SOYBEAN RESPONSE TO RUST AND SCLEROTINIA STEM ROT UNDER DIFFERENT BIOTIC AND ABIOTIC CONDITIONS

CHRISNA STEYN

Submitted in accordance with the requirements for the degree

PHILOSOPHIAE DOCTOR

In the Faculty of Natural and Agricultural Sciences
Department of Plant Sciences (Plant Pathology)
University of the Free State
Bloemfontein
South Africa

Promoter: Prof. N.W. McLaren

June 2015

TABLE OF CONTENTS

DECLARATION

"I declare that the thesis hereby is submitted by Chrisna Steyn for the degree Philosophiae Doctor in Agriculture at the University of the Free State represents my own independent work and has not previously been submitted by me at another university/faculty. I further cede copyright of the thesis in favour of the University of the Free State".

....

Chrisna Steyn Date

DEDICATION

I dedicate this thesis to my husband Jacques - it is a privilege to share my work, life and love with you; To my son, Rikus - watching you grow is a constant source of joy and pride; To my father Piet, for encouraging me to go on and not give up and to my mother, Velika who believes in me - always.

ACKNOWLEDGEMENTS

I would like to convey my sincere gratitude and deep appreciation to the following people and organisations for their contribution towards the success of this thesis:

- My Heavenly Father for the opportunity, strength, wisdom and abundant grace.
- My sincere gratitude and appreciation to my supervisor, Prof. Neal McLaren for his dedicated supervision, patience, input, valuable assistance, advice and motivation.
- To my husband Jacques Steyn for believing in me, encouragement, help, loyalty, love, support and taking care of our son, Rikus while writing up this thesis.
- My loving parents, Piet and Velika Botha for all their love, encouragement, guidance, prayers and support.
- To my brothers, Thys Botha and Marx Botha for support, love and encouragement.
- Department of Plant Sciences for providing research facilities.
- Dr Adré Minaar-Ontong for the technical assistance in conducting the AFLP analysis and assisting in writing of this thesis.
- To my colleagues for the technical assistance, research input and advice.
- To my colleagues at Plant Breeding for support, encouragement and valuable friendship.
- Protein Research Foundation for providing research funds and the opportunity to do this project.
- To all my precious friends for their encouragement, love and support during my studies.

GENERAL INTRODUCTION

Soybean (*Glycine max (L*.) Merr.) was first planted in Southeast Asia by Chinese farmers around 1100BC (Anonymous, 2011). Since then soybeans have become an important cash crop around the world, especially since it is a significant source of high quality, low cost protein (Shurtleff & Aoyagi, 2007). In 2013-2014 global soybean production was recorded at 283.7 million metric tons (O'Brien, 2015).

In South Africa soybean was first planted in 1903 on an experimental farm at Cedara, KwaZulu-Natal (Shurtleff & Aoyagi, 2007). Currently, main soybean production provinces include Mpumalanga, KwaZulu Natal and the Free State and primarily include the areas of Bergville, Bethal, Ermelo, Newcastle, Warden, Winterton, Vryheid and Vrede. In South Africa, consumption of soybean is estimated at 25% for oil and oil cake, 60% for animal feed and 20% for human consumption (Anonymous, 2010). Soybean oilseed production in 2012 was recorded at 850 000 metric tons and increased to 1084 500 metric tons in 2013/14 (Anonymous, 2013).

Due to the increased production of soybeans globally, factors influencing production such as diseases, insects and environmental factors have become a concern due to major yield losses reported each year. Species of the genus *Sclerotinia* cause destructive diseases on numerous plants affecting seedlings, mature plants and their harvested products (Agrios, 1997). *Sclerotinia sclerotiorum* (Lib.) de Bary in particular has a wide host range that contains 42 sub-species or varieties, 408 species, 278 genera and 75 plant families (Boland & Hall, 1994). *Phakopsora pachyrhizi* Syd. & P. Syd., the causal agent of soybean rust, has a broad host range and infects more than 95 plant species from more than 42 genera (Rytter *et al*., 1984; Ono *et al*., 1992) and is regarded as a serious yield limiting foliar disease of soybean prevailing in most soybean production areas.

Disease development of Sclerotinia stem rot is closely related to weather. The most important factor affecting disease development is moisture (Abawi & Grogan, 1975) as it is essential for the production, release and germination of ascospores. The latter are released and infect the aerial tissues resulting in stem blight, stalk rot, head rot, pod rot and blossom blight of plants (Bardin & Huang, 2001). The best germination of spores and optimum disease development conditions are created if adequate moisture is accompanied by moderate temperatures between 16°C and 25°C (Harikrishnan & Del Rio, 2006). Soybean rust infection starts with spore germination, the formation of appressoria,

penetration into the host tissue and colonization of the host plant after which the development of uredinia and sporulation occurs. Epidemics are more severe when long periods of leaf wetness and daily temperatures of less than 28°C occur (Sinclair & Hartman, 1999). Machetti *et al*. (1976) reported that a leaf wetness and temperature interaction of at least 6 hours of dew and temperatures of 20°C to 25°C are needed for infection.

Where favourable conditions for disease development persist, severe yield losses have been reported. Losses due to Sclerotinia stem rot result directly from loss in yield and indirectly from reduced grain quality due to a reduction in seed size, germination and loss in grade due to a reduction in oil content (Grau & Radke, 1984). Research has indicated that seed germination significantly decreased when Sclerotinia stem rot incidence increased (Hoffman *et al*., 1998). Losses due to soybean rust are the result of early senescence and defoliation resulting in reduced number of pods per plant and number of filled pods per plant (Ogle *et al*., 1979; Hartman *et al*., 1991) and thus reduced number of seeds per plant as well as 100 seed weight (Ogle *et al*., 1979; Kawuki *et al*., 2003). Losses due to soybean rust are closely related to the growth stage of the plant when infection occurs as well as severity of the disease (Ivancovich, 2005).

A proper understanding of the biology and genetic structure of the pathogen population is needed especially when devising disease control and resistance-screening strategies (Sexton & Howlett, 2004). In South Africa the occurrence of Sclerotinia stem rot is becoming more prominent, however information on this pathogen is still limited. Information collected on the genetic structure of isolates could assist in the breeding of cultivars with durable resistance (Zhao & Meng, 2003). It is also important to know if new genotypes have evolved that are more pathogenic than those occurring currently in the area of interest (Hambleton *et al*., 2002). However studies conducted around the world have failed to indicate a specific trend in *S. sclerotiorum* populations. High or low diversity was observed in studies done and results appear to vary between populations and continents. The fact that genetic variation is due to differences among isolates within populations, holds major implications for disease management strategies and development of new resistance cultivars.

Due to the occasional failure of fungicide applications to control the target disease(s) as well as factors such as cost and environmental hazards, the development of cultivars with disease resistance is the desired method for disease management as it is more economical and the environmental impact is reduced (Pham *et al*., 2010). Soybean is

highly susceptible to *S. sclerotiorum* and the impact of infection on the crop is high. To date, no known acceptable sources of resistance to Sclerotinia stem rot are available (Hoffman *et al*., 2002) and current knowledge of sources of resistance is limited because of the relatively low number of lines evaluated to date. The use of avoidance mechanisms such as upright and open plant structure, less dense canopies and branching patterns, elevated pod set and reduced lodging have been suggested to reduce the damage caused by *S. sclerotiorum*. However, identification of resistance is hampered by factors such as easy and reliable screening procedures and a little genetic variability available for resistance to Sclerotinia stem rot (Grafton, 1998). Although several methods have been evaluated for greenhouse testing, these methods are not always reliable for the identification of resistance and are poorly correlated with field data. A high correlation between greenhouse and field data is essential. Cultivars do not rank consistently which leads to problems with reproducibility of results as well as misinterpretation of results (Chun *et al*., 1987; Nelson *et al*., 1991; Pennypacker & Risius, 1999; Bradley *et al*., 2006).

Similar constraints exist in the development of resistant cultivars to soybean rust. Soybean genotypes show different resistance responses to soybean rust over time (Ribeiro *et al*., 2007) and therefore breeders need to combine early stage resistance with later stage resistance by carrying out assessment at different stages of host development. A problem in the recognition and evaluation of resistance, however, is the assessment of rust prevalence and severity throughout a planting season for a consecutive number of years (Bromfield, 1984). Significant genotype x micro-environment interactions observed in crosses suggested that the expression of soybean rust is dependent on specific combinations between the host and micro-environmental conditions (Ribeiro *et al*., 2007).

Three infection types on soybeans have been described by Bromfield *et al*. (1980), Bromfield and Hartwig (1980) and Bromfield (1984). After infection, leaf tissue surrounding the pustules turn into a reddish brown ("RB") type lesion in resistant plants representing infection type 1. A pale brown lesion in colour equivalent to the "Tan" type lesions in susceptible plants is referred to as infection type 2 while the absence of lesions indicates immunity or near-immunity known as infection type 3. In common bean trials, RB infection types were also associated with low sporulation while tan lesions had high sporulation (Miles *et al*., 2007). Twizeyimana *et al*., (2008) suggested that genotypes expressing RB lesions may be sources of partial resistance.

Due to the lack or limited resistant sources available in soybean germplasm, control of these diseases especially soybean rust remains a challenge and therefore chemicals are

still widely used. When disease is already present in fields, the application of fungicides will prevent further spread. However the application of chemicals prophylactically is still the primary recommendation. Fungicide protection against soybean rust is crucial during reproductive stages of crop development especially growth stages R1, also known as beginning of flowering through to R6 when the crop has reached the full seed stage. According to research, three fungicide applications are needed in areas with high rust severity to provide proper control. These applications should commence at first flowering and be followed by the other two sprays at 21 day intervals. In areas with a low risk of infection, two fungicide sprays have been shown to be sufficient (Levy, 2005). Although fungicides have proved to be effective, several factors need to be taken into consideration such as the product selected, especially since the efficacy differs among active ingredients used, while single ingredient or combinations remains important with reference to fungicide resistance.

Since plants are exposed to a number of pathogenic fungi and lack a circulating somatically adaptive immune system (Fritig *et al*., 1998) they have evolved several defence mechanisms to prevent the development of disease. These include the synthesis of low molecular compounds, proteins and peptides that have antifungal activity (Anguelova-Merhar *et al*., 2002). The expression of pathogenesis-related proteins and their synthesis during attack have been widely investigated in a variety of plant species. They have been classified as a diverse family due to the fact that they have displayed various biological activities in different species (Liu & Ekramoddoullah, 2006). Host plant resistance is expressed when it is affected by both abiotic and biotic factors (Bi *et al*., 1994) and therefore a better understanding of the role of chitinases, peroxidases, β-1,3-glucanase and other physiological compounds that play a role in disease tolerance against soybean rust will aid in the development of resistance breeding (Anguelova-Merhar *et al*., 2002).

Despite extensive research on the topics discussed above, Sclerotinia stem rot and soybean rust still remain a problem to soybean production in South Africa and many other countries.

The aim of this study was to:

1. Review literature available on Sclerotinia stem rot and soybean rust.
2. Compare and evaluate experimental lines for resistance to soybean rust in the greenhouse and field and compare epidemiological factors essential to this process.

3. Evaluate the physiological changes that occur in soybean plants and their influence on susceptibility to soybean rust.
4. Compare and evaluate yield losses caused by soybean rust on a short, medium and long maturity cultivars and the effect of chemical control on the disease.
5. Compare and evaluate experimental lines for resistance to Sclerotinia stem rot in the greenhouse and field.
6. Evaluate isolate and genetic variation of *S. sclerotiorum* isolates collected from local fields in South Africa using AFLP analysis.

References

Abawi, G.S. & Grogan, R.G., 1975. Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. Phytopathology 65: 300-309.

Agrios, G.N. 1997. Plant Pathology. 4th Edition. Academic press. New York.

Anguelova-Merhar, V.S., van der Westhuizen, A.J. & Pretorius, Z.A. 2002. Intercellular chitinase and peroxidase activities associated with resistance conferred by gene *Lr35* to leaf rust of wheat. Journal of Plant Physiology 159: 1259-1261.

Anonymous, 2010. Soya Beans - Production Guideline 2010. Department: Agriculture, Forestry & Fisheries. Republic of South Africa. http://www.nda.agric.za/docs/brochures/soya-beans.pdf. Accessed: April 2012.

Anonymous, 2011. History of soybeans - North Carolina soybeans producers association inc. www.ncsoy.org/ABOUT-SOYBEANS/History-of-soybeans. Accessed: June 2012.

Anonynous, 2013. Index Mundi - South Africa Soybean Oilseed Production by Year. http://www.indexmundi.com/agriculture/?country=za&commodity=soybean-oilseed&graph=production. Accessed: April 2014.

Bardin, S.D. & Huang H.C. 2001. Research on biology and control of *Sclerotinia* diseases in Canada. Canadian Journal of Plant Pathology 23: 88-98.

Bi, J.L., Felton, G.W. & Meuller, A.J. 1994. Induced resistance in soybean to *Helicoverpa zea*: Role of plant protein quality. Journal of Chemical Ecology 20: 183-1983.

Boland, G.J. & Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Canadian Journal of Plant Pathology 16: 93-108.

Bradley, C.A., Henson, R.A., Porter, R.A., LeGare, D.G., del Rio, L.E. & Knot, S.D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Disease 90: 215-219.

Bromfield, K.R. 1984. Soybean rust. Monograph no 11. American Phytopathological Society Press.. St. Paul, Minnesota, USA.

Bromfield, K. R., & Hartwig, E. E. 1980. Resistance to soybean rust and mode of inheritance. Crop Science 20:254-255.

Bromfield, K.R., Melching, J.S. & Kingsolver, C.H. 1980. Virulence and aggressiveness of *Phakopsora pachyrhizi* isolates causing soybean rust. Phytopathology 70: 17-21.

Chun, D., Kao, L.B. & Lockwood, J.L. & Isleib, T.G. 1987. Laboratory and assessment of resistance in soybean to stem rot caused by *Sclerotinia sclerotiorum*. Plant Disease 71: 811-815.

Fritig, B., Heitz, T. & Legrand, M. 1998. Antimicrobial proteins in induced plant defence. Current Opinion in Immunology 10: 16-22.

Grafton, K.F. 1998. Resistance to white mold in dry bean. Proceedings of the Sclerotinia workshop 1998

Grau, C.R. & Radke, V.L. 1984. Effects of cultivars and cultural practices on sclerotinia stem rot of soybean. Plant Disease 68: 56-58.

Hambleton, S., Walker, C. & Kohn, L.M. 2002. Clonal lineages of *Sclerotinia sclerotiorum* previously known from other crops predominate in 1999-2000 samples from Ontario and Quebec soybean. Canadian Journal of Plant Pathology 24: 309-315.

Harikrishnan, R. & Del Rio, L.E. 2006. Influence of temperature, relative humidity, ascospore concentration and length of dry bean flowers on white mold development. Plant Disease 90: 946-950.

Hartman, G.L., Wang, T.C. & Tschanz, A.T. 1991. Soybean rust development and the quantitative relationship between rust severity and soybean yield. Plant Disease 75: 596-600.

Hoffman, D. D., Diers, B. W., Hartman, G. L., Nickell, C. D., Nelson, R. L., Pedersen, W. L., Cober, E. R., Dorrance, A. E., Graef, G. L., Steadman, J. R., Grau, C. R., Nelson, B. D., del Rio, L. E., Helms, T., Poysa, V., Rajcan, I., & Stienstra, W. C. 2002. Selected soybean plant introductions with partial resistance to *Sclerotinia sclerotiorum*. Plant Disease 86:971-980.

Hoffman, D.D., Hartman, G.L., Meuller, D.S., Leitz, R.A., Nickell, C.D. & Pedersen, W.L. 1998. Yield and seed quality of soybean cultivars infected with *Sclerotinia sclerotiorum*. Plant Disease 82: 826-829.

Ivancovich, A. 2005. Soybean rust in Argentina. Plant Disease 89: 667-668.

Kawuki, R.S., Adipala, E. & Tukamuhabwa, P. 2003. Yield loss associated with soya bean rust (*Phakopsora pachyrhizi* Syd.) in Uganda. Journal of Phytopathology 151: 7-12.

Levy, C. 2005. Epidemiology and chemical control of soybean rust in Southern Africa. Plant Disease 89: 669-674.

Liu, J.J., & Ekramoddoullah, A.K.M. 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiological and Molecular Plant Pathology 68: 3-13.

Machetti, M.A., Melching, J.S. & Bromfield, K.R. 1976. The effects of temperature and dew period on germination and infection by uredospores of *Phakopsora pachyrhizi*. Phytpathology 66: 461-463.

Miles, M.R., Pastor-Corrales, M.A., Hartman, G.L & Frederick, R.D. 2007. Differential response of common bean cultivars to *Phakopsora pachyrhizi.* Plant Disease 91: 698-704.

Nelson, B.D., Helms, T.C. & Olson, M.A. 1991. Comparison of laboratory and field evaluations of resistance in soybean to *Sclerotinia sclerotiorum*. Plant Disease 75: 662-665.

O'Brien, D. 2015. Grain Market Outlook. Soybean Market Outlook in March 2015. March 20, 2015. http://www.agmanager.info/marketing/outlook/newletters/Soybeans.asp. Accessed: May 2015.

Ogle, H.J., Byth, D.E. & Mclean, R. 1979. Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in south-eastern Queensland. Australian Journal of Agricultural Research 30: 883-893.

Ono, Y., Buritica, P. & Hennen, J.F., 1992. Delimitation of *Phakopsora, Physopella* and *Cerotelium* and their species on Leguminosae. Mycological Research 96: 825-850.

Pennypacker, B.W. & Risius, M.L. 1999. Environmental sensitivity of soybean cultivar response to *Sclerotinia sclerotiorum.* Phytopathology 89: 618-622.

Pham, T.A., Hill, C.B., Miles, M.R., Nguyen, B.T., Vuong, T.D., Van Toai, T.T., Nguyen, H.T. & Hartman, G.L. 2010. Evaluation of soybean for resistance to soybean rust in Vietnam. Field Crops Research 117: 131-138.

Ribeiro, A.S., Moreira, J.U.V., Pierozzi, P.H.B., Rachid, B.F., de Toledo, J.F.F., Arias, C.A.A., Soares, R.M. & Godoy, C.V. 2007. Genetic control of Asian rust in soybean. Euphytica 157:15–25.

Rytter, J.L., Dowler, W.M. & Bromfield, K.R. 1984. Additional alternative hosts of *Phakopsora pachyrhizi*, causal agent of soybean rust. Plant Disease 68: 818-819.

Sexton, A.C. & Howlett, B.J. 2004. Microsatelite markers reveal genetic differentiation among populations of *Sclerotinia sclerotiorum* from Australian canola fields. Current Genetics 46: 357-365.

Shurtleff, W. & Aoyagi, A. 2007. History of world production and trade. Part 1 & 2. Chapter from: History of soybeans and soyfoods: 1100BC. to the 1980s. www.soyinfocenter.com. Accessed: January 2010.

Sinclair J.B. & Hartman G.L. 1999. Soybean rust. In: Compendium of Soybean Diseases, 4theds Hartman, G.L., Sinclair, J.B. & Rupe, J.C. p.25–26. American Phytopathological Society, St Paul, Minnesota.

Twizeyimana, M., Ojiambo, P.S., Ikotun, T., Dadipo, J.L., Hartman, G.L. & Bandyopadhyay, R. 2008. Evaluation of soybean germplasm for resistance to soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 92: 947-952.

Zhao, J. & Meng, J. 2003. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.) Theoretical and Applied Genetics 106: 759-764.

CHAPTER 1

AN OVERVIEW OF SCLEROTINIA STEM ROT AND SOYBEAN RUST IN SOUTH AFRICA

1.1 Introduction

The first record of soybean (*Glycine max* (L.) Merr.) production in South Africa is from 1903 (Shurtleff & Aoyagi, 2007). However, it is only relatively recently that soybean has developed into a major cash crop, globally (Pschorn-Strauss & Baijnath-Pillay, 2004). According to the Food and Agriculture Organization (FAO), soybean production in South Africa has increased from an estimated 153 472 tons (t) in 2000 to 218 000 t in 2008 and in 2009 this figure doubled to 516 000 t. In 2014, 948 000 t were produced (Sagis, 2014). Around the world, soybean is a significant source of income and factors influencing production may, therefore, affect the economic welfare of many countries (Wrather *et al*., 2001).

Diseases reduce yields as well as the quality of the product, increase production costs due to the need for the application of chemicals and can negatively affect cropping decisions. Disease severity, prevalence and yield loss are closely associated with environmental conditions, cropping practices and the susceptibility of soybean cultivars to infection by plant pathogens (Yang & Feng, 2001). Across the world several diseases are of particular concern to soybean production and emphasis has been placed on diseases, in particular soybean rust and Sclerotinia stem rot to ensure prevention or appropriate management.

In this study, the main focus was on Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary and the serious foliar disease, soybean rust, caused by *Phakopsora pachyrhizi* Syd. & P. Syd as these two diseases are the major biotic production constraints in South African soybean production areas.

1.2 Soybean production statistics

Around the world soybean is an important crop since seeds are an oil source and also used for protein meal. Soybeans are a significant source of high quality, low cost protein and, given the average daily requirement according to the FAO, 65 grams of soybean

protein per person per day can meet 34.9% of the protein needs of every person on the planet (Shurtleff & Aoyagi, 2007).

China, Brazil, Argentina, United States of America and India have all become major producers of soybean as the demand for food, oil and animal feed has increased annually. In 2012 soybeans represented 57% of oilseed production worldwide (Soystats, 2013). In 2005, China, Brazil, Argentina and the USA produced 200 000 000 t of soybeans, 90% of the global total for that specific year (Workman, 2007).

Soybeans have been grown in East Asia for more than a 1000 years and figures from as early as 1901 show the progression of soybean production in Asia (Markley, 1950). Records from 1909 through to 1913 indicated that China produced an estimated 71.5% of the world's soybeans (Shurtleff & Aoyagi, 2007). Today China is still one of the dominant factors in world soybean trade and as demand increases, it is projected that China will import more than half of the world's soybean production in future. This was the case in 2013/14 when 62.4% of the world soybeans were imported by China. In 2005, China produced 18 000 000 t of soybeans, while importing 27 000 000 t, 41% of the world soybean imports (Workman, 2007). In 2007, 14 300 000 t were produced in China (Soystats, 2011) and production figures from the 2012/13 production year indicated that China produced 12 600 000 t of soybeans (Statista, 2014) thus increasing a need to continue imports in order to meet local demand.

In 1970, soybean production in Brazil was recorded at 1509 t increasing to 11344 t by 1976 (Holz, 1977). However, production and demand increased and in 2003/04 Brazil produced 50 500 000 t, in 2005, 57 000 000 t (Workman, 2007) and 61 000 000 t in 2007 (Soystats 2008). In 2012/13, Brazil was a major soybean producing country in the world, producing 83 500 000 t which was a remarkable increase in production from the 2011/12 production year where 66 500 000 t were produced (Statista, 2014).

A similar trend has been reported from Argentina where production of soybean increased from 695 000 tons in 1975-76 to 36 000 000 tons in the 2002/03 season (Ivancovich, 2005). In 2005, Argentina produced 41 000 000 t (Workman, 2007) and in 2007, 47 000 000 t (Soystats, 2008). These figures continued to rise in the 2010/11 production year where 49 000 000 t were produced and during 2012/13, 53 000 000 t were recorded. South American soybean production consisting mainly of Brazil, Argentina and Paraguay produced 148 900 000 metric tons in 2013/2014 and made up 51.9% of the world production for that season.

In the USA soybean production grew from 30 675 t to 34 012 t in the 1970's (Holz, 1977). Since 2000 production has increased from 66 780 000 t in 2004 to 84 000 000 t in 2005 (Workman, 2007) and 70 400 000 t in 2007 (Soystats, 2008). Since then soybean production has kept rising in the USA making it a major soybean production country in the world with soybean production at 82 100 00 t in the 2012/13 production year (Statista, 2014). Soybeans are the second largest cash crop in the USA grown in more than 30 states (Soystats, 2013) and in 2013/14, 91 400 000 t were produced (Soystats, 2014).

South African soybean production has increased steadily over recent years. In the 2000/01 season a total of 209 705 tons were produced. By 2003/04, South Africa was ranked 18 in world soybean production with 220 000 t (Anonymous, 2009). In 2005/06 figures indicated that production almost doubled to 424 000 tons. In 2009/10, 516 000 tons were produced and a sharp increase was recorded in the 2012/13 season as a total production of 784 500 tons were recorded (Sagis, 2014). Soybeans are produced primarily in Mpumalanga, Kwazulu Natal and the Free State in areas surrounding Bergville, Bethal, Ermelo, Newcastle, Warden, Winterton, Vryheid and Vrede. In 2000/01, 72 000 ha of soybean were planted in Mpumalanga, 25 800 ha in Kwazulu Natal and 20 000 ha in the Free State. Over the past decade these figures have increased significantly to 145 000 ha in Mpumalanga and 95 000 ha in the Free State. Kwazulu Natal however has showed no significant growth over this period and has tended to stay stable with only 30 000 ha under production in 2009/10 (Sagis, 2011). Figures for the 2013 production year stated a high increase in area planted in the Free State (215 000 ha) followed by Mpumalanga (205 000 ha) with Kwazulu Natal still showing no significant increase in production area (30 000 ha) (Sagis, 2014).

1.3 Sclerotinia stem rot

1.3.1 Symptoms and signs

Symptoms vary according to host and environment. The disease first appears as wilted leaves scattered in the field and later soft, watersoaked lesions become visible on stems, pods and leaves, especially on soybeans where darker coloured areas with watersoaked margins appear then gradually enlarge. Infected stems and branches become wilted and as death of plant parts occurs, tissues take on a white bleached appearance which may be covered in white mycelium (Figure 1.1).

The fungus is able to colonize healthy tissue and produce new sclerotia in 10 to 14 days (Venette, 1998). These are white at first and later become black with a hard exterior (Agrios, 1997) (Figure 1.2). The epidermal layers become dried and cracked (Steadman, 1983) and the intervascular tissue in stems disintegrates (Thompson & van der Westhuizen, 1979).

Figure 1.1. Stems infected with *Sclertinia sclerotiorum* taking on a white, bleached appearance and covered in mycelium (Photo C. Steyn).

Figure 1.2. Formation of new sclerotia on plant parts infected with *Sclerotinia sclerotiorum* (Photo C. Steyn).

Death of the whole plant can occur as fungal activity continues within the infected plant parts and new sclerotia are formed as black elongated structures (Steadman, 1983) (Figure 1.3).

Undeveloped seedpods are visible in severely affected plants (Thompson & van der Westhuizen, 1979). Secondary spread of the disease is mostly associated with abundant mycelial growth from infected tissues and spread from diseased to healthy tissue occurs upon contact (Abawi & Grogan, 1979; Abawi & Hunter, 1979; Purdy, 1979; Steadman, 1983). Dispersal of severely infected and disintegrated tissue by environmental factors such as rain and wind can also contribute to secondary infections (Natti, 1971).

Figure 1.3. Formation of new sclerotia as black, elongated structures within soybean stems infected with *Sclerotinia sclerotiorum* (Photo C. Steyn).

1.3.2 Host range and distribution

In 1937, the pathogen was first described as *Peziza sclerotiorum* and subsequently renamed various times in honour of scientists involved in the identification of this fungus (Bolton *et al*., 2006). De Bary however used the name as early as 1884 and therefore, the proper name and authority for the fungus was accepted as *Sclerotinia sclerotiorum* (Lib.) de Bary (Purdy, 1979).

According to Boland & Hall (1994) the host index for *S. sclerotiorum* contains 42 sub-species, 408 species, 278 genera and 75 families of plants. Most reported hosts fall within Spermatophyta, specifically the Gymnospermae and Angiospermae. The largest number of hosts includes, in decreasing order, Asteraceae, Fabaceae, Brassicaceae, Solanaceae, Apiaceae and Ranunculaceae. Therefore, the wide host range makes *S. sclerotiorum* a

serious omnipresent pathogen and the widespread occurrence due to wind-blown ascospores that can travel as far as 10 to 50 m from the source (Steadman, 1983; Nelson, 1998) and dissemination from field to field on seed, in soil on farming equipment and by animals in the form of sclerotia (Adams & Ayers, 1979) make this pathogen a serious threat to susceptible crops.

The pathogen is known to cause severe losses in dicotyledonous agricultural crops such as sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry bean and lentils and in some monocotyledonous crops such as onions and tulips (Bolton *et al*., 2006). In South Africa *S. sclerotiorum* has been reported on brussels sprouts, cabbage, carrots, cauliflower, cotton, dry beans, green beans, lettuce, lupines, soybeans, sunflowers and tomatoes (Gorter, 1977; Philips & Botha, 1990).

Sclerotinia sclerotiorum has been reported on various crops worldwide. *Sclerotinia sclerotiorum* was reported in Canada on grass pea in 1990 (Zimmer & Campbell, 1990). In 2000 rotting grapevines were discovered in South Australia and the role of *S. sclerotiorum* was verified (Hall *et al*., 2002) with a subsequent report in 2001 of stem rot and wilt of Chick pea caused by *Sclerotinia minor* in Queensland (Fuhlbohm *et al*., 2003). In the United States, this pathogen was first reported on rosemary in 2002 (Putnam, 2003) and on peanut in Georgia in 2004 (Woodward *et al*., 2006). In North Dakota and Washington it was first reported on chickpea in 2005 (Chen *et a*l., 2006). In Texas it was reported for the first time on canola in 2007 (Isakeit *et al.,* 2010*).* In all major sunflower producing areas in Canada and the USA, *S. sclerotiorum* remains a major problem. In the major sunflower production areas of the USA namely North Dakota, South Dakota and Minnesota, Sclerotinia wilt was observed in 48% of fields in 1984 (Gulya & MacArthur, 1984).

In 2007 Sclerotinia rot on blueberries was identified for the first time in Argentina (Perez *et al*., 2011). In Italy, this pathogen was first reported on *Gazania* sp. hybrids in 2001 (Garibaldi *et al.*, 2001), on candytuft in 2004 (Garibaldi *et al*., 2007) and on citrus rootstock as Sclerotinia stem and twig blight in 2011 (Polizzi *et al*., 2011). In Turkey this pathogen was reported to cause wilt and collapse of sweet basil (Tok, 2008) and in 2009, was reported for the first time on potatoes in Iran (Ojaghian, 2009).

In 1979, Thompson and Van der Westhuizen, (1979) confirmed the presence of this fungus on soybeans in South Africa. The first severe epidemics of stem rot of soybeans occurred in the Gauteng area during the late 1970s and 1980s (Phillips & Botha, 1990). This disease

remains a major production constraint in South African fields and has increased in importance in the recent years.

1.3.3 Economic Importance

Species of the genus *Sclerotinia* cause destructive diseases on numerous plant species around the world, affecting seedlings, mature plants as well as harvested products (Agrios, 1997). *Sclerotinia sclerotiorum* is considered to be among the world's most omnipresent and successful plant pathogens, together with the closely related species, *S. minor* and *S. trifoliorum*. *Sclerotinia sclerotiorum* does the most damage to vegetables and oilseed species while *S. minor* occurs more frequently on peanuts and lettuce and *S. trifloliorum* on forage legumes (Steadman, 1983). *Sclerotinia minor* and other *Sclerotinia* spp. are known to cause root, stem, fruit and vegetable rots while *Sclerotium cepivorum* causes white rot of onions and other *Allium* spp. (Alexander & Stewart, 1994). An increase in the importance of Sclerotinia stem rot on soybeans is becoming a worldwide phenomenon due to changes in management practices, germplasm susceptibility and favourable weather conditions (Mueller *et al*., 2004).

Losses due to *S. sclerotiorum* on soybean result directly from loss in yield and indirectly from reduced grain quality and loss in grade. Grau & Radke (1984) found that disease severity and yield are statistically, inversely correlated ie. the greater the disease severity, the lower the yield. In Sclerotinia stem rot affected plants, seed quality characteristics such as a reduction in seed size, seed germination as well as a reduction in oil content are affected. Seed germination significantly decreases as Sclerotinia stem rot incidence increases (Hoffman *et al*., 1998).

Since 2002, collective annual losses due to *S. sclerotiorum* reported on crops in the USA has been as high as 100 000 000 $US for sunflowers, 70 000 000 $US for soybeans, 46 000 000 $US for dry edible beans and 24 000 000 $US for canola according to the National Sclerotinia Initiative (2014).

Wrather & Koenning (2009) reported that Sclerotinia stem rot was ranked among the top ten diseases suppressing soybean yields in the USA in six of the 12 years of evaluation from 1996 to 2007. In 1996, 354 105 t were lost due to this disease and dramatically increased to 957 687 t in 1997. In 1998, soybean yield losses in the USA due to Sclerotinia stem rot were estimated at $US 31 000 000 000 indicating a significant increase over a relative short time period when compared to losses in 1994 which were reported at $US 9

000 000 00 (Wrather *et al.,* 2001). Yield losses were the lowest during 2003 (56 635 t) but in 2004, 1 633 150 t in yield losses were recorded (Wrather & Koenning, 2009). According to Wrather *et al*. (2001), the reduction in soybean yields for the top ten soybean producing countries in the world during 1998 was greatest for the United States (509 000 t) followed by India (438 500 t) and Argentina (423 200 t). Seasonal variation in yield losses due to this disease makes the evaluation of losses difficult and therefore accurate and recent data are limited.

1.3.4 Epidemiology

Primary infection results from carpogenic germination (Steadman, 1983). Sclerotia buried in the soil need adequate moisture and temperatures between 4°C to 20°C to trigger the dormant sclerotia to become active. Upon activation, an apothecial fruiting body is produced from the sclerotia within 10 to14 days (Venette, 1998). Apothecial stalks are usually 5 cm in length meaning that only sclerotia present in the first 5 cm of the soil surface are regarded as being epidemiologically competent. Ascospores are formed within the apothecial disks and when ripe, large numbers ranging from 10 000 to 30 000 will mature and be released. A high relative humidity is a trigger for ascospore discharge which may continue over a period of several days. Spores are released and infect the aerial tissues resulting in stem blight, stalk rot, head rot, pod rot and blossom blight of plants (Bardin & Huang, 2001). Disease incidence increases in fields with a high inoculum density and high soil moisture as initiation of disease is favoured by cool, damp soil conditions (Tu, 1997).

Sclerotinia stem rot usually becomes evident in fields 10 to14 days after full bloom. The pathogen requires an exogenous energy source to infect healthy or green plant parts and senescent or injured organs provide the pathogen with the necessary energy (Steadman, 1983). Various reports state that senescent or dead flowers are the primary site of infection (Natti, 1971; Abawi & Grogan, 1979) as well as necrotic tissues resulting from injury or other pathogens (Tu, 1989). Dillard & Cobb (1995) investigated the effect of wounding on cabbage infection and results confirmed that injuries penetrating several leaf layers of cabbage plants resulted in cell damage and exudation that serves as a source of nutrients for the germination of ascospores and infection of the cabbage head by *S. sclerotiorum*.

The most important climatic factor affecting disease development is moisture (Abawi & Grogan, 1975) as it is essential for the production, release and germination of ascospores. Harikrishnan & Del Rio (2006) however, found that ascospores can cause infection at relative humidity as low as 25%. Plants inoculated with suspensions of dry ascospores all

developed disease after exposure to a range of wetnesss durations or RH at different temperatures, however the study failed to find a relationship between these criteria and the percentage of diseased plants (Young *et al*., (2004). Under continuous leaf wetness, ascospores inoculated on lettuce leaves germinated within 2-4h at 15 to 25°C, 10 h at 10°C with little to no germination at 30°C. Botha *et al*. (2011) found that under greenhouse conditions the optimum temperature for disease development was 20.95°C accompanied by a high RH period (>90%) of 8.95 days post-inoculation.

Tu (1989) stated that a free moisture period of 48 to 72 h is required for establishment of infection and lesion expansion. In the absence of free moisture, lesion development quickly stops and the fungus remains quiescent in the lesion until it can be reactivated upon the return of free moisture. This needs to be accompanied by temperatures between 16°C and 25°C that are best for germination (Harikrishnan & Del Rio, 2006). Abawi & Grogan (1979) found that no disease develops at temperatures below 5°C and above 30°C. After successful infection and under favourable conditions, the fungus proceeds from senescent blossoms to healthy tissue in about 16 to 24 h (Venette, 1998). Mycelium is most likely responsible for secondary infections in the field (Abawi & Grogan, 1979; Abawi & Hunter, 1979; Purdy, 1979; Steadman, 1983).

A second infection method, namely myceliogenic germination, is less common than carpogenic germination, however it plays a major role in the disease cycle of Sclerotinia wilt of sunflower. Sclerotia in the soil germinate in the presence of exogenous nutrients and produce hyphae that penetrate the host cuticle of roots and stems at the soil-air interface by mechanical pressure (Ferreira & Boley, 1992). This method of infection however is unlikely to take place if the plant is located more than 2 cm away from the sclerotium. Lesions develop and quickly expand upwards on the stem, resulting in wilting of the aerial parts of the plants and eventually death. Since plants are susceptible to *S. sclerotiorum* at any time after seedling emergence, this method of infection can result in damping-off symptoms (Purdy, 1979).

Myceliogenic germination of sclerotia occurs easily at temperatures ranging from 20°C to 25°C accompanied by high humidity (Huang & Kozub, 1993). Cook *et al*. (1975) found that *S. sclerotiorum* does not survive as mycelium in soil or residue, however Laemmlen (2006) reported that *S. sclerotiorum* and *S. minor* have the ability to survive between crops as mycelium in infected plant debris. Studies conducted by Natti (1971) showed that mycelium could serve as inoculum on beans (*Phaseolus vulgaris*). Survival of mycelia could be affected by humidity and microorganisms in the soil. Studies conducted in

Nebraska, USA, indicated that mycelium lost viability rapidly after 4 to 8 months in the field however when seedlings come into direct contact with surviving mycelia these can serve as primary inoculum for seedling infection (Huang & Kozub, 1993).

The most important environmental factors affecting mycelial survival is moisture and temperature and survival in air-dried stems was better at 10°C than at 20°C (Huang & Kozub, 1993). Mycelia are also more tolerant to desiccation than ascospores and therefore may be able to cause disease under lower RH conditions (Harikrishnan & Del Rio, 2006). Mycelium and ascospores proved equally effective on plants inoculated and maintained at 90% RH and at 18 and 22°C. When RH was lowered to 25%, disease still occurred when plants were inoculated with ascospores, however a lower germination rate was observed and thus less disease was recorded. In contrast, mycelia remained viable after 144 h of drying indicating that mycelia are more tolerant to desiccation than ascospores (Harikrishnan & Del Rio, 2006).

1.3.5 Genetic variability

An understanding of the genetic structure of the pathogen population is especially important when devising disease management and resistance screening strategies (Sexton & Howlett, 2004). Information on the genetic structure of *S. sclerotiorum* isolates could assist in the breeding and improving cultivars with durable resistance (Zhao & Meng, 2003). It is also important to know if new genotypes are evolving with increased aggressiveness (Hambleton *et al*., 2002).

Studies have confirmed various levels of genetic diversity in *S. sclerotiorum* populations. Noonan *et al*. (1996) studied isolates from New Zealand and the USA and given the large geographic difference between isolates, geographic and phytotypic differences were observed. However, a high level of genetic homogeneity was also observed. This homogeneity may be explained by the fact that the USA material was all derived from the same wild type. The New Zealand material was collected from three hosts which would suggest the presence of more variation, however the diversity was still low.

Genetic variability in *S. sclerotiorum* isolates ranged from moderate to high levels of differentiation in New South Wales and Victoria in Australia. This may be due to gene flow not being a regular phenomenon in these areas or the geographical separation between the two areas (Sexton & Howlett, 2004). In Iran isolates from four sites revealed that differentiation between isolates was possible and diverse populations of *S. sclerotiorum*

isolates were reported. High genetic diversity was found as well as high percentages of DNA mutation (Colagar *et al*., 2010). According to Attanayake *et al*. (2012) high genetic variability may also be explained by the new introduction of sclerotia from soil, seed machinery as well as wind-blown ascospores from surrounding areas.

Kohn *et al.* (1991) found that 63 *S. sclerotiorum* strains collected from two fields in Ontaria, were genetically heterogeneous when the mycelium compatibility grouping of these strains was evaluated. In Brazil a high level of genetic variability in 40 *S. sclerotiorum* isolates was reported. Isolates were representative of crop production regions in Brazil and the high level of variability suggests that sexual reproduction occurs in the tropical and subtropical regions as opposed to the clonal reproduction in the temperate regions (Litholdo Junior *et al*., 2011). A possible explanation for this may be that areas with milder winters enable the pathogen more favourable conditions for outcrossing (Atallah *et al.*, 2004). When samples from Brazil and USA were collected high diversity in the isolates present in the Brazil fields were reported and this might indicate some level of outcrossing in addition to the probable intra-clonal variation since each clone can accumulate mutations and generate new gentotypes (Koga *et al*., 2014). Gomes *et al*. (2011) attributed the high variability to the prevailing environmental conditions and crop rotation practices which may be favouring the sexual recombination in these populations.

A study conducted in Brazil involving 23 *S. sclerotiorum* isolates from winter bean fields indicated genetic uniformity. This may be attributed to *S. sclerotiorum* only recently being introduced into the studied areas and that the pathogen had limited time and opportunity to undergo genetic change (Meinhardt *et al*., 2002). Koga *et al*. (2014) also reported low genetic variability where 81% of isolates belonged to a single mycelium compatibility group. This is unexpected as samples were taken over an area covering 2000 km. Isolates infecting the recently planted crops may have originated from a common isolate present in contaminated seed or shared farm equipment.

According to Sexton & Howlett (2004), low genetic diversity present in the *S. sclerotiorum* population can be expected due to the homothallic nature of this fungus, resulting in a large amount of selfing and asexual propagation that occurs via myceliogenic germination of sclerotia. Factors such as clonal propagation through sclerotia production or homothallic ascospore production also contribute to the genetic structure of this pathogen (Attanayake *et al*., 2012).

1.3.5.1 Fingerprinting techniques

Since the success of a control strategy is dependent on an understanding of the genetic structure of the pathogen population, there has been increased progress regarding molecular biology techniques (Colager *et al*., 2010). These molecular techniques are based on the reliability of results and the majority require DNA based genetic markers. The usefulness of these markers is usually determined by the technology applied to reveal DNA based polymorphisms. The use of molecular markers especially in the characterisation of fungal species has increased and techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) have been widely used. All the above mentioned techniques have been widely used in the characterisation of *Sclerotinia* species.

RFLP (restriction enzyme-generated fragments of different lengths) is a molecular technique developed especially for the assessment of differences between and among species (Jeffreys *et al*., 1985). This technique was successfully implemented in the determination of diversity among *S. sclerotiorum* isolates from Brazil and a high level of genotypic diversity was reported (Gomes *et al.*, 2011). RFLP also proved useful as a technique in taxonomic characteristics due to the fact that RFLP is representative of the deviation that occurrs in the genome as a whole. Successful differentiation between nuclear and ribosomal DNA of *S. sclerotiorum*, *S. minor*, *S. asari*, *S. ficariae* and *Sclerotium cepivorum* was done using RFLP and characteristic fingerprints for species were detected (Kohn *et al*., 1988). However RFLP has the disadvantage of being expensive as well as time-consuming (Majer *et al*., 1996).

