot.

Keywords: *Sclerotinia sclerotiorum*, Sclerotinia stem rot, soybean, detached leaf assay, quantitative trait loci, simple sequence repeat markers, cultivar screening.

Footnotes:

M.A refers to soybean cultivar Maple Arrow

W82 refers to soybean cultivar Williams 82

1. Introduction

Soybean production in South Africa has increased over the last fifty years (Dlamini et al., 2014; FAO, 2014; Gasparri et al., 2016). In 1976, the area planted with soybean was approximately 22 000 hectares with an average yield of 0.81 tons per hectare (t/ha) (Dlamini et al., 2014). The recent soybean production figures show that approximately 787 200 hectares of soybean were planted in the 2017/18 season, with

an average yield of 1.97 t/ha (http://www.sagis.org.za/historicalhectares&production.html). This shows the growth potential of the soybean industry in South Africa. Production constraints like Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, can have devastating effects on South African soybean production, which will limit the expansion of this industry.

In South Africa, Sclerotinia stem rot was first recognised in soybean during the late 1970s in the Lydenburg district (Thompson and Van der Westhuizen, 1979), but has since spread across all local production regions (Botha et al., 2009). Losses due to Sclerotinia stem rot can be direct through yield loss when the crop is destroyed, or indirect through reduced grain quality (Ramusi and Flett, 2014). Dead infected soybean tissues within stems clog vascular bundles and prevent transport of water and minerals, slowing pod development and seed production (Hartman et al., 2011).

Soybean resistance has been reported as partial and quantitative (Arahana et al., 2001; Calla et al., 2009; Collard and Mackill, 2008; Kim and Diers, 2000; Kim et al., 2000; Zhao et al., 2015). This means that multiple genes each contribute a level of resistance resulting in the overall resistance of the plant. Resistance of a given soybean cultivar is also determined by the interaction between genes and the environment. Results from studies done in controlled environments have been found to show little to no correlation to those under field conditions (Kim et al., 2000; McLaren and Craven, 2008; Wegulo et al., 1998). This emphasises the importance of testing soybean cultivars for resistance to Sclerotinia stem rot under different environmental conditions where resistance genes can be expressed optimally. Various methods have been used to screen cultivars for susceptibility to *S. sclerotiorum*, but due to environmental influences, comparability of these methods is a limitation in most

instances (McLaren and Craven, 2008; Wegulo et al., 1998). Susceptibility ranking of cultivars has been found to vary between methods used, and even within the same method in different experiments (Wegulo et al., 1998). It has also been reported that response of cultivars may vary depending on disease incidence (Yang et al., 1999). Relative humidity and temperature play a role in disease establishment (Kim and Diers, 2000; McLaren and Craven, 2008), and therefore; these factors must be taken into consideration when screening for soybean resistance to Sclerotinia stem rot..

Eleven major quantitative trait loci (QTL) and two minor QTL regions that are significantly associated with Sclerotinia stem rot resistance have been reported (Bastien et al., 2014; Guo et al., 2008; Li et al., 2010; Vuong et al., 2008; Zhao et al., 2015). Molecular markers are an important part of QTL studies, as most of these are linked to many important QTLs and can be used as an indirect approach to identify resistant cultivars. Infection of soybean by *S. sclerotiorum* and the genetic aspects of soybean response have been studied in-depth in countries with large-scale soybean production; however, similar studies are limited for South African soybean cultivars.

Due to the ineffectiveness of management practices like chemical control, biological control and cultural practices, the identification and/or development of cultivars that show a level of resistance to *S. sclerotiorum* could provide more effective disease management options (Bastien et al., 2014; Cunha et al., 2010). In South Africa resistance of soybean cultivars to *S. sclerotiorum* was evaluated in the 2003/4 and 2005/6 seasons (McLaren and Craven, 2008), however limited information is available for cultivars currently planted. There is a need to screen soybean cultivars that are currently grown in South Africa for resistance to Sclerotinia stem rot, using methods that are not necessarily influenced by environmental factors. The aim of this study was to thus assess the possible genetic resistance of South African soybean cultivars to

Sclerotinia stem rot using detached leaf assays and simple sequence repeat (SSR) markers that are associated with resistance traits. Before testing soybean cultivar resistance in different environments, preliminary screening can be done using a method independent of environmental factors. The detached leaf assay is both cost effective and time-saving, and can provide information on the resistance of the tested cultivars. Arahana et al. (2001) used detached leaf assays together with identification of QTL associated with resistance in soybean to Sclerotinia stem rot to compare resistance across multiple cultivars. Huller et al. (2016) found that detached leaf assays alone could be used as an efficient method for the differentiation of soybean genotypes in terms of their susceptibility of *S. sclerotiorum*, with ratings strongly agreeing to other methods tested. Cunha et al. (2010) were able to show that transgenic soybean cultivars expressing the oxalic acid decarboxylase gene have delayed Sclerotinia stem rot symptom development compared to wild type genotypes, using only the detached leaf assay method. In other studies, in which detached leaf assays were used in conjunction with additional screening methods, the detached leaf assay was found to be one of the most effective and unbiased methods of screening soybean cultivars for resistance to Sclerotinia stem rot (Huller et al., 2016; Schwartz and Singh, 2013; Wegulo et al., 1998).

2. Materials and Methods

2.1. Plant material production

Twenty-nine commercial soybean cultivars currently grown in South Africa were obtained from the Agricultural Research Council of South Africa, Grain Crops, Potchefstroom, South Africa. These cultivars were used for SSR analysis and

detached leaf assays. The experimental layout was a completely randomized design. Four seeds were planted in each pot (20 cm diameter) in a sand-coir growth medium (composed of 0.7 mm washed silica sand and coconut coir in a 48:1 ratio). Each seed was inoculated with *Bradyrhizobium diazoefficiens* (strain WB 74-1) powder at 109 CFU g^{-1} (Soygro Bio-fertilizer Ltd, South Africa) and planted at a depth of approximately 0.5 cm. Plants used for detached leaf assays were kept in a greenhouse compartment at the University of Pretoria, at approximately 20 to 30^0 C. Plants used for DNA extraction were grown in a phytotron at 25^0 C / 16^0 C day / night temperatures, with artificial lighting providing a 13 hour photoperiod. Plants were watered daily with 250 mL of distilled water without any form of fertiliser.

2.2. Detached leaf assays

Sclerotia from pathogenic *S. sclerotiorum* isolate Excelsior, obtained from infected soybean fields in the Free State during the 2015/16 season, were surface sterilised for three minutes in a 1% sodium hypochlorite solution and then for two minutes in a 70% ethanol solution. Thereafter, sclerotia were rinsed twice in distilled water before drying on tissue paper in a laminar flow overnight. Sclerotia were then plated onto potato dextrose agar (PDA) and Petri dishes were sealed with parafilm. Plates were incubated at approximately 25^0 C. Sub-culturing was done by cutting fungal plugs out of growing mycelial cultures and then re-plating onto fresh PDA plates.

Detached leaf assays were done according to the method described by Wegulo et al. (1998). The experiment was repeated three times on different days, under two humidity conditions; high relative humidity (RH) and low RH, at 20^0 C and repeated at

25^0 C. One leaflet (the middle leaflet from a trifoliate) from each cultivar at the R1 to R3 growth stage (early flowering to early pod development, approximately 40 days after emergence) was detached, placed into a Petri dish containing three sterile filter paper discs moistened with sterilized water, and inoculated on the adaxial surface using an *S. sclerotiorum* mycelial plug (6 mm diameter) from a three-day old fungal culture. High RH was obtained by placing Petri dishes containing detached leaves onto plastic mesh in a 1.6 L plastic box filled with one litre of sterile water, inside a large container which was then sealed. Low RH was obtained by placing Petri dishes containing detached leaves into an empty 1.6 L plastic box, thus containing no water, and covered with plastic mesh, inside a large container that was then sealed. Different plants from each cultivar were used to obtain the leaves used for experiments under high and low RH for each replicate. Two completely randomised block design experiments were done at two different temperatures repeated three times on separate days. For all experiments the treatment design was a split-plot arrangement with two moisture conditions (high and low RH) as the main plots, and leaves from the 29 cultivars randomly arranged within each main plot as sub-plot factor.

Three days post-inoculation, each leaf was removed from the Petri dishes and placed onto black paper, for disease severity rating using the application Leaf Doctor (https://itunes.apple.com/us/app/leaf-doctor/id874509900?ls=1&mt=8). This application measures the percentage of tissue area that is diseased, based on an algorithm that recognises pixel colours for healthy plant tissues. Fungal agar plugs were removed from leaves to visualise leaf lesions on photographs. Leaves were photographed individually and collectively, and photos were subsequently imported into the application Leaf Doctor (Pethybridge and Nelson, 2015), to assess lesion size in relation to the leaf surface area (Cunha et al., 2010; Huller et al., 2016; Wegulo et

al., 1998). To improve the consistency of the assay, leaves used were of approximately uniform size, photos were taken at approximately the same distance from the leaf, and inoculum placement was constant (Wegulo et al., 1998). A control leaf was included for each replicate, from a randomly selected cultivar, which was inoculated with a clean PDA plug containing no mycelium. All data collected were subjected to an appropriate analysis of variance using the GenStat statistical system (Payne, 2009). Fisher's protected t-Least Significant Difference (LSD) was calculated to compare treatment means of significant effects (Snedecor and Cochran, 1980).

2.3. SSR Work

2.3.1. Selection of SSR markers from literature and database

Sclerotinia stem rot resistance DNA markers were selected within the regions or linkage groups of quantitative trait loci (QTLs) that have previously been reported to be significantly associated with soybean response to *S. sclerotiorum* infection. Eleven major and two minor QTL regions that are significantly associated with stem rot resistance in soybean were identified from literature. Thirty SSR markers (Table 1) were selected with the aid of the soybean composite interval maps on the Soy Base database (www.soybase.org). The selection was done by identifying the positions of a given QTL on the composite interval map and selecting markers that are mapped at a genetic distance of less than 50 centi-Morgans (cM) from a given QTL position. Fourteen of these are markers that have previously been reported in QTL regions with a strong association to partial resistance of soybean to Sclerotinia stem rot, while 16 are new markers that have not been reported before.

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Table 1. Thirty simple sequence repeat markers that are linked to stem rot resistance quantitative trait loci on soybean and their position on the soybean composite interval map (Song et al., 2010)

Chromosome no	Marker name	Motif	Position (cM)
1	Satt502	(TTTA)3agttttaaact(ATA)16	46,291
	Satt169	(AAT)16	44,788
	Sat_159	(TA)22	45,814
	Satt321	(TAA)14	48,254
7	Satt463	(AAT)13(GAT)17(AAT)19	46,268
	Satt323	(ATA)17	55,872
8	Satt233	(ATA)16	85,786
	Satt133	(AAT)10	110,379
	Satt525	(TTA)15	83,609
	Sat_233	(TA)14	72,782
	Sat_138	(TA)25	107,642
	Satt 089	(TAT)26	74,742
	Satt377	(TAA)14ta(TAT)5	77,51
	Sat_097	(AT)30	104,541
10	Satt581	(TAA)11	95,601
	Satt153	(TTG)4	106,322
11	Satt251	(TAT)15	38,802
	Satt638	(ATA)13	40,951
	Satt509	(ATA)31	37,47
	Sat_261	(AATA)4	38,042
13	Satt269	(ATT)11	27,45
	Satt145	(AAT)4c(ATA)7	27,606
	Satt149	(AAT)16	23,294
	Satt252	(TAT)23	22,623
	Satt423	(TAT)19	20,153
14	Satt126	(AAT)18	23,286
15	Satt369	(TAT)17	85,199
	Satt411	(TAT)11	13,66
	Satt685	(AAT)14	87,059
	Sat_124	(TA)35	50,773