RAPD is another technique commonly used especially since it can be applied to any organism from which DNA can be extracted and is described as a random DNA polymorphism assay. This is based on PCR developed with single short primers and is needed to specifically assist in the construction of genetic maps as well as for the evaluation of genetic diversity (Williams *et al*., 1990). According to Mandal *et al*. (2012) RAPD can be successfully implemented to determine genetic variability of *S. sclerotiorum* isolates and reproducible and scorable results can be obtained. Clear distinctions between species were seen and results indicated that *S. minor* and *S. trifoliorum* were more closely related to each other than to *S. sclerotiorum*. Variation within *S. sclerotiorum* populations was successfully determined using RAPD (Punja & Sun, 2001; Colager *et al*., 2010; Litholdo Junior *et al*., 2011). An advantage of RAPD is that a large number of isolates can

be compared, however a much smaller proportion of the genome is analysed (Majer *et al*., 1996). A disadvantage of RAPD is that it is not always able to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous, reproducibility, DNA quality and concentration as well as PCR cycling conditions may have an influence the results as this is an enzymatic reaction (Kumar & Gurusubramanian, 2011).

AFLP is defined as a selective PCR based amplification and has the ability to detect restriction fragments from a total digest of genomic DNA (Vos *et al.*, 1995). This method has been popular for pathotyping, mapping of quantitative trait loci used in population genetic studies and as an aid in the differentiation of closely related organisms (Muiru *et al*., 2008). AFLP has been used to detect genetic variability in *S. rolfsii* populations in South Africa (Cilliers *et al*., 2000). The diversity among 192 *Sclerotinia* isolates collected from 12 European countries was determined using AFLP analysis. Predominantly two species were identified namely *S. trifoliorum* and *S. sclerotiorum*. This technique was helpful in the determination of variation and 79.2% variation was detected within locations and 20.8% among locations (Vleugels *et al.,* 2012).

According to Majer *et al*. (1996) AFLP is a fast and easy method for the detection of polymorphisms especially when a large number of isolates are being evaluated. This method is reproducible and reliable and is able to detect variation over the entire genome without any prior knowledge of the genomic composition. A disadvantage of this technique however is that alleles are not recognised easily and this may lead to overestimation of variation. This technique is also associated with high costs and dominant effects (Muiru *et al*., 2008) ie. polymophism detected as either the band present or absent (Ali *et al*., 2004).

1.3.6 Chemical control

Due to limited resistant sources available in soybean germplasm to Sclerotinia stem rot control of this disease by means of chemicals is still widely applied. When disease is already present in fields, the application of fungicides will prevent further spread. However the degree of control remains dependent on several factors which affect the efficacy of chemicals such as the selection of the correct chemicals, proper timing of sprays, method of application, thorough coverage and penetration of the product into the crop canopy and affected areas.

Fungicides are still widely used to manage Sclerotinia stem rot as studies have shown that yield losses can be minimized with chemical control (Oplinger *et al*., 2007). Infection occurs mainly through senescent flower petals infected by ascospores and application of fungicides is aimed at coverage of infection sites and blossoms (Mueller *et al*., 2002). Hunter *et al.* (1978) reported results from a study done on snap bean (*Phaseolus vulgaris)* that desirable control was achieved when the entire plant or only the blossoms were sprayed. No control was achieved when all plant parts except the blossoms were sprayed indicating that these are crucial infection sites and need to be targeted during disease control strategies.

As with most diseases, control of Sclerotinia stem rot is best achieved when fungicides are applied prophylactically and best control is achieved when a crop in full bloom is sprayed before disease is visible (Mueller *et al.,* 2004). Benomyl proved to be effective against Sclerotinia stem rot on snap bean when applied three to five days before full bloom with proper coverage to ensure translocation of the product to crucial areas such as buds or blossoms ahead of the pathogen (Hunter *et al.*, 1978). According to Johnson & Atallah (2006), better control is achieved when chemicals are applied at full bloom compared to those applied at row closure ie. prior to the presence of flowers or after 20% blossom drop which was too late to provide effective control. Morton & Hall (1989) found that control was not improved by multiple sprays when applied to white bean (*Phaseolus vulgaris*) compared to only one spray applied on the appropriate time indicating how crucial timing of spray application is.

The interaction between fungicide efficacy and agronomic practice is crucial in achieving disease control. Agronomic practices such as early planting, narrow row spacing as well as high seeding rates are often used to optimize yields. These conditions are not only favourable for the development of Sclerotinia stem rot by creating a conducive microclimate, but also limit penetration into the canopy by fungicide sprays, leading to poor coverage of critical plant organs (Mueller *et al*., 2002). In studies done on beans, inadequate control of the disease and poor blossom coverage can be aggravated in varieties with an indeterminate growth pattern and thus, flowering covers an extended period (Steadman, 1979). Proper coverage of the whole plant as well as the blossoms also has additional advantages such as the fungicides reaching the soil surface which may aid in a reduction in the germination rate of sclerotia and production of apothecia (Mueller *et al*., 2002).

Several chemicals have been reported to be efficacious on Sclerotinia stem rot. Benomyl has been reported to provide effective control on soybeans, sunflower cabbage and lettuce (Ferreira & Boley, 1992). No symptoms were observed in greenhouse trials with Sclerotinia stem rot when Benomyl (Benlate™), thiophanate methyl (Topsin M™) and vinclozolin (Ronilan™) were applied (Mueller *et al*., 2002). The dicarboximide fungicides Rovral™, Ronilan™ and Procymidone™ were also reported to be efficacious in the control of Sclerotinia on lettuce and peanuts (Ferreira & Boley, 1992).

Resistance to fungicides remains an ongoing problem. During the 1980's, benzimidazole fungicides, in particular carbendazim, were widely used due to their systemic activity making them effective even after infection had occurred. This led to the rapid selection of resistant populations of *S. sclerotiorum* to this fungicide group (Kuang *et al*., 2011). The efficacy of fludioxonil, a non-systemic fungicide, against field populations of *S. sclerotiorum* was evaluated in China. Results indicated that fludioxonil was effective in controlling sclerotinia stem rot on rape. Laboratory results however identified fludioxonil mutants indicating that resistance to this fungicide could develop fairly rapidly although it was speculated that these will be too weak to survive in the field after the application of the fungicide. When applied in combination with other fungicides such as iprodione and dimethachlon, cross resistance was recorded which could increase the resistance of the pathogen to fludiozonil (Kuang *et al*., 2011). A mixture of thiram and azoxystrobin also provided good control as well as when combined with salicylhydroxamic acid. It was concluded that a mixture increases efficacy compared with the independent use of azoxystrobin and reduced the risk of in azoxystrobin-resistant isolates (Duan *et al*., 2012).

When peanut plants were transformed using a barley oxalate oxidase gene resistance to Sclerotinia blight was observed. However when the non-transgenic lines received two chemical treatments with fluazinam, similar disease incidence and yield were recorded as in the transgenic lines that did not receive chemical treatments. It was clear that there was no benefit in fungicide sprays when a resistant cultivar was planted however the application of fungicides to susceptible cultivars can still provide acceptable disease levels and yields (Partridge-Telenko *et al*., 2011). Therefore development of resistant cultivars may limit and reduce the use of fungicides.

Despite chemical control still being the most effective and widely used method of control against *S. sclerotiorum*, the cost of control is high and the risk of fungicide-resistance fungal strains is still a possibility that could minimize the efficacy of this control strategy (Steadman, 1979).

1.3.7 Breeding for resistance

Chemical and biological control are often expensive or ineffective and their efficacy is affected by environmental conditions and agricultural practices. Genetic control remains the most economic method of disease control as well as being more applicable to sustainable agriculture and minimizing harm to the environment (Phan *et al*., 2010). Identification of resistance, however is hampered by factors such as easy and reliable screening procedures and a minimum of genetic variation for resistance to Sclerotinia stem rot (Grafton, 1998). The goal of breeding for resistance should be to select cultivars with high resistance to all forms of *S. sclerotiorum* attack found in the region of cultivation (Castano, Vear & Tourvieille de labrouhe, 1993).

Despite the impact of the disease on soybeans and the numerous breeding and selection efforts, progress has been limited and acceptable levels of resistance to *Sclerotinia sclerotiorum* have not been forthcoming (Hoffman *et al*., 2002). Boland & Hall (1987) suggested that development of soybean cultivars with resistance to this pathogen appears feasible and may represent an effective and economic strategy for disease control in areas where this disease is prevalent.

Resistance to *Sclerotinia sclerotiorum* was reported in white bean by Tu & Beversdorf (1982). Initial disease incidence in cultivars ExRico 23 and Fleetwood was similar, equivalent in yield and maturity, but the subsequent progression of disease was considerably slower in the former. This cultivar demonstrated a higher cellular tolerance and impeded permeability to oxalic acid secreted by *S. sclerotiorum* compared to the susceptible cultivar. Oxalic acid is regarded as playing a major role in the pathogenicity of this pathogen (Tu, 1989).

Tu (1985) reported that in white bean seedlings susceptible to *S. sclerotiorum,* total collapse of the plant occurred by the seventh or eighth day after inoculation, the development of brown spots was rapid and the leaf tissue disintegrated in two to three days. In contrast the resistant cultivar, ExRico 23, only showed signs of infection on the fifth day after inoculation and the lesions on the hypocotyl and petiole were remarkably smaller than those on the susceptible cultivars. Tu (1989) confirmed that chloroplasts as well as other cellular organelles and the plasma membrane were disrupted by oxalic acid treatment in susceptible plants. This proves that the tolerance level of the plasma membrane and organelles to oxalic acid is an important factor in determining the rate of

disease progression in the tissue infected and therefore, also the resistance of the plant to *S. sclerotiorum.*

Similarly in a Virginia-type peanut transformed with the barley oxalate oxidase gene, reduced lesion size and severity was recorded compared with non-transgenic lines in the field. This demonstrated the effectiveness of the oxalate oxidase gene that has the ability to degrade oxalic acid secreted by the pathogen (Partridge-Telenko *et al*., 2011) and the importance of this degradation in resistance.

Differences in yield of cultivars affected with Sclerotinia stem rot show that different levels of tolerance exist among cultivars and this host characteristic could be an important factor when evaluating soybean cultivars for their response to *S. sclerotiorum* (Grau *et al*., 1982; Hoffman *et al*., 2002).

Grafton (1998) indicated that the nature of inheritance of resistance and the relative importance of physiological resistance, plant architecture and disease escape mechanisms under field conditions show potential for exploitation. According to Tu & Beversdorf (1982) resistance is controlled by several genes and the use of avoidance mechanisms such as upright and open plant structure, less dense canopies and branching patterns, elevated pod set and reduced lodging have been suggested to reduce the damage caused by *S. sclerotiorum*. They, however, were unclear whether the reduction in disease severity results from genetic or structural characteristics. Grafton (1998) reported that changing or incorporating these factors into a single host plant could result in better penetration of the canopy by sunlight and air circulation, making the microclimate within the plant canopy less favourable for disease development.

Identification of resistance is hampered by the absence of easy and reliable screening methods as well as limited genetic variability available for resistance to Sclerotinia stem rot (Grafton, 1998). Greenhouse testing is not a reliable method for identification of resistance and poor correlations between greenhouse and field data have generally been recorded. Cultivars do not rank consistently over trials which lead to problems with reproducibility of results as well as misinterpretation of results (Chun *et al*., 1987; Nelson *et al*., 1991; Pennypacker & Risius, 1999; Bradley *et al*., 2006). According to Wegulu *et al*. (1998) this may be due to differences in defence strategies among germplasm and physiological changes that occur upon infection, the interaction between these criteria and environmental conditions as well as the method of evaluation employed for example detached leaf assay, stem inoculations in the greenhouse or field evaluations. Botha *et al*.

(2009) reported that cultivars SNK 500 and CRN 5550 were ranked equally susceptible when evaluated using a spray mycelium method. However, ratings differed by 81.25% and 46.88% wilting, respectively, when a cut stem method was applied and the study indicated a clear differential response of germplasm to the six inoculation methods tested.

1.3.8 Mechanisms of resistance and pathogenicity

Oxalic acid (Ethanedioic acid – $H_2C_2O_4$) is a toxic metabolite produced by various microorganisms (Pierson & Rhodes, 1992) and its conjugate base, known as oxalate ($C_2O_4^{2-}$), is a chelating agent for metal cations. It occurs in nature as a free acid and commonly as a soluble potassium, sodium oxalate or insoluble calcium oxalate (Guimaraes & Stotz, 2004).

Oxalic acid commonly exists in plants, animals and microorganisms (Liu *et al*., 2009) and has been regarded as an inert end product of metabolism. It is also known as one of the strongest organic acid and the distribution and occurrence of oxalic acid varies considerably among organisms. Commonly in plants, the highest concentration of oxalic acid is found in leaves and the lowest in the roots. These concentrations however, also vary according to age of the plant, growing season, climate and type of soil (Caliskan, 2000).

Pathogenicity of *S. sclerotiorum* is closely related to the production of oxalic acid (Maxwell & Lumsden, 1970; Donaldson *et al*., 2001; Kim *et al*., 2008). Godoy *et al*. (1990) found oxalic acid to be an essential virulence factor and observed that mutant isolates, not able to produce oxalic acid, were non-pathogenic. Oxalic acid production by *S. sclerotiorum* wild-types were five times higher than in isolates that were oxalate deficient (Chipps *et al*., 2005). Identification of pathogenicity in *S. sclerotiorum* isolates has become increasingly important in order to develop management strategies to limit the spread of the disease and aid in breeding for resistance especially since other control measures such as application of fungicides, crop rotation and crop production strategies has been proven to provide limited control (Cessna *et al*., 2000).

The importance of oxalate in the pathogenicity of *Sclerotinia* species may be due to several factors. Either the acidity, which leads to cellular toxicity and aids in fungal invasion, leads to the development of a more suitable apoplastic pH for cell wall-degrading enzymes or the affinity for Ca_2 which weakens plant barriers by leaching the stabilizing cation from the host plant cell (Dutton and Evans, 1996). *Sclerotinia*-secreted oxalic acid is also an elicitor

of programmed cell death and is thus responsible for the induction of apototic-like structures in the plant during disease development as has been observed in tobacco plants inoculated with *S. sclerotiorum.* The induction of this fungal mediated programmed cell death process is required for pathogenic success. Observations also indicated that mutant inoculated plants had significant amounts of H_2O_2 which was not observed in plants inoculated with wild-types (Kim *et al*., 2008).

Studies have confirmed the disruption of chloroplasts, other cellular organelles and the plasma membrane by oxalic acid. Guimaraes & Stotz (2004) found that stomatal guard cells are disrupted due to the inhibition of cellular responses. Oxalate concentrations in leaves two days after inoculation with a virulent wild-type of *S. sclerotiorum* were 25 times higher than the leaves inoculated with an oxalate-deficient mutant. Open stomata were detected in advance of invading hyphae, suggesting that oxalic acid moves faster than fungal mycelium in the leaf tissues. According to Tu (1989), for the oxalic acid to affect the chloroplasts it must also change the semi-permeability of the plasma membrane. This was indicated when tolerance to Sclerotinia stem rot was noted in a moderately resistant bean cultivar, ExRico 23. Smaller lesions were observed in ExRico 23 than in other cultivars. According to Chipps *et al.* (2005), oxalate contributes to lesion expansion, therefore the observation of smaller lesions might be an indication that the plant has the ability to resist permeability of the plasma membrane and thus smaller lesions appear.

Oxalic acid is well documented to be one of the main factors responsible for the wilting symptoms resulting from *S. sclerotiorum* infection (Noyes & Hancock, 1981) as well as contributing to lesion development in susceptible cultivars (Chipps *et al*., 2005). Wilting due to *S. sclerotiorum* infection may also be due to the increase in stomatal conductance and transpiration which in turn results in a reduction in biomass. A decrease in dry weight was also reported and indirectly suggests that the fungus metabolizes host-derived carbohydrates (Guimaraes & Stotz, 2004). Studies have shown that isolates collected from different hosts differed in their ability to release acids, especially oxalic acid. These findings suggested the possibility that the host may have some selective pressure on *S. sclerotiorum* isolates (Durman *et al*., 2005).

Oxalate oxidase or oxalic acid oxidase (EC 1.2.3.4) is a protein that functions in catalyzing the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (Kanauchi *et al*., 2009). In transgenic peanut plants expressing an oxalic acid-degrading oxalate oxidase to *S. minor,* the expression of barley oxalate oxidase increased resistance to oxalic acid applied exogenously at concentrations from 0 to 200 millimolar. This emphasized the

importance of oxalic acid as a pathogenicity factor and the counteracting effects of oxalate oxidase on the expression of oxalic acid induced symptoms. Similarly it was shown that this molecule could enhance resistance to injury and infection by fungal pathogens (Livingstone *et al*., 2005).

According to Kim *et al*. (2008) programmed cell death is essential for fungal pathogenicity and involves reactive oxygen species termed oxidative burst. Many plant species can generate an oxidative burst to resist pathogens. Studies have shown that oxalic acid is effective in suppressing the burst in soybean and tobacco plants as stimulated by a variety of elicitors. If the pathogen manages to discharge sufficient quantities of oxalic acid before the plant can detect the intruder, a primary site of disease resistance will be compromised. Cessna *et al*. (2000) demonstrated a site of action of fungal oxalic acid in the facilitation of fungal infections and a suppression of the oxidative burst in the host plant in the mechanism used by which oxalate aids in the colonization of plant tissue.

1.4 Soybean rust

1.4.1 Symptoms and signs

Soybean rust is caused by *Phakopsora pachyrhizi* (Syd. & P. Syd). At first chlorotic spots are visible that gradually develop into coloured lesions usually on the abaxial leaf surface (Figure 1.4) (Bromfield, 1984; Hartman *et al*., 1999; Sconyers *et al*., 2006). These lesions develop into reddish brown lesions, characterised by dark red spots on the adaxial surface of the leaf (Figure 1.5) and the abaxial surface has been associated with few uredinia and reduced sporulation (Figure 1.6) (Miles et al., 2007). Tan coloured (Figure 1.7) lesions are the most common and are characterised by pale yellow-brown to tan lesions mainly due to the appearance of the abundant uredinia and beige coloured urediniospores on the abaxial surface of the leaf and these are often associated with susceptibility due to high sporulation (Figure 1.8).

Figure 1.4. Symptoms of leaves infected with *Phakopsora pachyrhizi* include development of chlorotic spots that gradually develop into coloured lesions (Photo C. Steyn).

Figure 1.5. The adaxial surface of a soybean leaf infected with *Phakopsora pachyrhizi* displaying the red-brown lesion type (Photo C. Steyn).

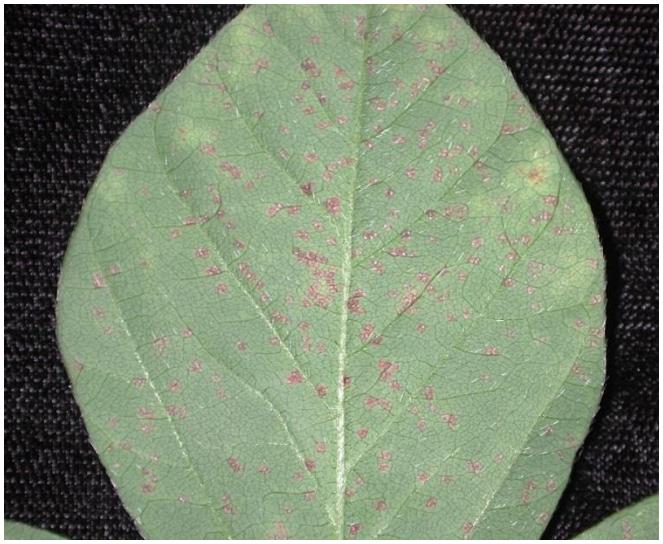

Figure 1.6. The abaxial surface of a soybean leaf infected with *Phakopsora pachyrhizi* displaying the red-brown lesion type and reduced uredinia and sporulation (Photo C. Steyn).

Figure 1.7. The adaxial surface of a soybean leaf infected with *Phakopsora pachyrhizi* displaying the tan-coloured lesion type (Photo C. Steyn).

Figure 1.8. The abaxial surface of a soybean leaf infected with *Phakopsora pachyrhizi* displaying the tan-coloured lesion type and ubundent uredinia and beige coloured urediniospores (Photo C. Steyn).

One to three rust pustules become visible in each lesion which are raised from the leaf surface. These lesions take on a polygonal shape and may be restricted by leaf veins. As the lesion ages, it becomes darker and individual lesions may enlarge and coalesce with time (Bromfield, 1984). Lesions may be present on leaves, stems, petioles or even pods in severe cases (Koenning *et al*., 2008). As the disease progresses, symptoms spread rapidly from the lower canopy to the middle and upper plant parts (Anonymous A, 2005). Approximately nine to ten days after infection, uredinia appear and urediniospores are produced for approximately three weeks (Figure 1.9) (Hartman *et al.,* 1999).

These uridiniospores serve as primary and secondary inoculum and are spread by wind or windblown rain (Sconyers *et al*., 2006). Spores take on a pale yellow-brown to colourless appearance with a surface ornamentation representing short spines (Rupe & Sconyers, 2008), however the colour of the lesions and spores depend on age as well as the interaction between the soybean genotype and race of the pathogen (Hartman *et al*., 1999). As the disease continues, premature yellowing, defoliation and early maturation occur (Figure 1.10 and 1.11) (Anonymous A, 2005).

Figure 1.9. Formation of the uredinia and urediniospores on the abaxial surface of the leaf infected with *Phakopsora pachyrhizi* (Photo C. Steyn).

As pustules age, teliospores form sub-epidermally among the uredinia and are dark brown to black at maturity (Hartman *et al*., 1999). These spores are crucial in the lifecycle of soybean rust as they are needed for overwintering of the fungus in the absence of a host as well as sexual reproduction (Rupe & Sconyers, 2008).

Figure 1.10. Premature yellowing of the plant as soybean rust progresses (Photo C. Steyn).

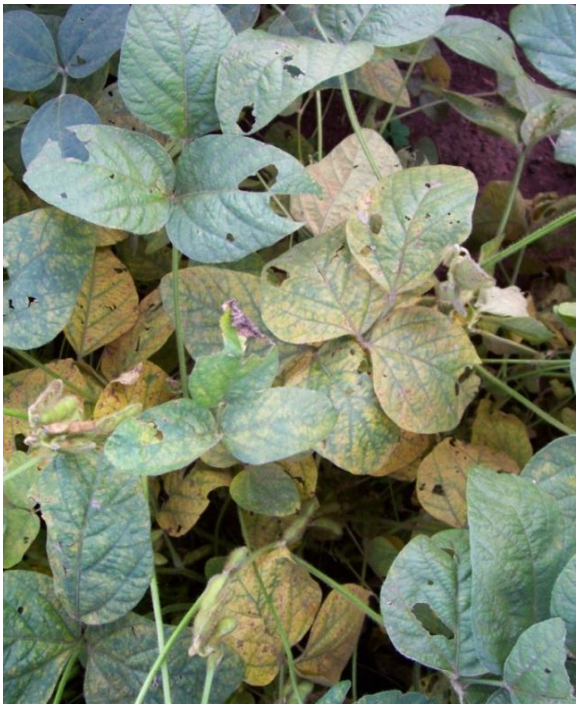

Figure 1.11. Yellowing, early maturation and defoliation of the host plant infected with soybean rust (Photo C. Steyn).

1.4.2 Host range and distribution

P. pachyrhizi is the most economically important pathogen within the genus *Phakopsora*. The fungus was first described in 1914 and was accepted as the name for the pathogen that initiates soybean rust (Bromfield, 1984).

Soybean rust has spread across the world since its first detection in 1902 in Japan (Kitani & Inoue, 1960). However the rapid and widespread occurrence of soybean rust can be attributed the increase in production of soybean, globally. Spores are well adapted for long distance dispersal and can be easily carried by wind which is ideal for the introduction of soybean rust to new rust-free areas (Figure 1.12) (Kawuki *et al*., 2003a; Rupe & Sconyers, 2008). According to Isard *et al*. (2006) however, the ability of an aerial pathogen to spread lies in its ability to withstand conditions such as UV irradiation encountered during movement and this may be a reason why this disease took more than 100 years to move globally.

P. pachyrhizi and the lesser important related species *P. meibomiae,* have a broad host range which is an unusual phenomena for rust fungi as most rusts have a narrow host

range limited to only a few plant species (Miles *et al*., 2003). *P. pachyrhizi* infects more than 95 plant species from more than 42 genera (Rytter *et al*., 1984; Ono *et al*., 1992) and is reported to occur on 34 natural hosts in Australia, Asia and Hawaii (Hartman *et al*., 1999). The ability of this fungus to infect numerous hosts raises concern as to their role in spread and survival of the pathogen (Ono *et al*., 1992; Hartman *et al*., 2005).

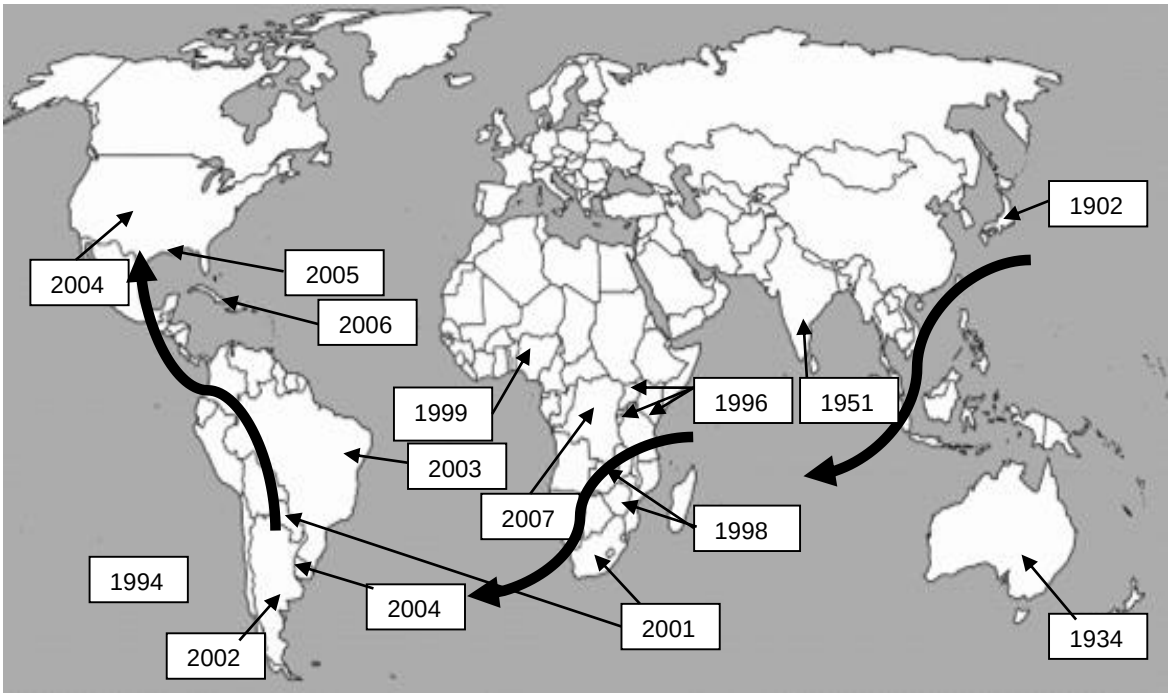

Figure 1.12. Soybean spread across the world since its first detection in 1902 in Japan (C. Steyn).

In 1934, the disease was reported in Australia as well as several Asian countries (Bromfield & Hartwig, 1980) and in 1951 the disease was first reported in India (Sharma & Mehta, 1996). With the arrival of the 20th century, new introductions of soybean rust continued and in 1994, it was reported in Hawaii (Killgore *et al.,* 1994).

The disease was absent in the western hemisphere until first detection in 1996 in Kenya, Rwanda and Uganda from where it spread to Zambia and Zimbabwe in 1998 and Nigeria in 1999 (Akinsanmi *et al*., 2001). In the 21st century the disease continued to spread and was detected on soybeans in 2001 in Paraguay (Morel *et al*., 2007), in 2002 in Argentina (Rossi, 2003) in 2003 Brazil and in 2004 in Uruguay (Stewart *et al*., 2005). In 2004 soybean rust was first reported in the continental USA (Schneider *et al*., 2005) and the assumption is that it was brought in by a hurricane from South America (Rupe & Sconyers, 2008). It was reported in Kentucky (Hershman *et al*., 2006) and Texas in 2005 (Isakeit *et al*., 2006) and continued to spread to Mexico (Rodriguez *et al*., 2006). In 2006 it was reported on

soybeans in Ghana (Bandyopadhyay *et al*., 2007), Cuba (Perez-Vicente *et al*., 2010), Argentina on *Phaseolus* sp. (Ivancovich *et al*., 2007) and in the Democratic Republic of the Congo on soybean during 2007 (Ojiambo *et al*., 2007).

In South Africa, the disease was first reported in 2001 on soybeans near Vryheid in northern KwaZulu-Natal (Pretorius *et al*., 2001). Epidemic levels were reached in Karkloof, Cedara, Howick and Greytown as the season progressed. From there the disease subsequently spread to major soybean production areas in the Highveld regions such as Amsterdam and Ermelo. In 2004, Du Preez *et al. (*2005) confirmed the presence of the pathogen on dry beans. Nunkumar *et al*. (2008) reported that *Cajanus cajan* (pigeon pea), *Glycine max* (vegetable soybean), *Lablab purpureus* (lablab), *Lupinus angustifolius* (lupin), *Phaseolus vulgaris* (common bean), *Pueraria lobata* (kudzu vine) and *Vigna unguiculata* (cowpea) were able to produce viable urediniospres when inoculated and can therefore serve as alternative hosts in South Africa. Kudzu especially is an important overwintering host. This is a concern as it an important souce of fresh urediniospores as well as being a source of selection pressure for the pathogen population (Kelly *et al*., 2015).

1.4.3 Economic importance

Soybean rust caused by *P. pachyrhizi* is an obligate parasite and is believed to be the most destructive foliar disease of soybean since its rapid spread over large geographic areas over the past few years (Miles *et al*., 2003; Isard *et al*., 2006). Soybean rust is caused by two fungal species namely *P. pachyrhizi* and *P. meibomiae (*Ono *et al., 1992)*. *P. pachyrhizi* is of greater importance since it is more aggressive and has spread throughout the western and eastern hemisphere since its first detection in Japan in 1902 (Kitani & Inoue, 1960). *P. meibomiae* is known to occur only in limited areas in the western hemisphere and no severe yield losses due to this pathogen have been documented (Miles *et al*., 2003).

According to Ivancovich (2005) losses due to rust are related to the phenological stage and the severity of symptoms. Losses are the result of early senescence and defoliation, resulting in reduced number of pods per plant and number of filled pods per plant (Hartman *et al*., 1991, Ogle *et al*., 1979) and thus reduced number of seeds per plant and 100 seed weight (Ogle *et al*., 1979; Kawuki *et al*., 2003b). No significant negative relationship has been reported between protein content of the grain and soybean rust while oil content was clearly reduced in plots that were exposed to long and severe rust infections (Ogle *et al*., 1979). Koch *et al*. (1987) reported a reduction in yield and 100 seed weight during cultivar

evaluation of 41.8% and 29.2% respectively in soybean rust trials done in Northern Thailand. Ogle *et al*. (1979) reported that soybean rust in field trials reduced seed yield by 60-70% in Asia. This was confirmed by Miles *et al*. (2003) who reported that in areas where the disease is common, losses of up to 80% have been reported.

The extent of losses may differ according to geographic area and cultivars planted, which in turn can be attributed to variation in inoculum pressure (Kawuki *et al*., 2003b). During 1994 soybean losses due to rust in major production countries such as China and Indonesia were 253 500 t and 200 t respectively (Wrather *et al*., 1997). In 1998 these countries reported losses of 198 900 t and 60 000 t respectively (Wrather *et al.,* 2001). After the introduction of soybean rust into South America, yield losses of 570 000 t during 2001/02 were recorded which increased to 3,4000 000 t in 2002/3. This is calculated at 6 % of total soybean production lost due to the disease (Livingston *et al*., 2004).

Soybean rust was first detected in the USA in 2004 and production and exports of soybeans showed a 8.4% and 26.3% decline, respectively after the introduction of the disease. This resulted in a price increase of soybeans of 23.4% and a welfare loss of 3.2% (Gomez *et al.,* 2009) highlighting the devastating effect diseases can have on the economic welfare of a country. According to Wrather & Koenning (2009) yield losses were estimated at 14 152 t in 2005, 24 521 t in 2006 and 14 968 t in 2007 due to this disease in the USA.

1.4.4 Epidemiology

Soybean rust causes severe epidemics when long periods of leaf wetness persist together with temperatures optimal for infection and development (Tschanz & Shanmugasundaram, 1985). Soybean plants are susceptible to rust during any stage of development, however the disease is most common from the mid- to late season (Hartman *et al*., 1999). Under favourable conditions, wind-blown urediniospores germinate to produce a single germtube. This germtube develops an appressorium which in turn develops an appressorial peg that penetrates directly into the cuticle and epidermal cells. This growth produces hyphae and within 10 to 14 days lesions containing pustules can be seen (Koch & Hoppe, 1987).

Considerable variation in the optimum requirements for disease development has been reported. Hartman *et al*. (1999) reported that epidemics are more severe when long periods of leaf wetness and daily temperatures of less than 28°C occur. Germination and penetration of host tissues by urediniospores require free water for 6 to 12 h and temperatures ranging from 15 to 28°C (Miles *et al*., 2003). According to Machetti *et al*.

(1976), a leaf wetness and temperature interaction occurs and at least 6 h of dew are needed for infection at 20 to 25°C, a minimum of 8 h at 15°C to 17.5°C while no infection occurs at temperatures above 27.5°C irrespective of the moisture period. Melching *et al*. (1989) found that 6 to 7 h of leaf wetness at temperatures of 18 to 26.5°C were required for soybean rust to develop. A minimum of 10°C was required for lesion development. Kochman (1979) also reported that temperatures ranging from 28.5°C to 42.5°C can inhibit germination and thus, high field temperatures can reduce inoculum concentrations in the field.

The spread of soybean rust is influenced by a variety of factors such as the availability of inoculum, the host growth stage and environmental factors such as moisture, wind, solar radiation and rainfall, the latter in particular being a good source of leaf wetness that directly affects infection and sporulation (Twizeyimana *et al.*, 2009). Although high temperatures and drought can limit survival, humid areas such as near water and within irrigated fields can create a microclimate favourable for survival and spread of the pathogen (Pivonia & Yang, 2004). The timing of rain during the growing season as well as the amount of rain plays a critical role in disease levels. Studies have indicated that soybeans grown with mist-type overhead or furrow irrigation still require rainfall to initiate the development of disease as seasons with low rainfall resulted in a delayed disease initiation and development rate. This is opposed to areas where daily precipitation occurred and early and rapid disease development was recorded (Wang & Hartman, 1992). Low rainfall seasons delay rust initiation and reduce the rate of development (Tschanz & Shanmugasundaram, 1985) and therefore the type of irrigation implemented should be carefully chosen, especially when it mimics rain as this can influence when an epidemic is initiated as well as how rapidly it develops.

Light is important in soybean rust disease development and urediniospores are adapted to darkness for germination. Light inhibits or delays germination (Shanmugasundaram, 1999) and this adaption helps to ensure dew and moderate temperatures during the infection process while minimising the risk of UV radiation and desiccation. According to Miles *et al*. (2003) aerial spread of urediniospores is crucial and therefore, spores need to be able to survive extreme environmental conditions. Isard *et al*. (2006) studied the exposure of urediniospores to solar radiation and found that viability was significantly reduced when uredionospores are exposed to solar radiation ≥27.3 MJ/m^2 and failed to germinate. When spores were exposed to 21 MJ/m^2 it resulted in the death of 63.2% of the urediniospore population. It was suggested that radiation could be valuable for aerobiological forecasts of the risk of this pathogen where inoculum is transported over

long distances over several days. The fact that spores are sensitive to ultraviolet radiation may indicate that the long distance spore movement is probably facilitated by storm systems where clouds are able to protect spores from sunlight (Rupe & Sconyers, 2008).

1.4.5 Genetic variability

Phakopsora pachyrhizi is highly variable and race patterns need to be established for each country so as to aid in the development of resistant cultivars (Kawuki *et al*., 2003a). Similarly soybean rust originated in Asia and analysis of variation in race populations from the world soybean production areas is important for understanding the evolution, distribution and genetic variation of *P. pachyrhizi* populations prevailing worldwide. In addition some degree of host specification has been reported. Yamaoka *et al*. (2002) also reported that races from wild host plants were less pathogenic on soybean than those isolated from the crop itself while Burdon & Lenne (1989) made the assumption that the *P. pachyrhizi* population in Australia is specialized on specific host species based on different patterns of susceptibility and resistance detected during inoculation trials.

High genetic variation was reported in previous studies (Freire *et al*., 2008; Twizeyimana *et al*., 2011; Yamaoka *et al*., 2014.) The uredinial-telial stage is spend on soybeans, however the spermagonial-aecial stage is unknown and it is therefore unknown if sexual reproduction occurs every year for the development of new races (Yamaoka *et al*., 2002). High genetic variation over a small geographic scale suggests rapid change within the pathogen population to a changing environment such as new resistance genes withing the host plant or resistance to fungicides (Twizeyimana *et al*., 2011).

Low genetic diversity over a wide geographic area, as reported by Twizeyimana *et al*. (2009) in Brazil, may suggest the occurrence of long distance dispersal (Twizeyimana *et al*., 2011). However Freire *et al.* (2008) reported that the pattern of introduction is not always compatible with the distribution of genetic diversity detected as was the case in Brazil where a high level of genetic variation was detected. These isolates showed characteristics with African and to a lesser degree with Asian isolates indicating that the disease was introduced to Brazil from Africa possibly through air currents.

1.4.6 Chemical control

Soybean rust is increasing in importance due to the formidable threat it poses to the worlds' soybean crop. This is mainly due to the loss of foliar area and severe reduction in grain yield. In areas where rust is endemic, losses as high as 80% have been reported on susceptible cultivars (Bromfield, 1984). Since durable and reliable sources of resistance have yet to be identified and despite the emphasis on the development and use of biological or alternative control methods due to concerns of the environmental impact, most control strategies remain dependent on fungicides due to their high level of efficacy (Budge & Whipps, 1991; Huang *et al*., 2000).

In most cases where fungicides have been used, these have been shown to be effective when applied correctly and a wide range of highly efficacious fungicides have been identified. Fungicide protection against rust is particularly crucial during the reproductive stages of crop development (growth stage beginning bloom (R1) to full seed (R6)). Two to three sprays applied 3 weeks apart, depending on disease onset was recommended by Galloway (2008). Levy (2005) suggested three fungicide applications in areas with high rust severity to provide proper control starting at first flowering with subsequent sprays at 21 day intervals. The number of sprays, however also depends on the maturity group of the host. In low risk areas two fungicide sprays were shown to be efficient.

In Dharwad, India, Patil & Basavaraja (2001) reported an interaction between levels of cultivar resistance and the number of fungicide sprays required to control soybean rust. Moderately resistance cultivars required only one spray applied at disease onset while susceptible genotypes needed two sprays applied at 15 day intervals to give normal yields.

A study conducted by Caldwell & McLaren (2004) in field trials during 2001-2003 indicated that timing of sprays is critical in soybean rust control. When chemical sprays were delayed to growth stage R5 (beginning seed), disease severity was minimally reduced. When sprays were applied at growth stage R2 (full bloom) a 63.4% disease reduction was recorded. Disease was reduced by 43 to 64% and up to 80% in trials when chemical sprays were applied during early flowering depending on spray efficacy (Caldwell & McLaren, 2004). The planting of indicator plots ie. 0.5 ha plots planted approximately 3 weeks prior to the main plots have proved a useful easy warning system to indicate when first sprays are needed and prevent the application of unnecessary sprays (Jarvie, 2009).

Glyphosate is a broad spectrum herbicide that is mainly used on genetically modified crop such as round-up ready soybeans. Glyphosate has been shown to be active against soybean rust with efficacies similar to fungicides such as carbendazim, flusilazole and tebuconazole. This was attributed to the systemic properties of glyphosate (Feng *et al*., 2008). Pilot studies have indicated that a pre-flowering application of Roundup® has no significant effect on soybean rust severity while post-flowering application resulted in a visible reduction in premature defoliation due to rust. However, since Roundup® is generally applied as a pre-flowering herbicide, this finding may be impractical (Jarvie, 2009).

Management of soybean rust is possible using a mixture of chemicals such as flusilazole and carbendazim and provides an alternative to the use of the contact fungicide, mancozeb (Galloway, 2008). Blum & Reis (2013) reported a 100% urediniospore germination inhibition after the application of myclobutanil and tebuconazole. After the application of mycolbutanil and flutriafol, 99.1% inhibition was observed, 99% for tetraconazole, 98.1% for cyproconazole and 98% germination inhibition for epoxiconazole. Scherm *et al.* (2009) found that triazoles performed better than strobilurins when applied individually while a combination of these two chemical groups provides more effective control. The mixture of chemicals also widens the spectrum of antifungal activity to control several diseases simultaneously and to provide additive and synergistic interactions between fungicides for optimum disease control. It is also advised to delay the selection of individuals in a pathogen population that are resistant to one component of the mixture (Duan *et al*., 2012).

Since no alternate reliable method of rust control, other than fungicide use is available, there is an ongoing fear that continued and constant use of chemical could promote the build-up of pathogen resistance to active ingredients that currently control soybean rust. In addition, this is not an environmentally friendly option and leads to an increase in production costs (Jarvie, 2009). Evaluation of soybean cultivars in South Africa for rust resistance has indicated that none of the cultivars show rust disease severity levels which could be of economic value and contribute significantly to reducing the dependence on chemical control (McLaren, 2008).

1.4.7 Breeding for resistance

The development of soybean cultivars with resistance is the desired method for controlling disease as it is more economical and the environmental impact, compared with chemical control, is reduced (Pham *et al*., 2010). Several important factors determine resistance

breeding methods such as the genetic distance between the cultivars evaluated for improvement and the resistant donor germplasm, the screening methods available, the genetics of resistance and the number of traits that need to be improved (Hassan *et al*., 2014). It is also crucial that resistant cultivars possess genes that are effective in the specific geographic region where the cultivars are most likely to be grown (Paul *et al*., 2015). Effectiveness of resistance genes, however is often short lived especially when dealing with an obligate parasite such as *P. pachyrhizi* which represents high virulence variability (Miles *et al*., 2011).

Sources of resistance in soybeans to rust have been widely sought. The usefulness of these sources depends on the ease of transfer of resistance genes to new varieties and the stability of these genes against different races of *P. pachyrhizi* associated with different environments (Politowski & Browning, 1978). A problem in the recognition and evaluation of resistance lies in consistent rust prevalence and severity throughout a planting season for a consecutive number of years (Bromfield, 1984). Soybean genotypes have been shown to have different resistance responses to soybean rust at various host growth stages (Ribeiro *et al*., 2007) and combined early stage and adult plant resistance need to be identified using assessment at different stages of host development. These authors also found that resistance to soybean rust does not rely on a few major genes but is mainly controlled by additive gene effects that are dispersed in the parental genotypes. Thus, recurrent selection in the presence of the pathogen could be valuable approach to resistance breeding.

Hartman *et al*. (1999) reported rate reducing resistance to soybean rust but found this difficult to evaluate because of the interdependence of this variable and plant development on disease severity. In theory rate-reducing resistance is effective against all races of a pathogen. It should however be taken into consideration that this pathogen, has evolved into multiple races over the years (Tshanz *et al*., 1985; Wang & Hartman, 1992). Lines showing traits of rate-reducing resistance have slower and more restricted hyphal growth, development of fewer haustoria and rapid, restricted collapse of host cells which are visible under the microscope as well as lesions producing less spores (McLean, 1979).