2.3.2. DNA extraction and SSR marker polymorphism pre-screening

DNA was extracted from three leaf samples from each of the 29 commercial soybean cultivars that are planted in South Africa. Maple Arrow (MA) is considered a partially resistant cultivar and Williams 82 (W82) is considered highly susceptible to *S. sclerotiorum* in North America (Arahana et al., 2001; Wegulo et al., 1998; Zhang and Xue, 2014; Zhao et al., 2015). For this reason, MA and W82 cultivars were included in this study, as tolerant and susceptible reference cultivars, respectively. Seeds for both cultivars were obtained from USDA Soybean germplasm collection, Illinois, USA. DNA extraction was done using the 2 X CTAB method described in Doyle and Doyle (1990). RNA contamination was removed from the DNA samples by adding one microliter of RN*ase*A (Thermo Fisher Scientific, United States) and incubating the samples for 15 minutes at 37^0 C. DNA samples were washed by adding 700 µL of 70% ethanol to each Eppendorf tube containing DNA. The tubes were centrifuged for one minute at 13000 rpm, and the supernatant was discarded. The samples were re-suspended in 30 µL of double distilled water for long-term storage.

Four cultivars were randomly selected from the 29 cultivars to perform a marker polymorphism pre-screening test. A multiplex PCR was done using the Platinum Multiplex PCR Kit according to the manufacturer's instructions (Applied Biosystems, Life Technologies, South Africa). The PCR cycle reaction was conducted in a BOECO TC-PRO thermocycler (Germany), under the following conditions: an initial denaturation step at 94^0 C for five minutes, denaturation step at 94^0 C for 30 seconds, primer annealing at 58^0 C for 30 seconds, an extension step at 72^0 C for one minute and a final extension step at 72^0 C for 10 minutes. The denaturation, primer annealing, and extension steps were repeated for 30 cycles in each PCR reaction. The resulting

PCR products were stored at 4^0 C and analysed using a 3% agarose gel electrophoresis, at 80 Volts for three hours.

2.3.3. SSR data analysis

All twenty-nine cultivars were screened by Multiplex PCR, using the selected SSR markers as above. The PCR cycling conditions and instrumentation were the same as in section 2.3.2. The products were analysed on a 3% agarose gel at 80 Volts for two hours and 30 minutes. Fragment length analysis was done using GeneScan™ 500 LIZ™ dye Size Standard Liz (Applied Biosystems, Life technologies, South Africa). Genotype data obtained after fragment length analysis was used to characterise the selected SSR markers using GenAlEx software version 6.5 (Peakall and Smouse, 2006). Allele frequency and allelic patterns were calculated for each SSR marker using all samples from South African commercial cultivars. This was done as the first step to determine if there is genetic differentiation among South African cultivars. The genetic relatedness and genetic diversity across cultivars were estimated by calculating fixation indices and the total fixation index. The polymorphic information content (PIC) was calculated according to Botstein et al. (1980), to determine the polymorphic content of the selected markers among South African cultivars.

Genetic relationships were evaluated between South African cultivars and MA; and compared with that of South African cultivars and W82, to estimate shared genetic resistance between MA and South African cultivars. This was done by constructing the pairwise matrices of Nei's genetic distance (NeiP) and Nei's genetic identity (Nei, 1972), Fixation index (FstP), as well as Shannon's pairwise index of diversity (SHuaP). The three matrices were used to specifically evaluate the genetic relatedness across

South African cultivars and their relatedness with W82 and MA. Nei's pairwise genetic distances were used to compare the genetic relatedness between South African cultivars and either MA or W82 as partially resistant and susceptible cultivars, respectively. A principal coordinate analysis (PCoA) was done to visualise the results of genetic distances between the stem rot resistant reference cultivar and South African cultivars, as well as to visualize the cultivars genetic clustering in a multidimensional space.

3. Results

3.1. Detached leaf assays

Lesion formation occurred at 20^0 C and 25^0 C on all inoculated soybean leaves, with green leaves discolouring to become brown in colour. Lesion size measured for each individual leaf (Supplementary Tables 1 and 2) revealed highly significant ($P<0.001$) differences in response to infection among soybean cultivars, although humidity ($P=0.096$) and temperature ($P=0.247$) means were not significantly ($P>0.05$) different from each other.

Results for percentage diseased leaf area under high and low RH (Fig. 1) revealed that cultivar LS 6444 R showed high susceptibility at high and low RH. Cultivars DM 6.8i RR and PAN 1583 R showed the most resistance under high and low RH, respectively. Percentage diseased leaf area at different temperatures showed that cultivars NS 6448 R and LS 6444 R were most susceptible at 20^0 C and 25^0 C, respectively (Fig. 2). Cultivars PAN 1614 R and LS 6466 R were the most resistant cultivars at 20^0 C and 25^0 C, respectively.