To date six different loci carrying dominant alleles have been identified namely *Rpp1* (Mclean & Byth, 1980), *Rpp2* (Bromfield & Melching, 1982), *Rpp3* (Bromfield & Hartwig, 1980), *Rpp4* (Hartwig, 1986), *Rpp5* (Garcia *et al*., 2008) and *Rpp6* (Li *et al*., 2012). Molecular resistance gene alleles have been mapped to *Rpp1*, *Rpp3* and *Rpp5* genes and the recently discovered *Rpp6* gene. The latter gene especially, is of great interest as it

was reported to provide a high level of resistance to numerous field populations and isolates from North and South America (Li *et al*., 2012).

Soybean lines derived from the original sources of these genes can produce intermediate reactions. Three infection types on soybeans were described by Bromfield *et al*. (1980), Bromfield & Hartwig (1980) and Bromfield (1984). After infection of resistant plants leaf tissue surrounding the pustules becomes reddish brown representing the "RB" type lesion or infection type 1 while in more susceptible plants, pale brown or "Tan" type lesions occur referred to as infection type 2. The absence of lesions indicates immunity or near-immunity known as infection type 3 and according to Miles *et al*. (2006) these limited infections may be due to the *Rpp1* gene. In common bean trials, RB infection types were also associated with low sporulation while tan lesions had high sporulation (Miles *et al*., 2007). Twizeyimana *et al*. (2008) suggested that genotypes expressing RB lesions may be sources of partial resistance. Limited fungal growth and sporulation associated with RB lesions is conferred to by *Rpp2, Rpp3* and *Rpp4* (Bonde *et al*. 2006). In 34 soybean accessions 56% produced "Tan" lesions and a strong interaction between the isolates used and the accessions screened emphasizes a differential response (Miles *et al*., 2011). Paul *et al.* (2015) reported that when similar PI lines were inoculated with different isolates, different reaction types were observed. When *Rpp3* genotype PI 462312 was inoculated with U.S isolates, nonsporulation red-brown lesions were observed while when inoculated with field populations less resistance was observed. Based on the criteria of disease severity and uredinia densities it is clear that isolates can be categorized into different groups of aggressiveness. When a study of five Brazilian isolates and two Japanese isolates which differed in pathogenicity were evaluated for the effect of pyramiding *Rpp2*, *Rpp4* and *Rpp5* on resistance to soybean rust, it was observed that these isolates differed in virulence and the Brazilian isolates displayed higher virulence due to the faster formation of lesions than the Japanese isolates (Yamanaka *et al*., 2013).

Koch *et al*. (1987) reported that lines displaying a RB-type have significantly less uredia and uredia primordia per lesion than lines showing Tan reactions. Larger lesion size, lesions expanding faster, a shorter latent period, a higher spore yield per unit area of tissue per lesion and a larger amount of infection resulting from a given amount of inoculum are all components indicating susceptibility to soybean rust (Kawuki *et al*., 2003b). Bonde (2006) evaluated the differences in numbers and sizes of uredinia in a susceptible, Tan reaction compared with a resistant, RB reaction using both *P. pachyrhizi* and *P. meibomiae* isolates. In a susceptible reaction, hyphae spread rapidly into leaf tissue after initial infection and subsequently produce uredinia over an extended period. This development

over time results in uredinia varying in maturity and size. In a resistant reaction the development of the pathogen is restricted, resulting in fewer and smaller uredinia. Miles *et al*. (2011) reported that the RB type of resistance resulted in incomplete sporulating uredinia within the lesions. The Tan lesions however indicated susceptibility and different levels were identified. These are referred to as partial resistance ie. when sporulation levels were equal or lower to those recorded in the RB lesions. Partial resistance should also be measured together with yield as the number of uredinia per leaf area was shown to be inversely correlated with yield.

Ribeiro *et al*. (2007) reported significant genotype x micro-environment interactions in the expression of soybean rust in crosses. Results indicated that *P. pachyrhizi* is dependent on specific combinations between the host and the micro-environment to enable it to manifest early infection. In the evaluation of 100 cultivars in India, Kumar *et al*. (1987) reported locality effects on the expression of rust resistance which were attributed to variable climatic conditions and differences in *P. pachyrhizi* races. In contrast Tschanz & Wang (1980) reported similar infection rates for cultivars evaluated in Taiwan and five other locations. Tschanz *et al*. (1985) also reported that rust severities at a particular growth stage were similar among the cultivars evaluated in the same environment, regardless of the cultivars maturity which suggested that plant growth or physiological stage is a primary driving variable in rust development. Thus, variation in rust development between cultivars grown at the same time and location could be a reflection of the cultivars level of resistance as well as developmental stage and maturation.

According to Melching *et al*. (1988) several factors such as plant and leaf age and maturity need to be taken into consideration for accurate evaluation. Exposure of plants to different photoperiods revealed a delay in rust development within cultivars which in turn was related to a delay in their development and maturation (Wang & Hartman, 1992). Oloka *et al*. (2009) also reported an increase in lesion density in lines from full bloom (R2) growth through to full pod (R4) and full seed (R6) confirming that lesion numbers increase after flowering and increase with maturity.

Shorter maturity cultivars tend to escape the effects of rust infection more readily than longer growers (Kawuki *et al*., 2004) which may be due to temporal differences in premature defoliation. Shorter maturity cultivars had the tendency to retain leaves for longer, relative to total growth period, than the longer maturity group cultivars (McLaren, 2008). Yang *et al*. (1990) found a significant relationship between disease severity and defoliation but not yield loss which was also influenced by the environment.

Gene pyramiding has been exploited, especially due to the high genetic diversity of *P. pachyrhizi.* Studies into gene pyramiding showed that *Rpp*-pyramided lines had higher and a more stable resistance compared to ancestor lines, however significant variability between pyramided lines were detected (Yamanaka *et al*., 2013). This poses promising breeding material for soybean resistance against soybean rust. Lemos *et al*. (2011) identified different genetic effects of *Rpp2*, *Rpp4* and *Rpp5* with the latter showing the largest contribution and it was concluded that this gene was the most useful in breeding programs.

Genes or alleles that could provide genetic variability for breeding programs have been identified but several factors still hamper the development of resistant cultivars. Resistance to soybean rust conferred by the *Rpp1* and *Rpp3* loci was overcome in only two years after the appearance of soybean rust making sustainable sources of resistance difficult to maintain (Garcia *et al*., 2008). According to Hartman *et al.* (2005) resistance conferred by the *Rpp4* locus lasted approximately 20 years in Asia. Studies have shown that multiple alleles with different modes of action of *Rpp* loci are present in soybean. Martins & Juliatti (2014) reported that rust resistance is a characteristic controlled by two to 23 genes that are dominant. Although varieties were released that indicated more tolerance to the disease, the stability of these sources is still a problem especially since the pathogen consists of a large number of races that have been identified, indicating great variability in the pathogen population (Hassan *et al*., 2014).

The use of cultivars with tolerance or partial resistance, in combination with the effective application of fungicides can effectively minimize the effects of soybean rust (Hartman *et al*., 1991). Politowski & Browning (1978) defined cultivars that were susceptible to soybean rust with an equivalent level of infection and reproduction rate of the pathogen, but still produced higher yield than other susceptible cultivars as tolerant. The yielding ability of soybean lines is a good indication of variation in tolerance. According to Kawuki *et al*. (2004) most variation in yield in early maturing lines can be attributed to the genotype as opposed to medium and late maturing lines where seasonal affect is more likely to contribute to yield. Tolerance to soybean rust can be more easily exploited than true resistance. According to Tschanz & Wang (1987) yield can be more easily and accurately assessed and related to tolerance as opposed to relating disease severity to resistance.

1.4.8 Mechanisms of resistance and pathogenicity

Plants are exposed to a number of pathogenic fungi and although they lack a circulating somatically adaptive immune system (Fritig *et al*., 1998) plants have evolved several defence mechanisms such as the synthesis of low molecular compounds, proteins and peptides that have antifungal activity (Anguelova-Merhar *et al*., 2002). Host plant resistance is expressed when it is affected by both abiotic and biotic factors (Bi *et al*., 1994). Several substances have been investigated that have been shown to be affected by disease development such as pathogenesis related proteins (PR-proteins) for example β-1,3-glucanases and chitinases, peroxidises, phenols and carbohydrates.

Elicitors are especially important in understanding the initial events of the host-parasite interactions that lead to plant defence responses. During elicitation, the process is preceded by gene activation which in turn leads to the synthesis of biosynthetic enzymes and the metabolites they produce (Gundlach *et al*., 1992). Graham *et al*. (2003) found that wounding and wall glucan elicitors treatment on soybean plants have profound and differential effects on the expression of representatives of the PR proteins of five families investigated. The expression of PR proteins and their synthesis during attack has been widely investigated in a variety of plant species. They have been classified as a diverse family in plants and show a complex expression pattern and a display of various biological activities in different species (Liu & Ekramoddoullah, 2006). β-1,3-glucanases are among the PR proteins that are the most well studied and characterized defence-related proteins (Mohammadi & Karr, 2002). Castresana *et al*. (1990) concluded in their study that β-1,3-glucanases and other defence chemicals act as a barrier controlling the size of the lesion and the spread of the pathogen. The β-1,3-glucanase activity evaluated in 18 soybean cultivars indicated that maturity group does not influence the activity of this enzyme and since cultivars were evaluated under similar environmental conditions the differences could be attributed to genetic differences. β-1,3-glucanase and chitinases are also associated with systemic acquired resistance in plants (Fritig *et al*., 1998; Edreva, 2005) as well as the degrading of the structural polysaccharides of fungal cell walls (Hammond-Kosack & Jones., 2000).

Peroxidase (EC 1.11.1.7) is an enzyme family that is known for diversity in form, function and distribution. An increase in peroxidase activity and expression of specific isoenzymes is believed to be a constitutive mechanism used by plants for protection against pathogen infection (De Souza *et al*., 2003). However the role of these in soybeans in particular in terms of soybean rust is still unclear.

1.5 Conclusion

Despite the vast volume of published research on the topics discussed in this literature review, Sclerotinia stem rot and soybean rust still remain a constraint to soybean production in many countries.

According to literature, defence responses have been extensively investigated in many plant-pathogen interactions, however, little is known about biochemical interactions in soybean-*P. pachyrhizi* interactions. Plants are sessile and have therefore evolved various mechanisms to protect themselves against biotic and abiotic stresses. Mechanisms include recognition of the pathogen and the activation of chemical processes such as the synthesis of phytoalexins, PR-proteins and other compounds that have antifungal activity. A detailed study of sources of resistance and tolerance so as to develop cultivars for use in the management of diseases as well as understanding the mechanisms involved in host-pathogen interactions will aid a more effective approach in control of these diseases.

1.6 References

Abawi, G.S. & Grogan, R.G., 1975. Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. Phytopathology 65: 300-309.

Abawi, G.S. & Grogan, R.G. 1979. Epidemiology of diseases caused by Sclerotinia species. Phytopathology 69: 899-903.

Abawi, G.S. & Hunter, J.E. 1979. White mold of beans in New York. New York's Food and Life Sciences Bulletin no 77. January, 1979.

Adams, P. & Ayers, W.A. 1979. Ecology of *Sclerotinia* species. Phytopathology 69: 896 899.

Agrios, G.N. 1997. Plant Pathology. Fouth Editions. Academic press. New York.

Akinasanmi, O.A., Ladipo, J.L. & Oyekan, P.O. 2001. First report of soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 85: 1288.

Alexander, B.J.R. & Stewart, A. 1994. Survival of *Sclerotinia* and *Sclerotium* spp in New Zealand horticultural soil. Soil Biology and Biochemistry 26: 1323-1329.

Ali, B.A., Huang, T.H., Qin, D.N. & Wang, X.M. 2004. A review of random amplified polymorphic DNA (RAPD) markers in fish. Reviews in Fish Biology and Fisheries 14: 4443–4453.

Anguelova-Merhar, V.S., van der Westhuizen, A.J. & Pretorius, Z.A. 2002. Intercellular chitinase and peroxidase activities associated with resistance conferred by gene *Lr35* to leaf rust of wheat. Journal of Plant Physiology 159: 1259-1261.

Anonymous. 2009. Agriculture statistics-Production-Soybean by country. Producion Estimates and Crop Assessment Division, FAS, USDA via NationMaster. www.nationmaster.com. Assessed September 2011.

Anonymous A. 2005. *Phakopsora pachyrhizi*: Asian soybean rust. www.eppo.int/QUARANTINE/Alert.../Phakopsora_pachyrhizi.doc. Accessed: September 2008.

Atallah, Z.K.., Larget, B., Chen, X. & Johnson, D.A. 2004. High genetic diversity, phenotypic uniformity, and evidence of outcrossing in *Sclerotinia sclerotiorum* in the Columbia Basin of Washington State. Phytopathology 94: 737-742.

Attanayake, R.N., Porter, L., Johnson, D.A. & Chen, W. 2012. Genetic and phenotypic diversity and random association of DNA markers of isolates of the fungal plant pathogen *Sclerotinia sclerotiorum* from soil on a fine geographic scale. Soil Biology and Biochemistry 55: 28-36.

Bandyopadhyay, R., Ojiambo, P.S., Twizeyimana, M., Asafo-Adjei, B., Frederick, R.D., Pedley, K.F., Stone, C.L. & Hartman, G.L. 2007. First report of soybean rust caused by *Phakopsora pachyrhizi* in Ghana. Plant Disease 91: 1057.

Bardin, S.D. & Huang, H.C. 2001. Research on biology and control of *Sclerotinia* diseases in Canada. Canadian Journal of Plant Pathology 23: 88-98.

Bi, J.L., Felton, G.W. & Meuller, A.J. 1994. Induced resistance in soybean to *Helicoverpa zea*: Role of plant protein quality. Journal of Chemical Ecology 20: 183-1983.

Blum, M.M.C. & Reis, E.M. 2013. *Phakopsora pachyrhizi in vitro* sensitivity to fungicides. Summa Phytopathologica 39: 215-216.

Boland, G.J. & Hall, R. 1987. Evaluation of soybean cultivars for resistance to *Sclerotinia sclerotiorum* under field conditions. Plant Disease 71: 934-936.

Boland, G.J. & Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Canadian Journal of Plant Pathology 16: 93-108.

Bolton., M.D., Thomma, B.P.H. & Nelson, B.D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molecular Plant Pathology 7: 1-16.

Bonde, M.R., Nester, S.E., Austin, C.N., Stone, C.L., Frederick, R.D., Hartman, G.L. & Miles, M.R. 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. Meibomiae* isolates. Plant Disease 90: 708-716.

Botha, C., McLaren, N.W. & Swart, W.J. 2009. Evaluation of greenhouse inoculation techniques used to screen for Sclerotinia stem rot resistance in soybeans. South African Journal of Plant and Soil 26:48-50.

Botha, C., McLaren, N.W. & Swart, W.J. 2011. The effect of temperature and period of high humidity on the development of Sclerotinia stem rot on soybeans in the greenhouse. South African Journal of Plant and Soil 28: 208-211.

Bradley, C.A., Henson, R.A., Porter, R.A., LeGare, D.G., del Rio, L.E. & Knot, S.D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Disease 90: 215-219.

Bromfield, K.R. 1984. Soybean Rust. Monograph no 11. American Phytopathological Society Press. St. Paul, Minnesota, USA.

Bromfield, K.R., & Hartwig, E. E. 1980. Resistance to soybean rust and mode of inheritance. Crop Science 20:254-255.

Bromfield K.R, & Melching, J.S. 1982. Sources of specific resistance to soybean rust. Phytopatology 72:S706.

Bromfield, K.R., Melching, J.S., & Kingsolver, C.H. 1980. Virulence and aggressiveness of *Phakopsora pachyrhizi* isolates causing soybean rust. Phytopathology 70:17-21.

Budge, S.P. & Whipps, J.M. 1991. Glasshouse trials of *Coniothyrium minitans* and *Trichoderma* species for the biological control of *Sclerotinia sclerotiorum* in celery and lettuce. Plant Pathology 40: 59-66.

Burdon, J.J. & Lenne, J.M. 1989. The *Phakopsora pachyrhizi-Kennedia rubicunda* host-pathogen association and its relation to leaf rust of *Glycine* spp. Australian Journal of Agricultural Research 40: 265-272.

Caldwell, P.M & McLaren, N.W. 2004. Soybean rust research in South Africa. VII World Soybean Research Conference Proceedings. IV International Soybean Processing and Utilization Conference. Brazil. 2004.

Caliskan, M. 2000. The Metabolism of oxalic acid. Turkish Journal of Zoology 24: 106-106.

Castresana, C., de Carvalho, F., Gheysen, D., Habets, M. Inze, D & Van Montagu, M. 1990. Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* β-1,3-glucanase Gene. The Plant Cell 2: 1131-1143.

Cessna, S.G., Sears, V.E., Dickman, M.B. & Low, P.S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. The Plant Cell 12: 2191-2199.

Chen, W., B. Schatz, B. Henson, K. E. McPhee and F. J. Muehlbauer. 2006. First report of Sclerotinia stem rot of chickpea caused by *Sclerotinia sclerotiorum* in North Dakota and Washington. Plant Disease 90:114.

Chipps, T.J., Gilmore, B., Myers, J.R. & Stotz, H.U. 2005. Relationship between oxalate, oxalate oxidase activity, oxalate sensitivity and white mold susceptibility in *Phaseolus coccineas*. Phytopathology 95: 292-299.

Chun, D., Kao, L.B. & Lockwood, J.L. & Isleib, T.G. 1987. Laboratory and assessment of resistance in soybean to stem rot caused by *Sclerotinia sclerotiorum*. Plant Disease 71: 811-815.

Cilliers, A.J., Herselman, L. & Pretorius, Z.A. 2000. Genetic variability within and among mycelial compatibility groups of *Sclerotium rolfsii* in South Africa. Phytopathology 90: 1026-1031.

Colagar, A.H., Saadati, M., Zarea, M. & Talei, S.A. 2010. Genetic variation of the Iranian *Sclerotinia sclerotiorum* isolates by standardizing DNA polymorphic Fragments. Biotechnology 9: 67-72.

De Souza, I.R.P., De Oliveira, E., Peres, M.A, De Oliveira, A.C. & Purcino, A.A.C. 2003. Peroxidase activity in maize inbred lines resistant or susceptible to maize dwarf mosaic virus. Revista Brasileira de Milho e Sorgo 2: 1-8.

Dillard, H.R. & Cobb, A.C. 1995. Relationship between leaf injury and colonization of cabbage by *Sclerotinia sclerotiorum*. Crop Protection 14: 677-682.

Donaldson, P.A., Anderson, T., Lane, B.G., Davidson, A.L. & Simmonds, D.H. 2001. Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. Physiological and Molecular Plant Pathology 59: 297–307.

Duan, Y., Liu, S., Ge, C., Feng, X., Chen, C. & Zhou, M. 2012. In vitro inhibition of *Sclerotinia sclerotiorum* by mixtures of azoxystrobin, SHAM and thiram. Pesticide Biochemistry and Physiology 103: 101-107.

Du Preez, E.D., van Rij, N.C., Lawrance, K.F., Miles, M.R. & Frederick, R.D. 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* on dry beans in South Africa. Plant Disease 89: 206.

Durman, S. B., Menendez, A.B. & Godeas, A.M. 2005. Variation in oxalic acid production and mycelium compatibility within field populations of *Sclerotinia sclerotiorum*. Soil Biology and Biochemistry 37: 2180-2184.

Dutton, M.V. & Evans, C.S. 1996. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology 42: 881–895.

Edreva, A. 2005. Pathogenesis-related proteins: Research progress in the last 15 years. General and Applied Plant Physiology 31: 105-124.

Feng, P.C.C., Clark, C., Andrade, G.C., Balbi, M.C. & Caldwell, P. 2008. The control of Asian rust by glyphosate in glyphosate-resistant soybeans. Pest Management 64: 353-359.

Ferreira, S.A. & Boley, R.A. 1992. *Sclerotinia sclerotiorum.* www.extento.hawaii.edu/kbase/crop/Type/s_scler.htm. Accessed: April 2013.

Freire, M.C.M., de Oliveira, L.O., de Almeida, A.M.R., Schuster, I., Moreira, M.A., Liebenberg, M.M. & Mienie, C.M.S. 2008. Evolutionary history of *Phakopsora pachyrhizi* (the Asian soybean rust) in Brazil based on nucleotide sequences of the internal transcribed spacer region of the nuclear ribosomal DNA. Genetics and Molecular Biology 31. 920–931.

Fritig, B., Heitz, T. & Legrand, M. 1998. Antimicrobial proteins in induced plant defence. Current Opinion in Immunology 10: 16-22.

Fuhlbohm, M.J., Tatnell, J.R. & Ryley, M.J. 2003. First report of stem rot and wilt of chickpea caused by *Sclerotinia minor* in Queensland Australia. Australasian Plant Pathology 32: 323-324.

Galloway, J. 2008. Effective management of soyabean rust and frogeye leaf spot using a mixture of flusilazole and carbendazim. Crop Protection 27: 566-571.

Garcia, A., Calvo, E.S., de Souza Kiihl, R., Harada, A., Hiromoto, D.M. & Vieira, L.G.E. 2008. Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. Theoretical applications of Genetics 117: 545-553.

Garibaldi, A., Minuto, A. & Gullino, M.L. 2007. First report of white mold caused by *Sclerotinia sclerotiorum* on *Iberis sempervirens* in Italy. Disease notes 91: 464:3.

Garibaldi, A., Minuto, A., Gilardi, G. & Gullino, M.L. 2001 First report of *Sclerotinia sclerotiorum* on *Gazania* sp. Hybrid in Italy. Plant Disease 85: 1207.

Godoy, G., Steadman, J.R., Dickman, M.B., & Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiological and Molecular Plant Pathology 37: 179-191.

Gómez, M.I., Nunez, H.M. & Önal, H. 2009. Economic impact of soybean rust on the US soybean sector. Selected Paper prepared for presentation at the Agricultural & Applied Economics Association 2009. AAEA & ACCI Joint Annual Meeting, Milwaukee, Wisconsin, July 26-29, 2009.

Gomes, E.V., Do Nascimento, L.B., De Freitas, M.A., Nasser, L.C.B. & Petrofeza, S. 2011. Microsatellite Markers Reveal Genetic Variation within *Sclerotinia sclerotiorum* populations in Irrigated Dry Bean Crops in Brazil. Journal of Phytopathology 159: 94-99.

Gorter, G.J.M.A. 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Department of Agricultural Technical Sevices Science, Bulletin no. 392.

Grafton, K.F. 1998. Resistance to white mold in dry bean. Proceedings of the Sclerotinia workshop 1998.

Graham, M.Y., Weidner, J., Wheeler, K., Pelow, M.J. & Graham, T.L. 2003. Induced expression of pathogenesis-related protein genes in soybean by wounding and the *Phytophthora sojae* cell wall glucan elicitor. Physiological and Molecular plant pathology 63: 141-149.

Grau, C.R. & Radke, V.L. 1984. Effects of cultivars and cultural practices on sclerotinia stem rot of soybean. Plant Disease 68: 56-58.

Grau, C.R., Radke, V.L. & Gillespie, F.L. 1982. Resistance of soybean cultivars to *Sclerotinia sclerotiorum*. Plant Disease 66: 506-508.

Guimaraes, R.L. & Stotz, H.U. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. Plant Physiology 136: 3703-3711.

Gundlach, H., Müller, M.J., Kutchan, T.M. & Zenk, M.H. 1992. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proceedings of the National Academy of science 89: 2389-2393.

Gulya, T.J. & MacArthur, R.A. 1984. Incidence and severity of sunflower diseases in the Dakotas and Minnesota during the 1984 growing season. Page 6 in: Proceedings of Sunflower Research Workshop, 10 December 1984, Fargo. North Dakota. USA.

Hambleton, S., Walker, C. & Kohn, L.M. 2002. Clonal lineages of *Sclerotinia sclerotiorum* previously known from other crops predominate in 1999-2000 samples from Ontario and Quebec soybean. Canadian Journal of Plant Pathology 24: 309-315.

Hammond-Kosack, K & Jones, J.D.G. 2000. Responces to Plant Pathogens. In: Buchanan, B.B., Gruissem, W. & Jones, R. L. 2000. Biochemisty & Molecular Biology of Plants. American Society of Plant Physiologists.

Harikrishnan, R. & Del Rio, L.E. 2006. Influence of temperature, relative humidity, ascospore concentration and length of dry bean flowers on white mold development. Plant Disease 90: 946-950.

Hassan, F.S.C., Fakheri, B. & Sattari, A. 2014. Review: Breeding for resistance to soybean rust. International Journal of Agricultural and Crop Sciences 7: 322-328.

Hartwig, E.E. 1986. Identification of a fourth major gene conferring resistance to soybean rust. Crop Science 26:1135-1136.

Hall, B.H., McMahon, R.L. & Wicks, T.J. 2002. First report of *Sclerotinia sclerotiorum* on grape (*Vitis Vinifera*) in South Australia. Australasian Plant Pathology 31: 417-418.

Hartman, G.L., Miles, M.R. & Frederick, R.D. 2005. Breeding for resistance to soybean rust. Plant Disease 89: 664-666.

Hartman, G.L., Sinclair, J.B. & Rupe. J.C. 1999. Compendium of soybean diseases. Soybean Rust. Fourth Edition. The American Phytopathological Society Press. 1999.

Hartman, G.L., Wang, T.C. & Tschanz, A.T. 1991. Soybean rust development and the quantitative relationship between rust severity and soybean yield. Plant Disease 75: 596-600.

Hershman, D.E., Bachi, P.R., Harmon, C.L., Harmon, P.F., Palm, M.E., McKemy, J.M., Zeller, K.A. & Levy, L. 2006. First report of soybean rust caused by *Phakopsora pachyrhizi* on Kudzu (*Pueraria Montana* var. *lobata*) in Kentucky. Plant Disease 90: 834.

Hoffman, D. D., Diers, B. W., Hartman, G. L., Nickell, C. D., Nelson, R. L., Pedersen, W. L., Cober, E. R., Dorrance, A. E., Graef, G. L., Steadman, J. R., Grau, C. R., Nelson, B. D., del Rio, L. E., Helms, T., Poysa, V., Rajcan, I., & Stienstra, W. C. 2002. Selected soybean plant introductions with partial resistance to *Sclerotinia sclerotiorum*. Plant Disease 86:971-980.

Hoffman, D.D., Hartman, G.L., Mueller, D.S., Leitz, R.A., Nickell, C.D. & Pedersen, W.L. 1998. Yield and seed quality of soybean cultivars infected with *Sclerotinia sclerotiorum*. Plant Disease 82: 826-829.

Holz, A. 1977. World outlook for soybean production, marketing. Excerpts from a talk by Alan E. Holz, agricultural economist, USDA Foreign Agricultural Service. Journal of the American oil Chemists' society 54: A208-A210.

Huang, H.C., Bremer, E., Hynes, R.K. & Erickson, R.S. 2000. Foliar application of fungal biocontrol agents for the control of white mold of cry beans caused by *Sclerotinia sclerotiorum*. Biological control 18: 270-276.

Huang, H.C. & Kozub, G.C. 1993. Influence of inoculum production temperature on carpogenic germination of sclerotia of Sclerotinia *sclerotiorum*. Canadian Journal of Microbiology 39: 548-550.

Hunter, J.E., Abawi, G.S. & Crosier, D.C. 1978. Effects of timing, coverage and spray oil on control of white mold of snap bean with Benomyl. Plant Disease Reporter 62: 633-637.

Isakeit, T., Miller, M.E., Saldana, R., Barnes, L.W., McKemy, J.M., Palm, M.E., Zeller, K.A., DeVries-Paterson, R. & Levy, L. 2006. First report of rust caused by *Phakopsora pachyrhizi* on Soybean and Kudzu in Texas. Plant Disease 90: 971.

Isakeit, T., Woodward, J.E., Niu, C. & Wright, R.J. 2010. First report of Sclerotinia stem rot of Canola caused by *Sclerotinia sclerotiorum* in Texas. Plant Disease 94: 792.2-792.2

Isard, S.A., Dufault, N.S., Hartman, GL., Russo, J.M., De Wolf, E.D. & Morel, W. 2006. The effect of solar irradiance on the mortality of *Phakopsora pachyrhizi* urediniospores. Plant Disease 90: 941-945.

Ivancovich, A. 2005. Soybean rust in Argentina. Plant Disease 89: 667-668.

Ivancovich, A.J., Botta, G., Rivandaneira, M., Saieg, E., Erazzu, L. & Guillin, E. 2007. First report of soybean rust caused by *Phakopsora pachyrhizi* on *Phaseolus* spp. in Argentina. Plant Disease 91: 111.

Jarvie, J.A. 2009. A review of soybean from a South African perspective. South African Journal of Science 105: 103-108.

Jeffreys, A.J., Wilson, V. & Thein, S.L. 1985. Hypervariable “minisatellite” regions in human DNA. Nature 314: 67-73.

Johnson, D.A. & Atallah, Z.K. 2006. Timing fungicide application for managing Sclerotinia stem rot of potato. Plant Disease 90: 755-758.

Kanauchi, M., Milet, J. & Bamforth, C.W. 2009. Oxalate and oxalate oxidase in malt. Journal of the Institute of Brewing 115: 232-237.

Kawuki, R.S., Adipala, E., Lamo, J & Tukamuhabwa, P. 2003a. Responding to the soybean rust epidemics in sub-saharan Africa: A review. African Crop Science Journal 11: 301-318.

Kawuki, R.S., Adipala, E. & Tukamuhabwa, P. 2003b. Yield loss associated with soya bean rust (*Phakopsora pachyrhizi* Syd.) in Uganda. Journal of Phytopathology 151: 7-12.

Kawuki, R.S., Tukamuhabwa, P. & Adipala, E. 2004. Soybean rust severity, rate of rust development and tolerance as influenced by maturity period and season. Crop Protection 23: 447-455.

Kelly, H.Y., Dufault, N.S., Walker, D.R., Isard, S.A., Schneider, R.W., Giesler, L.J., Wright. D.L., Marois, J.J. & Hartman, G.L. 2015. From select agent to an established pathogen: The response to *Phakopsora pachyrhizi* (soybean rust) in North America. Phytopathology 105: 905-916.

Killgore, E., Heu, R. & Gardner, D.E. 1994. First Report of Soybean rust in Hawaii. Plant Disease 78: 1216.

Kim, K.S., Min, J. & Dickman, M.B. 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Molecular Plant Microbe Interactions 21: 605-612.

Kitani, K., & Inoue, Y. 1960. Studies on the soybean rust and its control measure (Part 1). Studies on the soybean rust. Bulletin of the Shikoku National Agricultural Experiment Station 5: 319-42.

Koch, E. & Hoppe, H.H. 1987. Effect of light on uredospore germination and germ tube growth of soybean rust (*Phakopsora pachyrhizi* Syd). Journal of Phytopathology 119: 64-74.

Koch, E., Hoppe, H.H. & Smutkupt, S. 1987. Characterization of rust resistance in soybean mutant breeding lines. Soybean Rust Newsletter 8: 5-8.

Kochman, J.K. 1979. The effect of temperature on development of soybean rust (*Phakopsora pachyrhizi*). Australian Journal of Agricultural Research 30: 273-277.

Koenning, S., Mueller, J., Kemerait, R. & Phipps, P. 2008. Soybean Rust: Soybean disease information note 8. http://www.ces.ncsu.edu/depts/pp/notes/Soybean/soy008/soy008.htm. Accessed: June 2012.

Koga, L.J., Bowen, C.R., Godoy, C.V., de Oliveira, M.C.N. & Hartman, G.L. 2014. Mycelium compatibility and aggressiveness of *Sclerotinia sclerotiorum* isolates from Brazil and the United States. Pesquisa Agropecuária Brasileira 49: 265-272.

Kohn, L.M., Petsche, D.M., Bailey, S. R., Novak, L.A. & Anderson, J.B. 1988. Restriction fragment length polymorphisms in nuclear and mitochondrial DNA of Sclerotinia species. Phytopathology 78: 1047-1051.

Kohn, L.M., Stasovski, E., Carbone, I., Royer, J. & Anderson, J.B. 1991. Mycelial incompatibility and molecular markers identify genetic variability in field populations of *Sclerotinia sclerotiorum*. Phytopathology 81: 480-485.

Kumar, S., Chandra, S., Verma, R.N. & Sharma, B.K. 1987. Reaction of soybean cultivars to soybean rust (*Phakopsora pachyrhizi* Syd.). Soybean Rust Newsletter 8: 9-10.

Kuang, J., Hou, Y-P., Wang, J-X. & Zhou, M-G. 2011. Sensitivity of *Sclerotinia sclerotiorum* to fludioxonil: In vitro determination of baseline sensitivity and resistance risk. Crop Protection 30: 876-882.

Kumar, N.S. & Gurusubramanian, G. 2011. Random amplified polymorphic DNA (RAPD) markers and its applications. Science Vision 11: 116-124.

Lemos, N.G., Braccini, A.L., Abdelnoor, R.V., Oliveira, M.C.N., Suenaga, K. & Yamanaka N. 2011. Characterization of genes *Rpp2*, *Rpp4*, and *Rpp5* for resistance to soybean rust. Euphytica 182: 53-64.

Levy, C. 2005. Epidemiology and chemical control of soybean rust in Southern Africa. Plant Disease 89: 669-674.

Li, S., Smith, J.R., Ray, J.D. & Frederick, R.D. 2012. Identification of a new soybean rust resistance gene in PI 567102B. Theoretical and Applied Genetics 125: 133-142.

Litholdo Júnior, C.G., Gomes, E.V., Lobo Júnior, M., Nasser, L.C., Petrofeza, S. 2011. Genetic diversity and mycelial compatibility groups of the plant-pathogenic fungus *Sclerotinia sclerotiorum* in Brazil. Genetics and Molecular Research 10:868-877.

Liu, J.J., & Ekramoddoullah, A.K.M. 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiological and Molecular Plant Pathology 68: 3-13.

Liu, E-E., Luo, W., Zhou, H. & Peng, X-X. 2009. Determination of oxalate in plant tissues with oxalate oxidase prepared from wheat. Biologia Plantarum 53: 129-132.

Livingstone, D.M., Hampron, J.L., Phipps. P.M. & Grabau, E.A. 2005. Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. Plant Physiology 137: 1354-1362.

Livingston, M., Johansson, R., Daberkow, S., Roberts, M., Ash, M. & Breneman, V. 2004. Economic and policy implications of wind-borne entry of Asian soybean rust into the United States. Economic Research Service/USDA. http://www.utcrops.com/soybean/diseases/disease_images/ERSreportOnSBR.pdf. Accessed: September 2012.

Machetti, M.A., Melching, J.S. & Bromfield, K.R. 1976. The effects of temperature and dew period on germination and infection by uredospores of *Phakopsora pachyrhizi*. Phytpathology 66: 461-463.

Majer, D., Mithen, R., Lewis, B.G., Vos, P. & Oliver, R.P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100: 1107-1111.

Mandal, A.K. & Dubey, S.C. 2012. Genetic diversity analysis of *Sclerotinia sclerotiorum* causing stem rot in chickpea using RAPD, ITS-RFLP, ITS sequencing and mycelia compatibility grouping. World Journal of Microbiological Biotechnology 28: 1849-1855.

Markley, K.S. 1950. Soybeans and Soybean Products. New York: Interscience Publishers, Inc.

Martins, J.A.S. & Juliatti, F.C. 2014. Genetic control of partial resistance to Asian soybean rust. Acta Scientiarum 36: 11-17.

Maxwell, D.P. & Lumsden, R.D. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60: 1395-1398.

McLean, R.J. 1979. Histological studies of resistance to soybean rust, *Phakopsora pachyrhizi* Syd. Australian Journal of Agricultural Research 30:77-84.

McLean, R.J., & Byth, D.E. 1980. Inheritance of resistance to rust (*Phakopsora pachyrhizi*) in soybeans. Australian Journal of Agricultural Research 31:951-956.

McLaren, N.W. 2008. Reaction of South African cultivars to rust caused by *Phakopsora pachyrhizi*. South African Journal of Plant and Soil. 25: 49-54.

Melching, J. S., Dowler, W.M., Koogle, D.L. & Royer, M.H. 1988. Effect of plant and leaf age on susceptibility of soybean to soybean rust. Canadian Journal of Plant Pathology 10: 30-35.

Melching, J. S., Dowler, W.M., Koogle, D.L. & Royer, M.H. 1989. Effects of duration, frequency and temperature of leaf wetness periods on soybean rust. Plant Disease 73: 117-122.

Meinhardt, L.W., Wulff, N.A., Bellato, C.M. & Tsai, S.M. 2002. Telomere and microsatellite primers reveal diversity among *Sclerotinia sclerotiorum* isolates from Brazil. Fitopatologia Brasileira 27: 211-215.

Miles, M.R., Bonde, M.R., Nester, S.E. Berner, D.K., Frederick, R.D. & Hartman, G.L. 2011. Characterizing resistance to *Phakopsora pachyrhizi* in soybean. Plant Disease 95: 577-581.

Miles, M.R., Frederick, R.D. & Hartman, G.L. 2003. Soybean Rust: Is the U.S. Soybean Crop At Risk? APSnet Features. Online. DOI: 10.1094/APSnetFeature-2003-0603.

Miles, M.R., Frederick, R.D. & Hartman, G.L. 2006. Evaluation of soybean germplasm for resistance to *Phakopsora pachyrhizi*. Plant Health Progress DOI: 10.1094/PHP-2006-0104-01-RS.

Miles, M.R., Pastor-Corrales, M.A., Hartman, G.L & Frederick, R.D. 2007. Differential response of common bean cultivars to *Phakopsora pachyrhizi.* Plant Disease 91: 698-704.

Mohammadi, M & Karr, A.L. 2002. β -1,3-glucanase and chitinase activities in soybean root nodules. Journal of plant physiology 159: 245-256.

Morel, W., Miles, M.R., Hernandez, J.R., Stone, C.L., Frederick, R.D. 2007. First report of *Phakopsora pachyrhizi*, cause of Soybean rust on *Neonotonia wightii* in Paraguay. Plant Disease 91: 325.

Morton, J.G. & Hall, R. 1989. Factors determining the efficacy of chemical control of white mold in white bean. Canadian Journal of Plant Pathology 11: 297-302.

Mueller, D.S., Bradley, C.A., Grau, C.R., Gaska, J.M., Kurle, J.E, & Pederson, W.L. 2004. Application of thiophanate-methyl at different host growth stages for management of Sclerotinia stem rot in soybean. Crop Protection 23: 983-988.

Mueller, D.S., Dorrance, A.E., Derksen, R.C., Ozkan, E., Kurle, J.E., Grau, C.R., Gaska, J.M., Hartman, G.K., Bradley, C.A. & Pedersen, W.L. 2002. Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. Plant Disease 86: 26-31.

Muiru, W.M., Koopmann, B., Tiedemann, A.V., Mutitu, E.W. & Kimenju, J.W. 2008. A review of the Amplified Fragment Length Polymorphism (AFLP) technique in genotyping and DNA fingerprinting studies. Journal of Applied Biosciences 9: 389-395.

National Sclerotinia Initiative. 2014. U.S. Canola Association. www.uscanola.com/site/epage/102383_956.htm. Accessed: February 2014.

Natti J.J. 1971. Epidemiology and control of Bean White Mold. Phytopathology 61: 699-674.

Nelson, B. 1998. Biology of Sclerotinia. Proceedings of the Sclerotinia Workshop. January 1998.

Nelson, B.D., Helms, T.C. & Olson, M.A. 1991. Comparison of laboratory and field evaluations of resistance in soybean to *Sclerotinia sclerotiorum*. Plant Disease 75: 662-665.

Noonan, M.P., Glare, T.R., Harvey, I.C. & Sands D.C. 1996. Genetic comparison of *Sclerotinia sclerotiorum* isolates form New Zealand and USA. Proceedings of the 49th New Zealand Plant Protection Conference. 126-131.

Noyes, R.D. & Hancock, J.G. 1981. Role of oxalic acid in the sclerotinia wilt of sunflower. Physiological Plant Pathology 18: 123–132.

Nunkamar, A., Caldwell, P.M. & Pretorius, Z.A. 2008. Alternative hosts of Asian soybean rust (*Phakopsora pachyrhizi*) in South Africa. South African Journal of Plant and Soil 25: 62-63.

Ogle, H.J., Byth, D.E. & Mclean, R. 1979. Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in south-eastern Queensland. Australian Journal of Agricultural Research 30: 883-893.

Ojaghian, M.R. 2009. First report of *Sclerotinia sclerotiorum* on potato plants in Iran. Australasian Plant Disease Notes 4: 39-41.

Ojiambo, P.S., Bandyopadhyay, R., Twizeyimana, M., Lema, A., Frederick, R.D., Pedley,K.F., Stone, C.L. & Hartman, G.L. 2007. First report of rust caused by *Phakopsora pachyrhizi* on soybean in Democratic Republic of Congo. Plant Disease 91; 1204.

Oloka, H.K., Tukamuhabwa, P., Sengooba, T., Adipala, E. & Kabayi, P. 2009. Potential for soybean rust tolerance among elite soybean lines in Uganda. Crop Protection 28: 1076-1080.

Ono, Y., Buritica, P. & Hennen, J.F., 1992. Delimitation of *Phakopsora, Physopella* and *Cerotelium* and their species on Leguminosae. Mycological Research 96: 825-850.

Oplinger, E.S., Grau, C.R., Kurle, J.E., Gaska, J.M. & Kurtzweil, N. 2007. Foliar treatments for control of white mold in soybean.

www.soils.wisc.edu/extension/FAMP/proceedings/3B.oplinger.pdf. Accessed: August 2007.

Patil, P.V. & Basavaraja, G.T. 2001. Management of soybean rust by fungicides and host plant resistance. In. T. Lumpkin, ed. 2001. Second International Vegetable Soybean Conference. Pullman. Washington: Washington State University.

Partridge-Telenko, D.E., Hu, J., Livingston, D.M., Shew, B.B., Phipps, P.M. & Grabau, E.A. 2011. Sclerotinia blight resistance in Virginia-type peanut transformed with a barley oxalate gene. Phytopathology 101: 786-793.

Paul, C., Frederickm R.D., Hill, C.B., Hartman, G.L. & Walker, D.R. 2015. Comparison of pathogenic variation among *Phakopsora pachyrhizi* isolates collected from the United States and international locations and identification of soybean genotypes resistant to the U.S. Isolates. Plant Disease 99: 1059-1069.

Pennypacker, B.W. & Risius, M.L. 1999. Environmental sensitivity of soybean cultivar response to *Sclerotinia sclerotiorum.* Phytopathology 89: 618-622.

Perez, B.A., Farinon, O.M. & Berretta, M.F. 2011. First report of Sclerotinia rot on Blueberry caused by *Sclerotinia sclerotiorum* in Argentina. Plant Disease 95: 774.1-774.1

Perez-Vicente, L., Martinez-de la Parte, E., Perez-Miranda, M., Martin-Triana, E.L., Borras-Hidalgo, O. & Hernandez-Estevez, I. 2010. First report of Asian rust of soybean caused by *Phakopsora pachyrhizi* in Cuba. Plant Pathology 59: 803.

Pham, T.A., Hill, C.B., Miles, M.R., Nguyen, B.T., Vuong, T.D., VanToai, T.T., Nguyen, H.T. & Hartman, G.L. 2010. Evaluation of soybean for resistance to soybean rust in Vietnam. Field Crops Research 117: 131-138.

Phillips, A.J.L & Botha, W.J. Sclerotinia stem rot of soybeans. Farming in South Africa, Soybeans F.1/1990.

Pivonia, S. & Yang, X.B. 2004. Assessment of the potential year-round establishment of soybean rust throughout the world. Plant Disease 88: 532-529.