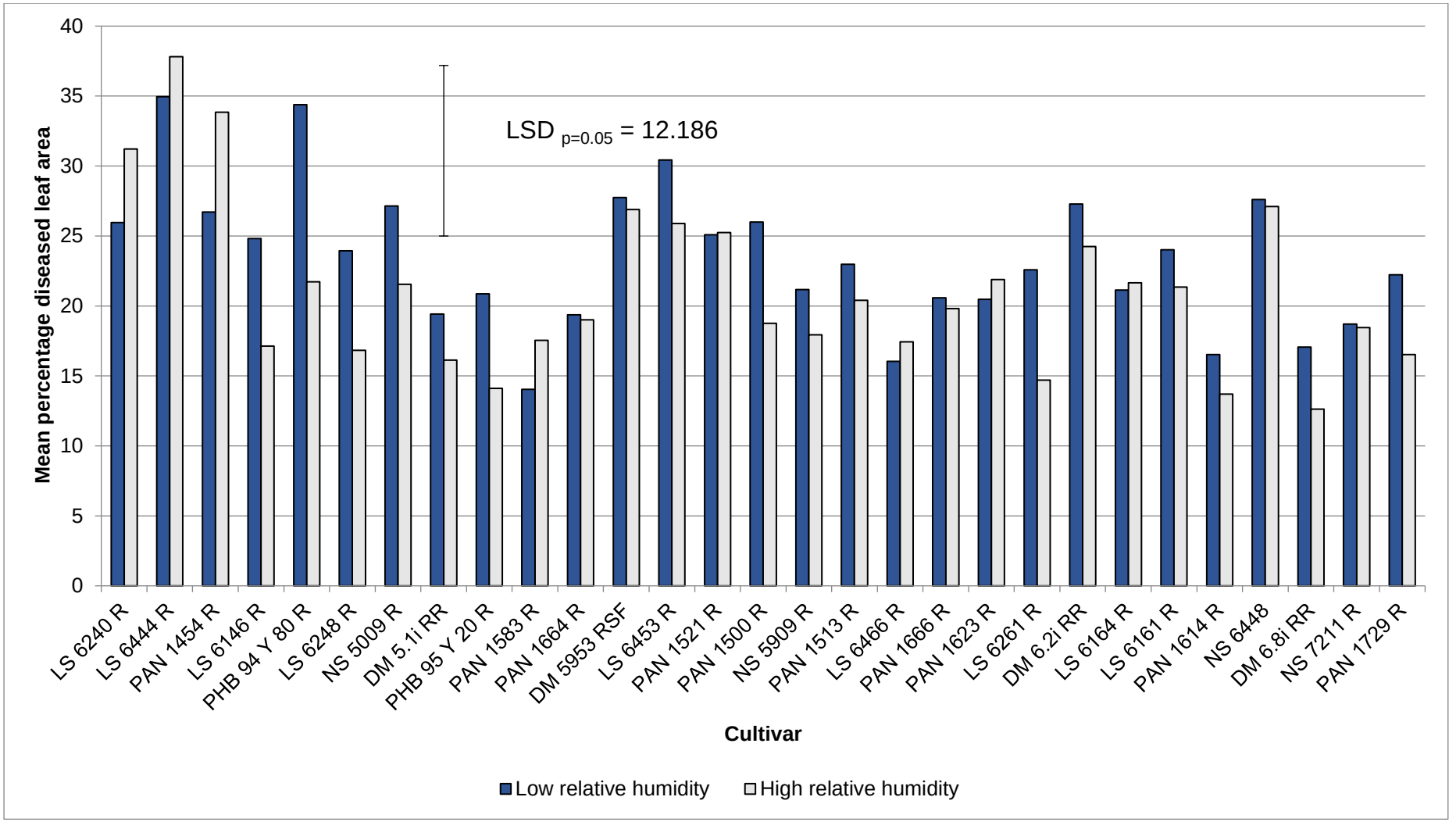

Fig. 1. Mean percentage diseased leaf area for each cultivar at high and low relative humidity.

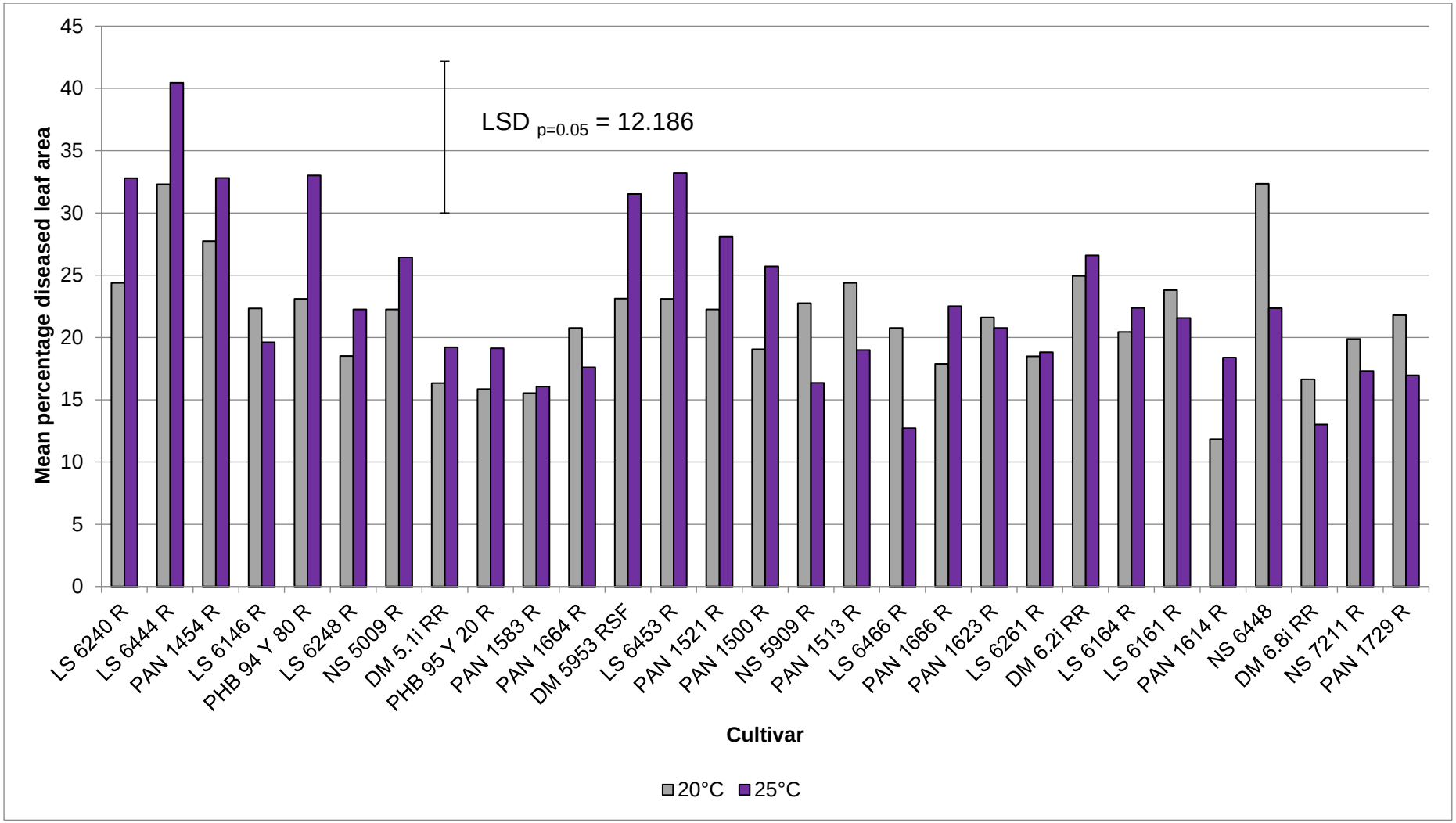

Fig. 2. Mean percentage diseased leaf area for each cultivar at two different temperatures.

3.2. SSR work

3.2.1. DNA extraction and SSR marker polymorphism pre-screening

The extracted DNA was stored at -20°C and used for all subsequent SSR work. A 3% agarose gel showed size polymorphism of twenty SSR markers (Fig. 3, m1 to m20); however, only 11 markers could distinguish the cultivars used for pre-screening based on estimated fragment size on the agarose gel.

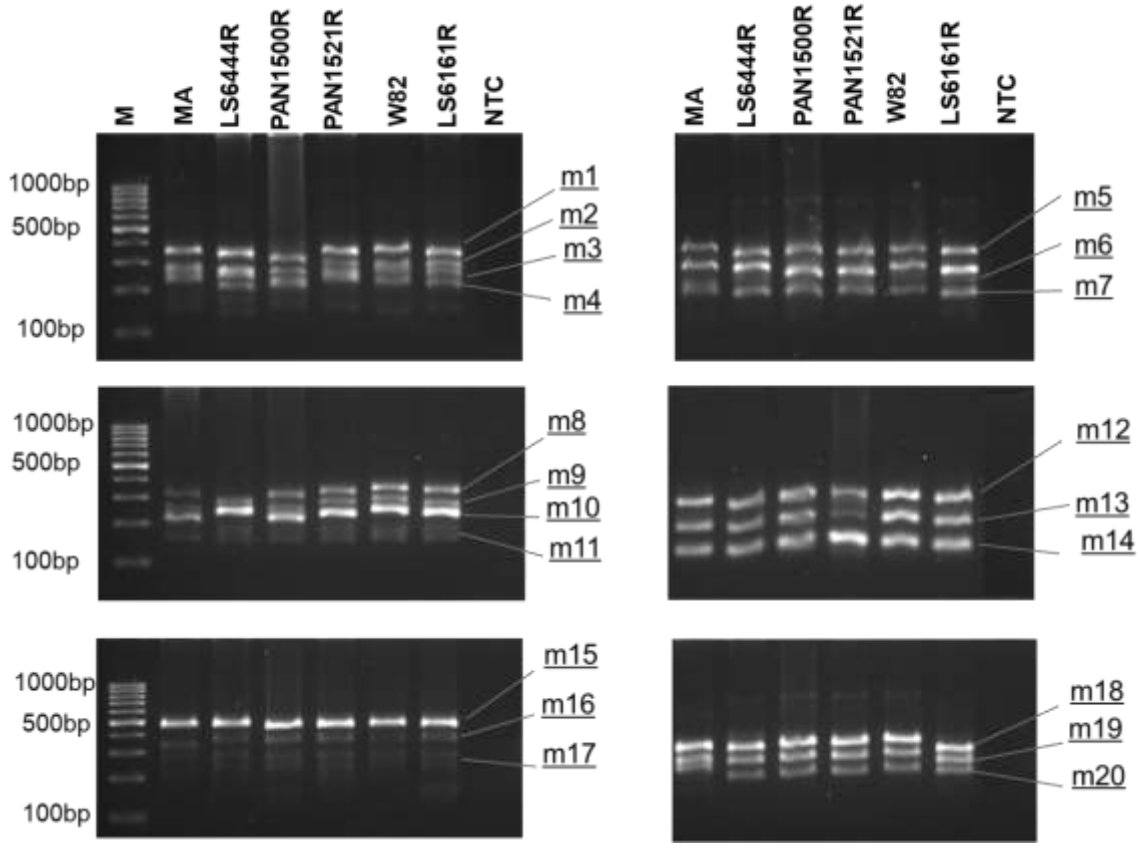

Fig. 3. A 3% agarose gel showing size polymorphism of 20 selected simple sequence repeat markers (m1 to m20) of four South African soybean cultivars (SA) as well as a positive control cultivar for Sclerotinia stem rot resistance (MA) and a negative control cultivar for Sclerotinia stem rot resistance (W82). A molecular size marker (M) and a non-template control (NTC) are also shown.

3.2.2. SSR data analysis

After PCR optimisation, only 19 of 20 SSR markers that have been previously reported to be associated with soybean resistance to stem rot were evaluated further in the South African cultivars. Allele size in the evaluated cultivars ranged from 93bp to 360bp. The evaluated SSR markers had 55 alleles in total in the 29 South African soybean cultivars as well as in W82 and MA. The average number of alleles was 2.87 per locus. The loci containing the highest number of alleles per locus were Satt369 and Satt252, both with an average of 5.0 alleles per locus. The percentage polymorphic information content (PIC) was calculated to evaluate the usefulness of these markers specifically to South African cultivars (Table 2); five out of 19 markers were not polymorphic in these cultivars. Sat_233 was the marker with the highest percentage PIC when calculated using allele frequencies.

Allele diversity per locus over all cultivars was measured by heterozygosity, as well as Wright's F-statistics. Satt323 had the highest allele diversity per locus (0.202), while Satt133, Satt411, Satt685, Satt126, and Satt638 had the lowest allele diversity of all South African cultivars in this study. Satt323, however, also had a positive inbreeding coefficient (Fis), which may indicate the allele relationships on Satt323 locus, for this specific sample of soybean cultivars. The mean gene diversity over all cultivars was 0.079. The inbreeding coefficient and estimate of gene flow over all cultivars were 0.567 and 0.077, respectively. Finally, the Fixation index, which also measures heterozygosity and genetic diversity, was 0.791.