Politowski, K. & Browning, J.A. 1978. Tolerance and resistance to plant diseases: An epidemiological study. Phytopathology 68: 1177–1185.

Polizzi, G., Alello, D., Scuderi, G. & Cirvilleri, G. 2011. First report of Sclerotinia stem and twig blight caused by *Sclerotinia sclerotiorum* on Citrus Volkameriana rootstock in Italy. Plant Disease 95: 1,030.3-1,030.3.

Pretorius, Z.A., Kloppers, F.J. & Frederick, R.D. 2001. First report of Soybean rust in South Africa. Plant disease 85: 1,288.3.

Pschorn-Strauss, E. & Baijnath-Pillay, N. 2004. Genetically engineered soya–an industrial wasteland. http://www.biowatch.org.za/docs/briefs/2004/briefing05.pdf. Accessed: October 2013.

Punja, Z.K. & Sun, L-J. 2001. Genetic diversity among mycelia compatibility groups of *Sclerotium rolfsii* (teleomorph *Athelia rolfsii*) and *S. delphinii*. Mycological Research 105: 537-546.

Purdy, L.H. 1979. *Sclerotinia sclerotiorum*: History, disease and symptomatology, host range, geographic distribution and impact. Phytopathology 69: 875-880.

Putnam, M.L. 2003. First report of stem rot of rosemary caused by *Sclerotinia sclerotiorum* in the United States. New Disease Report 8: 7.

Ribeiro, A.S., Moreira, J.U.V., Pierozzi, P.H.B., Rachid, B.F., de Toledo, J.F.F., Arias, C.A.A., Soares, R.M. & Godoy, C.V. 2007. Genetic control of Asian rust in soybean. Euphytica 157:15–25.

Rodriguez, A.C., Rios, J.A. & Hernandez, J.R. 2006. First report of Asian soybean rust caused by *Phakopsora pachyrhizi* from Mexico. Plant Disease 90: 1260.

Rossi, R.L. 2003. First report of *Phakopsora pachyrhizi*, the causal organism of soybean rust in the Province of Misiones, Argentina. Plant Disease 87: 102.

Rupe, J. & Sconyers, L. 2008. Soybean Rust. The Plant Health Instructor. American Phytopathological Society.

Rytter, J.L., Dowler, W.M. & Bromfield, K.R. 1984. Additional alternative hosts of *Phakopsora pachyrhizi*, causal agent of soybean rust. Plant Disease 68: 818-819.

Sagis. 2011. South African Grain Information Service – Crop Estimates committee www.sagis.org.za/CEC.

Sagis. 2014. South African Grain Information Service – Crop Estimates committee www.sagis.org.za/CEC.

Scherm, H., Christiano, R.S.C., Esker, P.D. Del Ponte, E.M. & Godoy, C. V. 2009. Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil. Crop Protection 28: 774-782.

Schneider, R.W., Hollier, C.A., Whitam, H.K., Palm, M.E., McKenny, J.M., Hernandez. J.R., Levy, L. & DeVries-Paterson, R. 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* in the Continental United States. Plant Disease 89: 774.

Sconyers, L.E., Kemerait, R.C., Brock, J., Phillips, D.V., Jost, P.H., Sikora, E.J. Gutierrez-Estrada, A., Mueller, J.D., Marois, J.J. Wright, D.L. & Harmon, C.L. 2006. Asian soybean rust development in 2005: A perspective from South Eastern United States. www.apsnet.org/publications/apsnetfeatures/Pages/SoybeanRustDev.asp.

Sexton, A.C. & Howlett, B.J. 2004. Microsatellite markers reveal genetic differentiation among populations of *Sclerotinia sclerotiorum* form Australian canola fields. Current Genetics 46: 357-365.

Shanmugasundaram, S. 1999. Epidemiology and control strategies for soybean rust-A knowledge base for Africa. In: Proceedings of workshop on soybean rust (*Phakopsora pachyrhizi*), (ed.) M. A. Smit, ARC Summer Grain Centre, Potchefstroom, South Africa.

Sharma, N.D. & Mehta, S.K. 1996. Soybean rust in Madhya Pradesh. Acta Botanica Indica 24:115-116.

Shurtleff, W. & Aoyagi, A. 2007. History of world production and trade. Part 1 & 2. Chapter from History of soybeans and soyfoods: 1100BC. to the 1980s.

www.soyinfocenter.com/HSS/production_and_trade1.php. Accessed: October 2012.

Soystats. 2008. A reference guide to important soybean facts and figures. www.soystats.com. Accessed: September 2008.

Soystats. 2011. A reference guide to important soybean facts and figures. www.soystats.com. Accessed: September 2011.

Soystats. 2013. A reference guide to important soybean facts and figures. www.soystats.com. Accessed: January 2014.

Soystats. 2014. A reference guide to important soybean facts and figures. www.soystats.com. Accessed: November 2014.

Statista. 2014. Major soybean producing countries worldwide from 2008/09 -2012/13 (in million metric tons). www.statista.com/statistics/267270/production-of-soybeans-by-countries-since-2008/ Accessed: May 2014.

Steadman, J.R. 1979. Control of plant diseases caused by *Sclerotinia* species. Phytopathology 69: 904-907.

Steadman, J.R. 1983. White mold – A serious yield limiting disease of bean. Plant Disease 67: 346-350.

Stewart, S., Guillin, E.A. & Diaz, L. 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* in Uruguay. Plant Disease 89: 909.

Thompson, A.H. & Van Der Westhuizen, C.A. 1979. *Sclerotinia sclerotiorum* (Lib.) De Bary on soybean in South Africa. Phytophylactica 11: 145-148.

Tok, F.M. 2008. First report of white mold caused by *Sclerotinia sclerotiorum* on sweet basil in Turkey. Plant Disease 92: 1,471.3-1,471.3.

Tschanz, A. T. *&* Shanmugasundaram, S. 1985*:* Soybean rust*. In:* Shibles, S. *(ed.), Proceedings of 3rd World Soybean Research Conference*, Ames, IA, 12–17, August 1984 pp. *562–567* West view Press, Boulder, CO, USA.

Tschanz, A.T. & Wang, T.C. 1980. Soybean rust development and apparent infection rates at five locations in Taiwan. Protection Ecology 2: 247-250.

Tschanz, A.T. & Wang, T.C. 1987. Interrelationship between soybean development, resistance and *Phakopsora pachyrhizi*. Soybean Rust Newsletter 8: 14-18.

Tschanz, A.T., Wang, T.C. & Tsai, B.Y. 1985. Recent advances in soybean rust research. In: Shanmugasundaram, S., Sulzberger, E.W. & Mclean, B.T. (Eds.). Soybean in tropical and sub tropical cropping systems. Shanhua, Taiwan, AVRDC.

Tu, J.C. 1985. Tolerance of white bean (*Phaseolus vulgaris*) to white mold (*Sclerotinia sclerotiorum*) associated with tolerance to oxalic acid. Physiological Plant Pathology 26: 111-117.

Tu, J.C. 1989. Oxalic acid induced cytological alterations differ in beans tolerant or susceptible to white mold. New Phytologist 112: 519-525.

Tu, J.C. 1997. An integrated control of white mold (*Sclerotinia sclerotiorum*) of beans, with emphasis on recent advances in biological control. Botanical Bulletin of Academia Sinica 38:73-76.

Tu, J.C. & Beversdorf, W.D. 1982. Tolerance to white mold (*Sclerotinia sclerotiorum* (Lib.) De Bary) in Ex Rico 23, a cultivar of white bean (*Phaseolus vulgaris* L.). Plant Science 62: 65-69.

Twizeyimana, M., Ojiambo, P.S., Haudenshield, J.S., Caestano-Anolles, G., Pedley, K.F., Bandyopadhyay, R. & Hartman, G.L. 2011. Genetic structure and diversity of *Phakopsora pachyrhizi* isolates from soybean. Plant Pathology 60: 719-729.

Twizeyimana, M., Ojiambo, P.S., Ikotun, T., Ladipo, J.L., Hartman, G.L. & Bandyopadhyay, R. 2008. Evaluation of soybean germplasm for resistance to soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 92: 947-952.

Twizeyimana, M., Ojiambo, P.S., Ikotun, T., Hartman, G.L. & Bandyopadhyay, R. 2009. Pathogenic variation of *Phakopsora pachyrhizi* infecting soybean in Nigeria. Phytopathology 99: 353-361.

Venette, J. 1998. *Sclerotinia* spore formation, transport and infection. Proceedings of the *Sclerotinia* workshop 1998. www.ndsu.nodak.edu/plantpath/sclero.htm. Accessed: September 2006.

Vleugels, T., De Riek, J., Heugens, K., Van Bockstaele, E. & Baert, J. 2012. Genetic diversity and population structure of *Sclerotinia* species from European red clover crops (*Trifolium pratense* L.). Journal of Plant Pathology 94: 493-503.

Vos, P., Hogers, R., Bleeter, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: a new concept for DNA fingerprinting. Nucleic Acids Research 23: 4407- 4414.

Wang, T.C. & Hartman, G.L. 1992. Epidemiology of soybean rust and breeding for host resistance. Plant Protection Bulletin 34: 109-124.

Wegulu, S.N., Yang, X.B. & Martinson, C.A. 1998. Soybean cultivar responses to *Sclerotinia sclerotiorum* in field and controlled environment studies. Plant Disease 82: 1264-1270.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531–6535.

Woodward, J.E., Brenneman, T.B., Kemerait jr, R.C. & Culbreath, A.K. 2006. First report of Sclerotinia blight caused by *Sclerotinia sclerotiorum* on Peanut in Georgia. Plant Disease 90: 111.

Workman, D. 2007. Top soybean countries. www.Internationaltrade.suite101. com/article.cfm/top_soybean_countries. Accessed: October 2009.

Wrather, J.A., Anderson, T.R., Asrsyad, D.M., Gai, J. Ploper, L.D., Porta-Puglia, A., Ram, H.H., Yorinori, J.T. 1997. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. Plant Disease 81: 107-110.

Wrather, J.A., Anderson, T.R., Arsyad, D.M., Tan, Y., Ploper, L.D., Porta-Puglia, A., Ram, H.H. & Yorinori, J.T. 2001. Soybean disease loss estimates for the top ten soybean-producing countries in 1998. Canadian Journal of Plant Pathology 23: 115-121.

Wrather, A. & Koenning, S. 2009. Effect of diseases on soybean yields in the United States 1996-2007. Plant Health Progress DOI:10.1094/PHP-2009-0401-01-RS.

Yamanaka, N., Lemos, N.G., Uno, M., Akamatsu, H., Yamaoka, Y., Abdelnoor, R.V., Braccini, A.L., & Suenaga, K. 2013. Resistance to Asian soybean rust in soybean lines with the pyramided three *Rpp* genes. Crop Breeding and Applied Biotechnology 13: 75-82, 2013.

Yamaoka, Y., Fujiwara, Y., Kakishima, M., Katsuya, K., Yamada, K. & Hagiwara, H. 2002. Pathogenic races of *Phakopsora pachyrhizi* on soybean and wild host plants collected in Japan. Journal of Genetic Plant Patholology 68:52-56.

Yamaoka, Y., Yamanaka, N., Akamatsu, H. & Suenaga, K. 2014. Pathogenic races of soybean rust *Phakopsora pachyrhizi* collected in Tsukuba and vicinity in Ibaraki, Japan. Journal of Genetic Plant Pathology 80:184–188.

Yang, X.B. & Feng, F. 2001. Ranges and diversity of soybean fungal disease in North America. Phytopathology 91: 769-775.

Yang, X.B., Royer, M.H., Tschanz, A.T. & Wang, T.C. 1990. Analysis and Quantification of soybean rust epidemics from seventy-three sequential planting experiments. Phytopathology 80: 1421-1427.

Young, C.S., Clarkson, J.P., Smith, J.A., Whatling, M., Phelps, K. & Whipps, J.M. 2004. Environmental conditions influencing *Sclerotinia sclerotiorum* infection and disease development in lettuce. Plant Pathology 53: 387-397.

Zhao, J. & Meng, J. 2003. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.) Theoretical and Applied Genetics 106: 759-764.

Zimmer, R.C. & Campbell. C. 1990. First report of *Sclerotinia sclerotiorum* on *Lathyrus sativus*. Canadian Plant Disease Survey 70: 1.

CHAPTER 2

EPIDEMIOLOGICAL FACTORS TO BE CONSIDERED DURING THE EVALUATION OF SOYBEAN LINES FOR RESISTANCE TO SOYBEAN RUST CAUSED BY *PHAKOPSORA PACHYRHIZI* IN SOUTH AFRICA

2.1 Abstract

An increase in soybean production over the past decade has made this crop a significant source of income worldwide. As the demand for soybean and soybean products has increased, so has interest in improved production technologies as well as cultivars with disease resistance. Soybean rust caused by *Phakopsora pachyrhizi* is a serious yield limiting foliar disease of soybean prevailing in most soybean production areas. In this study experimental lines were evaluated for resistance to soybean rust in the greenhouse and in field trials at Cedara during 2008/09 and 2009/10. In the greenhouse lines were planted in seed trays filled with steam sterilized soil and grown to V3 growth stage before inoculation. Freshly harvested urediniospores from stock cultures were suspended in a light mineral oil and sprayed onto three plants per line. Inoculated plants were placed in a dew chamber for 16 hours and subsequently transferred to the greenhouse set to a 25/20°C day/night regime until ratings were conducted. Greenhouse ratings were conducted using a disease diagram to assess rust severity as well as infection type. Field assessments were dependent on natural infection and were conducted at two week intervals subsequent to flowering until maturity. Disease severity was expressed as affected leaf area based on visual assessment of rust severity and degree of defoliation. Rust severity in lines evaluated in the greenhouse differed significantly and the majority of lines showed Tan-type lesions suggesting susceptibility to soybean rust. In the field, significant differences in disease severity between lines were recorded as well as in relative live time (RLT) at disease onset, RLT at which 100% disease was reached, apparent rate of infection and area under disease progress curve (AUDPC). Delayed onset of the disease proved a major component of reduced disease severity and was negatively correlated with AUDPC. Correlations between greenhouse and field results were poor and consistency over seasons and locality remains a major constraint in cultivar evaluation. Although lines differed in disease severity further research is needed to determine the economic value of the levels of disease resistance recorded.

2.2 Introduction

Soybean (*Glycine max* (L.) Merr.) is an important crop in many countries. During 2011, 258,4 million metric tons of soybeans were produced worldwide making soybean a significant source of income (American Soybean Association, 2012). In South Africa specifically, soybean production has shown a steady increase in recent years. In the 2000/01 season 209 705 tons were produced and this increased to 948 000 tons in 2014 (Sagis, 2015).

Soybean rust caused by *Phakopsora pachyrhizi* Syd. & P. Syd is a serious yield limiting foliar disease in most soybean production areas (Miles *et al*., 2003; Isard *et al*., 2006; Mueller *et al*., 2009). The disease was first recorded in Japan in 1902 and has subsequently spread throughout Asia (Bromfield, 1984) and Africa (Akinasanmi *et al*., 2001) Uganda (Kawuki *et al*., 2003b), Zimbabwe (Shanmugasundaram, 1999) and South Africa (Pretorius *et al*., 2001). More recently it was recorded in North and South America (Isakeit *et al*., 2006; Rupe & Sconyers, 2008).

Common symptoms of soybean rust include initial small watersoaked lesions on the lower leaf surfaces. As the disease progress, lesions increase in size and change from gray to tan or reddish brown (Anonymous, 2008). Uredia develop in lesions and when mature, urediniospores are released initiating secondary infections. As lesion number increase, affected leaf areas show signs of yellowing and premature defoliation occurs (Rupe & Sconyers, 2008). Pod-fill, which includes a reduction in the number of seeds per pod, number of filled seeds per pod and 100 seed weight, is affected by soybean rust (Ogle *et al*., 1979, Kawuki *et al*., 2003b).

The increasing demand for soybeans has generated a greater interest in production technologies as well as cultivars with disease resistance (Ribeiro *et al*., 2008). Breeding for resistance still remains the most economic method of disease control. It is more applicable to sustainable agriculture, reduces the need for chemical applications and thus, environmental damage while serving as the best long term solution for disease management (Grafton, 1998). Although sources of resistance to soybean rust have been identified, to date, no soybean cultivars with stable resistance have been released (Silva *et al*., 2008). To date six different loci carrying dominant alleles have been identified namely *Rpp*1 (Mclean & Byth, 1980), *Rpp*2 (Bromfield & Melching, 1982), *Rpp*3 (Bromfield & Hartwig, 1980), *Rpp*4 (Hartwig, 1986), *Rpp*5 (Garcia *et al*., 2008) and *Rpp*6 (Li *et al*., 2012). None of the identified rust resistance genes have been deployed due to matching

avirulence genes in races of *P. pachyrhizi* which overcome resistance (Hartman, 2005; da Silva *et al*., 2011).

Soybean germplasm with rate reducing resistance to soybean rust has been reported. This type of resistance is effective against most races of a pathogen (Tschanz *et al*., 1985). Hartman (1991) used latent period and number of uredia to identify lines with partial resistance or slow rusting. However, developing lines with rate-reducing resistance is constricted by the difficulty of disease evaluation, especially in lines from segregating populations or lines with different maturities (Tschanz *et al*., 1985; Wang & Hartman, 1992). Bromfield & Hartwig (1980) described three infection types for *P. pachyrhizi* on soybeans namely reddish brown (RB) lesions, Tan coloured lesions and an immune reaction characterised by whitish or pale coloured lesions. These authors, as well as Bonde *et al*. (2006), attributed susceptibility to the Tan reaction and resistance to the RB reaction. Twizeyimana *et al*. (2008) suggests that genotypes expressing RB lesions may be sources of partial resistance.

This study was conducted to determine the reaction of artificially inoculated experimental soybean lines to soybean rust under controlled conditions in the greenhouse as well as under South African field conditions where they were subjected to natural infection in order to identify lines with possible resistance for further commercial exploitation.

2.3 Materials and methods

2.3.1 Greenhouse evaluation

2.3.1.1 Inoculum production

Infected leaves from the field were sampled and spores were collected using a mechanical vacuum pump. Spores were transferred to a cryo vial and stored at -80°C until further use. Inoculum for greenhouse trials was produced on plants of the commercial cultivar “Stork” which is known to be susceptible to rust. Four pots (500ml) were filled with steam sterilized soil:peat mixture (1:1) and ten seeds were planted per pot. Pots were watered daily with regular tap water. When primary leaves were fully developed plants were treated with 50 ml maleic hydrazide (3g/l water) per pot. Maleic hydrazide is a plant growth regulator that acts by inhibiting cell division in plants and thus, restricts growth to keep plants small and manageable. Two weeks after emergence, plants were inoculated with *P. pachyrhizi* spores collected in the field. When sporulation commenced, a single pustule was collected

to remove the possibility of a mixture of races, and bulked by inoculating a new batch of greenhouse grown plants as described above. Spores isolated from these plants were used to inoculate experimental lines and maintained at -80°C as the stock culture for further use in the study during 2008/09 and 2009/10 evaluation seasons.

2.3.1.2 Plant production

Lines were planted in seed trays (98, 10x4 cm seed cones) filled with a steam sterilized soil:peat mixture (1:1). Two seeds were planted per cone and thinned to one plant per cone after emergence. Three cones per line were planted. A randomized block design was used and the trial was done in triplicate. Seed trays were watered daily with tap water and maintained at a 25/20°C day/night regime with a 14/10 hour light/dark cycle. Plants were grown to V 3 growth stage before inoculation.

In 2009, 39 lines (Table 2.1) were evaluated using the above methodology. These selected lines were also planted in field trials. Subsequently in 2010, 17 lines (Table 2.2) were selected from the 39 lines based on greenhouse and field reaction and re-evaluated in the greenhouse.

2.3.1.3 Inoculation procedure

Freshly harvested urediniospores from the stock culture (8 mg) were suspended in a light mineral oil (800 µl) (Soltrol 170®) and placed in a gelatine capsule. Urediniospores were sprayed onto leaves of the test lines using a mechanical compressor pump. Four capsules were applied per seedling tray to ensure even coverage of the leaves. Plants were allowed to dry at room temperature before being transferred to a dew chamber for 16 hours. Plants were subsequently transferred to the greenhouse and maintained at a 25/20°C day/night regime with a 14/10 hour light/dark cycle.

2.3.1.4 Ratings and data analysis

Ratings were conducted 14 days after inoculation. Rust severity was assessed with the aid of a disease diagram with six severity levels (0.6, 2, 7, 18, 42 and 78.5%) (Godoy *et al*., 2006). The infection type was also recorded. Where both a RB and TAN reactions were recorded these were considered a RB reaction for analysis purposes (Miles *et al*., 2006).

Data were analysed using Number Cruncher Statistical Systems (NCSS) (Hintze, 2007). A General Linear Model (GLM) analysis of variance (ANOVA) was conducted to determine the effects of line genotype on severity and infection type. Means were compared using Fisher's Least Significance Difference (LSD) at $P < 0.05$.

2.3.2 Field evaluation

2.3.2.1 Field plots

Field trials were conducted at Cedara, KwaZulu-Natal (GPS coordinates: -29.533°S, 30.283°E) where soybean rust is endemic. During the 2008/09 growing season, blocks consisting of 39 lines corresponding with those used in greenhouse experiments, were planted in a randomised block experimental design. The 2008/09 trial consisted of five blocks sequentially planted on 13 and 25 November and 4, 12 and 18 December 2008. During the 2009/10 planting season, 22 lines selected during the preceding season were planted in a randomised block design trial sequentially planted on 10 and 26 November as well as 3 and 14 December 2009. Plantings of replicates in this manner were used to minimize the risk of escape potentially associated with natural infection.

Prior to planting, fields were fertilized with 300 kg ha^{-1} superphospate and 50 kg ha^{-1} KCl. Lines were hand-planted in single row plots, 10m in length with a 45 cm inter-row spacing on each planting date. The plant density was equivalent to 360 000 plants ha^{-1}. Commercial rhizobium (*Bradyrhizobium japonicum*) was mixed with water (100g/15ℓ water) and approximately 150 ml were added to each planting furrow at planting before rows were closed by raking. Plots were sprayed with 1920 ml (a.i) ha^{-1} Alachlor for pre-emergence grass control. Hand weeding was done when necessary.

2.3.2.2 Ratings and data analysis

Infection of lines by *P. pachyrhizi* was dependent on natural inoculum. Plots were monitored for rust development at two week intervals subsequent to flowering (R1 to R2) until maturity (R7/R8).

Rust was visually assessed as the percentage leaf area covered with rust lesions based on a disease diagram (Godoy *et al*., 2006). Estimated percentage defoliation was also

rated. Since both rust and defoliation are responsible for decreased photosynthetic area of plants, an integrated rating of disease severity and defoliation as a measure of disease was used ie. affected leaf area (ALA)=defoliation (%)+rust (%) on remaining leaves (McLaren, 2008). Thus, ALA=defoliation + ((100-defoliation) * (Rust severity/100)). This was based on the assumption that leaves discoloured by rust lesions no longer photosynthesize and contribute to yield. Days after planting on each assessment date was used to calculate the relative life time (RLT) of varieties as RLT=(days after planting/days to maturity)*100 (Tschanz, 1984) so as to standardize the growth periods of lines with differing maturity periods.

Disease progress curves were fitted for each line after calculated ALA were converted to a proportion and linearised according to the logistic equation Y=Ln(X/(1-X) (van der Plank, 1963) where RLT was used as the independent-variable and transformed ALA as the dependent variable. Data were analysed using simple regression analysis (NCSS, 2007). The apparent infection rate (van der Plank, 1963) ie. disease increase per time increment expressed as per unit per day (pupd), was recorded from the regression output. The regression model was back-transformed and used to estimate disease onset and RLT at which 100% disease occurred. Area under the disease progress curve (AUDPC) was calculated according to the model of Campbell & Madden (1990) where AUDPC = Σ_i=1 n-1 [(t_i+1 – t_i) (y_i + y_i+1)/2] using RLT as the independent variable and ALA as the dependent variable. Linear regression and correlation analyses were used to determine the relationships between onset and AUDPC. Multiple regression analysis was used to determine the interaction of apparent rate of infection and onset as independent variables on AUDPC as dependent variable.

2.4 Results

2.4.1 Greenhouse evaluation

During 2009, significant differences in disease severity were observed in the greenhouse between the 39 lines evaluated (Table 2.1). Mean values ranged from 1.53 % in line Selection 16 to 26.00% in line PI 445844 with only 7 lines showing less than 5% disease severity. Lesion types for lines were also recorded and the majority of the lines showed Tan-type lesions (71.80%) while 25.60% had red-brown lesions and 2.50% immune-type lesions. In some cases mixed reactions were detected where red-brown (RB) and Tan lesions or red-brown and immune lesions were present on a single leaf. In both cases these were considered red-brown.

Significant differences in rust severity in lines selected for re-evaluation in 2010 were recorded within the range of 3.20% in line PI 603909A to 29.51% in line PI 507133 (Table 2.2). Spearman rank correlations (r=0.17, 15df) indicated that relative responses of the lines were not repeatable over the two experiments. The majority of lines showed Tan-type lesions (76.50 %), 17.70% of the lines RB-type lesions and 5.90% immune-type lesions. However, only 11 of the 17 reaction types corresponded with the 2009 greenhouse trials data, indicating inconsistency in this criterion. In PI 507133, for example a tan (susceptible) response was recorded in 2009 and an immune response in 2010. This cultivar also had a high disease severity in 2009 and the highest disease severity in 2010 reinforcing the questionability of these criteria as an indication of resistance.

2.4.2 Field evaluation

During field evaluations in 2008/09 and 2009/10, disease was recorded in all lines indicating sufficient levels of natural inoculum. Analysis of variance indicated significant differences between lines using the assessment criteria RLT at onset of disease, the RLT at which 100% disease severity was reached, apparent rate of infection and AUDPC. Rust onset was determined as the RLT at which 5% disease severity was reached when expressed on a standardized physiological scale and ranged from a RLT of 68.33% in PI 419043 to a high of 85.21% in line Selection 43 during field trials in 2008/2009 (Table 2.3). The majority of lines had mean onset values ranging between 69% and 79%. During the 2010 planting season line PI 499957 showed the earliest onset (66.57%) and PI 445845 had the latest onset at a RLT of 79.83 % (Table 2.4). Onset in lines common to both seasons was poorly correlated however, in PI 567614C, PI 603160 and PI 594411 and onset was significantly later than the remaining lines, suggesting merit in this as a rust resistance selection criterion.

Relative lifetime of lines at which 100% disease severity was recorded indicated that during the 2008/2009 season, PI 603486 reached 100% disease severity first at a RLT of 75.23% which may be attributed to the early onset of disease at 72.41% RLT. In 2010, 100% disease severity was earliest in Line PI 499957 at a RLT of 74.16%. This figure was expected as onset of disease was the earliest recorded at RLT of 66.57%. Apparent infection rates ranged from 0.11 to 0.46 per unit per day (pupd) during 2008/2009 which were recorded in lines PI 603486 and PI 587723A respectively. During the 2009/2010 season, infection rates were generally higher compared to the 2008/2009 season, ranging from 0.22 pupd in PI 499957 to a high of 0.35 pupd in lines PI 603166 and PI 567614 C respectively.

Significant differences in AUDPC were recorded between lines in both 2008/09 and 2009/10. Line PI 603160 had the lowest AUDPC value (280.97) and PI 603486 the highest (2352.36) in 2009. During 2010, line PI 603160 had the lowest AUDPC value (1209.20) and line PI 499957 the highest (2604.64). A significant ($P<0.05$) negative relationship was recorded between AUDPC and disease onset although this relationship was weak during 2008/09 ($R^2=0.37$). A stronger relationship was observed in 2009/10 ($R^2=0.93$) (Figure 2.1). A significant relationship ($R^2=0.88$) was also observed between AUDPC and the apparent infection rate during 2009/10 trial (Figure 2.2) but not during 2008/09 (data not shown).

A combined analysis of variance was conducted on disease severity data from the 22 lines common to both seasons (Table 2.5). A significant interaction between genotype and season for the RLT at onset of rust development, apparent infection rate and AUDPC criteria indicate that the reaction of genotypes differed over seasons. Onset of disease was first recorded in line PI 603486 at a RLT of 67.40% and last in line PI 594411 at 79.93% while the rest of the lines all had onset values within the narrow range of 71 to 77%. The RLT at which 100% ALA was recorded differed significantly between the 22 lines but no season or interaction effect was recorded. Line PI 603755E had the slowest rate of infection over the two seasons and line Selection 36 the highest with 0.22 pupd and 0.37 pupd respectively. Line PI 419043 had the lowest AUDPC value (778.43) and line PI 499957 the highest (2327.00). Line PI 499957 reached 100% disease levels first at a RLT of 77.05% and since line PI 594411 had the latest onset of disease, the RLT when 100% isease levels was recorded was the highest at 90.71%. Apparent rate of infection was poorly correlated with AUDPC (r=-0.047). Correlation between disease onset and AUDPC showed a high negative correlation (r=-0.79). Apparent infection rates over the two seasons were also poorly correlated (r=-0.13). Correlation coefficients between disease onset, AUDPC and RLT at 100% disease was reached were also low (r=0.22, r=0.37 and r=0.38 respectively).

2.5 Discussion

Deployment of cultivars with resistance to soybean rust remains the most viable disease management strategy (Twizeyimana *et al*., 2008; Pham *et al.*, 2010; Maposa *et al.,* 2012). In the current greenhouse studies, rust severity ratings covered a wide range of values but none of the lines was immune to the disease. These data also illustrated the high variation in disease response from one trial to the next. A typical example of this is the line PI

603909A which had a disease severity rating of 14.33%, significantly above average in 2009 but displayed a disease severity of only 3.2% in 2010 during greenhouse evaluations.

Bromfield & Hartwig (1980) proposed that red-brown lesions may be an indication of resistance to soybean rust and lesion type was therefore, also recorded during this study. Most entries showed TAN lesions which according to these authors, suggests susceptibility. During 2009 a tan (susceptible) response in Selection 6/43 displayed a disease severity rating of 2.0% while in PI 603909A a red-brown (resistant) response displayed 14.33% disease severity. Similar discrepancies were recorded during 2010. These observations suggests that lesion type is not an indication of resistance but rather an indication of lesion maturity, especially since mixed reactions where both TAN and RB lesions appeared on a single leaf, were recorded. The possibility of this reaction due to a mixture of races was not investigated since a single pustule isolate was used. According to Bonde (2006) differences in the numbers and sizes of uredinia as well as hyphal spread and development are an indication of resistance or susceptibility and this in turn could be related to lesion types. Miles *et al*. (2007) and Maphosa *et al*. (2012) indicated that RB lesions were associated with low sporulation and TAN lesions with high sporulation suggesting that lesion type may be more closely related to epidemiological aspects of inoculum potential, rather than colonisation and leaf tissue damage when inoculum is provided as in the case of the greenhouse studies. Twizeyimana *et al*. (2008) reported that a longer latent period, a lower rate of increase in pustule numbers as well as smaller lesions were recorded with RB infection reactions although these were not observed here.

The use of relative life time (RLT) instead of days after planting, and thus the percentage of the soybean growth cycle that has been completed on a particular date (Kawuki *et al*., 2003a) has been used widely in resistance screening studies as an accurate assessment of phenological stage at which assessments are made. This is especially important in disease systems that are driven by host physiology which appears to be crucial in soybean rust development. A study by Moreira *et al*. (2015) confirmed that cultivar maturity as well as planting dates ie. environmental factors had significant effects on leaf area index as well as soybean rust progress.

McLaren (2008) showed that the physiological stage at which disease development accelerated varied between cultivars within the range of 68 to 82% RLT and that this was a cultivar attribute that affects rust onset and development (Hartman *et al*., 1991; Tschanz & Tsai, 1982; Melching *et al,* 1988). In the current study, onset in lines common to the two seasons of evaluation were poorly correlated in contrast to the earlier cultivar study

(McLaren, 2008) although those cultivars with a high percentage RLT at onset maintained a higher onset value over seasons. Disease onset can be affected by factors such as weather, cloud cover, precipitation and photoperiods (Yang *et al*., 1991). Tschanz & Tsai (1982) observed that rust development is delayed on plants under extended photoperiods compared to those subjected to reduced photoperiods. These conditions, although not measured in this study and no doubt others, could have contributed to poor correlations between seasons. Ribeiro *et al*. (2007) suggested that the expression of soybean rust is dependent on specific combinations of host and micro-environmental conditions. Jarvie & Shanahan (2009) emphasized the importance of seasonal variability in lines especially when assessing yield loss in soybean tolerant lines. Studies confirmed that mean relative area under the soybean rust progress curve decreased as planting was delayed and the length of the growing season was a driving variable affecting leaf area index. The relative area under leaf area index progress curve was the highest for the planting dates with the longest growing seasons (Moreira *et al*., 2015). Bromfield (1984) and Kawuki *et al*. (2004) suggested the assessment of rust prevalence and severity throughout a planting season for a number of consecutive years in order to ascertain the stability of assessment criteria.

Subsequent to disease onset, the apparent infection rate was rapid with the result that, with the exception of five lines in 2008/09, all lines reached 100% disease severity before 90% RLT. Only Selection 43 reached 100% RLT and this was associated with a later disease onset (RLT=85.2% and slow apparent infection rate (rate=0.16). A similar tendency was observed in 2009/10 season and all the lines reached 100% disease severity before 90% RLT. The rapid rate of infection could be attributed to the moderate temperatures at Cedara. In 2008/09 the average maximum temperature was 29.6°C, the minimum temperature 20.2°C, a maximum RH of 92.61% and rainfall was measured at 3.64 mm. During the 2009/10 season, the average maximum temperature was 25.4°C, the minimum temperature 14.5°C, a maximum RH of 94.6% and 4.11 mm of rain was recorded. A significant line x season interaction was recorded and therefore the cooler temperatures and higher RH as well as rainfall may explain the differences between the two seasons as well as the earlier onset, higher apparent infection rate and high AUDPC values obtained in the 2009/10 season. This highlights the importance of these variables in the selection and identification of resistance (McLaren, 2008). Moreira *et al*. (2015) also indicated that different soybean rust assessments among cultivars and planting dates may be attributed to weather conditions and specifically photoperiods on plant physiology as longer day lengths associated with early plantings would result in extended solar radiation and thus, a greater influence on plant physiology and growth.

Although yield loss was not assessed in the current study, the early onset of disease and subsequent rapid infection rate could be assumed to have impacted on the critical stages of yield components (see Chapter 3). Ogle *et al.* (1979) found that the longer the period of exposure to soybean rust, the greater are losses due to its effect on pod-set and pod-fill. Rust severity from pod formation (R4) to pod filling stage (R4 to R5) appear most critical in yield losses (Handaningsih *et al*., 1986 see Kawuki *et al*., 2004) and it can also thus be anticipated that late onset and reduced rate e.g. Selection 43 (85.21% RLT and 0.16 pupd) and PI 567619 (83.52% RLT and 0.19 pupd) will result in reduced rust severities during these critical growth stages and thus, reduced yield loss.

Results of this study emphasize the need for multiple evaluation criteria when assessing germplasm for resistance. Furthermore, these assessment criteria should not be seen as independent from each other. Lines with similar onsets but different rates will yield different rust severities or RLT at which 100% ALA is reached and this will all need to be assessed and integrated before a final evaluation of lines performance is made.

Although differences in resistance to rust were recorded between lines used in this study, levels were such that farmers would still have to rely on fungicides for protection against this pathogen. However some lines, in particular Selection 43, showed characteristics such as late onset as well as low rate of infection and could contribute to a resistance breeding program (Hartman *et al*., 1991). However, inheritance of these traits still requires study. In addition, the later onset and reduced rate of infection could contribute to reducing the number of sprays required to minimize yield loss, thus reducing costs associated with soybean production. Studies to determine the extent to which these criteria can be exploited in breeding programs or fungicide regimes are still required.

2.6 References

Akinasanmi, O.A., Ladipo, J.L. & Oyekan, P.O. 2001. First report of soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 85: 97.

American Soybean Association. 2012. www.soygrowers.com. Accessed: December 2013.

Anonymous. 2008. Soybean rust: *Phakopsora pachyrhizi* and *P. meibomiae*.

National Pest Alert. www.aphis.usda.gov/publications/plant_health/content/printable_version/Soybean_Rust_22.pdf. Accessed: August 2008.

Bromfield, K.R. 1984. Soybean Rust. Monograph no 11. American Phytopathological Society Press. St. Paul, Minnesota, USA.

Bromfield, K.R., & Hartwig, E.E. 1980. Resistance to soybean rust and mode of inheritance. Crop Science 20:254-255.

Bromfield K.R, & Melching, J.S. 1982. Sources of specific resistance to soybean rust. Phytopathology 72:706.

Bonde, M.R., Nester, S.E., Austin, C.N., Stone, C.L., Frederick, R.D., Hartman, G.L. & Miles, M.R. 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. meibomiae* isolates. Plant Disease 90:708–716.

Campbell, C.L. & Madden, L.V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, New York. USA.

Da Silva, J.V.C., Juliatti, F.C., da Silva, J.R.V. & Barros, F.C., 2011. Soybean cultivar performance in the presence of soybean Asian rust, in relation to chemical control programs. European Journal of Plant Pathology 131: 409-418.

Garcia, A., Calvo, E.S., de Souza Kiihl, R., Harada, A., Hiromoto, D.M. & Vieira, L.G.E. 2008. Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. Theoretical and Applied Genetics 117: 545-553.

Godoy, C.V., Koga, L.J. & Canteri, M.G. 2006. Diagrammatic scale for assessment of soybean rust severity. Fitopatologia Brasileira 31: 063-068.

Grafton, K.F. 1998. Resistance to white mold in dry bean. Proceedings of the Sclerotinia workshop 1998.

Handaningsih, S., Pusposendjojo, N. & Sudarmadi, N. 1986. The relationship between the incidence of soybean rust (*Phakopsora pachyrhizi Sydow*) and crop yield. Panelitian Palawija 12: 72-78.

Hartman, G.L., Miles, M.R. & Frederick, R.D. 2005. Breeding for resistance to soybean rust. Plant Disease 89: 664-666.

Hartman, G.L., Wang, T.C. & Tschanz, A.T. 1991. Soybean rust development and the quantitative relationship between rust severity and soybean yield. Plant Disease 75: 596-600.

Hartwig, E. E. 1986. Identification of a fourth major gene conferring resistance to soybean rust. Crop Science 26:1135-1136.

Hintze, J.L. 2007. Number cruncher statistical systems (NCSS). Kaysville, UT: Number Cruncher Statistical Systems.

Isakeit, T., Miller, M.E., Saldana, R., Barnes, L.W., McKemy, J.M., Palm, M.E., Zeller, K.A., DeVries-Paterson, R. & Levy, L. 2006. First report of rust caused by *Phakopsora pachyrhizi* on Soybean and Kudzu in Texas. Plant Disease 90: 971.

Isard, S.A., Dufault, N.S., Hartman, GL., Russo, J.M., De Wolf, E.D. & Morel, W. 2006. The effect of solar irradiance on the mortality of *Phakopsora pachyrhizi* urediniospores. Plant Disease 90: 941-945.

Jarvie, J.A. & Shanahan, P.E. 2009. Assessing tolerance to soybean rust in selected genotypes. Field Crops Research 114: 419-425.

Kawuki, R.S., Adipala, E., Lamo, J & Tukamuhabwa, P. 2003a. Responding to the soybean rust epidemics in sub-saharan Africa: A review. African Crop Science Journal 11: 301-318.

Kawuki, R.S., Adipala, E. & Tukamuhabwa, P. 2003b. Yield loss associated with soya bean rust (*Phakopsora pachyrhizi* Syd.) in Uganda. Journal of Phytopathology 151: 7-12.

Kawuki, R.S., Thukamuhabwa, P. & Adipala, E. 2004. Soybean rust severity, rate of rust development and tolerance as influenced by maturity period and season. Crop Protection 23: 447-455.

Li, S., Smith, J.R., Ray, J.D. & Frederick, R.D. 2012. Identification of a new soybean rust resistance gene in PI 567102B. Theoretical and Applied Genetics 125: 133-142.

Maposa, M., Talwana, H., Gibson, P. & Tukamuhabwa, P. 2012. Combining ability for resistance to soybean rust in F2 and F3 soybean populations. Field Crops Research 130: 1-7.

McLaren, N.W. 2008. Reaction of South African cultivars to rust caused by *Phakopsora pachyrhizi*. South African Journal of Plant and Soil. 25: 49-54.

McLean, R.J., and Byth, D.E. 1980. Inheritance of resistance to rust (*Phakopsora pachyrhizi*) in soybeans. Australian Journal of Agricultural Research 31:951-956.

Melching, J.S., Dowler, W.M., Koogle, D.L. & Royer, M.H. 1988. Effect of plant and leaf age on susceptibility of soybean to soybean rust. Canadian Journal of Plant Pathology 10: 30-35.

Mueller, T.A., Miles, M.R., Morel, W., Marois, J.J., Wright, D.L., Kemerait, R.C., Levy, C. & Hartman, G.L. 2009. Effect of fungicide and timing of application on soybean rust severity and yield. Plant Disease 93: 243-248.

Miles, M.R., Frederick, R.D. & Hartman, G.L. 2003. Soybean Rust: Is the U.S. Soybean Crop At Risk? APSnet Features. Online. DOI: 10.1094/APSnetFeature-2003-0603.

Miles, M.R., Pastor-Corrales, M.A., Hartman, G.L. & Frederick, R.D. 2007. Differential response of common bean cultivars to *Phakopsora pachyrhizi*. Plant Disease 91: 698-704.

Moreira, E.N., Vale, F.X.R., Paul, P.A., Rodrigues, F.A., & Jesus Júnior, W.C. 2015. Temporal dynamics of soybean rust associated with leaf area index in soybean cultivars of different maturity groups. Plant Disease 99: 1216-1226.

Ogle, H.J., Byth, D.E. & Mclean, R. 1979. Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in south-eastern Queensland. Australian Journal of Agricultural Research 30: 883-893.

Pham, T.A., Hill, C.B., Miles, M.R., Nguyen, B.T., Vu, T.T., Vuong, T.D., Van Toai, T.T., Nguyen, H.T. & Hartman, G.L. 2010. Evaluation of soybean for resistance to soybean rust in Vietnam. Field Crops Research 117: 131-138.

Pretorius, Z.A., Kloppers, F.J. & Frederick, R.D. 2001. First report of soybean rust in South Africa. Plant disease 85: 1,288.3.

Ribeiro, A.S., de Toledo, J.F.F., Arias, C.A.A., Gdody, C.V., Soares, R.M., Moreira, J.U.V., Pierozzi, P.H.B., Goncalves-Vidigal, M.C., & de Oliveira, M.F. 2008. Genetic control of soybean (*Glycine max*) yield in the absence and presence of the Asian rust fungus (*Phakopsora pachyrhizi*). Genetics and Molecular Biology 3: 98-105.

Ribeiro, A.S., Moreira, J.U.V., Pierozzi, P.H.B., Rachid, B.F., de Toledo, J.F.F., Arais, C.A.A., Soares, R.M. & Godoy, G.V. 2007. Genetic control of Asian soybean rust in soybean. Euphytica 157: 15-25.

Rupe, J. & Sconyers, L. 2008. Soybean rust. The Plant Health Instructor. DOI: 10.1094/PHI-I-2008-0401-01.

Sagis. 2015. South African Grain Information Service – Crop Estimates committee www.sagis.org.za/CEC. Accessed: April 2015.

Shanmugasundaram, S. 1999. Epidemiology and control strategies for soybean rust-A knowledge base for Africa. In: Proceedings of workshop on soybean rust (*Phakopsora pachyrhizi*), (ed.) M. A. Smit, ARC Summer Grains Centre, Potchefstroom, South Africa.