Table 2. Average allele size ranges, numbers, and percentage polymorphism on stem rot resistance simple sequence repeat markers determined from average allele frequency of South African commercial soybean cultivars

Marker locus	Size range (bp)	Number of alleles per locus	Percentage polymorphism (%)
Satt323	145-170	4	59
Satt502	251-260	2	38
Satt233	186-207	4	50
Satt369	221-251	5	53
Satt581	132-146	4	44
Satt153	188-209	3	51
Satt169	185-224	4	37
Satt251	204-211	3	43
Satt525	302-304	2	8
Satt411	93-96	2	0
Satt269	251-258	2	10
Satt133	181-190	2	0
Satt145	142-146	3	47
Satt685	213	1	0
Satt126	149	1	0
Satt149	251-275	3	46
Satt638	176	1	0
Sat_233	242-360	4	70
Satt252	207-224	5	64

Nei's pairwise genetic distance and genetic identity were the first parameters evaluated to understand genetic relatedness among cultivars. Nei's pairwise genetic distances ranged between 0.027 and 1.189. The highest genetic distance and the

lowest genetic identity was between controls W82 and MA, while for South African cultivars the lowest genetic distance and highest genetic identity was between cultivar NS 6448 R and LS 6248 R. The Shannon index was calculated to determine the pairwise genetic differentiation among individual South African cultivars, W82, and MA. The lowest genetic differentiation of 0.037 was between NS 6448 R and LS 6248 R, while the highest genetic differentiation of 0.682 was between W82 and MA. Nei's genetic distances between South African cultivars and W82 were compared with the genetic distances between South African cultivars and MA (Table 3). Based on Nei's pairwise genetic distance comparison, cultivars were divided into two groups; one group representing South African cultivars that are genetically closer to W82 than to MA and the second group representing cultivars that are closer to MA than to W82. Most South African cultivars were genetically closer to W82 than to MA, with only ten cultivars closer to MA than to W82. Using the groupings from genetic distances, a principal coordinate analysis (PCoA) was constructed to visualise the genetic relationships in a two-dimensional space (Fig. 4). The mean percentage diseased leaf area column in Table 3 shows that cultivars LS 6444 R, PAN 1454 R, LS 6240 R, LS 6453 R and PAN 1500 R were grouped as being significantly ($P<0.001$) more susceptible to *S. sclerotiorum* compared to other cultivars (cultivars shown in bold, Table 3). The data in column 4 in Table 3 was constructed by combining all temperature and RH data for each soybean cultivar. All South African cultivars that were classified as susceptible in the detached leaf assay experiment were closely related to W82 and grouped with W82 on the PCoA. All cultivars classified as resistant in the detached leaf assay were closely related with MA.

Table 3. The mean percentage diseased leaf area and the average Nei's pairwise genetic distance between Williams 82 (W82), Maple Arrow (MA) and South African cultivars show possible Sclerotinia stem rot resistance in South African cultivars

Cultivar	Average genetic distance between W82 and South African cultivars (NeiP)	Average genetic distance between MA and South African cultivars (NeiP)	Mean % diseased leaf area
W82	0.000	1.189	
LS 6453 R	0.111	0.887	**28.16**^{abcd}
LS 6240 R	0.226	0.576	**28.59**^{abc}
PAN 1623 R	0.244	0.545	21.18^{cdefghi}
PAN 1521 R	0.286	0.738	25.17^{bcdefg}
NS 6448	0.287	0.549	27.35^{bcde}
LS 6444 R	0.318	0.576	**36.38**^{a}
PAN 1500 R	0.318	0.413	22.38^{bcdefghi}
NS 5909 R	0.343	0.430	19.55^{defghi}
LS 6248 R	0.370	0.565	20.39^{cdefghi}
NS 5009 R	0.372	0.630	24.34^{bcdefgh}
PAN 1614 R	0.378	0.566	15.12^{i}
PHB 94 Y 80 R	0.379	0.655	**28.06**^{abcd}
DM 5953 RSF	0.396	0.459	27.32^{bcde}
PAN 1513 R	0.423	0.767	21.69^{bcdefghi}
PAN 1729 R	0.441	0.768	19.37^{efghi}
DM 6.2i RR	0.442	0.556	25.77^{bcdef}
NS 7211 R	0.460	0.588	18.59^{fghi}
LS 6146 R*	0.470	**0.389**	20.98^{cdefghi}
PAN 1454 R	0.471	0.481	**30.28**^{ab}
LS 6164 R*	0.512	**0.329**	21.40^{cdefghi}
LS 6466 R*	0.547	**0.385**	16.73^{ghi}
DM 6.8i RR	0.552	0.637	14.84^{i}
DM 5.1i RR*	0.560	**0.371**	17.78^{fghi}
LS 6261 R*	0.566	**0.309**	18.65^{fghi}
PAN 1666 R*	0.580	**0.559**	20.20^{cdefghi}
PHB 95 Y 20 R*	0.588	**0.475**	17.50^{fghi}

PAN 1664 R*	0.597	**0.484**	19.19^{efghi}
LS 6161 R*	0.642	**0.556**	22.68^{bcdefghi}
PAN 1583 R*	0.684	**0.454**	15.79^{hi}
MA	1.189	0.000	

* the asterisk indicates cultivars that are more closely related to MA than W82 due to shared Sclerotinia stem rot resistance alleles.

The genetic distances that indicate shared resistance alleles between MA and South African cultivars are written in bold.

P=0.05 for mean percentage diseased leaf area. Cultivars with mean percentage diseased leaf area in bold were grouped as being significantly (P<0.001) more susceptible to *S. sclerotiorum* compared to other cultivars.