Silva, D.C.G., Yamanaka, N., Brogin, R.L., Arias, C.A.A., Nepomuceno, A.L., Di Mauro, Pereira, S., Nogueira, L.M., Passianotto, A.L.L. & Abdelnoor, R.V. 2008. Molecular mapping of two loci that confer resistance to Asian rust in soybean. Theoretical and Applied Genetics 117:57-63.

Tschanz, A.T. 1984. Soybean rust epidemiology, Final Report. Asian Vegetable Research and Development Centre. Shanhau, Taiwan. AVRDC.

Tschanz, A.T. & Tsai, B.Y. 1982. Effects of maturity on soybean rust development. Soybean rust Newsletter 5: 38-41.

Tschanz, A.T., Wang, T.C. & Tsai, B.Y. 1985. Recent advances in soybean rust research. In: Shanmugasundaram, S., Sulzberger, E.W. & Mclean, B.T. (Eds.). Soybean in tropical and sub tropical cropping systems. Shanhua, Taiwan, AVRDC.

Twizeyimana, M., Ojiambo, P.S., Ikotun, T., Ladipo, J.L., Hartman, G.L. & Bandyopadhyay, R. 2008. Evaluation of soybean germplasm for resistance to soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 92: 947-952.

Twizeyimana, M., Ojiambo, P.S., Ikotun, T., Hartman, G.L. & Bandyopadhyay, R. 2007. Comparison of field, greenhouse and detached-leaf evaluations of soybean germplasm for resistance to *Phakopsora pachyrhizi*. Plant Disease 91: 1161-1169.

Van der Plank, J.E. 1963. Plant diseases, epidemics and control. Academic Press, New York, USA.

Wang, T.C. & Hartman, G.L. 1992. Epidemiology of soybean rust and breeding for host resistance. Plant Protection Bulletin 34: 109-124.

Yang, X.B., Dowler, W.M. & Tschanz, A.T. 1991. A simulation model for assessing soybean rust epidemics. Journal of Phytopathology 133: 187-200.

Table 2.1. Rust severity and infection type recorded in 39 lines evaluated in the greenhouse during 2009.

Entry	Rust severity (%)	Infection type*
PI 445844	26.00	Tan
PI 594437	18.00	Tan
PI 606376	18.00	Tan
PI 423833A	14.33	Tan
PI 499957	14.33	Tan
PI 506870	14.33	Tan
PI 5-07523	14.33	RB
PI 567616	14.33	Tan
PI 567741	14.33	Tan
PI 587999B	14.33	Tan
PI 594411	14.33	Tan
PI 594884	14.33	Tan
PI 603909A	14.33	RB
PI 603160	12.67	Tan
PI 567614C	10.70	Tan
PI 603486	10.70	Tan
Selection 3/1/2	10.70	RB
Selection 44	10.70	Tan
PI 507133	10.67	Tan
PI 567619	10.67	Tan
PI 419043	7.33	Tan
Selection 5/132	7.33	RB
PI 587723A	7.00	IM
PI 603158	7.00	Tan
PI 603458S	7.00	Tan
PI 603755E	7.00	Tan
Selection 40	7.00	RB
Selection 43	7.00	RB
Selection 5/60	7.00	RB
Selection 6/105	7.00	Tan
Selection 6/86	7.00	Tan
PI 445845	6.96	Tan
PI 603166	5.33	Tan
Selection 36	5.33	RB
Selection 6/12	5.33	Tan
Selection 6/977	5.33	RB
Selection 13	2.00	RB
Selection 6/43	2.00	Tan
Selection 16	1.53	Tan
Mean	10.10	
LSD (P<0.05)	10.00	

* RB - Red brown lesions; TAN - Tan coloured lesions; IM - Immunity lesions

Table 2.2. Rust severity and infection type recorded in 17 lines evaluated in the greenhouse during 2010.

Entry	Rust severity (%)	Infection type*
PI 507133	29.51	IM
PI 567614 C	19.00	Tan
PI 587999B	19.00	Tan
PI 423833A	18.00	RB
PI 445844	18.00	Tan
PI 603755E	18.00	Tan
Selection 36	17.50	Tan
PI 506870	14.33	Tan
PI 603158	14.33	Tan
PI 603166	14.33	RB
PI 445845	13.70	Tan
PI 567619	13.69	Tan
PI 594411	13.69	RB
PI 603160	8.53	Tan
PI 567616	8.40	Tan
PI 603458S	7.00	Tan
PI 603909A	3.20	Tan
Mean	14.72	
LSD (P<0.05)	10.40	

* RB - Red brown lesions; TAN - Tan coloured lesions; IM - Immunity lesions

Table 2.3. Calculated parameter (apparent rate of infection, soybean rust onset potential, area under the disease progress curve and relative life time at 100% disease) for soybean rust lines planted in field trials at Cedara during 2008/2009.

Entry	Onset (RLT)	Apparent rate of infection (pupd)	RLT at 100% disease	AUDPC
Selection 43	85.21	0.16	100.1	642.70
PI 567619	83.52	0.19	95.50	758.45
PI 587723A	79.58	0.46	84.40	1597.21
PI 567741	77.80	0.19	89.85	1208.51
PI 507133	76.00	0.31	83.11	1734.78
PI 603160	75.52	0.18	94.08	280.97
PI 445844	75.33	0.34	82.78	1768.63
PI 567614C	74.67	0.20	90.93	1080.03
Selection 5/132	74.55	0.21	90.54	1137.54
Selection 13	73.67	0.26	85.39	1510.60
PI 445845	73.50	0.24	86.46	1418.11
PI 603909A	73.46	0.23	82.92	1762.55
PI 603458S	73.33	0.21	84.20	1649.42
PI 603166	73.24	0.23	85.58	1492.91
Selection 5/60	73.24	0.24	85.47	1499.18
Selection 36	73.17	0.26	84.73	1573.79
Selection 6/977	73.08	0.18	84.61	1614.31
PI 594411	72.99	0.23	85.55	1494.15
Selection 6/86	72.67	0.25	85.04	1550.02
Selection 6/105	72.58	0.19	87.45	1331.82
PI 594437	72.52	0.25	81.71	1888.56
PI 603486	72.41	0.11	75.23	2352.36
PI 507523	72.05	0.22	86.16	1459.60
Selection 16	71.50	0.23	83.28	1724.16
PI 587999B	71.46	0.25	83.48	1703.30
Selection 40	71.17	0.36	77.37	1896.02
PI 603158	71.08	0.40	78.49	2190.80
PI 594884	71.05	0.22	85.02	1571.23
Selection 6/12	70.99	0.35	79.72	2056.06
Selection 44	70.58	0.15	89.32	1183.35
Selection 3/1/2	70.50	0.20	86.22	1488.73
PI 603755E	70.46	0.24	84.67	1590.55
PI 567616	70.33	0.23	83.31	1721.58
PI 423833A	69.91	0.23	82.28	1806.35
PI 499957	69.67	0.23	83.13	1745.31
PI 594437	69.41	0.21	83.17	1732.36
PI 606376	69.41	0.21	83.17	1732.33
Selection 6/43	69.41	0.21	83.17	1732.33
PI 506870	69.00	0.28	79.93	2049.37
PI 419043	68.33	0.18	85.58	1635.34
Mean	72.96	0.24	85.08	1559.13
LSD (P<0.05)	3.89	0.08	5.17	322.60

Table 2.4. Calculated parameters (apparent rate of infection, soybean rust onset potential, area under the disease progress curve and relative life time at 100% disease) for soybean rust lines planted in field trials at Cedara during 2009/2010.

Line	Onset RLT	Apparent rate of infection (pupd)	RLT at 100% disease	AUDPC
PI 445845	79.83	0.31	86.40	1398.37
PI 603160	79.67	0.34	88.26	1209.20
PI 567614C	79.50	0.35	88.12	1223.10
Selection 36	79.17	0.34	87.93	1241.95
PI 419043	79.00	0.34	87.59	1275.90
PI 567619	79.00	0.34	87.58	1277.34
PI 603909A	79.00	0.34	87.90	1244.93
PI 603755E	78.83	0.34	87.53	1282.43
PI 507523	78.67	0.34	87.37	1298.00
PI 445844	78.33	0.34	87.14	1320.12
PI 506870	78.33	0.34	87.16	1318.98
PI 603458S	78.17	0.34	87.00	1334.78
PI 567616	78.00	0.33	86.83	1351.48
PI 423833A	78.00	0.32	87.37	1300.67
PI 58799B	77.83	0.34	86.74	1360.46
PI 594411	76.33	0.31	85.91	1445.63
PI 606376	75.83	0.32	85.26	1509.68
PI 603166	75.50	0.35	84.12	1619.58
PI 507133	71.50	0.28	82.43	1797.75
PI 603158	71.33	0.28	82.22	1818.86
PI 499957	66.57	0.22	74.16	2604.64
PI 603486	64.33	0.24	77.57	2287.35
Mean	76.49	0.32	85.57	1478.24
LSD (P<0.05)	6.21	0.06	5.50	340.30

Table 2.5. Mean apparent rate of infection, soybean rust onset potential, area under the disease progress curve and relative life time at 100% disease obtained from pooled data for soybean rust lines planted in field trials at Cedara during 2008/2009 and 2009/2010 planting season.

Line	Onset RLT	Apparent rate of infection (pupd)	RLT at 100% disease	AUDPC
PI 594411	79.93	0.25	90.71	1102.04
PI 419043	77.26	0.26	90.83	778.43
PI 567614C	77.08	0.27	89.53	1151.56
PI 603160	76.42	0.30	86.50	1391.50
PI 567619	76.17	0.28	85.89	1463.38
PI 603458S	75.83	0.29	86.73	1376.45
PI 567616	75.73	0.28	84.88	1557.01
PI 445844	75.66	0.29	86.35	1407.14
PI 445845	75.65	0.28	84.94	1550.83
PI 423833A	75.62	0.28	86.48	1396.79
PI 603755E	75.62	0.22	81.38	1817.39
PI 603166	75.42	0.34	83.45	1694.11
Selection 36	75.12	0.37	83.21	1716.37
PI 58799B	74.94	0.28	86.45	1410.03
PI 603909A	74.67	0.29	85.61	1483.26
PI 507523	74.29	0.29	84.82	1552.17
PI 506870	74.00	0.28	85.14	1532.14
PI 603158	73.67	0.30	82.67	1776.82
PI 606376	72.62	0.27	84.22	1621.01
PI 507133	69.92	0.23	84.01	1716.55
PI 499957	68.08	0.25	77.05	2327.00
PI 603486	67.40	0.24	81.12	1938.95
Means	74.60	0.28	85.09	1534.59
LSD (P<0.05)	4.41	0.06	4.43	414.14

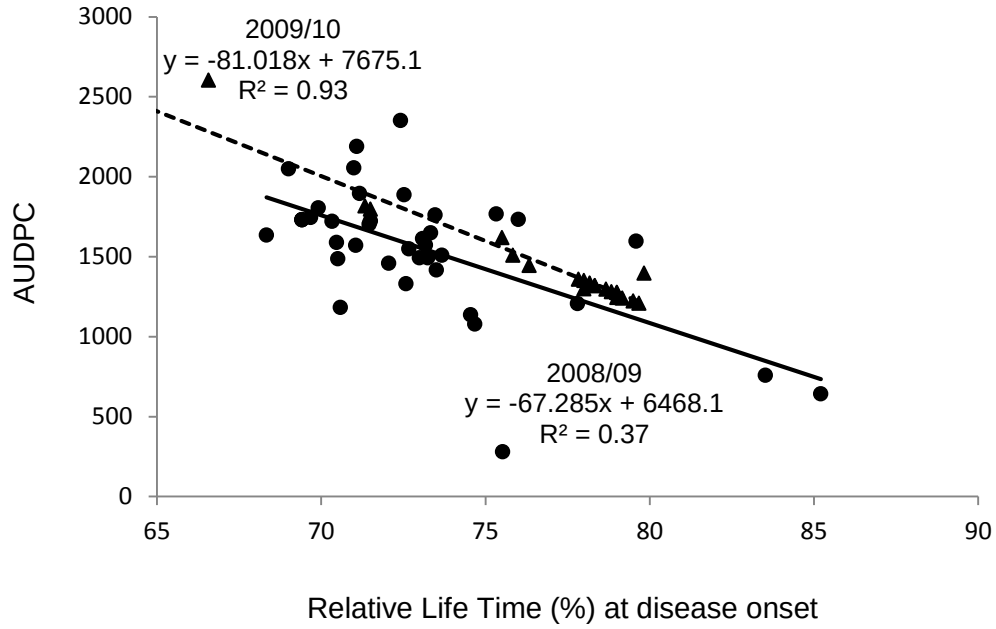

Figure 2.1. Relationship between relative life time at disease onset and area under disease progress curve (AUDPC) in soybean lines for 2008/09 and 2009/10 seasons.

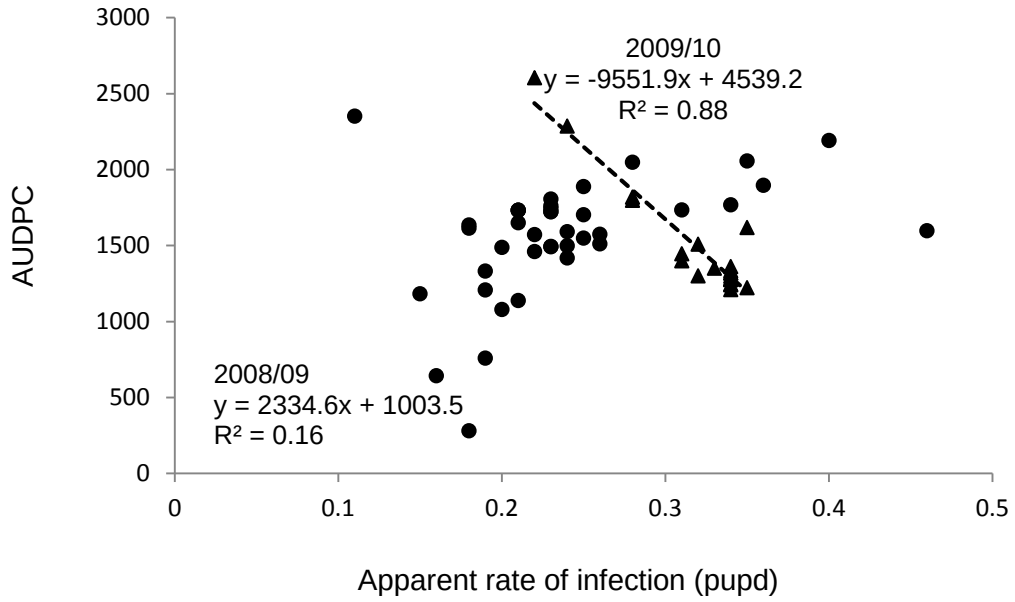

Figure 2.2. Relationship between apparent rate of infection ie. disease increase per time increment (per unit per day (pupd)) and area under disease progress curve (AUDPC) in soybean lines for 2008/09 and 2009/10 seasons.

CHAPTER 3

VARIATION IN YIELD ASSOCIATED WITH SOYBEAN RUST EPIDEMICS IN SOUTH AFRICA

3.1 Abstract

Soybean rust caused by *Phakopsora pachyrhizi* is a serious global foliar disease of soybeans that causes severe yield losses in major production areas of the world. Yield losses are mainly due to a decrease in the number of pods and a reduction in seed weight. The aim of this study was to quantify losses associated with soybean rust in South Africa in varieties from different maturity groups and determine the effects of planting date, row spacing and fungicide sprays on disease severity and concomitant yield. Three cultivars ie. PAN 494 (short maturity), LS 666 (medium maturity) and SCS 1 (long maturity) were planted over six seasons at Cedara and two seasons at Piet Retief. Two inter-row spacings were used (90 cm and 45 cm) and plots were split to accommodate fungicide sprayed and unsprayed plots. Plots were visually assessed for infection on a weekly basis and affected leaf area was determined. Disease onset, apparent rate of infection, area under disease progress curve (AUDPC), spray efficiency, variation in yield as well as protein and oil content were determined. Results indicated that row spacing was not a significant variable in disease development. No significant effect on disease onset was recorded and row spacing had a limited effect on AUDPC, oil and protein content. However, a significant difference due to row spacing and apparent rate of infection was recorded in some treatments, including LS 666 which had an apparent rate of infection of 0.09 pupd in sprayed plots with 45cm row spacing while in 90 cm unsprayed plots, an infection rate of 0.46 pupd was recorded. Fungicide treatments had a significant effect on disease and host parameters. Disease onset, AUDPC as well as yield differed significantly between sprayed and unsprayed plots and disease onset in sprayed plots of PAN 494 and SCS 1 was 5 to 10 days later than unsprayed plots. Significant differences in protein content were recorded between sprayed and unsprayed plots but not in oil content. Although differences in cultivar maturity groups had an effect on yield and quality losses with reduced loss in the short season cultivar in particular, this was not sufficient to eliminate the need for fungicides.

3.2 Introduction

Soybean rust caused by *Phakopsora pachyrhizi* Syd. & P. Syd. is a serious yield limiting disease of soybean (*Glycine max* (L.) Merr.) due to the ability of the pathogen to spread and infect the host rapidly. The disease was initially recorded in Japan in 1902 and spread throughout most of Asia (Bromfield, 1984). In Africa it was first detected in Uganda in 1996 and spread to Zimbabwe in 1998 (Akinsanmi *et al*., 2001). It was reported in South Africa three years later in 2001 in the Vryheid area of KwaZulu-Natal (Pretorius *et al.*, 2001) and subsequently spread throughout the soybean production areas east of the escarpment.

Yield losses of up to 80% due to soybean rust have been reported in major soybean production areas around the world in areas where this disease commonly occurs (Miles *et al*., 2003). Sinclair & Hartman (1995) reported losses of up to 90 % in India. In South Africa, losses of 40 to 60% have been reported, however in severe cases yield losses of up to 100% were recorded (Caldwell & McLaren, 2004). The severity of disease and extent of losses is closely associated with time of disease onset, severity and the yield components affected (Ogle *et al.*, 1979) which can vary according to the growth stage of host plant as well as the host variety (McLaren, 2008). Initial symptoms include premature yellowing and defoliation, the latter which is often associated with an increase in lesion density (Sinclair, 1982). Yield losses are mainly due to a decrease in the number of normal pods per plant, reduction in seed weight per plant as well as 100-seed weight (Bromfield, 1984; Sinclair & Hartman, 1999).

Various studies have indicated that soybean rust, as well as the reduction in yield loss, can be effectively managed with the application of fungicides and this is therefore, still the primary method of control. Several constraints associated with the application of fungicides still remain despite the efficacy and cost effectiveness of this control measure. These include correct timing and application methods, proper coverage and penetration of the host canopy. Proper crop growth stage and detection systems reduce the number of fungicide applications and the number of unnecessary sprays (Mueller *et al*., 2009).

Du Preez (2005) concluded that a mixture of products is more effective especially if fungicides from two chemical classes with different modes of action are applied. These provide proper protection as well as reducing the risk of resistance to a specific fungicide group. Galloway (2008) found that flusilazole and carbendazim can effectively manage soybean rust in the field. Piamonte & Quebral (1980) reported a significant increase in yield from sprayed plots that received one or two fungicide treatments consisting of a

mixture of Dithane M-45® (mancozeb) and Plantvax 75W® (oxycarboxin) compared to unsprayed plots. Da Silva *et al*. (2011) applied a mixture of azoxystrobin and cyproconazole and confirmed that losses up to 77% were recorded in untreated plots compared to those subjected to a chemical control program. Miles *et al*. (2007) confirmed that untreated control plots had 30% lower yield compared with plots treated with flusilazole, carbendazim, oxycarboxin or triflumizole.

Correct application is crucial for effective management of rust with fungicides. Soybean rust starts in the lower canopy and moves upwards to the middle and upper canopy as the plant matures and the epidemic progresses (Piamonte & Quebral, 1980). Temperature fluctuations in the bottom canopy are minimal, leading to a favourable environment for spore germination and infection. This especially, is achieved in soybean fields planted at high plant densities or narrow row spacing or in some cases both (Balardin *et al*., 2010). Failure of chemicals to reach the lower leaves result in the continuous production of inoculum and the spread of rust into unprotected fields (da Silva *et al*., 2011). As a result it is important for fungicides to penetrate into the canopy as deeply as possible and ensure uniform deposition. To ensure this, proper air pressure, nozzle selection and droplet size is crucial (Kemmit *et al*., 2008). An increase in air pressure as well as increased water volume per hectare is necessary to ensure proper penetration into the canopy (Miles *et al*., 2003).

Considerable research has been done on the number of sprays required to keep soybean rust to below the critical yield loss level. Patil & Basavaraja (2001) concluded that moderately resistant cultivars required only one spray at onset to give acceptable yields whereas susceptible cultivars needed two sprays at 15 days intervals starting at disease onset. Miles & Hartman (2006) found significant differences between two and three applications in trials where plots were severely infected and defoliation was high. According to Du Preez (2005) disease severity decreased with an increase in the number of applications. Timing of application is also crucial and Mueller *et al*. (2009) found that fungicides applied prior to first detection of rust gave higher yields compared to those applied 14 to 20 days after first detection. However, the response to number of applications depends on the type of chemical applied as well as the cultivar planted. Despite studies on the control of rust with the use of fungicides, factors such as cost, the number of applications, correct application methods as well as the persistence of chemicals on the host plant still requires clarity.

The aim of the current study was to quantify losses associated with soybean rust in varieties from different maturity groups and determine the effects of disease severity associated with planting date and row spacing. The effect of chemical sprays on yield losses associated with soybean rust was quantified.

3.3 Materials and Methods

3.3.1 Field trials

Field experiments were conducted over six seasons at Cedara, Kwazulu Natal (GPS coordinates: -29.533°S, 30.283°E) and two seasons at Piet Retief, Mpumalanga (GPS coordinates: 27.0000°S, 30.8000°C). Localities were selected based on a history of high disease pressure and infection was dependent on natural inoculum.

Experiments at each locality consisted of three sequential plantings (split replications) spaced from early-November to late-December of each season in order to create a range of environment x plant physiological stage interactions. Experimental plots consisted of 10 rows, 8 m in length with a plant population equivalent to 360 000 plants ha^{-1}. Two inter-row spacings were used ie. 90 cm and 45 cm, with three commercial varieties ie. PAN 494 (short maturity) LS 666 (medium maturity) and SCS 1 (long maturity). Each plot was repeated to accommodate both fungicide sprayed and unsprayed plots. Plots were arranged in a randomised split-plot design with fungicide treatment as a whole plot, cultivars as a sub-plot and row-width as a sub-sub plot.

Prior to planting plots were fertilized with 300 kg superphosphate and 50 kg KCl ha^{-1}. Rhizobium (*Bradyrhizobium japonicum*) was added in a water suspension (100g/15 ℓ water) to each row subsequent to seeding but prior to closing of the planting furrow. Subsequent to planting, plots were sprayed with 1920 ℓ (a.i) Alachlor for grass control. Additional weeding was done by hand as required. Between growth stages R1 and R2 when plants were at 50 % flowering, plots designated for fungicide application were sprayed with carbendazim/flusilazole (250/125 g $ℓ^{-1}$) at a rate of 800 ml ha^{-1} using a backpack sprayer with a flat fan nozzle, size 0.4mm. This was done subsequently at 21 day intervals until maturity.

3.3.2 Crop and disease assessment

Plots were monitored weekly for rust onset and the days after planting to first detection were recorded in each plot. Subsequent rust development was visually assessed as the estimated percentage diseased leaf area. The percentage defoliation was also estimated. Since both rust and defoliation are responsible for decreased photosynthetic area of plants, an integrated rating of disease severity and defoliation was used as a measure of the total affected leaf area (ALA) where ALA=defoliation (%)+rust (%) on remaining leaves. Thus, ALA=D+((100-D)*(rust/100)) (McLaren, 2008). The assumption was made that leaves discoloured by rust are no longer photosynthetic. Days to maturity and premature defoliation were recorded. In order to compare varieties from the different maturity groups, days after planting at which assessments were made were converted to a relative life time (RLT) scale (Tschanz, 1984) calculated as RLT=days after planting/days to maturity. Days to maturity were determined from sprayed plots.

Disease progress curves were fitted for each variety after calculated percentage ALA was converted to a proportion, linearised according to the linearised format of the logistic equation Y=Ln(X/(1-X)) (Van der Plank, 1963) and used as the dependent variable; RLT was used as the independent variable. Data were analysed using simple regression analysis using NCSS (Hintze, 2007). The apparent infection rate (van der Plank, 1963) was recorded from the regression coefficients. Area under the disease progress curve (AUDPC) was calculated according to Campbell and Madden (1990) $AUDPC=\Sigma_i=1\ n-1\ [(t_i+1-t_i\ )(y_i+y_i+1)/2]$ using RLT as the independent variable and ALA as the dependent variable. The difference in AUDPC values between sprayed and unsprayed plots was recorded. Spray efficiency was calculated using the following formula: Spray efficiency=((AUDPC difference/AUDPC unsprayed plots)*100).

At physiological maturity yield was determined on the central six rows of each plot. Grain from two years from Piet Retief trials and four years from Cedara trials were collected from the determination of grain protein and oil content. This was done by the ARC-Grain Crops Institute grain quality laboratory in Potchefstroom.

3.3.3 Data analysis

Data were analysed using Number Cruncher Statistical Systems (NCSS) (Hintze, 2007). Variables used during assessment are as listed in Table 3.1 and Table 3.2. Mean values

of measured variables from sprayed and unsprayed plots between 45 cm and 90 cm row spacing over all seasons were compared using Students t-test assuming equal variances. Correlation and linear regression analysis was used to determine relationships between onset, apparent rate of infection ie. disease increase per time increment expressed as per unit per day (pupd), yield variation, AUDPC, spray efficiency, total protein concentration and oil concentration.

3.4 Results

Pooled mean values were derived from combining the data from all of the trials. Disease and host parameters as well as the comparison of the sprayed and unsprayed plots at each row width using Fishers t-test are presented in Table 3.1 and Table 3.2. The current study indicated that row width had a limited effect on disease and host parameters. Rust onset in 45 cm and 90 cm row widths only differed significantly in the short season and smaller canopied PAN 494 unsprayed plots with onset being an average of two days earlier in the wider row spacing. Although not significant this tendency was also evident in the other two varieties. It is evident from the data that row width is not a primary driving variable affecting disease onset in these cultivars.

Subsequent to onset, the mean apparent infection rate differed significantly between the two row widths in sprayed blocks of PAN 494, unsprayed blocks of SCS 1 and in both sprayed and unsprayed plots of LS 666. Apparent infection rate was the lowest for LS 666 sprayed plots planted in 45 cm row widths (0.09 pupd) and the highest in 90 cm unsprayed plots (0.46 pupd).

Row width had a limited effect on AUDPC. Only LS 666 and SCS 1 showed significant differences in AUDPC values between 45 cm and 90 cm row widths in unsprayed plots and no significant differences were observed in PAN 494. No significant differences in maturity were detected between 45 cm and 90 cm row widths in all three cultivars. Only the long maturity group cultivar, SCS 1, showed a significant difference in yield between 45 cm and 90 cm row widths.

Protein content was only affected by row width in PAN 494 and no row width effect was observed in oil content. Highest protein content was recorded in SCS 1 planted in 90 cm unsprayed row widths (41.03%) and the lowest were in PAN 494 in unsprayed 45 cm row width plot (36.63%).

In contrast to row width, fungicide treatments had a significant effect on disease and host parameters. Disease onset was significantly earlier in unsprayed compared with sprayed plots in PAN 494 and SCS 1 but not in LS 666. In the 45 cm row width, onset in unsprayed plots was the earliest in SCS 1 (RLT=72.77%) and latest in LS 666 (RLT=75.47%). In sprayed plots onset was earliest in LS 666 with means of RLT=76.72% and RLT=74.17% in 45 cm and 90 cm plots respectively and the latest in SCS 1 with means of RLT=80.46 % and RLT=81.86%.

Results indicated that the application of fungicides plays a crucial role in slowing down the apparent rate of infection by as much as 80%. Fungicides had a significant effect on the apparent infection rate in all row widths and all three cultivars. Mean apparent infection rate was highest in the short growing cultivar PAN 494 in both row widths (rate=0.34 pupd in 45 cm and rate=0.29 in 90 cm rows) and the lowest in SCS 1 (rate=0.20 pupd in both 45 cm and 90 cm rows).

AUDPC differed significantly between sprayed and unsprayed plots in both 45 cm and 90 cm row widths in all three cultivars. In sprayed plots, AUDPC means ranged from 512.55 to 705.55 in 45 cm row spacing and 582.79 to 621.88 in 90 cm row spacing. AUDPC values of unsprayed plots ranged from a mean of 1433.13 in LS 666 to 1758.61 and 1805.87 in PAN 494 and SCS 1 respectively in 45 cm row width. In 90 cm row widths, LS 666 had the highest AUDPC value (1683.91) and SCS 1 the lowest (1456.31).

The difference in mean AUDPC values, calculated as the difference in value between sprayed and unsprayed plots served as an indication of spray efficacy in reducing total disease intensity. AUDPC difference was greatest in SCS 1 in the 45 cm rows and the lowest in LS 666 (816.34). In 90 cm rows however, SCS 1 had the lowest AUDPC difference and LS 666 the highest with 892.31 and 1062.04 respectively (Table 3.3).

Spray efficiency in 45 cm row width was the highest in SCS 1 (69.87%) followed by PAN 494 (60.38%) and LS 666 (57.09%). In 90 cm rows SCS 1 had the lowest spray efficiency (59.60%) compared to PAN 494 and LS 666 with mean values of 63.45% and 63.51% respectively.

In unsprayed plots, maturity of cultivars was reached earlier compared with sprayed plots. Differences in maturity between sprayed and unsprayed plots was the greatest in 45 cm row spacing ranging from 5.58 to 6.13 days. Fungicide applications had a significant effect

on grain protein quality in all row widths except PAN 494 at 90 cm row width and oil content at both row widths in LS 666 and SCS 1.

Yield of cultivars differed significantly between sprayed and unsprayed plots within each row width. In fungicide treated plots, highest yield were observed in SCS 1 with 45 cm row width (3.02 t/ha). In untreated plots the lowest yield were recorded in SCS 1 with 90 cm row spacing (0.97 t/ha). Variation in yield in cultivars had mean values of 33.62%, 27.37% and 62.48% in PAN 494, LS 666 and SCS 1 respectively, with 45 cm row width. In 90 cm row widths LS 666 had the lowest variation in yield (31.95%) followed by PAN 494 (34.79%) and SCS 1 (56.11%). The overall yield difference between sprayed and unsprayed plots was 1.13 t per ha.

Regression analysis showed a positive relationship between disease onset and AUDPC in all treatments associated with unsprayed plots (Figure 3.1) with early onset resulting in higher AUDPC than later onset. In contrast, no relationship was recorded between disease onset and AUDPC in fungicide treated plots (regression not shown).

A poor relationship between apparent rate of infection and AUDPC (Figure 3.2) in unsprayed plots of PAN 494 was recorded in both row widths while no relationship was recorded in SCS 1. LS 666 however, showed significant relationships between these two variables in 45 cm and 90 cm row widths (R^2=0.64 and R^2=0.61 respectively). A poor relationship between apparent rate of infection and AUDPC was observed in sprayed plots in both row widths (data not shown) in all three cultivars. Significant relationships between a gain in yield and difference in AUDPC were observed in all three cultivars (Figure 3.3). The results indicate that yield gain is closely related to the amount to which disease suppressed and the AUDPC is reduced by fungicide sprays. The greater the difference in AUDPC values between sprayed and unsprayed plots, the greater the yield gain.

3.5 Discussion

In order to devise economical management strategies such as the use of fungicides for the control of soybean rust, it is important to quantify the extent of damage attributable to this disease (Kawuki *et al*., 2003) as well as to determine the relationship between the variance components of the epidemic and yield. Soybean diseases and in particular soybean rust, reduce yields by limiting plant growth and seed quality and quantity. This is due to premature defoliation, low percentage pod formation and filling and a reduction in grain

weight in severely infected plants (Yang *et al*., 1991). According to Oloka *et al*. (2009) yield decreases as rust severity increases and the application of fungicides improved yield by an average of 9.4% over three seasons.

The efficacy of fungicide applications as a disease management strategy depends on the timing of applications and the extent to which the chemical reaches the critical infection points within the host canopy. In this study, losses ranged from 30-60% depending on cultivar, row spacing and the response of epidemiological driving variables targeted. Fungicides were particularly effective in the late maturity group SCS1, where losses were highest in unsprayed plots and almost double those of the earlier maturity groups. Indications from other studies are that longer growing cultivars have a better response to fungicide applications and yield losses also tend to be greater in these cultivars when not treated (da Silva *et al*., 2011). Similar results were reported by Oliviera *et al*. (2005) and Rodrigues *et al*. (2013) who found that early cultivars performed better when no control was used compared with medium or late cultivars, while in the presence of rust and the application of fungicides, medium to late maturing cultivars performed considerably better. This was also confirmed by Moreira *et al*. (2015) who found that mean soybean rust severity was lower in the early, compared with late maturing cultivars mainly due to the extended growth period of the latter and therefore extended exposure to the pathogen.

According to Bromfield (1984) variation in yield in early maturing lines is mostly attributed to genotype while season contributed to variation in yield in medium and late maturing lines. Kawuki *et al*. (2004) reported that 41.2% of variation in early maturing lines was attributed to genotype while in medium and late maturing lines 49.4% and 48.6% of variation was due to seasonal influences. These observations were attributed to a more rapid loss of green leaf area relative to physiological growth stage and thus, a reduction in photosynthetic area during critical grain accumulation phases of development ie. from pod formation to pod filling stage. Since late maturing lines spend more time in this phase, they are more subjected to higher yield losses (Oliveira *et al*., 2005).

Row spacing plays a crucial role in the penetration of the product into the canopy. The spray efficiency in the 45 cm plots was lower compared to the 90 cm plots which may be attributed to improved penetration of the chemical in the wider rows. Da Silva *et al*. (2011) confirmed that efficacy of fungicide sprays in the lower canopy is less due to insufficient penetration of the product suggesting that plots planted to narrower rows are expected to show more disease. However, despite the poor penetration in the current study, the ratio of AUDPC values in unsprayed plots compared to sprayed plots indicate that fungicides

have a more significant effect on disease than row width and that the contribution of this variable is minimal.

The application of fungicides has been reported to delay the onset of rust in treated plots although physiological age of the plant is a major factor that determines disease onset and development (Melching *et al.,* 1988). Studies showed that it can take up to 20 days longer for soybean rust severity to reach 30% threshold in longer growing cultivars than a short maturity cultivar and therefore, fungicides need to be applied earlier to the short maturing cultivars than the former mentioned (Moreira *et al*., 2015). According to Ogle *et al*. (1979) yield loss is dependent on the growth stage at which infection occurs. Variations in onset have been attributed to weather conditions where warm, low humidity conditions delayed onset and cooler, high humidity conditions promoted onset (Tschanz & Tsai, 1982; Yang *et al.*, 1991; Wang & Hartman, 1992).

Mueller *et al.* (2009) reported that delaying the initiation of symptoms with prophylactic fungicide applications as opposed to applications made once symptoms have been observed, resulted in significant decreases in disease severity and yield loss differences. This was also observed in the current study where targeting disease onset such that the disease was delayed by 5 to 10 days in sprayed plots of PAN 494 and SCS 1 resulted in a significant effect on the final disease intensities as indicated in the respective AUDPC's.

Fungicides also significantly reduced the apparent rate of infection in the current study although this effect appeared more pronounced in the shorter maturity groups. This reduction, together with the delayed onset, also contributed significantly to reduced AUDPC and delayed total defoliation. Notably, the former variable was also significantly related to variation in yield. Board & Harville (1998) reported that defoliation and thus, loss of leaf area that increased to 66 % during the first three weeks of seed fill, resulted in a yield loss of 40-50% indicating that the retention of photosynthetic area during grain fill is essential. This too, is highlighted in the current study where yield losses ranged from an average of 30 to 60 % depending on cultivar and maturity group. These differences were also reflected in protein and oil content although these were relatively small, corresponding with the findings of Ogle *et al.* (1979).

Spray efficacy remains the greatest obstacle to rust management and this was clearly reflected in Figure 3.3 where the percentage yield gain was high when canopy penetration and thus AUDPC was significantly reduced, as opposed to where spray efficacy was poor. Balardin *et al*. (2010) found that a wider plant spacing will maximize the residual effect of

the chemical product applied. Fungicide applied to fields with a plant spacing of 60 cm space row opposed to 45 cm and 30 cm, showed a higher residual and resulted in an extended period of green foliage which in turn resulted in higher yield. Hanna *et al.* (2008) found that fungicide coverage decreased with depth in the canopy with resultant increases in disease development. A paradox too, is that canopy closure is one of the prerequisites for maximum seed yield (Shibles & Weber, 1966; Tanner & Hume, 1978) and this will vary with season, cultivar, fertilization, row spacing and planting density. Maximum yield is achieved by equidistant plant spacing however, this also leads to an increase in canopy leaf area and thus, potentially reduced coverage by fungicide sprays.

The efficacy associated with a single application methodology could vary depending on agronomic traits of the crop, weather prevailing, especially since rain has a significant effect on the residual activity, efficacy of the chemical product applied to foliage (Balardin *et al*., 2010) and timing of chemical application. It was observed that applications made prior to first detection resulted in greater yields and better control than applications made after the first detection (Mueller *et al*., 2009), as well as fungicide application methods to optimize efficacy, such as nozzle type and pattern, reduction in droplet size and spraying speed. Studies have found that fan nozzles are more effective than cone nozzles. Two angled nozzles are more effective than a single nozzle spraying vertically. Efficacy was also increased when spraying was done as low speeds (Ozkan *et al.,* 2015). Air- assist spraying is more effective in achieving penetration of the chemical product into the lower canopy than a conventional flat fan sprayer, drift fan sprayer or core-hollow cone sprayer (Ozkan *et al*., 2015) and a smaller droplet size showed higher plant absorption and higher amount of chemical product per cm^2. The flow rate of the fungicide and the pressure of the liquid is also important to ensure proper penetration into the canopy (Balardin *et al*., 2010). Thus, although not addressed in the current study, potential methods of improved canopy penetration could improve spray efficacy, reduce AUDPC even further and reduce losses significantly.

In conclusion, soybean rust remains a serious yield limiting foliar disease. Since resistant cultivars still need to be developed, the main control method still remains the application of fungicides. Control is possible if the correct fungicide is applied with the most effective equipment to ensure canopy penetration. However, timing is crucial and continued scouting during the growing season is crucial to ensure that applications are applied as soon as infection is observed. Together with the adaptation of agronomical practices such as wider row spacing and lower plant densities to ensure proper penetration of the product, better control of the soybean rust can be achieved and desired yields can be obtained.

3.6 References

Akinasanmi, O.A., Ladipo, J.L. & Oyekan, P.O. 2001. First report of soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Disease 85: 97.

Balardin, R., Madalosso, M., Debortoli, M. & Lenz, G. 2010. Factors affecting fungicide efficacy in the tropics, Fungicides, Odile Carisse (Ed.), ISBN: 987-953-307-266-1, InTech. www.intechopen.com/books/fungicides/factors-affecting-fungicide-efficacy-in-the-tropics.

Board, J.E. & Harville, B.G. 1998. Late-planted soybean yield response to reproductive source sink stress. Crop Science 38: 763-771.

Bromfield, K.R. 1984. Soybean Rust. Monograph no 11. American Phytopathological Society Press. St. Paul, Minnesota, USA.

Caldwell, P.M., & Mclaren, N.W. 2004. Soybean rust research in South Africa. Pages 354-360 in: Proc. VII World Soybean Res. Conf. IV Int. Soybean Processing and Utilization Conference, III Brazilian Soybean Congress, Foz do Iguassu, PR, Brazil.

Cambell, C.L. & Madden, L.V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, New York, USA.

Da Silva, J.V.C., Juliatti, F.C., da Silva, J.R.V. & Barros, F.C. 2011. Soybean cultivar performance in the presence of soybean Asian rust, in relation to chemical control programs. European Journal of Plant Pathology 131:409-418.

Du Preez, E.D. 2005. Chemical control of soybean rust (*Phakopsora pachyrhizi*) on soybeans. M.Sc. dissertation. University of Kwazulu Natal. March 2005.

Galloway J. 2008. Effective management of soyabean rust and frogeye leaf spot using a mixture of flusilazole and carbendazim. Crop Protection 27:566–571.

Hanna, S.O., Conley, S.P., Shaner, G.E. & Santini, J.B. 2008. Fungicide application timing and row spacing effect on soybean canopy penetration and grain yield. Agronomy Journal 100: 1488-1492.

Hintze, J.L., 2007. Number cruncher statistical systems (NCSS). Kaysville, UT: Number Cruncher Statistical Systems.

Kawuki, R.S., Adipala, E. & Tukamuhabwa, P. 2003. Yield loss associated with soya bean rust (*Phakopsora pachyrhizi* Syd.) in Uganda. Journal of Phytopathology 151: 7-12.

Kawuki, R.S., Tukamuhabwa, P. & Adipala, E. 2004. Soybean rust severity, rate of rust development and tolerance as influenced by maturity period and season. Crop Protection 23: 447-455.

Kemmit, G.M., DeBoer, G., Quimette, D. & Lamauti, M. 2008. Systemic properties of myclobutanil in soybean plants, affecting control of Asian soybean rust (*Phakopsora pachyrhizi*). Pest Management Science 64: 1285-1293.

Levy, C. 2005. Epidemiology and chemical control of soybean rust in Southern Africa. Plant Disease 89: 699-674.

McLaren, N.W. 2008. Reaction of South African soybean cultivars to rust caused by *Phakopsora pachyrhizi*. South African Journal of Plant and Soil 25: 49-54.

Melching, J.S., Dowler, W.M., Koogle & Roger, M.H. 1988. Effect of plant and leaf age on susceptibility of soybean to soybean rust. Canadian Journal of Plant Pathology. 10:30-35.

Miles, M.R. & Hartman, G.L. 2006. Control of soybean rust in a determinate cultivar at the Rattray Arnold Research Station, Zimbabwe, 2004-05. Fungicide & Nematicide Tests 61: F003.

Miles, M.R, Hartman, G.L., Levy, C. & Morel, W. 2003. Current status of soybean rust control by fungicides. Pesticide Outlook 14: 197-200.

Miles, M.R., Levy, C., Morel, W., Mueller, T., Steinlage, T., van Rij, N., Frederick, R.D. & Hartman, G.L. 2007. International fungicide efficacy trials for the management of soybean rust. Plant Disease 91: 1450-1458.

Moreira, E.N., Vale, F.X.R., Paul, P/.A., Rodrigues, F.A., & Jesus Júnior, W.C. 2015. Temporal dynamics of soybean rust associated with leaf area index in soybean cultivars of different maturity groups. Plant Disease 99: 1216-1226.

Mueller, T.A., Miles, M.R., Morel, W., Marois, J.J., Wright, D.L., Kemerait, R.C. Levy, C. & Hartman, G.L. 2009. Effect of fungicide and timing of application on soybean rust severity and yield. Plant Disease 93: 243-248.

Ogle, H.J., Byth, D.E. & McLean, R. 1979. Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in south-eastern Queensland. Australian Journal of Agricultural Research 30: 883-893.