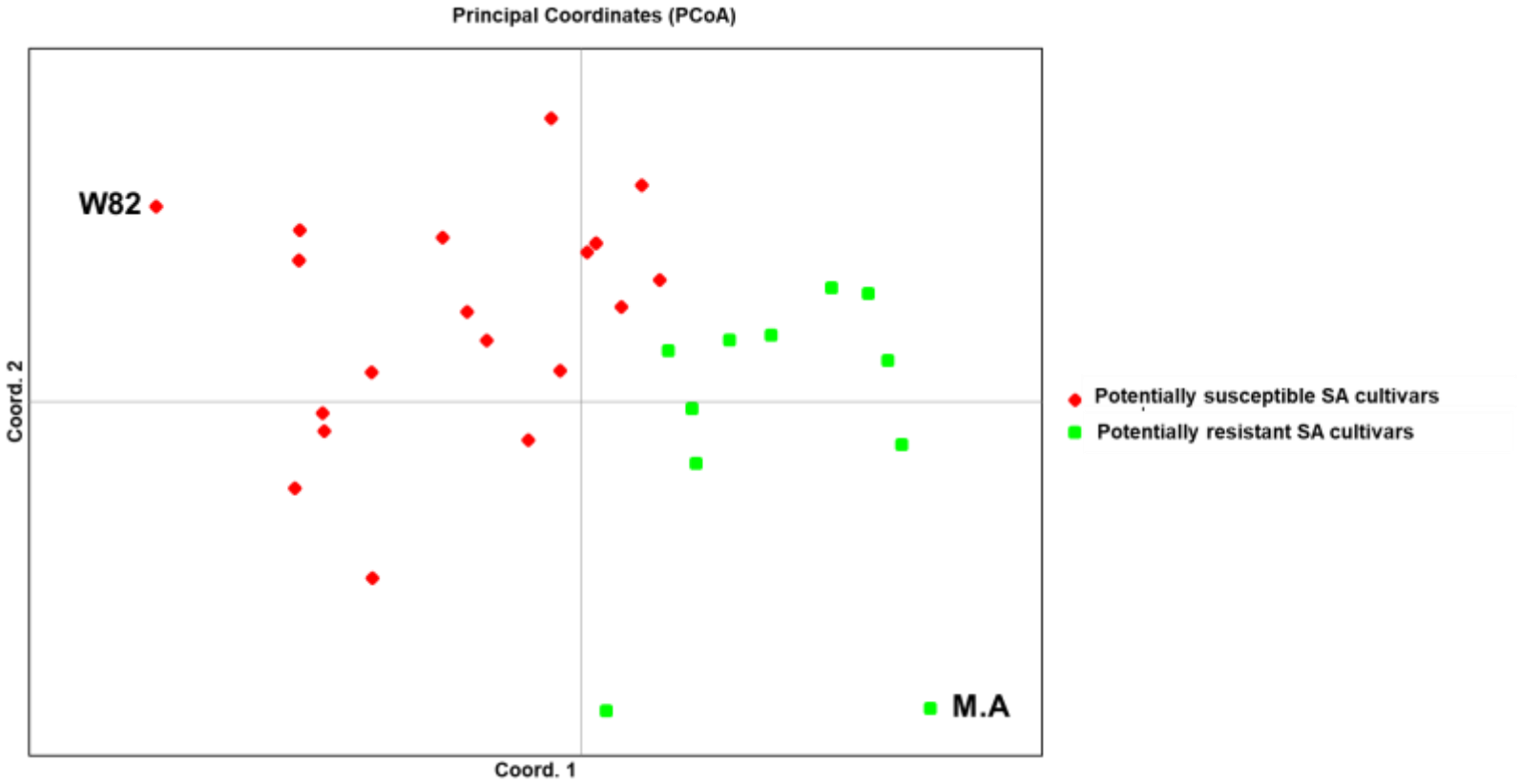

Fig. 4. A scatter plot of principal coordinate analysis (PCoA) showing South African cultivars that cluster towards W82 (red diamonds) and those that cluster towards MA (green squares). The first and the second axes explained 21.75% and 16.04% variation respectively. This figure is based on the average genetic distance of three samples representing each cultivar.

4. Discussion and Conclusion

In this study we combined detached leaf assays and SSR markers to screen for possible Sclerotinia stem rot resistance in 29 commercial soybean cultivars from South Africa.

Detached leaf assays revealed highly significant differences ($P<0.001$) between disease severity of different soybean cultivars, suggesting that the cultivars tested differ in their susceptibility to *S. sclerotiorum*. This is consistent with other studies evaluating soybean cultivar responses to *S. sclerotiorum* (Kim et al., 2000; McLaren and Craven, 2008; Wegulo et al., 1998). Cultivars NS6448 and LS6444R were most susceptible at 20°C and 25°C; while PAN1614R and LS6466R were most resistant at 20°C and 25°C. Cultivar DM6.8i RR showed the most resistance under high RH, while cultivar PAN1583 R showed the most resistance under low RH. Overall, cultivars LS 6444 R, PAN 1454 R, LS 6240 R, LS 6453 R and PAN 1500 R were significantly more susceptible to *S. sclerotiorum* than other cultivars. The susceptible cultivars were all more closely related to the susceptible W82 than resistant MA, suggesting shared susceptibility between these cultivars and W82. LS 6444 R, PAN 1454 R, and LS 6240 R are short growing cultivars, and LS 6453 R and PAN 1500 R are classified as medium growing cultivars (De Beer and Prinsloo, 2013). In choosing which cultivar to plant, the most important factor to take into consideration is the length of the growing season, as soybean is sensitive to day length (De Beer and Bronkhorst, 2015). The results from this study show that short to medium growing cultivars are more susceptible to *S. sclerotiorum* than longer growing cultivars. It was, however, communicated by soybean growers, at a Grain SA Sunflower- and Soybean-specialist work-group meeting, that shorter growing cultivars are normally not infected by *S. sclerotiorum* (Grain South Africa, 2017). This confirms the findings of Yang et al.

(1999), where cultivars with higher maturity groups showed higher disease incidences. These observations could be explained by the possibility that shorter growing cultivars escape disease in the field. In South Africa, environmental conditions during flowering of short growers are not conducive to infection, since *S. sclerotiorum* requires a cool, wet environment at the flowering stage for infection (Abawi and Grogan, 1975; Cline and Jacobsen, 1983; Grau et al., 1982; Purdy, 1979). The use of disease escape mechanisms relating to flowering date, growing season, and physiological architecture has been reported for soybean plants, making this a feasible assumption (Boland and Hall, 1988; Kim et al., 1999; Kim and Diers, 2000; Nelson et al., 1991).