Oliveira, A.C.B., Godoy, C.V. & Martins, M.C. 2005. Avaliacao da tolerancia de cultivares de soja a ferrugem asiatica no oeste da Bahia (Tolerance assessment of soybean cultivars to Asian rust caused by *Phakopsora pachyrhizi* in western Bahia). Fitopatologia Brasileira 30: 658-662.

Oloka, H.K., Tukamuhabwa, P., Sengooba, T., Adipala, E. & Kabayi, P. 2009. Potential for soybean rust tolerance among elite soybean lines in Uganda. Crop Protection 28: 1076-1080.

Ozkan, H.E. 2015. Spraying recommendations for soybean rust. The Ohio State University Extention Fact Sheet. www.ohioline.osu.edi/aex-fact/pdf/0526.pdf. Accessed: May 2015.

Patil, P.V. & Basavaraja. G.T. 2001. Management of soybean rust by fungicides and host plant resistance. In: T. Lumpkin, ed. 2001. Second international Vegetable Soybean conference. Pullman, Washington: Washington State University.

Piamonte, A.L. & Quebral, F.C. 1980. The influence of rust control on the yield of soybean. Phillippine Phytopathology 16: 38-41.

Pretorius, Z.A., Kloppers, F.J. & Frederick, R.D. 2001. First report of soybean rust in South Africa. Plant Disease 85: 1288.

Rodrigues, R.A., Juliatti, F.C., Pedrini. J.E., Fernandes, J.M.C., Justino, F.B., Heinemann, A.B., Fraisse, C.W., Farias, J.R.B., Pavan, W., Costa, L.C., Vale, F.X. R. & Rezende,

A.A. 2013. Asian soybean rust: Modeling the impact on soybean grain yield in the Triangula Mineiro/Alto Paranaiba region, Minas Gerais, Brazil. Bioscience Journal Uberlândia 29: 264-279.

Shibles, R.M. & Weber, C.R. 1966. Interpretation of solar radiation and dry matter production by various soybean planting patterns. Crop Science 6: 55-59.

Sinclair, J.B. 1982. Compendium of Soybean Diseases, Second Edition. APS Press, St. Paul, Minnesota. 104pp.

Sinclair, J.B. & Hartman, G.L., eds. 1995. Effect of the pathogen on soybeans. In: Proceedings of the Soybean Rust Workshop, 9-11 Augustus 1995. National Soybean Research Laboratory Publication Number 1, Urbana, Illinois.

Sinclair J.B. & Hartman G.L. 1999. Soybean rust. In: Compendium of Soybean Diseases, 4theds Hartman, G.L., Sinclair, J.B. & Rupe, J.C. American Phytopathological Society, St Paul, Minnesota.

Tanner, J.W. & Hume, D.J. 1978. Management and production of soybeans. In A.G. Norman (ed.) Soybean physiology, agronomy and utilization. Academic Press, New York.

Tschanz, A.T. 1984. Soybean rust epidemiology: Final Report. Asian Vegetable Research and Development Centre. Shanhau, Taiwan.

Tschanz, A.T. & Tsai, B.Y. 1982. Effects of maturity on soybean rust development. Soybean Rust Newsletter 5:38-41.

Van der Plank, J.E. 1963. Plant diseases, epidemics and control. Academic Press, New York, USA.

Wang, T.C. & Hartman, G.L. 1992. Epidemiology of soybean rust and breeding for host resistance. Plant Protection Bulletin 34: 39-54.

Yang, X.B., Dowler, W.M. & Tschanz, A.T. 1991. A simulation model for assessing soybean rust epidemics. Journal of Phytopathology 133:187-200.

Table 3.1. The effect of cultivars, row width and fungicide applications on soybean rust parameters (onset, apparent rate of infection and area under disease progress curve AUDPC).

		Cultivar											
		PAN 494				LS 666				SCS 1			
Row width (cm)		**45 cm**	**90 cm**	**Mean (rows)**	**t-value (rows)**	**45 cm**	**90 cm**	**Mean (rows)**	**t-value (rows)**	**45 cm**	**90 cm**	**Mean (rows)**	**t-value (rows)**
Onset (RLT)	**Sprayed**	77.41	77.27	77.34	**0.40**	76.72	74.17	75.45	**1.22**	80.46	81.86	81.16	**0.52**
	Unsprayed	73.78	71.63	72.71	**1.99***	75.47	72.81	74.14	**1.87**	72.77	70.69	71.73	**0.82**
	Mean (columns)	75.60	74.45	75.02		76.10	73.49	74.79		76.62	76.28	76.45	
	t-value (columns)	**2.25***	**3.14***			**0.08**	**0.63**			**6.67***	**5.82***		
Apparent rate of infection (pupd)	**Sprayed**	0.27	0.15	0.21	**4.53***	0.09	0.13	0.11	**2.46***	0.13	0.15	0.14	**1.29**
	Unsprayed	0.41	0.43	0.42	**0.73**	0.34	0.46	0.40	**3.67***	0.26	0.25	0.26	**3.05***
	Mean (columns)	0.34	0.29	0.31		0.21	0.30	0.25		0.20	0.20	0.20	
	t-value (columns)	**8.47***	**14.65***			**9.66***	**12.84***			**10.73***	**14.13***		
AUDPC	**Sprayed**	705.55	582.79	644.17	**1.00**	512.55	621.88	567.22	**1.35**	541.62	613.53	577.58	**0.53**
	Unsprayed	1758.61	1586.58	1672.60	**1.41**	1433.13	1683.91	1558.52	**3.54***	1805.87	1456.31	1631.09	**4.09***
	Mean (columns)	1232.08	1084.69	1158.38		972.84	1152.90	1062.87		1173.75	1034.92	1104.33	
	t-value (columns)	**7.05***	**8.98***			**6.68***	**7.86***			**14.54***	**14.13***		

P(0.05) = 1.99 *= significant difference

Table 3.2. The effect of cultivars, row width and fungicide applications on soybean physiological parameters (maturity, yield, variation in yield protein- and oil content).

		Cultivar											
		PAN 494				LS 666				SCS 1			
Row width (cm)		45 cm	90 cm	Mean (rows)	t-value (rows)	45 cm	90 cm	Mean (rows)	t-value (rows)	45 cm	90 cm	Mean (rows)	t-value (rows)
Maturity (d)	Sprayed	139.06	138.50	138.78	**0.41**	146.69	147.59	147.14	**0.50**	154.69	152.18	153.44	**0.71**
	Unsprayed	133.38	135.19	134.29	**1.42**	140.84	143.23	142.04	**0.84**	148.56	151.55	150.06	**0.81**
	Mean (columns)	136.22	136.85	136.53	0.92	143.77	145.41	144.59	0.67	151.63	151.87	151.75	0.76
	t-value (columns)	**4.31***	**2.46***			**2.72***	**2.50***			**2.93***	**1.08***		
Yield (t/ha)	Sprayed	2.50	2.70	2.60	**0.50**	2.70	2.61	2.66	**0.93**	3.02	2.29	2.66	**5.23***
	Unsprayed	1.59	1.60	1.60	**0.12**	1.95	1.79	1.87	**0.32**	1.15	0.97	1.06	**1.85**
	Mean (columns)	2.05	2.15	2.10		2.33	2.20	2.26		2.09	1.63	1.86	
	t-value (columns)	**5.00***	**7.93***			**8.97***	**13.85***			**15.39***	**2.90***		
Yield variation (%)		33.62	34.79			27.37	31.95			62.48	56.11		
Protein content (%)	Sprayed	36.01	37.24	36.63	**2.46***	36.60	37.33	36.97	**1.52**	37.90	39.02	38.46	**1.52**
	Unsprayed	37.50	36.63	37.07	**0.26**	38.85	39.37	39.11	**0.93**	39.87	41.03	40.45	**0.93**
	Mean (columns)	36.76	36.94	36.85		37.73	38.35	38.04		38.89	40.03	39.46	
	t-value (columns)	**2.87***	**0.69**			**4.43***	**3.80***			**3.44***	**5.68***		
Oil content (%)	Sprayed	19.03	19.56	19.30	**0.56**	20.22	20.11	20.17	**0.31**	18.85	18.72	18.79	**0.31**
	Unsprayed	19.25	19.02	19.14	**0.11**	17.97	17.63	17.80	**0.91**	16.80	16.47	16.64	**0.91**
	Mean (columns)	19.14	19.29	19.22		19.10	18.87	18.98		17.83	17.60	17.71	
	t-value (columns)	**0.51**	**0.67**			**6.80***	**6.22***			**3.37***	**4.20***		

P(0.05) = 1.99 *= significant difference

Table 3.3. Area under disease progress curve (AUDPC) means for unsprayed and sprayed plots and difference in AUDPC (calculated as the difference between unsprayed plots and sprayed plot AUDPC values).

	Cultivar								
	PAN 494			LS 666			SCS 1		
Row width (cm)	**45 cm**	**90 cm**	**Mean (rows)**	**45 cm**	**90 cm**	**Mean (rows)**	**45 cm**	**90 cm**	**Mean (rows)**
AUDPC - unsprayed	1758.61	1586.58	1672.60	1433.13	1683.91	1558.52	1805.87	1456.31	1631.09
AUDPC - sprayed	705.55	582.79	644.17	512.55	621.88	567.22	541.62	613.53	577.58
Mean (columns)	1232.08	1084.69	1158.38	972.84	1152.90	1062.87	1173.75	1034.92	1104.33
AUDPC - difference	1094.89	1003.80	1049.35	816.34	1062.04	939.19	1264.25	892.31	1078.28
Spray efficiency	60.38	63.45	61.92	57.09	63.51	60.30	69.87	59.60	64.74

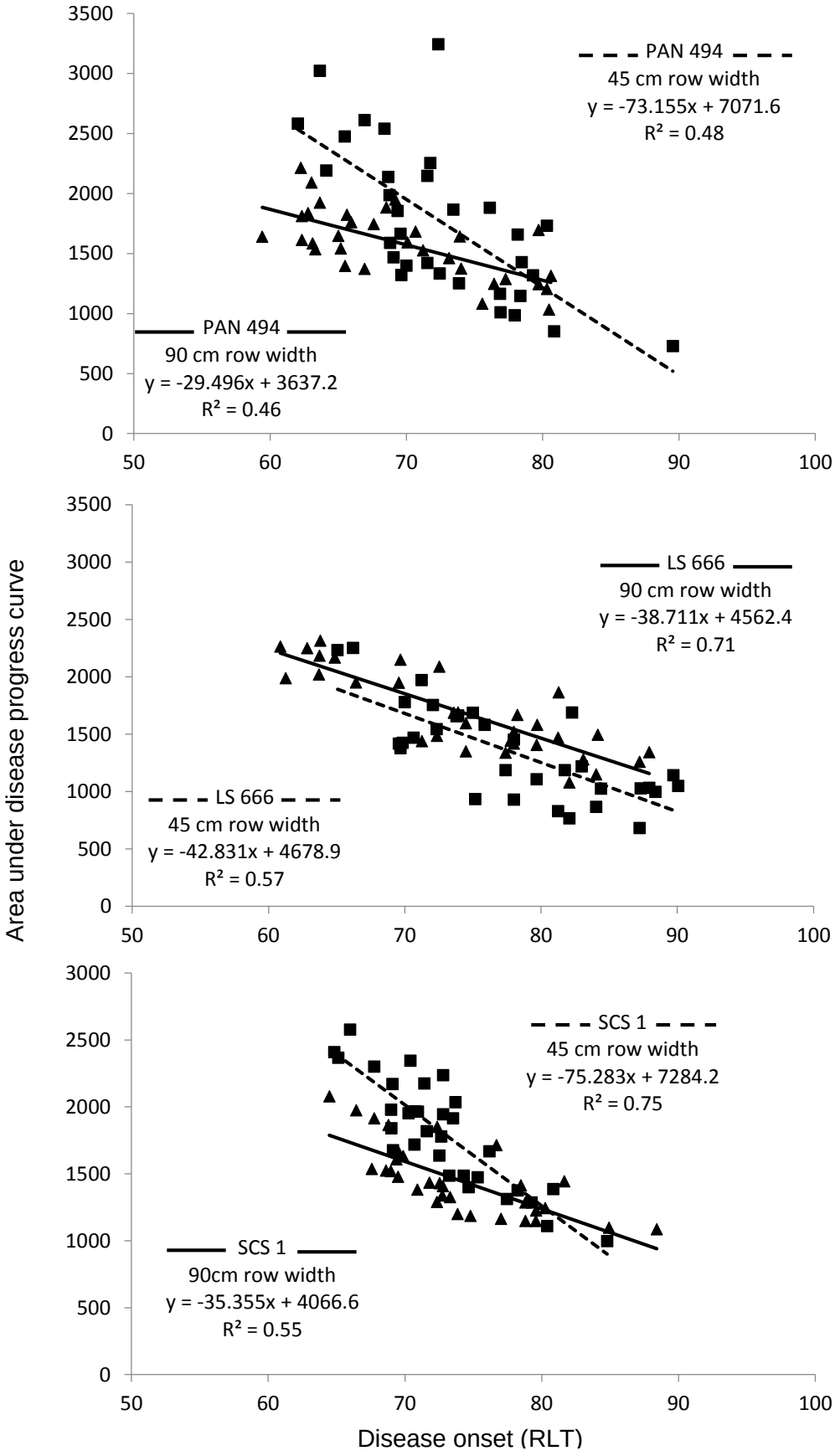

Figure 3.1. Relationship between disease onset and area under disease progress curve (AUDPC) in unsprayed plots.

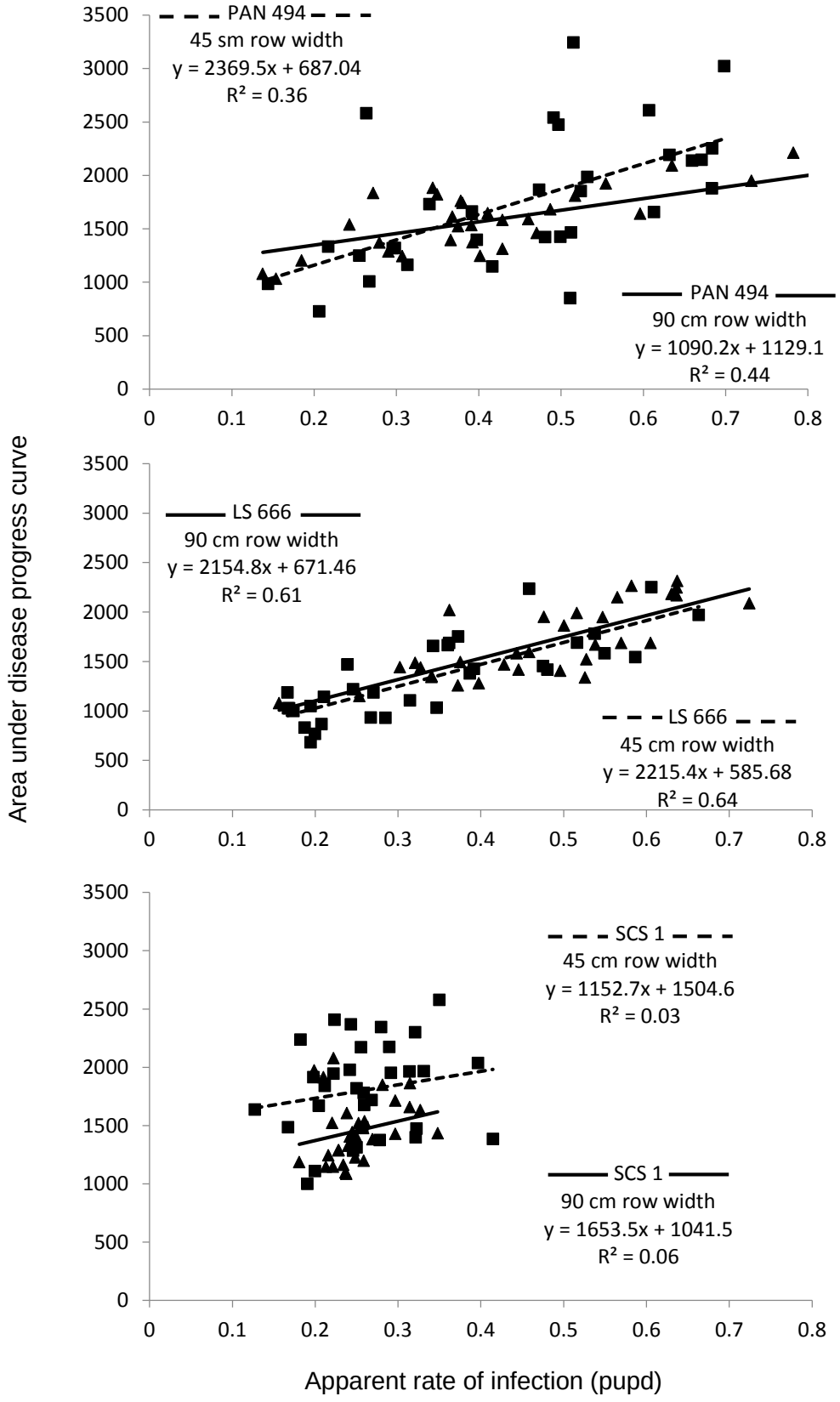

Figure 3.2. Relationship between apparent rate of infection ie. disease increase per time increment expressed as per unit per day (pupd) and area under disease progress curve (AUDPC) unsprayed plots.

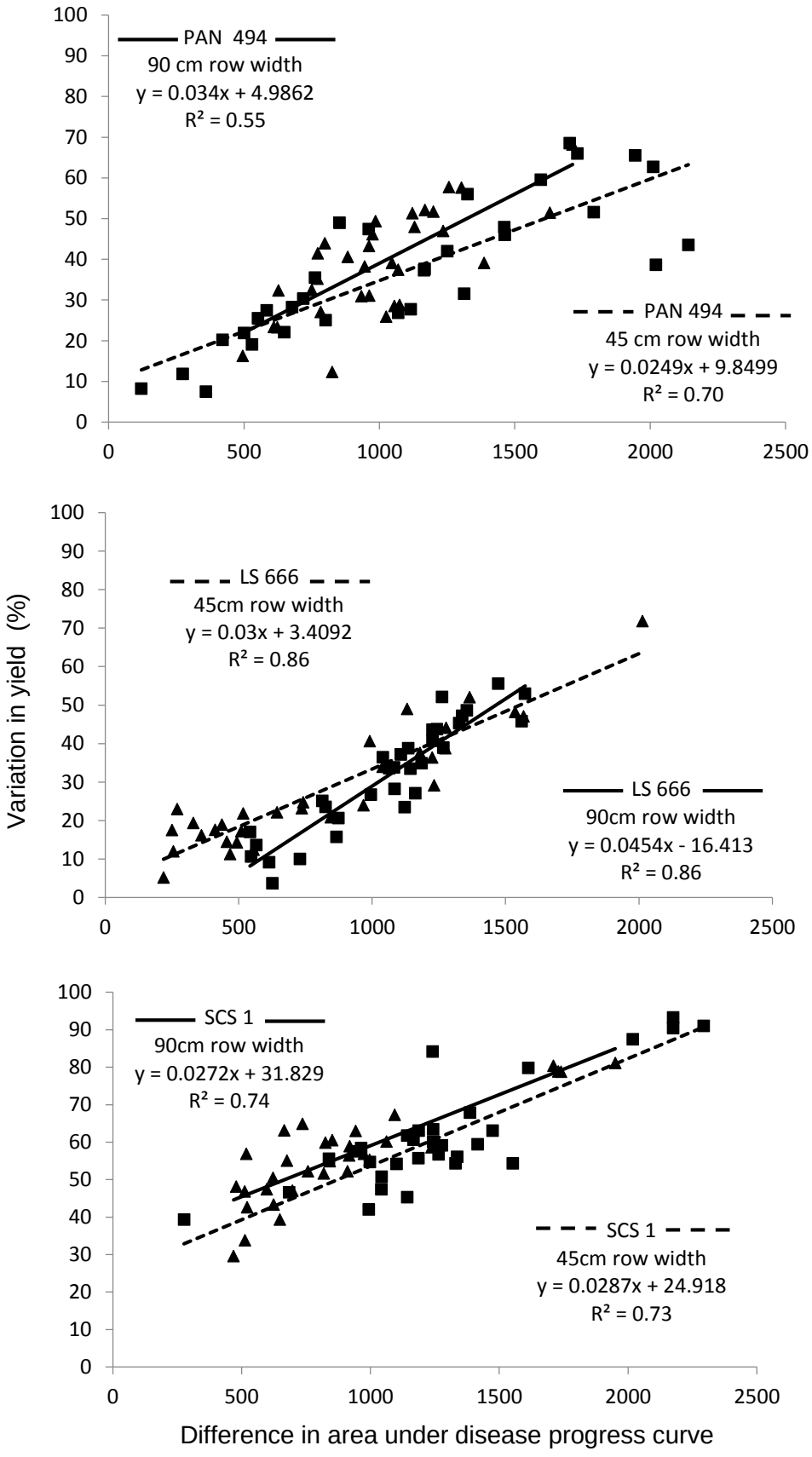

Figure 3.3. Relationship between difference in area under disease progress curve (AUDPC) and variation in yield.

CHAPTER 4

PHYSIOLOGICAL CHANGES DURING THE GROWTH OF SOYBEAN AND THEIR EFFECT ON SUSCEPTIBILITY TO SOYBEAN RUST CAUSED BY *PHAKOPSORA PACHYRHIZI*

4.1 Abstract

Soybean rust caused by *Phakopsora pachyrhizi* is regarded as one of the most destructive soybean diseases and yield losses of up to 80% has been recorded. To date no resistant cultivars have been released. Information on the biochemical events related to rust resistance, especially in soybeans, is limited. The aim of this study was to monitor disease development in field experiments and relate changes in host susceptibility to soybean rust to host physiology. During 2008/09, field trials with experimental blocks consisting of three commercial varieties, namely PAN 1454RR (short maturity), SNK 500 (medium maturity) and LS 678 (long maturity) were planted at Cedara. In addition, plots of SNK 500, where plants received fertilizer treatments of LAN (28) and Urea (42), were also planted in three-replicate randomized blocks. Trials were repeated during 2009/10. Rust development was visually assessed during the growing season. Leaves were collected at R3 to R7 growth stages. Intercellular wash fluids were collected and protein concentration, total phenolics and carbohydrates, β-1,3-glucanase, chitinase and peroxidise activities were determined. Disease onset in all plots was late (mean onset =78% Relative Life Time) and high subsequent rate of disease development was recorded. F-test indicated no significant differences in the disease progress curves of cultivars in the two seasons. In fertilizer amended plots a later onset was recorded indicating a prolonged growth period due to increased nitrogen levels. Pooled data to determine the relationship between host physiological stage and the compounds of interest indicated that the rapid decline of 52% in chitnase activity and 60% decline in *β* -1,3- glucanase corresponds with the time of disease onset. Plots that received different fertilizer levels indicated that chitinase, *β* -1,3- glucanase, peroxidase and phenol content were related to the form of nitrogen received. Higher chitinase activity was also associated with lower disease severity. It was evident in the current study that reductions in the enzyme concentrations related to changes in host physiology are closely associated with rapid onset and development of soybean rust.

4.2 Introduction

Phakopsora pachyrhizi Syd. & P.Syd. the causal organism of soybean rust was first reported and described in South Africa in 2001 (Pretorius *et al*., 2001). Since then, severe epidemics have occurred in most soybean (*Glycine max* (L.) Merr.) production areas of Kwazulu-Natal and Eastern Highveld (Jarvie, 2009). The ability of this pathogen to cause large scale epidemics worldwide is to a large extent dependent on weather, especially temperature and moisture and host plant resistance.

Host plants resistance is expressed when plants are affected by both abiotic and biotic stresses (Bi *et al*., 1994). However, in order to defend themselves against these attacks, plants need to have a fully functional, sophisticated surveillance system able to distinguish between self-generated signals and those send from invaders (Hammond-Kosack & Jones, 2000). This must therefore, be capable of activating a complex set of defense mechanisms to prevent pathogen invasion and ensure genetic incompatibility between the pathogen and the host (Talarczyk & Hennig, 2001). In addition pre-existing defense mechanisms that include structural characteristics that act as physical barriers, such as the cuticle, epidermis, lignin and thrichomes that inhibit the pathogen from entering and spreading throughout the host as well as biochemical reactions that occur in cells and tissues of the plant that inhibit penetration and colonization of host tissues, can contribute to plant health. These reactions may be toxic to the pathogen or just inhibit growth and thus, limit spread of the pathogen in the host plant (Agrios, 1997). These include defense responses such as the hypersensitive response, production of reactive oxygen species, pathogenesis-related (PR) proteins, secondary metabolites and antifungal enzymes (Selitrennikoff, 2001).

The expression of PR-genes in plants when attacked by pathogens has been widely investigated in a variety of plant species. They have been classified as a diverse family in plants that show a complex expression pattern and a display of various biological activities in different plant species (Liu & Ekramoddoullah, 2006). These substances are available in small amounts in plants but are produced in massive quantities after pathogen attack and stress and can be found in the intracellular and extracellular spaces of the host plant cells (Agrios, 1997). They can be constitutive or the result of induced resistance to fungal attack. Several antifungal proteins are involved in the inhibition of the synthesis of the fungal cell wall or the disruption of the cell wall structure and/or function. The mode of action of these proteins varies considerably as they have been classified according to

their function, serological relationship, amino acid sequence and molecular weight (Selitrennikoff, 2001).

A better understanding of the role of physiological compounds that play a role in disease resistance will help in the development of resistant varieties in breeding programmes and provide additional selection criteria which can add to the stability of host responses to infection (Anguelova-Merhar *et al*., 2002). Various studies claim that resistant genotypes show higher levels of antifungal substances than susceptible genotypes for example De Souza *et al* (2003), reported resistant maize seedlings that had higher levels of peroxidase activity than the susceptible group.

Information on biochemical events related to rust resistance in soybean is currently limited and this study was undertaken to increase the current understanding of the resistance response in this host-pathogen interaction. The aim of the study was to monitor disease development in field experiments and relate changes in the rate of disease development and host susceptibility to host physiology. Detection of changes in host physiology, in particular anti-fungal substances and the variation within soybean populations for these fungal inhibitors, could lead to the identification and exploitation of partial resistance to soybean rust. During 2006/07 and 2007/08 soybeans that were planted subsequent to a high potential maize trials at Cedara showed rust suppression. The assumption that residual N may have influenced disease development was therefore investigated.

4.3 Materials and Methods

4.3.1 Plant production

4.3.1.1 Evaluation of resistance mechanisms to soybean rust in commercial cultivars from different maturity groups

Field trials were conducted during the 2008/09 and 2009/10 planting seasons at Cedara, KwaZulu-Natal (GPS coordinates: -29.533°S, 30.283°E) were soybean rust is endemic. Experimental plots of three rust susceptible commercial cultivars ie. PAN 1454RR (130 days to maturity), SNK 500 (135 days to maturity) and LS 678 (140 days to maturity) were planted sequentially with selected planting dates, 13 and 25 November and 4, 12 and 18 December 2008. During 2009/10 plots were planted on 10 and 26 November, 3 and 14 December 2009. Plots consisted of 10 rows, 10 m in length with a 45 cm row spacing. The plant density was equivalent to 360 000 plants ha^{-1}.

Prior to planting plots were fertilized with 300 kg superphosphate and 50 kg KCl ha^{-1}. Rhizobium (*Bradyrhizobium japonicum*) was added in a water suspension (100g/15 ℓ water) to each row subsequent to seeding but prior to closing of the planting furrow. Subsequent to planting, plots were sprayed with 1920 ℓ (a.i) Alachlor for grass control. Additional weeding was done by hand as required.

4.3.1.2 Nitrogen treatments

During these two planting seasons, 10 plots of SNK 500 consisting of 10 rows, 5 m in length with 45 cm row spacing were also planted. Each plot received a different level of limestone ammonium nitrate (LAN (28)) or Urea (42) fertilizer as indicated in Table 1. Levels were calculated to the equivalents of 40kg, 30kg, 20kg and 10kg per ha. and transformed to fit the smaller plot size of 12.5m^2. The two control plots i.e. a plot amended with commercial rhizobium (*Bradyrhizobium japonicum*) and a plot without were also included. Trials were maintained using the above herbicide regime and hand weeding.

4.3.2 Disease measurement and data analysis

Rust onset and development was studied in all experimental plots during 2008/09 and 2009/10. Rust was visually assessed as the percentage of leaf area infected using the rating scale of Godoy et al. (2006). Percentage defoliation was also estimated. Affected leaf area per plant was then calculated as ALA=defoliation+((100leaf drop)*(rust/100)) (McLaren, 2008). Days after planting on each assessment date was used to calculate relative life time (RLT) of varieties as days after planting/days to full maturity (Tschanz, 1984) so as to enable comparisons between cultivars with differing maturity dates. Disease progress curves were fitted for each cultivar using the linearised form of the logistic growth model (y=x/(1-x)) (Van der Plank, 1963) where RLT was used as the independent-variable and ALA as the dependent-variable. Data were analysed using simple regression analysis (NCSS, 2007). The apparent infection rate (Van der Plank, 1963) was recorded from the regression output and the regression model was back-transformed to the non-linear logistic model and used to estimate disease onset and RLT when 100% disease severity was reached. Onset of rust was set at 5% affected leaf area.

Area under the disease progress curve (AUDPC) was calculated according to the model of Campbell & Madden (1990) where AUDPC = Σ_i=1 n-1 [(t_i+1 – t_i) (y_i + y_i+1)/2] using RLT as the independent variable and ALA as the dependent variable. Linear and correlation analyses were used to determine the relationship between RLT at which disease onset occurred, rate of disease development and AUDPC and physiological variables investigated in this study. Data were analysed using Number Cruncher Statistical Systems (NCSS) (Hintze, 2007).

4.3.3 Collection of Intercelluar wash fluids (IWF)

During the 2008/09 and 2009/10 seasons leaves were sampled at three growth stages ie. R3, R5 and R7. Fifteen plants per cultivar or fertilizer block were collected. Three trifoliate leaves were collected from the top, middle and bottom part of each plant, cut into separate pinnas and combined. The leaves were washed in regular tap water to remove intracellular contamination due to mechanical wounding and dried with blotting paper. A fresh sample (5 g) was frozen and kept at -74 °C for the measurement of total phenolics and carbohydrates.

Intercellular wash fluids (IWFs) were extracted from the remaining leaves of the collected leaves using a modified method from Jung *et al*. (1993). Fresh leaves were placed into a glass tube and covered with 50 mM sodium acetate buffer (pH 5.2), containing 15 mM mercaptoethanol. These were vacuum-infiltrated for 5 min using a water jet pump and infiltrated with the buffer by slowly releasing the vacuum. The leaves were blotted dry with paper, placed in a centrifuge tube containing a perforated plastic disk at the bottom to provide space for the extract to collect at the bottom. The tubes were centrifuged at 2000 rpm for 10 min at 4 °C.

The resulting IWFs were collected and frozen at -74 °C until used for the determination of proteins, β-1,3-glucanase, chitinase, peroxidase activity as well as total phenols present.

4.3.4 Determination of protein concentration

The protein concentrations of collected IWF samples were determined using the dye-binding assay technique of Bradford (1976). The assay mixture consisted of 150 µl distilled water, 40 µl ul BioRad and 10 µl enzyme extract or in the case of the standard

curve 10 µl *γ*-globulin (0.5 µg µl^{-1} *γ*-globulin). Samples were placed into a Biorad microplate reader (model 3550) and read at a wavelength of 595 nm as described by Rybutt & Parish (1982). The sample was read in triplicate. Total proteins were expressed as mg^{-1} protein h^{-1}.

4.3.5 Determination of enzyme activities

The IWF samples collected were used to test for three Pathogenesis Related enzymes namely β-1,3-glucanase activity, chitinase activity and peroxidase activity, which were determined spectrophotometrically with a TG 60 UV visible spectrophotometer from PG Instruments. All activities, as well as component amounts, were determined in triplicate.

4.3.5.1 β-1,3-glucanase activity

Measurements for ß-1,3-glucanase activity were done using a modified technique of Fink *et al*. (1988). The assay mixture contained 250 µl Laminarin (Sigma), 240 µl sodium acetate buffer, (pH 4.5) and 10 µl enzyme extract. After a 10 min incubation period at 37°C, 500 µl Somogyi's reagent (1952) was added. Thereafter the samples were boiled for 10 min at 100°C and cooled on ice. Nelson's reagent (500 µl) (1944) was added and the absorbance of the samples read was at 540 nm against a blank prepared with the same components but with buffer replacing the enzyme extract. A prepared glucose standard curve was used to estimate the amount of glucose in the samples. Specific β-1,3-glucanase activity was expressed as mg glucose mg^{-1} protein min^{-1}.

4.3.5.2 Chitinase activity

Chitinase activity was assessed using a modification of the method of Wirth & Wolf (1990), which is based on the precipitability of carboxymethyl-chitin-remazol brilliant violet 5R (CM-chitin-RBV; Loewe Biochemica GmbH) in buffered solutions with hydrochloric acid. The mixture consisted of 600 µl 0.1 M sodium acetate buffer (pH 5.2), 200 µl (2 mg/ml) CM-chitin-RBV, 10 µl enzyme extract and 2 N hydrochloric acid to stop the reaction. The reaction mixture was incubated at 37°C for 30 min, after which 2 N HCl was added. The reaction was left on ice for 10 min to allow complete precipitation. The sample was then centrifuged at 14 000 rpm for 5 min and the absorbance measured against a blank, at 550 nm. The enzyme activity is given in relative enzyme units mg^{-1} protein h^{-1}.

4.3.5.3 Peroxidase activity

Peroxidase activity was determined using the method described by Zieslin & Ben-Zaken (1991). The reaction is based on the use of guaiacol as a substrate in the presence of H_2O_2. The assay mixture consisted of of 50 mM guaiacol, 80 mM phosphate buffer (pH 5.5), 8.2 mM H_2O_2 and 10 µl enzyme extract in a total volume of 1 ml. The increase in absorbance was measured at 470 nm for 180 s against a blank prepared in the same way but with buffer substituting the enzyme component. The linear part of the curve during the last 60 s was used for the calculation of the enzyme activity. The specific activity was expressed as µmol tetraguaiacol mg^{-1} protein min^{-1}, after calculating the molar extinction coefficient ($\varepsilon = 2.66 \times 10^4$ mol^{-1} cm^{-1}) of tetraguaiacol.

4.3.6 Determination of total phenolics

Total phenolics were determined for each of the freshly frozen leaf samples, using a modified prussian blue method (Budini *et al*., 1980). Sample was frozen separately in liquid nitrogen and homogenised to a fine powder. The homogenate was then hydrolyzed for 30 min at 95°C in 2 N HCl, and then cooled in an ice bath. The sample was then centrifuged at 10000 rpm for 15 min at 4°C and made up to 15 ml with ddH_2O. The mixture consisted of homogenate, 0.08 M $K_3Fe(CN)_6$ and 0.1 M $FeCl_3$ in 0.1 M HCl. Optical density was read after 5 min at 700 nm. Total phenolic content is expressed as nmol gallic acid equivalents g^{-1} fresh mass.

4.3.7 Determination of total carbohydrates

Total soluble carbohydrate tests were carried out for each of the frozen upper and lower leaf samples using a modified anthrone method (Yemm & Willis, 1954). A sample of 1 g of frozen soybean leaves was homogenised for 1 min in 10 ml acetone and centrifuged at 10 000 for 15 min at 0 °C. The supernatant was pipetted into McCartney bottles and evaporated to dryness in a water bath at 50 °C. The residue was made up to 20 ml with dH_2O. Five ml anthrone (0.2 g anthrone in 100 ml 2 N H_2SO_4) was pipetted into a thick walled glass test tube in an ice bath, after which 1 ml test solution was layered on the acid, cooled for a further 5 min and vortexed. The mixture was heated for 10 min in a 90°C water bath and returned to the ice for 5 min. Absorbance was measured at 630 nm.

4.4.1 Evaluation of resistance mechanisms to soybean rust in commercial cultivars from different maturity groups

Outputs from analysis of variance of linearised disease progress curves where Y (assessed as Affected Leaf Area)=LnY/(1-Y) and X=Relative Life Time at which disease severity was assessed, were used to compare regression models using the F-test where

$$F=[\{(SS_{combined}(SS_{reg1}+SS_{reg2}))/(DF_{combined}(DF_{reg1}+DF_{reg2}))\}/\{(SS_{reg1}+SS_{reg2})/(DF_{reg1}+DF_{reg2})\}]$$

F-values proved non-significant indicating that disease onset, rate of disease development and the stage at which 100% ALA was reached between cultivars and seasons did not differ significantly between cultivars and seasons. Figure 4.1 illustrates the back-transformed logistic probability model and the parameter values for onset, rate of disease development, relative life time at which 100% affected leaf area was reached as well as AUDPC.

As a result of the absence of significant differences between cultivars, data within seasons was initially pooled to determine the relationships between host physiological stage and targeted compounds within leaves. Seasonal differences were minimal as illustrated for chitinase in Figure 4.2, with the result that data from the two seasons were pooled in the calculation of relationships for this enzyme as well as ß-1,3- glucanase and total protein. Figure 4.2 indicates a significant, negative relationship between chitinase activity and host relative life time. The rapid decline in chitinase activity subsequent to 72% RLT from 561 to 266 mg^{-1} protein h^{-1}, ie. 52%, corresponds with the time of disease onset (mean onset over all cultivars and seasons=78.5%) and subsequent rapid increase in disease severity.

Figure 4.3 shows a similar decline in ß-1,3- glucanase content corresponding to the same relative life time scale from a high of 1619 mg glucose mg^{-1} protein min^{-1} at 72% RLT to 654 mg glucose mg^{-1} protein min^{-1} at 78% RLT corresponding with a 60% decline over a 4% relative life time period. Concomitant with this and chitinase activity is the increase in total protein content over this period from 3.6 to 9.4 h^{-1} mg^{-1} protein h^{-1} an almost three-fold increase (Figure 4.4).

Peroxidase activity differed significantly over seasons (F-value= 4.61) as indicated in Figure 4.5. The increase in peroxidase activity increased at an overall rate of 1094 µmol tetraguaiacol mg^{-1} protein min^{-1} per percentage increase in RLT compared with only 484 µmol tetraguaiacol mg^{-1} protein min^{-1} per percentage increase in RLT during 2008/09 and 2009/10 respectively. This seasonal difference in the activity of this defense response protein corresponds with the slightly earlier, albeit non-significant, onset and reduced rate of disease development in 2009/10 compared with 2008/09. This reaction also suggests that this enzyme is dependent on external biotic and abiotic factors compared to chitinase and β-1,3-glucanase which appear to be host growth stage related.

No significant relationship was recorded between RLT and host total phenol content or total carbohydrates indicating that the level of these compounds in not a function of host growth stage.

Figures 4.6, 4.7 and 4.8 illustrate the relationships between the tested compounds and disease severity as measured by affected leaf area. Significant, negative relationships were recorded with chitinase and β-1,3-glucanase activity clearly showing that disease severity is closely linked to the levels of these compounds in leaves. Concomitant with this is the increase in disease severity with protein content (Figure 4.8). It can therefore, be assumed that the reductions in the concentration of these enzymes with changes in host physiology are associated with the rapid onset and development of soybean rust. No relationships were recorded with disease severity and total carbohydrates, phenols or peroxidase activity.

4.4.2 Nitrogen treatments

Analysis of variance indicated significant differences in ALA as well as the leaf compounds tested for rust suppression associated with N-treatments and season with significant treatment x season interactions. However, with the exception of ALA (Figure 4.9), no direct relationship between the concentration of the respective compounds and N-level was recorded.

In Table 1 a distinct decrease in ALA is evident with increasing LAN-levels although a threshold level appears to be evident. This relationship was also consistent over the two seasons. In both the LAN and Urea treatments, a significantly higher level of disease was recorded in control treatments that received no N, irrespective of the addition of rhizobium or not, and 10 kg-N ha^{-1} compared with those that received 20 kg-N ha^{-1} and

higher levels of N. The latter treatments did not differ significantly from one another. No differences in disease severity were associated with the form of N applied. This is in contrast with chitinases where the level of chitinase activity tended to be higher with Urea compared with LAN at the lower 10 and 20 kg-N ha^{-1} levels and higher in the LAN treatment compared to Urea at the higher application rates of 30 and 40 kg-N ha^{-1}. Notable, too is the difference in chitinase activity associated with the presence or absence of rhizobium where the latter showed significantly higher chitinase activity. The higher chitinase activity during 2009/10 is also associated with lower disease severity during this season. These tendencies were also evident with β-1,3- glucanase (Table 1).

Peroxidase activity and total phenol content of leaves was also related to the form of N (Table 2). Peroxidase activity in the respective LAN treatments were significantly higher than the Urea treatments. Although there was a tendency for higher peroxidase activity at the lower N-levels, this was not consistent over all N-levels. No consistency was evident in the relationship with N-form or levels and total phenol content of leaves or protein content (Table 2) or total carbohydrate content (Table 3).

4.5 Discussion

A better understanding of host physiology and the biochemical activities in plants during infection and colonization of tissues and relating these to resistance responses can add to the improvement of disease resistance selection criteria and improve the chances of identifying stable resistance to disease in breeding programs (Anguelova-Merhar *et al*., 2002). These data can also prove useful in the development of genetically engineered crops with enhanced plant resistance responses (Edreva, 2005; Fritig *et al*., 1998).

Several studies have indicated that PR proteins, more specifically β-1,3-glucanases, chitinases and peroxidases, have been detected in infected plant tissue in which disease severity is reduced, however, their specific function in the defense responses of plants is still somewhat unclear. According to Hammond-Kosack & Jones (2000) chitinases and β-1,3-glucanases function in the degrading of structural polysaccharides of fungal cell walls and also reduce fungal growth. These may also act as a barrier in controlling the size of the lesion and pathogen spread as was observed in a study when a *Nicotiana plumbaginidolia* gn 1 gene encoding a β-1,3-glucanase isoform was incorporated into tobacco plants (Castresana *et al.*, 1990). In wheat plants inoculated with *Puccinia reconditia* f.sp*. tritici* an increase in β-1,3-glucanase activity was observed as well as

necrosis indicating a hypersensitive response. A high proportion of the infection sites also did not develope beyond the substomatal phase emphasizing a reduction in fungal growth and development (Kemp *et al*., 1999).

Several stresses have been shown to influence the production of defense substances especially β-1,3-glucanase and chitinase activity (Carr & Klessing, 1989; Bowles, 1990). Krishnaveni *et al*., (1999) provided evidence that fungal infection, insect infestation and mechanical wounding increased the levels of chitinases and β-1,3-glucanases. Hou *et al.* (1998) also reported that chitinase activity is induced systemically by insect damage. Boller *et al*. (1983), on the other hand, found that mechanical wounding of bean leaves *in situ* resulted in no increase in chitinase activity in the wounded leaves. Krishnaveni *et al.* (1999) also failed to report a comparison of the levels of chitinase and β-1,3-glucanase between resistant and susceptible sorghum genotypes. In most studies β-1,3-glucanase and chitinase seem to act synergistically as near similar patterns were observed and the positive correlation observed in the current study confirms this.

It is generally accepted that recognition of pathogens in order to activate constitutive or active defense mechanisms is crucial and this has been the focus of numerous host x pathogen interaction studies (Graham *et al*., 2003; Saikia *et al*., 2005; Melgar *et al*., 2006). In the current study, however, patterns displayed by the enzyme activities indicate that activity may be more closely linked to the growth and development of the host plant rather a response to infection, at least in the genotypes used in the current study. This response was particularly evident with chitinase and β-1,3-glucanase activity and these appear to be the primary factors in the onset and subsequent rapid rate of disease development. Prior to the recorded decline in these compounds at approximately 78% RLT, disease development was limited and only significant subsequent to this growth stage. It may be for this reason why the pattern of disease development over seasons is fairly constant and that in contrast to host physiology, environmental influences appear to have a relatively small effect on rust severity except at extreme conditions beyond the adaptation of the pathogen and especially once infection has been initiated. Optimum environmental conditions for rust development is reported to be in temperatures ranging from 8 to 28°C and the availability of free water is crucial for spore germination and penetration. A leaf wetness and temperature interaction however was reported and in conditions less optimal for disease a longer period of free moisture is needed for infection i.e. 6 hours of dew were needed for infection at 20°C to 25°C and a minimum of 8 hours moisture period at 15° to 17.5°C. No infection was observed at temperatures of 27.5°C (Machetti *et al*., 1976) and this was confirmed by Kochman (1979) who stated that temperatures ranging

from 28.5°C to 42.5°C can inhibit germination. Extreme temperatures can thus reduce inoculum concentrations in the field.

By implication the identification of genotypes in which the decline in these enzymes is delayed or maintained above a critical threshold (200 mg^{-1} protein h^{-1} and 500 mg glucose mg^{-1} protein min^{-1} for chitinase and β-1,3-glucanase respectively according to Figures 4.2 and 4.3) for an extended period could contribute to increasing resistance to this disease.

According to Snoeijers *et al.* (2000) nitrogen level can affect disease development as ammonia sources provide essential building blocks for amino acids, proteins, chlorophyll and nucleotides. In the current study, chitinase and β-1,3- glucanase activity were the only compounds tested that showed any significant response to N-fertilization in relation to rust severity, again confirming the probable role of these in the response of plants to the rust pathogen. However, the reduction in disease was not directly significantly related to the levels of these enzymes in the fertilizer trial and the potential role of N-levels in stimulating these enzymes and suppressing rust severity significantly as a result of any interaction with these enzymes is questionable. Instead other potential mechanisms for the reduction in disease severity observed in this study should be sought. Examples of this include a delay in host senescence which has been shown to delay disease onset due to a delay in the susceptible host growth stage rather than directly effects key enzymes. Nitrogen delayed senescence as a key factor in reduced disease severity has been recorded in a number of host x pathogen interactions (Bockus & Davis, 1993; Westerveld *et al*., 2008) while a nitrogen deficiency results in earlier, rapid senescence of the host which, in turn is correlated with an increase in disease severity.

Although peroxidase activity was seen to increase subsequent to flowering, no relationship with disease development was recorded. This is despite this enzyme family being known for diversity in form, function and distribution and involvement in lignification, production of reactive oxygen species and suberin synthesis. Peroxidase also plays a crucial role in wound responses and pathogen attack (Mohan & Kolattukudy, 1990; De Souza *et al*., 2003). The release of active oxygen species, also known as the oxidative burst is a common phenomenon prior to the hypersensitive response occurring in most plant pathogen interactions (Mehdy, 1994). The absence of a gene-for-gene interaction in the host x pathogen interaction in the current study may also explain the absence of a disease x peroxidase relationship being recorded.

Carbohydrates favour the colonization of the host plant by pathogens and are important for the production of antibiotic compounds in the host as well the inhibition of enzyme production by the pathogen (Horsfall & Cowling, 1980). Previous studies have shown a decline in sugar concentrations in soybeans during different stages and towards maturity (MacMasters *et al*., 1941; Kuo *et al*., 1997; Abood & Lösel, 2003). However in the current study varying levels of carbohydrate concentration were detected but these could not be successfully related to any trend in host plant growth or disease development.

The current study has indicated the probable role of chitinase and β-1,3- glucanase activity in the onset and development of soybean rust. This activity appears to be linked to the normal phenological stages in the host's development rather than being influenced by external factors except those beyond the tolerance levels of the pathogen. In the cultivars used in the current study the decline in the activity of these key enzymes occurred over a very narrow range of growth stages and a wider range of genotypes is required to determine the potential range of critical stages, especially beyond flowering or pod formation to determine their potential for exploitation in resistance breeding programs. Results of this study, however suggest that chitinase and β-1,3-glucanase could be a useful additional selection criterion to enhance resistance.

4.6 References

Abood, J.K. & Löser, D.M. 2003. Changes in carbohydrate composition of cucumber leaves during the development of powdery mildew infection. Plant Pathology 52: 256-265.

Agrios, G.N. 1997. Plant Pathology. Fouth Editions. Academic press. New York.

Anguelova-Merhar, V.S., van der Westhuizen, A.J. & Pretorius, Z.A. 2002. Intercellular chitinase and peroxidase activities associated with resistance conferred by gene Lr35 to leaf rust of wheat. Journal of Plant Physiology 159: 1259-1261.

Bi, J.L., Felton, G.W. & Meuller, A.J. 1994. Induced resistance in soybean to Helicoverpa zea: Role of plant protein quality. Journal of Chemical Ecology 20: 183-1983.

Bockus, W.W. & Cavis, M.A. 1993. Effect of nitrogen fertilizers on severity of tan spot of winter wheat. Plant Disease 77: 508-510.

Boller, T., Gehri, A., Mauch, F. & Vögeli, U. 1983. Chitinase in bean leaves: Induction by ethylene, purification properties and possible function. Planta 157: 22-31.

Bowles, D-J. 1990. Defense-related proteins in higher plants. Annual Review of Biochemisty 59: 873-907.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Analytical Biochemistry 72: 248-254.

Budini, R., Tonelli, D. & Girotti, S. 1980. Analysis of total phenols using the Prussian blue method. Journal of Agricultural and Food Chemistry 28: 1236-1238.

Cambell, C.L. & Madden, L.V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, New York, USA.

Carr, J.P. & Klessig, D.F. 1989. The pathogenesis-related proteins in plants. In: Genetic Engineering. J.K. Setlow, ed. Plenum Press, New York.

Castresana, C., de Carvalho, F., Gheysen, D., Habets, M. Inze, D & Van Montagu, M. 1990. Tissue-specific and pathogen-induced regulation of a Nicotiana plumbaginifolia β-1,3-glucanase gene. The Plant Cell 2: 1131-1143.

De Souza, I.R.P., De Oliveira, E., Peres, M.A, De Oliveira, A.C. & Purcino, A.A.C. 2003. Peroxidase activity in maize inbred lines resistant or susceptible to maize dwarf mosaic virus. Revista Brasileira de Milho e Sorgo 2: 1-8.

Edreva, A. 2005. Pathogenesis-related proteins: Research progress in the last 15 years. General and Applied Plant Physiology 31: 105-124.

Fink, W., Liefland, M. & Mendgen, K. 1988. Chitinases and β-1,3-glucanases in the apoplastic compartment of oat (Avena sativa L.). Plant Physiology 88: 270-275.

Fritig, B., Heitz, T. & Legrand, M. 1998. Antimicorbial proteins in induced plant defense. Current Opinion in Immunology 10: 16-22.

Graham, M.Y., Weidner, J., Wheeler, K., Pelow, M.J. & Graham, T.L. 2003. Induced expression of pathogenesis-related protein genes in soybean by wounding and the Phytophthora sojae cell wall glucan elicitor. Physiological and Molecular Plant Pathology 63: 141-149.

Hammond-Kosack, K & Jones, J.D.G. 2000. Responces to Plant Pathogens. In: Buchanan, B.B., Gruissem, W. & Jones, R. L. 2000. Biochemisty & Molecular Biology of Plants. American Society of Plant Physiologists.

Hintze, J.L. 2007. Number cruncher statistical systems (NCSS). Kaysville, UT: Number Cruncher Statistical Systems.

Horsfall, J.G. & Cowling, E.B. 1980. Plant disease: An advanced treatise. Volume V. How plants defend themselves. Academic Press, New York.

Hou, W-C., Chen, Y-C & Lin, Y-H. 1998. Chitinase activity of sweet potato (Ipomoea batatas (L) Lam var. Tainong 57). Botanical Bulletin of Academia Sinica 39: 93-97.

Jarvie, J.A. 2009. A Review of soybean rust from a South African perspective. South African Journal of Science 105: 103-108.

Jung, J-L., Fritig, B. & Hahne, G. 1993. Sunflower (Helianthus annuus L.) pathogenesis-related proteins. Plant Physiology 101:873-880.

Kemp, G., Botha, A-M., Kloppers, F.J. & Pretorius, Z.A. 1999. Disease development and β-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings. Physiological and Molecular Plant Pathology 55: 45-52.

Kochman, J.K. 1979. The effect of temperature on development of soybean rust (*Phakopsora pachyrhizi*). Australian Journal of Agricultural Research 30: 273-277.

Kuo, T.M., Lowell, C.A. & Nelsen, T.C. 1997. Occurrence of pinitol in developing soybean seed tissues. Phytochemistry 45: 29-35.

Krishnaveni, S., Muthukrishnan, S., Liang, G.H., Wilde, G. & Manickam, A. 1999. Induction of chitinases and β-1,3-glucanases in resistant and susceptible cultivars

of sorghum in response to insect attack, fungal infection and wounding. Plant Science 144: 9-16.

Liu, J.J., & Ekramoddoullah, A.K.M. 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiological and Molecular Plant Pathology 68: 3-13.

Machetti, M.A., Melching, J.S. & Bromfield, K.R. 1976. The effects of temperature and dew period on germination and infection by uredospores of *Phakopsora pachyrhizi*. Phytpathology 66: 461-463.

MacMasters, M.M., Woodruff, S. & Klaas, H. 1941. Studies on soybean carbohydrates. Industrial and Engineering Chemistry 13: 471-474.

Mehdy, M.C. 1994. Active oxygen species in plant defense against pathogens. Plant Physiology 105: 467-472.

Melgar, J.C., Abney, T.S. & Vierling, R.A. 2006. Peroxidase activity in soybeans following inoculation with *Phytophthora sojae*. Mycopathologia 161: 37-42

Mohan, R & Kolattukudy, P.E. 1990. Differential activation of expression of suberization-associated anionic peroxidase gene in near-isogenic resistant and susceptible tomato lines by elicitors of *Verticillium albo-atratrum*. Plant Physiology 92: 276-280.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153: 375-380.

Pretorius, Z.A., Kloppers, F.J. & Frederick, R.D. 2001. First report of Soybean rust in South Africa. Plant disease 85: 1,288.3.

Rybutt, D.B. & Parish, C.R. 1982. Protein determination on an automatic spectrophotometer. Analytical Biochemistry 121: 213-214.

Saikia, R., Singh, B.P., Kumar, R., & Arora, D.K. 2005. Detection of pathogenesis-related proteins chitinase and β-1,3-glucanase in induced chickpea. Current Science 89: 659-663

Seletrennikoff, C.P. 2001. Antifungal proteins. Applied and Environmental Microbiology 67: 2883-2894.

Snoeijers, S.S., Perez-Garcia, A., Joosten, M.H. & De Wit, P.J. 2000. The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. European Journal of Plant Pathology. 106: 493–506.

Somogyi, M. 1952. Notes on sugar determination. Journal of Biological Chemistry 195: 19-23.

Talarczyk, A. & Hennig, J. 2001. Early defense responses in plants infected with pathogenic organisms. Cellular and Molecular Biology Letters 6: 955-970.

Tschanz, A.T. 1984. Soybean rust epidemiology. Final Report Asian Vegetable Research and Development Centre. Shanhau, Taiwan. AVRDC.

Westerveld, S.M., McKeown, A.W. & McDonald, M.R. 2008. Relationship between nitrogen fertilization and Cercospora leaf spot and Alternaria leaf blight of carrot. Hortscience 43:1522–1527.

Van der Plank, J.E. 1963. Plant diseases, epidemics and control. Academic Press, New York, USA.

Wirth, S.J. & Wolf, G.A. 1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. Journal of Microbiological Methods 12: 197-205.

Yemm, E.W. & Willis, A.J. 1954. The estimation of carbohydrates in plant extracts by anthrone. Biological Journal 57: 508-514.

Zieslin, N. & Ben-Zaken, R. 1991. Peroxidases, phenylalanine ammonia lyases and lignification of rose flowers. Plant Physiology and Biochemistry 29: 147-151.

Table 4.1. Effect of nitrogen formulations and application rates on rust severity as indicated by mean affected leaf area, chitinase and ß-1,3-glucanase activity in soybean leaves assessed for rust severity.

Treatment	**Affected leaf area**			**Chitinase**			**B 1,3 Glucanase**		
	Season 1	**Season 2**	**Mean**	**Season 1**	**Season 2**	**Mean**	**Season 1**	**Season 2**	**Mean**
10 kg-N (LAN)	58.67	52.00	**55.33 ^{d}**	192.90	200.06	**196.48^{b}**	640.29	677.36	**658.83^{cd}**
10 kg-N (Urea)	56.50	49.00	**52.75^{c}**	200.67	211.27	**205.97^{bcd}**	663.33	751.22	**707.28^{d}**
20 kg-N (LAN)	54.67	45.00	**49.83^{abc}**	172.95	223.62	**198.28b^{c}**	410.36	527.80	**469.08^{b}**
20 kg-N (Urea)	47.50	46.20	**46.85^{a}**	202.01	221.92	**211.97^{cd}**	713.01	525.83	**619.42^{c}**
30 kg-N (LAN)	52.83	39.83	**46.33^{a}**	184.05	291.35	**237.70^{e}**	598.52	801.22	**699.87^{d}**
30 kg-N (Urea)	52.67	45.50	**49.08^{abc}**	181.67	215.64	**198.65^{bc}**	647.08	795.30	**721.19^{d}**
40 kg-N (LAN)	50.50	41.25	**45.87^{a}**	174.59	249.95	**212.27^{cd}**	588.95	822.94	**705.95^{d}**
40 kg-N (Urea)	47.00	42.00	**44.50^{a}**	177.95	203.82	**190.88^{b}**	586.72	617.69	**602.20^{c}**
Control + R	59.00	49.50	**54.25^{c}**	166.79	175.35	**171.07^{a}**	441.35	302.36	**371.85^{a}**
Control - R	62.67	48.00	**55.33^{d}**	180.56	247.73	**214.14^{d}**	441.13	772.34	**606.73^{c}**
Mean	**54.20 ^{b}**	**45.82 ^{a}**		**183.41^{a}**	**224.07^{b}**		**573.07 ^{a}**	**659.41 ^{b}**	
LSD cult x season			7.89			21.39			88.76

Table 4.2. Effect of nitrogen formulations and application rates on peroxidase activity, total phenols and protein content in soybean leaves assessed for rust severity.

Treatment	Peroxidase			Total Phenols			Protein content		
	Season 1	**Season 2**	**Mean**	**Season 1**	**Season 2**	**Mean**	**Season 1**	**Season2**	**Mean**
10 kg-N (LAN)	14373.21	7333.13	**10853.17^{f}**	3945.78	4047.38	**3996.58^{b}**	10.69	10.81	**10.75^{f}**
10 kg-N (Urea)	9371.46	4419.31	**6895.38^{bc}**	3396.79	2231.28	**2814.03^{a}**	10.87	8.74	**9.80^{cde}**
20 kg-N (LAN)	14817.13	2417.05	**8617.09^{de}**	4023.13	3640.98	**3832.05^{b}**	10.30	8.29	**9.29^{ab}**
20 kg-N (Urea)	9901.78	5981.05	**7941.41^{c}**	4300.22	3782.41	**4041.31^{bc}**	10.63	8.72	**9.67^{bcde}**
30 kg-N (LAN)	10419.41	5582.45	**8000.93^{cd}**	4640.23	4307.15	**4473.69^{c}**	9.31	8.85	**9.07^{a}**
30 kg-N (Urea)	8334.85	4285.66	**6310.26^{b}**	4378.15	3447.01	**3912.58^{b}**	10.58	9.22	**9.90^{de}**
40 kg-N (LAN)	10644.74	8010.85	**9327.80^{e}**	3983.88	2240.51	**3112.19^{a}**	10.28	9.96	**10.12^{e}**
40 kg-N (Urea)	9585.72	5674.99	**7630.35^{c}**	4325.04	3290.57	**3807.81^{b}**	9.83	8.87	**9.34^{abc}**
Control + R	4202.03	5438.78	**4820.41^{a}**	3380.05	2335.18	**2857.62^{a}**	11.18	10.55	**10.86^{f}**
Control - R	8772.92	2327.75	**5550.34^{a}**	3453.36	3983.88	**3718.62^{b}**	10.55	8.54	**9.54^{abcd}**
Mean	**10042.32^{a}**	**5147.10^{a}**		**3982.66^{b}**	**3330.63^{a}**		**10.42^{b}**	**9.25^{a}**	
LSD cult x season			1696.06			654.73			0.67

Table 4.3. Effect of nitrogen formulations and application rates on total carbohydrates in soybean leaves assessed for rust severity.

Treatment	**Total Carbohydrates**		
	Season 1	**Season 2**	**Mean**
10 kg-N (LAN)	80.04	30.26	**24.68** [a]
10 kg-N (Urea)	32.81	23.41	**27.65** [a]
20 kg-N (LAN)	29.91	19.44	**52.04** [b]
20 kg-N (Urea)	30.33	24.96	**32.39** [a]
30 kg-N (LAN)	22.70	81.37	**83.01** [c]
30 kg-N (Urea)	30.70	34.07	**48.08** [b]
40 kg-N (LAN)	95.80	70.22	**28.11** [a]
40 kg-N (Urea)	56.00	40.17	**48.08** [b]
Control + R	34.37	19.44	**31.98** [a]
Control - R	32.43	31.54	**55.15** [b]
Mean	**44.51**	**37.49**	
LSD treatment x season			16.79

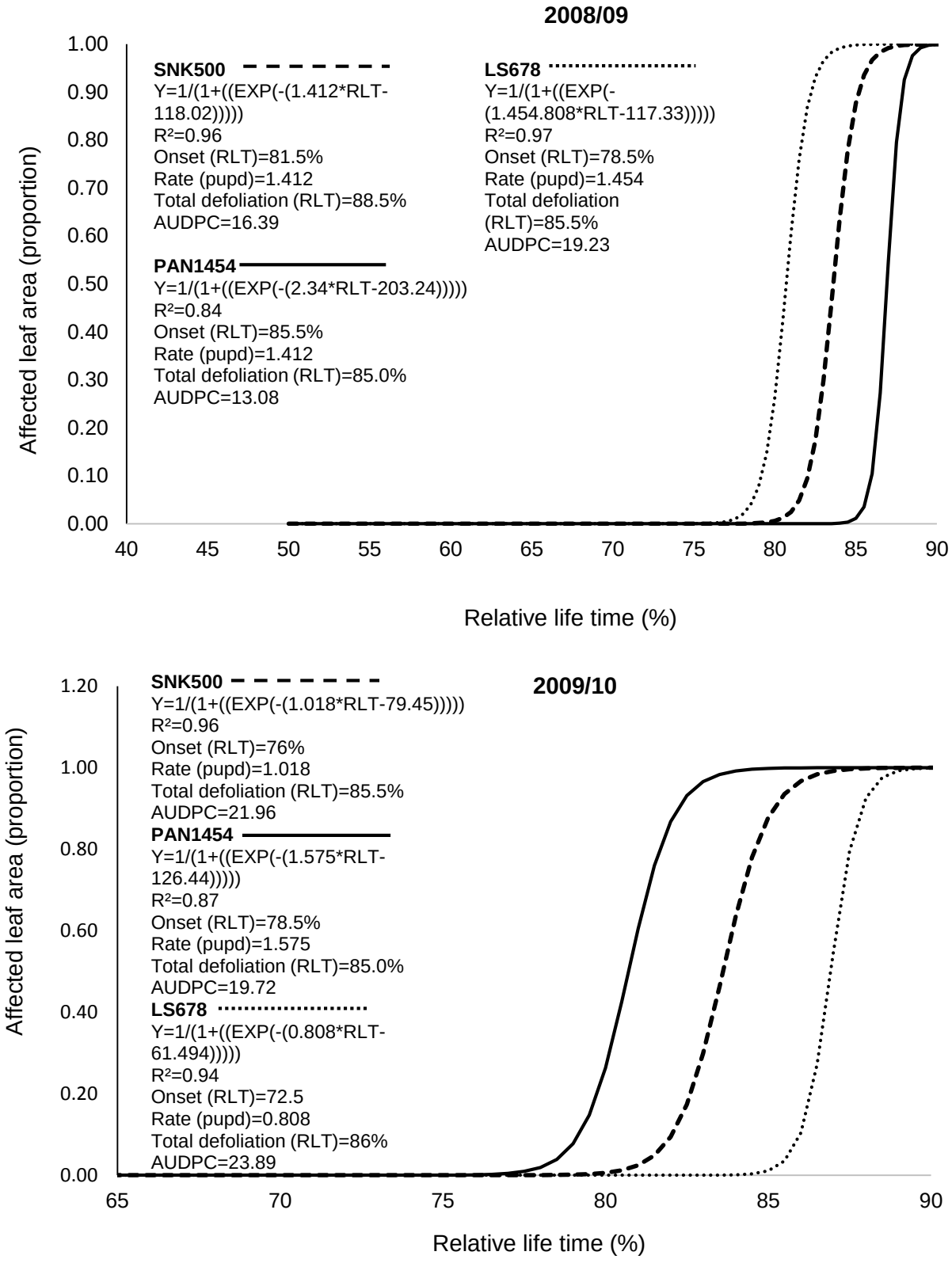

Figure 4.1. Rust disease progress curves in three cultivars grown at Cedara during 2008/09 and 2009/10, determined using the logistic probability model.

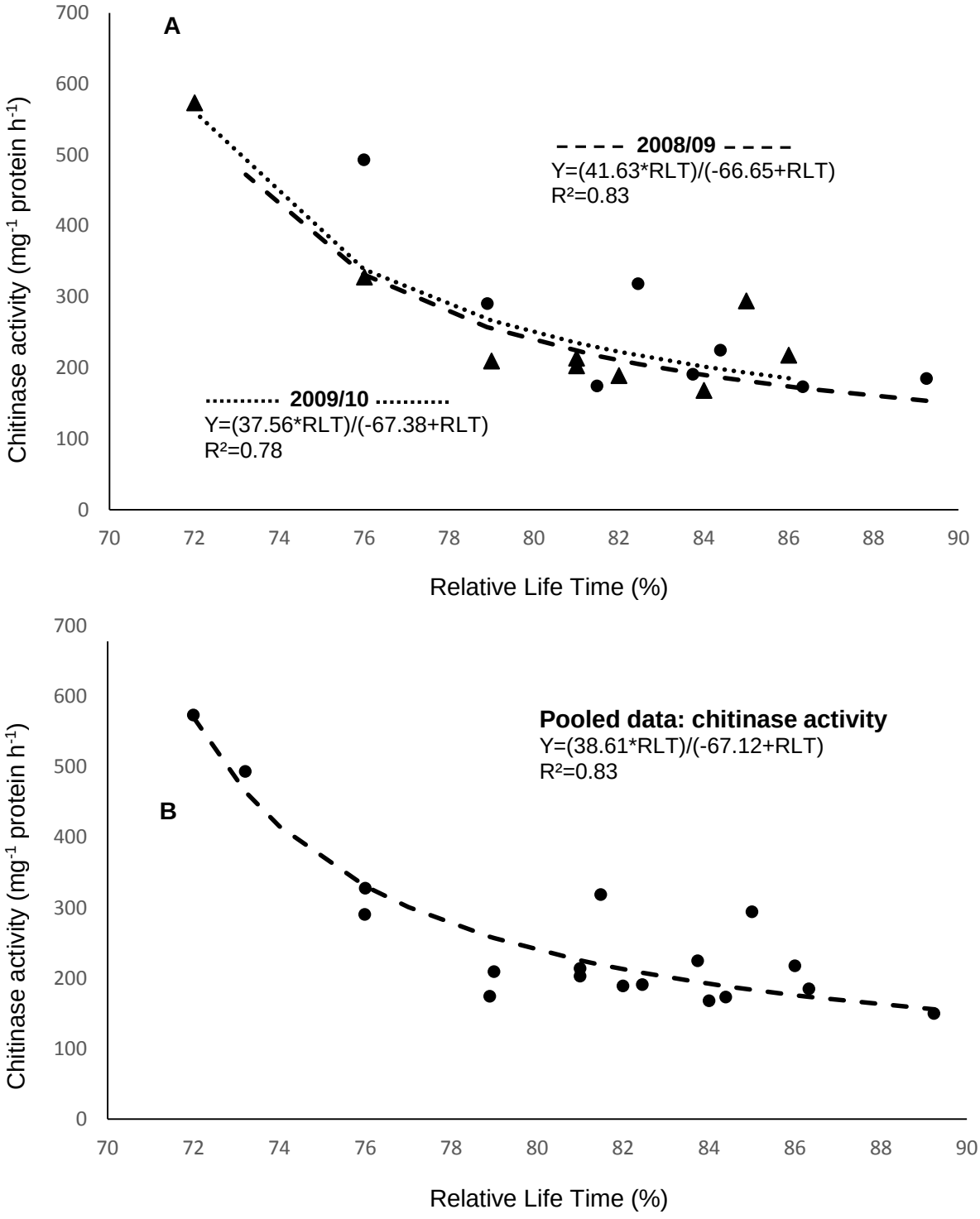

Figure 4.2. Chitinase activity relative to physiological growth stage expressed as Relative Growth Stage in three commercial cultivars planted during 2008/09 and 2009/10 planting season (A) and data pooled over both seasons (B).

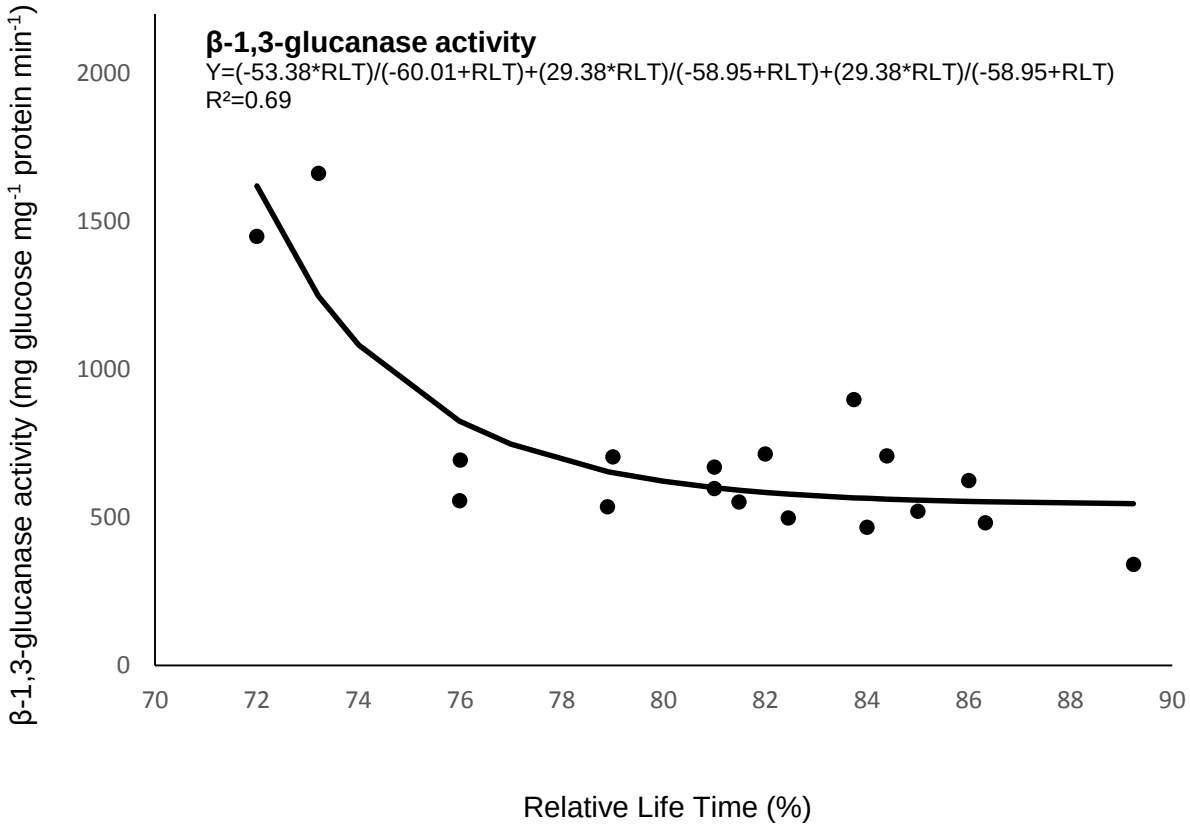

Figure 4.3. β-1,3-glucanase activity relative to physiological growth stage expressed as Relative Growth Stage in three commercial cultivars planted during 2008/09 and 2009/10 at Cedara – data pooled over both seasons.

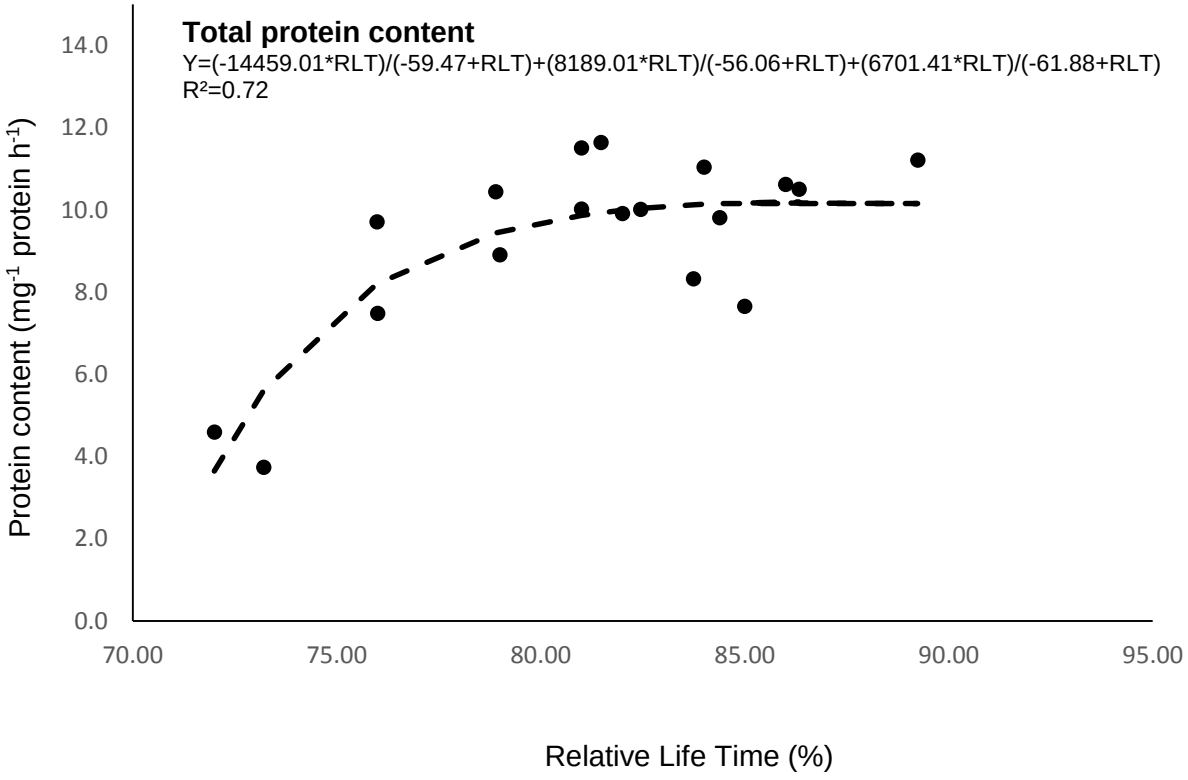

Figure 4.4. Leaf protein content relative to physiological growth stage expressed as Relative Growth Stage in three commercial cultivars planted during 2008/09 and 2009/10 at Cedara – data pooled over both seasons.

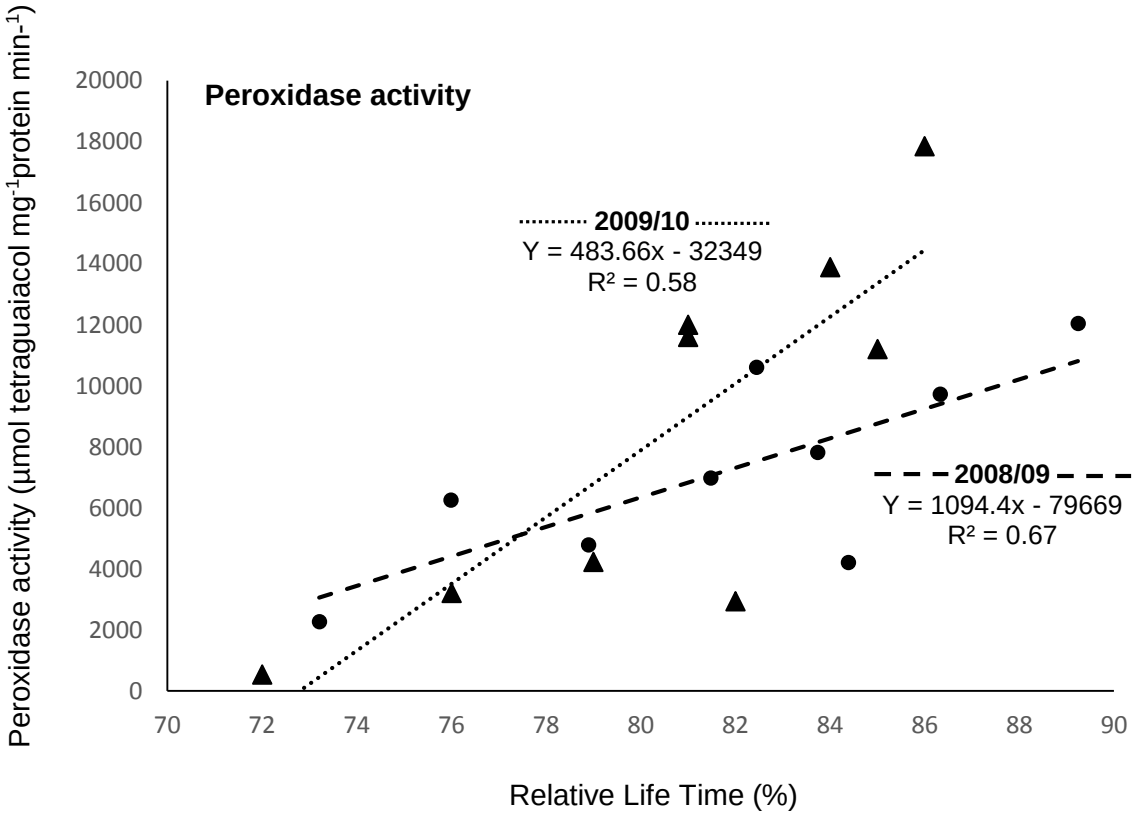

Figure 4.5. Peroxidase activity in three commercial cultivars planted during 2008/09 and 2009/10 planting season at Cedara.

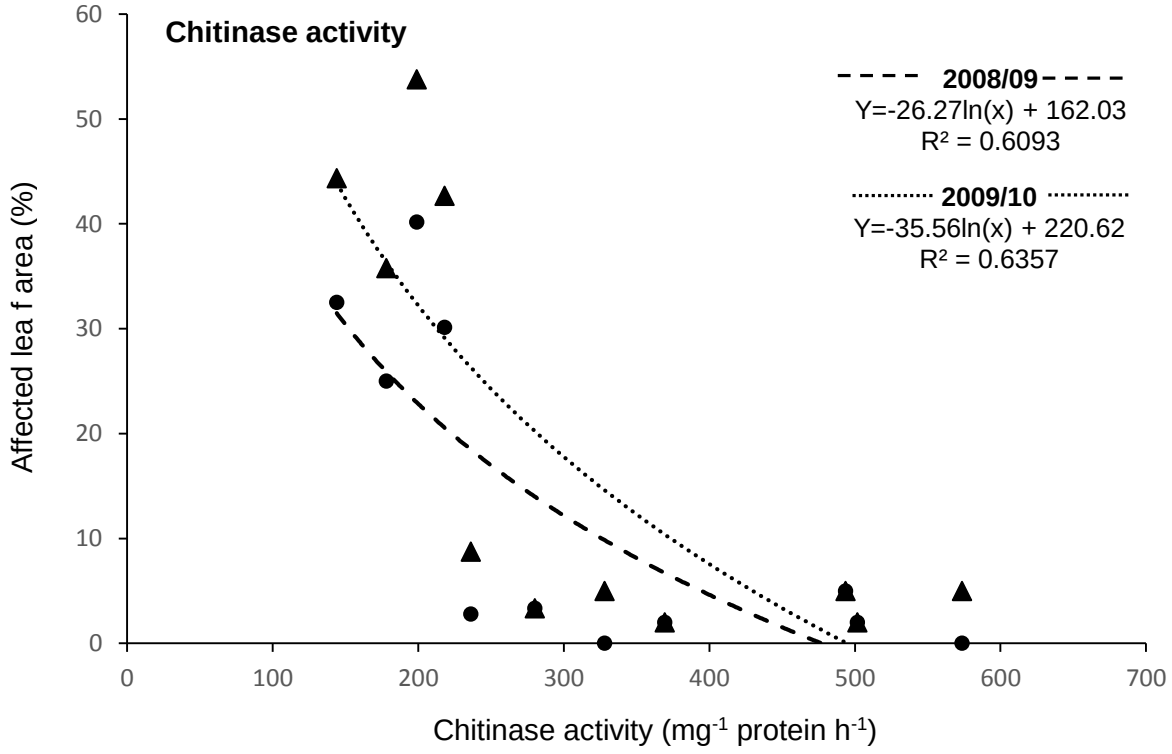

Figure 4.6. Relationship between chitinase and rust severity as indicated by affected leaf area in three commercial cultivars planted during 2008/09 and 2009/10 at Cedara.

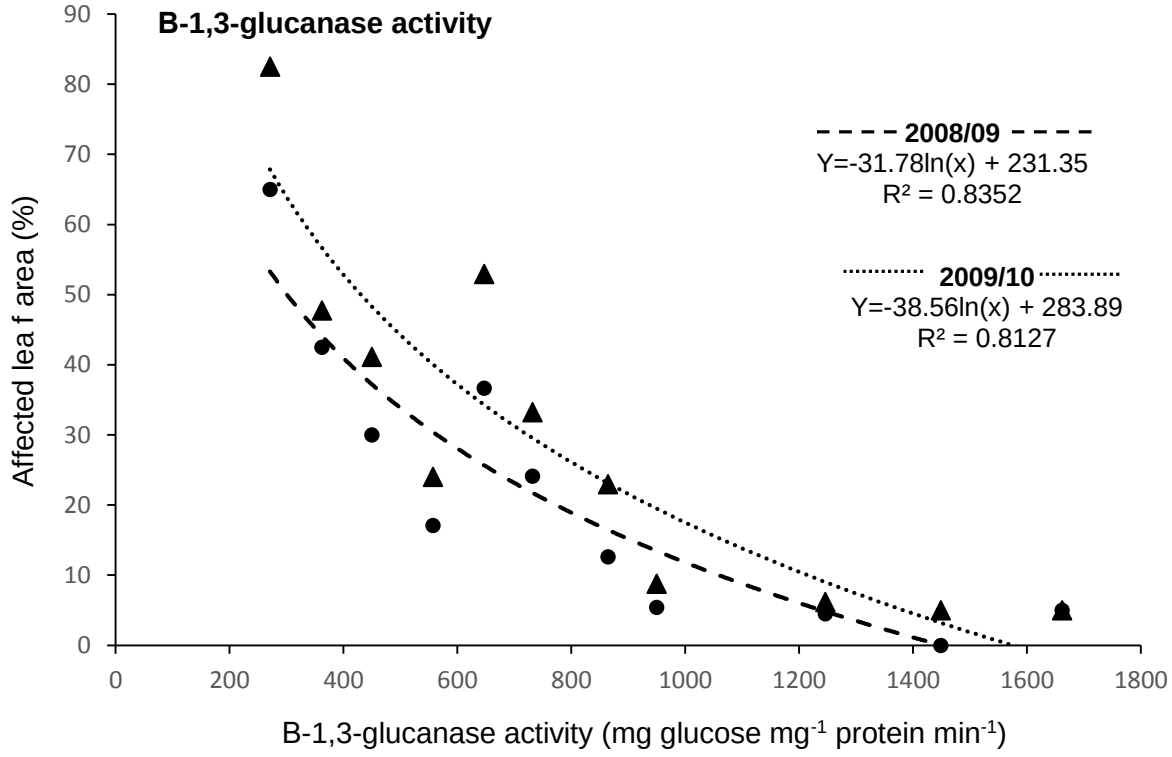

Figure 4.7. Relationship between β-1,3-glucanase activity and rust severity as indicated by affected leaf area in three commercial cultivars planted during 2008/09 and 2009/10 at Cedara.

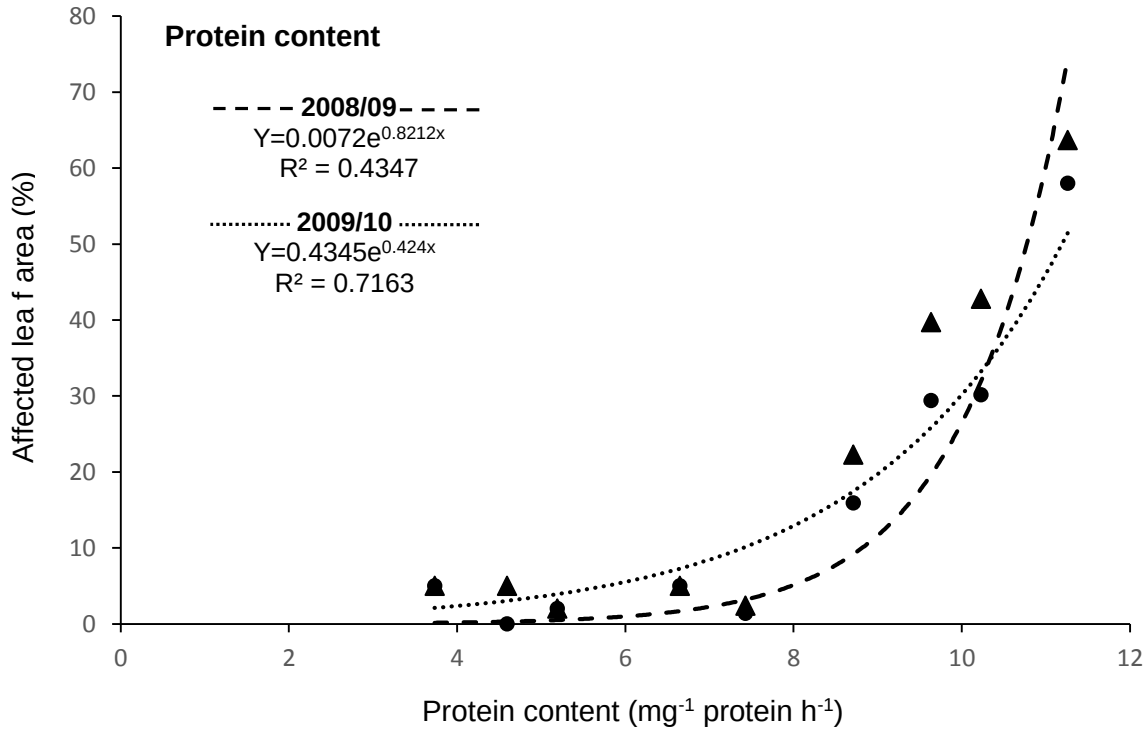

Figure 4.8. Relationship between protein and rust severity as indicated by affected leaf area in three commercial cultivars planted during 2008/09 and 2009/10 at Cedara.