Certain soybean attributes like canopy and plant density are strongly influenced by the environment and genotype, and therefore cannot be used to describe specific soybean varieties (Jarvie, 2017). Leaf shape in soybean is, however, strongly controlled genetically, thus not influenced by environmental factors, meaning that leaf shape can be used in breeding for Sclerotinia stem rot resistance. It has been found that lanceolate leaves allow for better light penetration into the crop canopy than ovate leaves (Wells et al., 1993), thus lowering leaf wetness and RH, reducing ideal environmental conditions for Sclerotinia stem rot development. Leaf shape is therefore one of the important traits that can be explored further in future work. Indeterminate cultivars that flower for between one and five weeks could provide more opportunities for *S. sclerotiorum* infection, even with more than one fungicide application per season (Mueller et al., 2002), making the consideration of flowering pattern important for future work and breeding.

It should be noted that the results from this study, however, do not suggest that farmers should refrain from planting short growing cultivars, but rather that they should ensure correct planting time, such as planting short growing cultivars earlier in the

season, to increase the probability of disease escape. The two most susceptible long growing cultivars were found to be DM 6.2i RR and NS 6448 R, and thus these two cultivars should be avoided, particularly in South African fields with a history of Sclerotinia stem rot. Since it is known that soybean maturity group significantly ($P<0.01$) influences disease incidence, the relationship between yield loss and disease incidence for different cultivars needs to be better characterized in the future, to assist producers in making sound economical farming decisions (Yang et al., 1999).

To confirm that the cultivars that showed resistance to *S. sclerotiorum* in the detached leaf assay contain the quantitative trait loci for Sclerotinia stem rot resistance, the 19 SSR markers that are linked to Sclerotinia stem rot resistance were characterized across all 29 South African cultivars. Fourteen of the markers selected in this study had previously been reported as Sclerotinia stem rot resistance markers (Bastien et al., 2014; Guo et al., 2008; Li et al., 2010; Vuong et al., 2008; Zhao et al., 2015), while the remaining seventeen markers were either linked to one of the eleven major or two minor QTLs that have been reported to have an association with soybean resistance to Sclerotinia stem rot. Williams 82 has been reported as a susceptible cultivar while MA was previously reported as a partially resistant cultivar (Li et al., 2010; Zhang and Xue, 2014; Zhao et al., 2015). Using this information, W82 and MA cultivars were used as references for susceptibility and resistance to Sclerotinia stem rot, respectively. Seven markers out of 19 had low polymorphic information content and allele diversity; five of these, namely Satt411, Satt133, Satt685, Satt126, and Satt638 were not polymorphic across South African cultivars. Satt411 however, was polymorphic between W82 and MA. The low genetic diversity of these markers could be attributed to low genetic diversity in South African cultivars because all these markers have been reported with substantial gene diversity in cultivars other than

those grown in South Africa (Song et al., 2010). A study by Holla et al. (2014) showed that markers that appear monomorphic in a given set of genotypes were closely linked to functional genes that control important characteristics. This restricts mutations that render polymorphism to a given marker thus making that marker monomorphic in populations that contain the gene involved. Fourteen out of 19 markers were highly polymorphic across South African cultivars. Of the 14 polymorphic markers, 10 have previously been reported to be associated with either soluble stem pigments (Li et al., 2010; Zhao et al., 2015) or lesion length (Bastien et al., 2014; Guo et al., 2008; Vuong et al., 2008). The observed heterozygosity is also lower than what would be expected under Hardy-Weinberg equilibrium; the low genetic diversity of these cultivars might be due to inbreeding. This has important implications for the use of these cultivars as sources of resistance in breeding programs against Sclerotinia stem rot. The overall fixation index of the South African cultivars also indicates that most of these cultivars have been subjected to artificial selection, perhaps for other agronomic traits, which might be the reason for the reduced genetic diversity. Many genetic diversity studies have found that wild soybeans generally have more diversity than cultivated soybeans (Hwang et al., 2008; Kuroda et al., 2009; Zhang et al., 2016). It might therefore be worth exploring the use of wild soybeans to increase genetic diversity in cultivated soybeans (Ji et al., 2010; Kim et al., 2011). The improvement of cultivated crop varieties using their wild relatives has been explored in rice (Marjee et al., 2004) and wheat (Peleg et al., 2009). According to the latest review of wild relatives of domesticated crops as potential genetic resources for breeding against pests and diseases, the three species that are wild relatives of soybean with potential resistance to Sclerotinia stem rot are *Glycine tabacina*, *Glycine tomentella* and *Glycine lalifolia* (Mammadov et al., 2018). Using genetic relationships based on shared alleles, and

the relatedness of South African cultivars with W82 and MA, we estimated which South African cultivars share Sclerotinia stem rot resistance with MA. Ten South African cultivars were more closely related to MA than to W82; these cultivars also had low mean percentage diseased leaf area in detached leaf assays. South African cultivars that are closely related to MA could therefore potentially have some genetic resistance to Sclerotinia stem rot.

Considering the low polymorphism in markers Satt638, Satt133, Satt411, Satt685 and Satt323, these are not recommended for use in indirect screening methods for Sclerotinia stem rot resistance in current South African commercial cultivars. Markers that were polymorphic in the South African cultivars tested were Satt502, Satt233, Satt525, Satt251, Satt369, Satt269, Satt153, Satt581, Satt149, Satt323, Satt252, Satt169, Satt145, and Sat_233. These markers are good candidates to use in indirect screenings for soybean resistance to Sclerotinia stem rot, specifically in South African soybean cultivars. This is the first study to indicate the possible genetic resistance to Sclerotinia stem rot in cultivars LS 6146 R, LS 6261 R, LS 6164 R, LS 6161 R, DM 5.1i RR, PHB 95 Y 20 R, PAN 1583 R, PAN 1664 R, PAN 1666 R and LS 6466 R, which are grown in South Africa; providing an important step towards South African soybean breeding for Sclerotinia stem rot resistance.

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