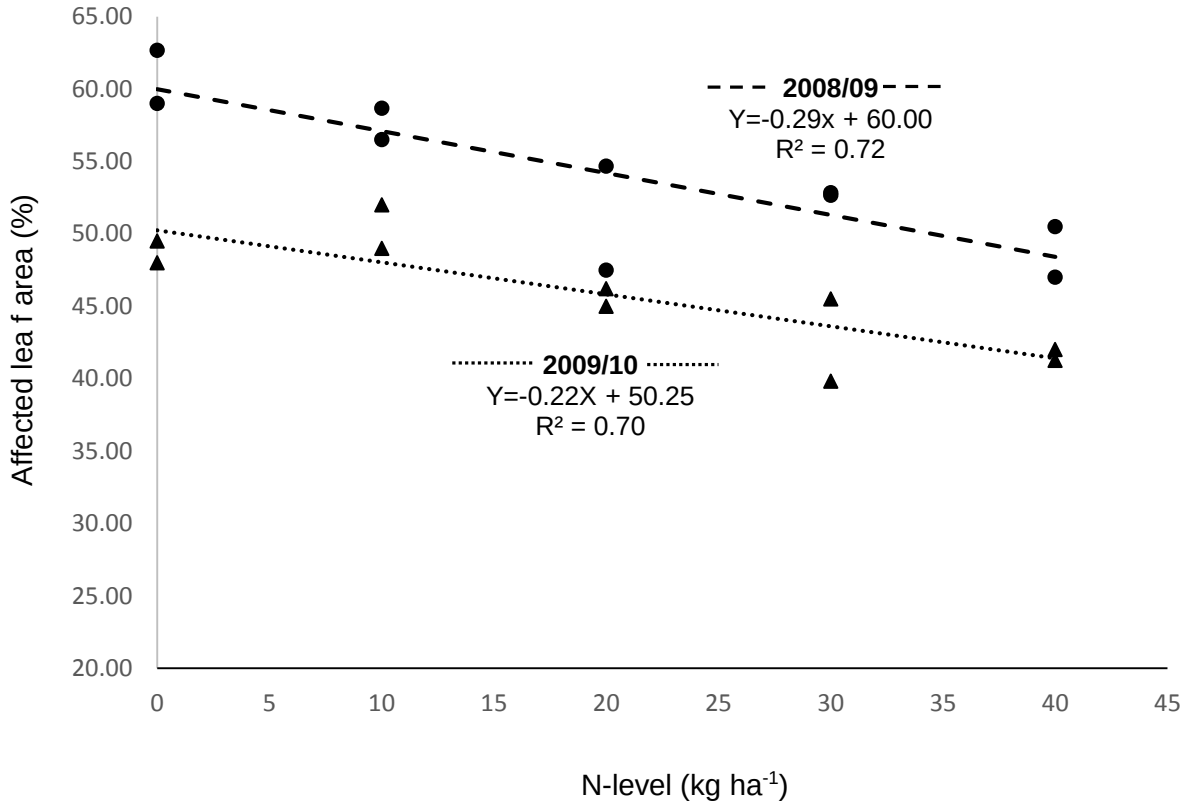

Figure 4.9: Relationship between nitrogen application rate and soybean rust severity in three soybean cultivars grown at Cedara in 2008/09 and 2009/10.

CHAPTER 5

EVALUATION OF SOYBEAN CULTIVARS UNDER FIELD AND GREENHOUSE CONDITIONS FOR RESISTANCE TO SCLEROTINIA STEM ROT IN SOUTH AFRICA

5.1 Abstract

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* is a widespread problem on soybeans. The development of resistant cultivars is still the most effective and economic control strategy for this disease. This study was done to evaluate differences in susceptibility of soybean cultivars to Sclerotinia stem rot in the greenhouse and under field conditions as well as the reassessment of a regression methodology applied to diseases such as sorghum ergot, Stenocarpella ear rot of maize and Sclerotinia stem rot. Soybean cultivars were planted in the greenhouse and grown to V3 growth stage. Plants were inoculated using a spray mycelium method and scored using leaf damage and wilting incidence as criteria. In field trials cultivars were evaluated for resistance at Bethlehem and Greytown, South Africa. Use was made of sequential plantings spaced from mid-November to late-December to create a range of environmental conditions during the early post-flowering period. Plots were inoculated at flowering using colonized sorghum grains and wilting was quantified at the R7 growth stage. Greenhouse evaluations indicated no significant differences between cultivars regarding scoring criteria of leaf damage and wilting incidence indicating that under these conditions all cultivars were susceptible to Sclerotinia stem rot. This may be due to high disease potentials and optimum disease development conditions that prevailed in the greenhouse. Field trials indicated that cultivar responses to the pathogen were poorly correlated over seasons and localities with only 11 of the 37 correlation coefficients proving significant. Cultivar response to changing disease potential was quantified using the regression model Y=AX^b where Y is the observed disease severity in the test cultivar and X is the disease potential within each planting quantified as the mean disease incidence over all cultivars within the respective planting. This model allowed the potential required to initiate disease to be determined as well as the response rate of the cultivar to changing disease potential to be qualified. Cultivar responses could be placed in three distinct response categories based on the regression b-parameter ie. those linearly related to changing disease potential, those with high susceptibility even at low disease potentials and cultivars with

various degrees of resistance to changing disease potentials. CRN 5550, LS 580 and PHB95B33 were the only cultivars that displayed any significant resistance to the disease, requiring potentials in excess of 20% to initiate disease, whereas most cultivars required a potential of less than 10% to initiate infection. The study emphasizes the fact that locally adapted cultivars still lack the desired level of resistance to Sclerotinia stem rot. However some cultivars may be rated as lower risk than others.

5.2 Introduction

Soybeans (*Glycine max* (L.). Merr.) are a significant source of income globally and the economic welfare of people can be severely affected by factors that reduce yields such as diseases, insects, weeds and adverse weather conditions (Wrather *et al*., 2001). Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is widespread on soybeans (Mueller *et al*., 2004) although its incidence may be sporadic, depending on environmental conditions during flowering ie. the critical growth stage of plant growth (Morral & Thompson, 1991). Losses due to this disease are substantial and from 1996 to 2007 the disease was ranked among the top ten diseases that suppress yields of soybeans in the USA (Wrather & Koenning, 2009). Collective annual losses due to Sclerotinia stem rot in the USA has been as high 70 million dollars for soybeans since 2002 according to the National Sclerotinia Initiative (2014). Losses result from reduced seed weight, a reduction in the number of pods, seeds per pod as well as reductions in seed oil and protein content (Danielson *et al*., 2004).

The increase in the incidence of Sclerotinia stem rot has been attributed to the expansion of soybean production into areas with a history of Sclerotinia stem rot (Boland & Hall, 1986) as well as changes in management practices such as narrower row spacing, higher plant populations and optimum fertilisation which increase canopy density and creating microclimate conditions that favour infection and disease development (Mueller *et al*., 2004). Development of resistant soybean cultivars appears to be a feasible, effective and economic management tool for the control of Sclerotinia stem rot (Boland & Hall, 1987; Lithourgidis *et al*., 2005; Mei *et al*., 2011) and cultivars with field resistance have become increasingly important in the management of Sclerotinia stem rot (Yang *et al*., 1999).

Numerous crop and pathogen variables contribute to the severity of Sclerotinia stem rot. Cultivar characteristics such as plant height, lodging, plant architecture, date of flowering and

maturity have been associated with disease escape but are difficult to evaluate in a controlled environment (Boland & Hall, 1987; Nelson *et al*., 1991). Disease severity associated with these host characteristics vary according to environmental conditions and could therefore influence the ranking of the disease reactions of genotypes to the pathogen (McLaren & Craven, 2008). Several authors have highlighted that laboratory, greenhouse and field screening results are not correlated and this has impeded the development of soybean cultivars with Sclerotinia stem rot resistance (Boland & Hall, 1987; Chun *et al*., 1987; Nelson et al., 1991; Pennypacker & Risius, 1999).

Variation in cultivar reactions under field conditions increases the difficulty of identifying sources of field resistance to Sclerotinia stem rot (Boland & Hall, 1987). This variation is exacerbated by Sclerotinia stem rot being highly seasonal, making field site selection that ensures consistent infection, a constraint that influences selection of resistant cultivars (Boland & Hall, 1986). Significant differences in disease ratings of cultivars across field environments as well as significant genotype x environment interactions suggest that results obtained at one locality or during one season cannot be extrapolated to another (Kim *et al.,* 2000; McLaren & Craven, 2008). These interactions emphasize the need to develop a reliable and economic inoculation method for testing and evaluating cultivars so as to aid a breeding effort, especially in a recurrent selection program (Boland & Hall, 1986). High genotype x environment interactions also limit the value of controlled environment screening methodologies (McLaren & Craven, 2008). Greenhouse and laboratory screening methods cannot detect disease escape which contributes to the poor correlation between field and greenhouse results (Pennypacker & Risius, 1999).

This study was conducted to evaluate differences in the susceptibility of soybean cultivars to Sclerotinia stem rot in the greenhouse and under field conditions as well as the reassessment of a regression methodology applied to sorghum ergot (McLaren, 1992), Stenocarpella ear rot of maize (Flett & McLaren, 1994) and sclerotinia stem rot of soybean (McLaren & Craven, 2008).

5.3.1 Greenhouse trials

5.3.1.1 Plant production

Cultivars from the National Soybean Cultivar trials, received from the ARC-Grain Crops Institute, Potchefstroom were evaluated in the greenhouse during 2009 and 2010 for resistance to Sclerotinia stem rot. Twenty-eight cultivars were planted in seed trays (containing 98, 10x4 cm seed cones) filled with a steam sterilized soil:peat in a 1:1 mixture. Two seeds per cone were planted with three cones per replicate. A complete randomized design with 3 replicates per entry was used. Seed trays were watered daily with tap water and maintained at a 25/20°C day/night regime with 14/10 hours light/dark cycle. After emergence plants were fertilized weekly with Multifeed P® soluble fertilizer for two weeks after which WonderSR 3:2:1 (28) was applied once a week until inoculation. Plants were grown to V 3 growth stage ie. three nodes on the main stem with fully unfolded leaflets, before inoculation.

5.3.1.2 Inoculum production and inoculation procedure

Sclerotia of *S. sclerotiorum,* collected in infected soybean fields in Greytown were stored in an airtight container at 4°C. Sclerotia were surface sterilized in 1% sodium hypochloride for 3 minutes and rinsed twice in sterile distilled water. Surface sterilized sclerotia were placed onto potato dextrose agar (PDA) and incubated for three days at 22°C. Pure growing colonies were sub-cultured after 2 days to water agar. Hyphal tips were then isolated after 1 to 2 days, using a dissecting microscope and transferred to PDA containing streptomycin (3ml/ℓ). Pure colonies were stored on agar slants in the cold room at 5°C for preservation. This mycelial culture was used as inoculum. Inoculum was subsequently prepared using the method of Chen & Wang (2005). Potato dextrose broth (PDB) was prepared by cutting 200g of unpeeled, washed potatoes into cubes and boiling these in 1 ℓ of water until soft (approximately 25 min). The potatoes were drained and water was added to make 1 ℓ. Dextrose (20 g) was added and shaken until dissolved. The suspension was autoclaved for 20 min at 121 kpa and left to cool. Three agar plugs were cut from the three day old colony of *S. sclerotiorum* using a sterile 5 mm cork borer and placed into each of five 0.5 ℓ Erlenmeyer

flask containing 200 ml of the broth. Flasks were placed in a shaker for 3 days at room temperature and the resulting fungal colonies were homogenized for 15–20 seconds using a Heidolph Silentcrusher M to ensure that mycelium was evenly suspended.

The mycelium suspension was placed in a hand spray bottle and the plants in the trays were sprayed with 150 ml of inoculum. Trays were subsequently placed in a plastic tent with small holes at the top to ensure optimum humidity for 9 days while allowing respiratory gas exchange (Botha *et al*., 2011).

5.3.1.3 Rating and data analysis

After nine days the plastic tents were removed and leaf damage on plants was assessed as 0=no visible symptoms on leaves; 1=leaves showing small symptoms (spots, browning of leaves and curling up); 2=Some leaves are yellow, large brown lesions visible or lesions visible moving downward on the stem; 3=infected leaves and petioles are brown, mycelium growth visible in some cases, lesions moved further down stems and leaves start to die; 4=90% of leaves are dead, curled up or shed. Leaves and apical meristem are wilted and lodging occurs. Mycelial growth may be visible; 5=all leaves are infected, dead or shed. Mycelial growth and sclerotia are visible. In some cases the whole plant is dead.

Wilting was assessed as follows; 0%=no wilting are visible; 10%=only infected leaves show signs of wilting; 30%=aerial plants parts are wilted (apical meristem and upper leaves); 50%= infected leaves and petioles are wilted; lesion extended downwards towards the middle of the stem; 80%=main stem and primary leaves are wilted; 100%=whole plant and main stem are wilted, leaves are dead or shed.

Data were analyzed using Number Cruncher Statistical Systems (NCSS) (Hintze, 2007). A general linear model (GLM) analysis of variance (ANOVA) was conducted and assessment means were separated using Fisher's LSD ($P<0.05$).

5.3.2 Field trials

5.3.2.1 Plant production

Cultivars from the National Soybean Cultivar Trials were evaluated in field trials at Bethlehem, Free State (GPS coordinates: -28.163°S, 28.297°E) over three growing seasons and a single growing season at Greytown, KwaZulu-Natal (GPS coordinates: -28.970°S, 30.379°E). Blocks of cultivars were sequentially planted in Bethlehem in mid- and late November and early December during the first two seasons with additional late December plantings in Bethlehem and Greytown during the third season to provide 14 diverse environments.

Cultivar plots consisted of four rows, 8 m in length with row spacing of 45 cm and a plant density equivalent to 360 000 plants ha^{-1}. Prior to planting plots were amended with 300 kg ha^{-1} super phosphate and 50 kg ha^{-1} KCl. During planting commercial rhizobium (*Bradyrhizobium japonicum*) was mixed with water (100 g /15 ℓ^{-1} water) and approximately 150 ml per furrow was applied at seeding.

5.3.2.2 Inoculum production and inoculation procedure

Sclerotia of *S. sclerotiorum,* collected in infected soybean fields in Greytown were stored in an airtight container at 4°C. Sclerotia were surface sterilized in 1% sodium hypochloride for 3 minutes and rinsed twice in sterile distilled water. Surface sterilized sclerotia were placed onto potato dextrose agar (PDA) and incubated for three days at 22°C. The resultant mycelial culture was used as inoculum. Glass flasks (500 ml) were filled with 400 ml sorghum grain and tap water was added to fill jars and allow the grain to soak for 24h. The water was then removed and 30 ml of modified Fries-Bosal medium was added (Chambers, 1987). Jars were autoclaved for 30 min periods at 121°C left to cool and autoclaved again after 24 h. After final autoclaving, jars were cooled and inoculated with a single mycelium plug of the actively growing colony. Jars were incubated at 22°C and shaken every two days to ensure proper colonization of the substrate. Flasks were left until the sorghum grains were fully colonized. Once colonized, grains were removed, air dried for five days at room temperature, milled and stored at 4°C until needed.

Twenty five grams of the prepared inoculum was distributed by hand over each test plot at flowering. A second application was made after 14 days.

5.3.2.3 Ratings and data analysis

Ratings of disease severity were conducted on the two centre rows of each plot just prior to beginning maturity (growth stage R7). Disease was quantified as the percentage of wilted plants per plot. Wilt data was analysed using the two factor-unreplicated analysis sub-routine in Excel 2013. Respective factors were planting/Sclerotinia potential and cultivars. Spearman rank correlation analysis was used to determine ranking of cultivars within each planting and over localities (NCSS, Hintze, 2007). Regression analysis was applied using the model Y=AX^b. Sclerotinia stem rot potential associated with each planting was used as the independent variable and the mean observed disease incidence within the test cultivar within a specific planting date as the dependent variable. A and b are regression parameters.

The Sclerotinia stem rot potential within each planting was determined as the mean disease severity over all cultivars. The disease potential required to initiate disease (arbitrary taken at 10% disease incidence) was determined from regression parameters of each cultivar. The rate of disease increase at the point of disease onset was determined from r=a*b*(Soybean disease potential$^{\wedge(b-1)}$). The calculation of the area under the disease potential curve (AUDPoC) served as an indication of the total host response to changing disease potentials. Area under the disease potential curve was calculated according to the model of Campbell & Madden (1990) where AUDPoC=Σ n-1$_i$=(t_i+1-t_i) [(y_i +y_i+1)/2] using Relative lifetime(RLT) as the independent variable and wilting incidence the dependent variable. Multiple regression analysis was used to determine the relationship between Sclerotinia stem rot potential and rate of response to changing potential after disease onset and AUDPoC.

5.4 Results

5.4.1 Greenhouse evaluations

The responses of cultivars to *S. sclerotiorum* in the greenhouse are shown in Table 5.1. No significant differences between leaf damage and wilting incidence for the 28 cultivars evaluated in the greenhouse during the two trials were recorded. Trial 1, mean values for

wilting incidence ranged from 16.67% to a high of 100.00%. Leaf damage values were the lowest for PAN 535 (1.67) followed by PAN 809 (1.96) while Dundee showed the highest leaf damage value (5.00). During evaluations in Trial 2, A 5409 RG had the lowest wilting incidence as well as leaf damage values, 26.16 and 2.00 respectively. LS 6150 R had the highest wilting incidence (93.33%) and leaf damage (4.67).

5.4.2 Field evaluations

Symptoms of Sclerotinia stem rot include watery spots or lesions on leaves and stems. These are usually followed by soft watery rot and in severy cases cottony mycelium grows over the affected tissues (Purdy, 1979). The lesions progress along and around the stem, restricting vascular flow and results in wilting of aerial plant parts. Severe infection weakens the plant which results in total wilting, lodging and eventually death (Peltier, 2012).

Analysis of variance (Table 5.2) indicated that cultivars evaluated at the different locality-plantings during the respective seasons differed significantly in their responses to Sclerotinia stem rot. PHB95B33 yielded the lowest overall mean Sclerotinia stem rot incidence with 2.05% wilted plants while Stork was the most susceptible at 33.70% wilted plants per plot (Table 5.3). Only 9 of the 37 cultivars showed less than 10% Sclerotinia stem rot per plot. Partitioning of sum of squares indicated that cultivar and locality-planting accounted for 9% and 70% of the variation in Sclerotinia stem rot potential respectively indicating that environment is the primary driving variable in the Sclerotinia stem rot epidemic. Responses over locality-plantings were not consistent as indicated by Spearman rank correlation with only 11 of 37 possible correlations being significant ($P<0.05$) (Table 5.4).

Application of regression parameters (Table 5.3) enabled the cultivar responses to changing disease potential to be quantified. The b-parameter indicates a response type where $b>1$ indicates a more stable response to increasing disease potential with an initial degree of resistance, $b<1$ indicates susceptibility of cultivars despite low disease potentials. A linear relationship between Sclerotinia stem rot potential and observed stem rot within cultivars is indicated by $b=1$. These responses are illustrated by selected cultivars in Figure 5.1. In the present study the b-parameter ranged from 0.62 in Dumela, indicating a highly susceptible response to 3.33 in LS 580 which indicates a more stable response despite changing disease potentials.

Rearranging the model parameters to X=EXP((ln(Y)-LN(A))/b) allows the calculation of the Sclerotinia stem rot potential required to induce disease onset in each cultivar. Onset was arbitrarily set at Y=10% and the disease potential required to induce this disease incidence was referred to as the Sclerotinia onset potential (McLaren & Craven 2008). In the current study this ranged from 3.63% in A 5409 RG to 50.80% in CRN 5550 (Table 5.3). Only 5 of the 37 cultivars had onset potentials greater than 20% suggesting that most cultivars are highly susceptible to Sclerotinia stem rot.

A similar rearrangement of the model, ie. $Y=AbX^{(b-1)}$ was used to calculate the rate of response to increases in disease potential at the point of disease onset. Cultivar CRN 5550 had a rate of 0.13 per increase in potential unit compared with cultivars A 5409 RG and PAN 535RR with rates of 1.85 and 1.80 per increase in potential unit respectively. Both disease onset and rate of increase at disease onset were significantly related to AUDPoC (Figure 5.2 and 5.3). AUDPoC was high in PAN 535RR (AUDPoC=1953) compared to CRN 550 (AUDPoC=275). Inclusion of these variables into a multiple regression analysis ie. $y=A+B_1X_1+B_2X_2+E$ where X_1=disease onset and X_2=rate, yielded the model AUDPoC=399.68-9.98(onset potential)+946.86(rate)(R^2=0.86). Model analysis of variance indicated only the rate parameter to be significant indicating the rate of response to changing disease potential to be a more critical disease variable of AUDPoC than onset potential. Mean wilting percentage and AUDPoC were significantly correlated (r=0.57).

5.5 Discussion

No sources of good and stable resistance to sclerotinia stem rot in soybeans have been reported. Similarly resistance to this pathogen remains elusive in many other economically important crops such as peas (Porter, 2011), canola (Bradley *et al*., 2006) and faba beans (Lithourgidis *et al*., 2005). Continuous evaluation of germplasm is needed to identify and improve resistance levels in available germplasm (Hoffman *et al*., 2002). Identification of resistance however is hampered by factors such as easy and reliable screening procedures and a minimum of genetic variability (Grafton, 1998).

This study failed to identify sources of resistance in the 28 cultivars evaluated in the greenhouse. This could be attributed to the high disease potentials associated with artificial inoculation in the greenhouse and optimum humidity provided in the greenhouse tents with

the result that under these conditions subtle differences in disease responses could not be detected. Greenhouse conditions appear to override the expression of disease escape usually associated with field evaluations. Although various studies have focused on developing a greenhouse inoculation method that has the necessary consistency and reliability to predict expected responses under field conditions, repeatability of resistance screening results under greenhouse conditions still remains a problem (Pennypacker & Risius, 1999, Vuong *et al*., 2004; Bradley *et al*., 2006).

The disparity between greenhouse and field trials is illustrated by the response of cultivars to inoculation with *S. sclerotiorum*. In the greenhouse, LS 6050 R proved to be highly susceptible with mean ratings of 68.33% wilting incidence and 3.67 leaf damage while in the field, this was one of the best performing cultivars. In contrast, A 5409 RG showed low wilting incidence as well as leaf damage in the greenhouse, however in the field this cultivar was highly susceptible with onset potential and rate of increase subsequent to disease onset of 3.63% and 1.85%. A strong correlation between field and controlled environment studies is desired as the main use of an artificial screening method is to identify germplasm that will perform similarly in the field as in the greenhouse and this was clearly not evident in the current study (Nelson *et al*., 1991).

Evaluating plants under field conditions remains a more reliable indicator of disease response however, it is not always possible to ensure even exposure of the plants to the pathogen and thus get uniform infection (Davar *et al*., 2011). According to Falkhof *et al*. (1988) sufficient inoculum or inoculum density is needed before plant defense responses are activated and resistance is observed. Although cultivar resistance has been used to reduce the risk of Sclerotinia stem rot, Mueller *et al*. (2002) found that the response of germplasm changes with inoculum potential. The ranking of cultivars over seasons and localities was inconsistent in the current study and indicates that cultivars react differently to changing environmental conditions and disease potentials. Similar results were reported by Boland & Hall (1987) who, in a cultivar resistance study, found high variation between trials where cultivars ranged from relatively resistant in one year to highly susceptible during the second year of evaluation. Differences in ranking may be attributed to differential interactions of host genotypes with environmental variables and inoculum potential (McLaren & Craven, 2008).

Kim & Diers (2000) reported highly significant genotype x year and genotype x locality interactions and emphasized the need to include an environmental factor into the quantification of resistance to the disease. In the current study, most cultivars yielded a b-parameter < 1 indicating poor stability in their response to *S. sclerotiorum* as disease potential increased. Similarly, most cultivars had an onset potential of less than 10% indicating that the majority of the cultivars can become infected even under sub-optimal disease-favourable conditions. Only cultivars CRN 5550, LS 580 and PHB95B33 had onset potentials that exceeded 20%, suggesting that these cultivars had some degree of disease resistance that could contribute to risk reduction and reduced disease. However, the high response rate of 1.11% per potential unit subsequent to disease onset indicates that this resistance cannot be relied upon as the sole disease control strategy, particularly in LS 580. This is reflected in AUDPoC's where, despite similar onset potentials, the AUDPoC in LS 580 was higher than PHB95B33. This also illustrates the importance of multiple criteria when screening cultivars rather than relying solely on a single evaluation at a single growth stage (McLaren & Craven, 2008).

Resistance is controlled by several genes and the use of avoidance mechanisms such as upright and open plant structure, less dense canopies and branching patterns, elevated pod set and reduced lodging have been suggested to reduce the damage by *S. sclerotiorum* (Grafton, 1998). Wegulo *et al.* (1998) tested ten screening methods in the field and greenhouse and attributed the poor correlations between methods to differences in the defense and disease escape mechanisms in cultivars. Factors such as cultivar height, lodging severity, maturity, temperature and relative humidity are important in spore survival and infection. Environmental and genetic variability may explain variation in disease pressure over locations and different planting seasons (Yang *et al*., 1999; Bradley *et al*., 2006) again emphasizing that these two aspects cannot be considered in isolation. Results obtained in these and the current study emphasizes the difficulties associated with identifying and selecting stable, resistant cultivars. These data also illustrate that a resistant reaction at one locality cannot be extrapolated to another. According to Bradley *et al*. (2006) disease pressure that differs in the field may also lead to misinterpretation of results. According to Wegulu *et al*. (1998) differences in cultivars may be due to differences in the defense mechanisms among germplasm evaluated. These differences in defense strategies among cultivars may be due to environmental conditions prevailing or the method of resistance evaluation employed. The goal of breeding for resistance should be to select cultivars with

high resistance to all forms of *S. sclerotiorum* attack found in the region of cultivation (Castano *et al*., 1993).

The above results also imply that resistance only provides a limited degree of Sclerotina stem rot control and cannot be depended on solely to reduce the risk of the disease. It is thus essential that the use of lower risk varieties be integrated with broader crop management practices in a systems approach to disease reduction *(*Kranz & Hau, 1980). Numerous cultural and chemical control strategies have been proposed for Sclerotinia stem rot including crop rotation (Steadman, 1983), tillage practices (Tu, 1986, 1997), row spacing (Steadman, 1979), sanitation (Tu, 1989), weed control (Philips, 1992) and chemical control (Ferreira & Boley, 1992; Mueller *et al.,* 2004; Tu, 1989, 1997). It is evident from the large volume of literature on *S. sclerotiorum* that adaptations to crop production systems can be used to reduce the disease potential and enhance the stability and effectiveness of the limited resistance in soybean varieties.

The present study indicates that locally adapted cultivars still lack the desired level of resistance to Sclerotinia stem rot and stability to changing disease potentials. The study also emphasizes the need to consider multiple evaluations in order to determine the expected response of a cultivar under different conditions associated with locality and season. The regression methodology applied in this study (McLaren, 1992; Flett & McLaren, 1998; McLaren & Craven, 2008) provides a useful technique to quantify cultivar stability and this is supported by the high R^2 values (Table 5.3) that indicate that cultivar response to disease potential can be accurately modeled. However, the methodology requires at least five diverse epidemics in order to conduct the regression analyses and can be labour intensive.

5.6 References

Boland, G.J. & Hall, R. 1986. Growth room evaluation of soybean cultivars for resistance to *Sclerotinia sclerotiorum*. Canadian Journal of Plant Science 66: 559-564.

Boland, G.L. & Hall, R. 1987. Evaluating soybean cultivars for resistance to *Sclerotinina sclerotiorum* under field conditions. Plant Disease 71: 934-936.

Botha, C., McLaren, N.W. & Swart, W.J. 2011. The effect of temperature and period of high humidity on the development of Sclerotinia stem rot on soybeans in the greenhouse. South African Journal of Plant and Soil 28: 208-211.

Bradley, C.A., Henson, R.A., Portor, P.M., LeGare, D.G., Del Rio, L. E. & Khot, S.D. 2006. Response of canola to *Sclerotinia sclerotiorum* in controlled and field environment. Plant Disease 90: 215-219.

Campbell, C.L. & Madden, L.V., 1990. Introduction to plant disease epidemiology. John Wiley and Sons, New York.

Castano, F., Vear, F. & Tourvieille de Labrouhe, D. 1993: Resistance of sunflower lines to various forms of attack by *Sclerotinia sclerotiorum* and relations with some morphological characters. Euphytica 68: 85-98.

Chambers, K.R. 1987. Ability of fungal isolates from maize and sorghum to infect roots and reduce seedling emergence of two maize hybrids. Plant Disease 71: 736-739.

Chen, Y. & Wang, D. 2005. Two convenient methods to evaluate soybean for resistance to *Sclerotinia sclerotiorum*. Plant Disease 89: 1268-1272.

Chun, D., Kao, L.B., & Lockwood, J.L. 1987. Laboratory and field assessment of resistance in soybean to stem rot caused by *Sclerotinia sclerotiorum*. Plant Disease 71: 881-815.

Danielson, G.A., Nelson, B.D. & Helms, T.C. 2004. Effect of Sclerotinia stem rot in yield of soybean inoculated at different growth stages. Plant Disease 88: 297-300.

Davar, R., Darvishzadeh, R. & Majd, A. 2011. Genotype-isolate interaction for resistance to *Sclerotinia sclerotiorum* in sunflower. Phytopathologia Mediterranea 50: 442-449.

Falkhof, A.G., Dehne, H.W. & Schonbeck F. 1988. Dependence of the effectiveness of induced resistance on environmental conditions. Journal of Phytopathology 123:311-321.

Ferreira, S.A. & Boley, R.A. 1992. *Sclerotinia sclerotiorum.* www.extento.hawaii.edu/kbase/crop/Type/s_scler.htm. Accessed: April 2005

Flett, B.C. & McLaren, N.W. 1994. Optimum disease potential for evaluating *Stenocarpella maydis* ear rot resistance in corn hybrids. Plant Disease 71: 736-739.

Grafton, K.F. 1998. Resistance to white mold in dry bean. Proceedings of the Sclerotinia workshop 1998.

Hintze, J.L., 2007. Number cruncher statistical systems (NCSS). Kaysville, UT: Number Cruncher Statistical Systems.

Hoffman, D.D., Diers, B.W., Hartman, G.L., Nickell, C.D., Nelson, R.L., Pederson, W.L., Cober, E.R., Graef, G.L., Steadman, J.R., Nelson, B.D., Del Rio, L.E., Helms, T., Anderson T., Poysa, V. Rajcan, I. & Stienstra, W.C. 2002. Selected soybean plant introductions with partial resistance to *Sclerotinia sclerotiorum*. Plant Disease 86: 971-980.

Kim, H.S. & Diers, B.W. 2000. Inheritance of partial resistance to Sclerotinia stem rot in soybean. Crop Science 40: 55-61.

Kim, H.S., Hartman, G.L., Manadhar, J.B., Graef, G.L., Steadman, J.R. & Diers, B.W., 2000. Reaction of soybean cultivars to Sclerotinia stem rot in field, greenhouse and laboratory evaluations. Crop Science 40: 665–669.

Kranz, J. & Hau, B. 1980. Systems analysis in epidemiology. Annual Review of Phytopathology 18:67-83.

Lithourgidis, A.S., Roupakias, D.G. & Damalas, C.A. 2004. Inheritance of resistance to sclerotinia stem rot (*Sclerotinia trifoliorum*) in faba beans (*Vicia faba* L.). Field Crops Research 91: 125-130.

McLaren, N.W., 1992. Quantifying resistance of sorghum genotypes to the sugary disease pathogen (*Claviceps africana*). Plant Disease. 76, 986-988.

McLaren, N.W. & Craven, M. 2008. Evaluation of soybean cultivars for resistance to Sclerotinia stalk rot in South Africa. Crop Protection 27: 231-235.

Mei, L., Qian, L., Disi, J.O., Yand, X., Li, Q., Li, J., Frauen, D., Cai, D. & Qian, W. 2011. Identification of resistant sources against *Sclerotinia sclerotiorum* in Brassica species with emphasis on *B. oleracea*. Euphytica 177: 393-399.

Morrall, R.A.A. & J.R. Thomson. 1991. Petal test manual for *Sclerotinia* in canola. University of Saskatchewan, Saskatoon, SK.

Mueller, D.S., Bradley, C.A., Grau, C.R., Gaska, J.M., Kurle, J.E, & Pederson, W.L. 2004. Application of thiophanate-methyl at different host growth stages for management of Sclerotinia stem rot in soybean. Crop Protection 23: 983-988.

Mueller, D.S., Dorrance, A.E., Derksen, R.C., Ozkan, E., Kurle, J.E., Grau, C.R., Gaska, J.M., Hartman, G.K., Bradley, C.A. & Pedersen, W.L. 2002. Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. Plant Disease 86: 26-31.

Nelson, B.D., Helms, T.C. & Olson, M.A. 1991. Comparison of laboratory and field evaluations of resistance in soybean to *Sclerotinia sclerotiorum*. Plant Disease 75: 662-665.

Peltier, A.J., Bradley, C.A., Chilvers, M.I., Malvick, D.K., Mueller, D.S., Wise, K.A. & Esker, P.D. 2012. Biology, yield loss and control of sclerotinia stem Rot of soybean. *Journal of Integrated Pest Management* **3**:1-7.

Pennypacker, B.W. & Risius, M.L. 1999. Environmental sensitivity of soybean cultivar response to *Sclerotinia sclerotiorum*. Phytopathology 89: 619-622.

Phillips, A.J.L. 1992. Some common weed species as alternative hosts for *Sclerotinia sclerotiorum*. Phytophylactica 24: 207-210.

Porter, L. 2011. Selection of pea genotypes with partial resistance to *Sclerotinia sclerotiorum* across a wide range of temperatures and periods of high relative humidity. Euphytica. DOI 10.1007/s10681-011-0531-x.

Purdy, L.H. 1979. *Sclerotinia sclerotiorum*: History, disease and symptomatology, host range, geographic distribution and impact. Phytopathology 69: 875-880.

Steadman, J.R. 1979. Control of plant diseases caused by *Sclerotinia* species. Phytopathology 69: 904-907.

Steadman, J.R. 1983. White mold – A serious yield limiting disease of bean. Plant Disease 67: 346-350.

Tu, J.C. 1986. Integrated disease control of white mold (*Sclerotinia sclerotiorum*) in navy bean (*Phaseolus vulgaris*). International Symposium of Crop Protection 39: 731-740.

Tu, J.C. 1989. Management of white mold of white beans in Ontario. Plant Disease 73: 281-285.

Tu, J.C. 1997. An integrated control of white mold (*Sclerotinia sclerotiorum*) of beans, with emphasis on recent advances in biological control. Botanical Bulletin of Academia Sinica 38:73-76.

Vuong, T.D., Hoffman, D.D., Diers, B. W., Miller , J.F., Steadman, J. R. & Hartman, G.H. 2004. Evaluation of soybean, drybean and sunflower for resistance to *Sclerotinia sclerotiorum*. Crop Science 44: 777-783.

Wegulo, S.N., Yang, X.B. & Martinson, C.A. 1998. Soybean responses to *Sclerotinia sclerotiorum* in field and controlled environment studies. Plant Disease 82:1264-1270.

Wrather, J.A., Anderson, T.R., Arsyad, D.M., Tan, Y., Ploper, L.D., Porta-Puglia, A., Ram, H.H. & Yorinori, J.T. 2001. Soybean disease loss estimates for the top ten soybean-producing countries in 1998. Canadian Journal of Plant Pathology 23: 115-121.

Wrather, J.A. & Koenning, S.R. 2009. Effects of diseases on soybean trials in the United States in 1996 to 2007. Online. Plant Health Progress. DOI:10.1094/PHP-2009-0401-01-RS.

Yang, X.B., Lundeen, P. & Uphoff, M.D. 1999. Soybean varietal response and yield loss caused by *Sclerotinia sclerotiorum*. Plant Disease 83: 456-461.

Table 5.1. Wilting incidence (%) and leaf damage ratings in soybean cultivars evaluated for resistance to Sclerotinia stem rot in the greenhouse.

Line	Wilting incidence		Mean	Leaf Damage		Mean
	Trial 1	Trial 2		Trial 1	Trial 2	
LEX 1235R	93.33	88.30	90.82	4.67	4.45	4.56
Egret	83.33	86.67	85.00	4.33	4.33	4.33
PHB 96B01	100.00	70.00	85.00	5.00	3.67	4.33
PHB 95B53	100.00	66.67	83.33	5.00	3.67	4.33
LS 6150R	70.00	93.33	81.67	4.00	4.67	4.33
PAN 1666R	80.00	70.00	75.00	4.00	3.67	3.83
ISIS 2000	70.00	78.30	74.15	3.67	3.95	3.81
LS 6164R	63.30	83.33	73.32	3.45	4.33	3.89
Dundee	100.00	40.54	70.27	5.00	3.03	4.01
Heron	83.33	56.67	70.00	4.33	3.33	3.83
LS 6162R	53.33	86.67	70.00	3.00	4.33	3.67
LS 6050R	60.00	76.67	68.33	3.33	4.00	3.67
LS 6161R	60.00	76.67	68.33	3.33	4.00	3.67
PAN 1664R	76.67	60.00	68.33	4.00	3.33	3.67
PAN 1867	76.67	60.00	68.33	4.00	3.33	3.67
PAN 1652	53.33	80.00	66.67	3.33	4.00	3.67
LEX 2257R	76.67	53.30	64.98	4.00	2.95	3.47
LS 678	50.54	75.54	63.04	3.03	4.03	3.53
PAN 660	36.67	76.67	56.67	2.33	4.00	3.17
LEX 1233R	83.33	26.67	55.00	4.33	2.00	3.17
PAN 809	20.00	86.67	53.33	1.96	4.33	3.14
PAN 1583R	23.33	76.67	50.00	2.33	4.00	3.17
PAN 626	20.00	76.67	48.33	2.00	4.00	3.00
PAN 535	16.67	78.92	47.79	1.67	3.94	2.80
PAN 1454R	20.00	73.30	46.65	2.00	3.95	2.97
LEX 2685R	33.33	50.00	41.67	2.67	3.00	2.83
A 5409 RG	50.54	26.16	38.35	2.53	2.00	2.28
PAN 737R	30.00	43.33	36.67	2.33	2.67	2.50
Mean	60.16	68.49	64.32	3.42	3.68	3.55
LSD (P<0.05)			30.58			1.16

Table 5.2. Analysis of variance of cultivars evaluated at the different locality-plantings during the respective planting seasons.

Sources	Degree of Freedom	Sum of Squares	Mean Sum of Squares	F-Ratio	Prob Level	Percentage of Sum of Squares
A: Locality	13	77612.94	5970.226	100.54	0.000000*	70.28330497
B: Cultivar	36	10729.88	298.0522	5.02	0.000000*	9.71656824
Residual	330	19595.41	59.3800			
Total (Adjusted)	379	110428.70				
Total	380					
* Term significant at alpha = 0.05		LSD cultivar = 2.91				

Table 5.3. Cultivars evaluated and parameters calculated for the relationship between Sclerotinia stem rot potential and observed disease incidence in the field.

Cultivar	Sclerotinia stem rot incidence (%)		A-parameter	b-parameter	R^2	Soybean disease potential	Rate of break-down	Area under sclerotinia potential curve
Stork	33.70	r	1.06	1.11	0.91	7.61	1.46	1747.46
AG 5603	26.53	q	1.88	0.80	0.98	8.10	0.99	1099.46
Dumela	26.22	q	4.04	0.62	0.81	4.33	1.43	1299.36
AG 6101	22.37	pq	0.12	1.60	0.85	15.68	1.02	1114.20
A 5409 RG	21.48	op	4.20	0.67	0.91	3.63	1.85	1611.78
LS 555	19.66	nop	0.70	1.23	0.83	8.62	1.43	1785.56
Wenner	18.67	mnop	0.82	1.16	0.85	8.60	1.35	1631.61
LS 677	18.66	mnop	1.76	0.92	0.96	6.64	1.38	1529.60
SNK 500	18.05	lmnop	2.05	0.87	0.92	6.19	1.40	1514.44
Marula	17.87	lmnop	1.49	0.97	0.94	7.13	1.36	1539.79
PAN 660	17.40	klmnop	1.13	1.04	0.91	8.15	1.28	1481.73
LS 444	17.18	klmnop	1.79	0.74	0.87	10.14	0.73	876.11
SNK 440	16.96	klmnop	0.57	1.29	0.99	9.12	1.42	1797.23
LS 678	16.45	jklmno	0.63	1.21	0.93	9.78	1.24	1491.18
Sonop	16.06	ijklmno	1.95	0.84	0.88	7.07	1.18	1287.05
PAN 535RR	15.90	hijklmno	2.23	0.92	0.85	5.10	1.80	1953.07
PAN 809	15.89	hijklmno	0.96	0.90	0.91	13.62	0.66	779.13
SCS 1	14.78	ghijklmn	0.13	1.46	0.94	19.02	0.77	760.40
Knap	14.51	fghijklmn	0.22	1.49	0.89	13.14	1.13	1334.60
LS 666	14.05	fghijklmn	0.98	1.02	0.91	9.79	1.04	1195.40
Highveld Top	13.75	fghijklm	0.23	1.43	0.83	14.02	1.02	1152.15
PAN 421RR	13.49	efghijklm	1.58	0.85	0.89	8.67	0.99	1109.59
PAN 626	12.38	defghijkl	0.15	1.56	0.94	14.59	1.07	1216.21
Tamboti	11.71	defghijk	0.19	1.36	0.92	18.57	0.73	743.73
LS 580	11.20	cdefghij	0.00	3.33	0.97	30.01	1.11	596.16
LS6050R	10.61	cdefghi	3.82	0.65	0.79	4.40	1.47	1359.24
Maruti	10.23	cdefgh	0.05	1.87	0.92	17.96	1.04	1075.60
PAN1643RR	10.12	cdefg	1.00	1.11	0.96	7.94	1.40	1683.15
Prima 2000	9.05	bcdef	0.52	0.92	0.93	24.63	0.38	459.80
PAN 522RR	8.95	bcdef	1.46	0.74	0.87	13.34	0.56	707.57
AG 5601	7.88	bcde	0.01	2.15	0.96	23.31	0.92	746.66
PAN 538RR	7.11	abcd	0.57	1.13	0.92	12.75	0.88	1004.40
PAN 737RR	6.80	abcd	1.02	0.90	0.80	12.76	0.70	826.14
LS6162R	6.67	abcd	0.24	1.48	0.93	12.66	1.17	1396.69
CRN 5550	5.53	abc	0.73	0.67	0.83	50.80	0.13	274.96
Mukwa	4.40	ab	0.40	1.18	0.96	15.65	0.75	832.45
PHB 95B33	2.05	a	0.20	1.13	0.87	31.60	0.36	360.96

Table 5.4: Spearman Correlations (excluding 0-potentials) between plantings of 37 soybean cultivars at different localities and seasons.

Potential	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14
4	1.00	0.56*	-0.13	0.07	0.26	0.17	0.15	0.25	0.32*	0.31	0.30
5		1.00	-0.04	0.24	0.23	0.21	0.42*	0.08	0.03	-0.14	-0.13
6			1.00	0.44*	0.26	-0.17	0.20	0.31	0.33*	0.12	0.16
7				1.00	0.45*	0.01	0.66*	0.63*	-0.03	0.27	0.10
8					1.00	0.08	0.77*	0.59*	0.02	0.50*	0.33*
9						1.00	0.12	0.03	0.16	-0.28	-0.35*
10							1.00	0.73*	0.10	0.46*	0.22
11								1.00	0.25	0.71*	0.57*
12									1.00	0.38*	0.16
13										1.00	0.80*
14											1.00

*Significant (P>0.05)

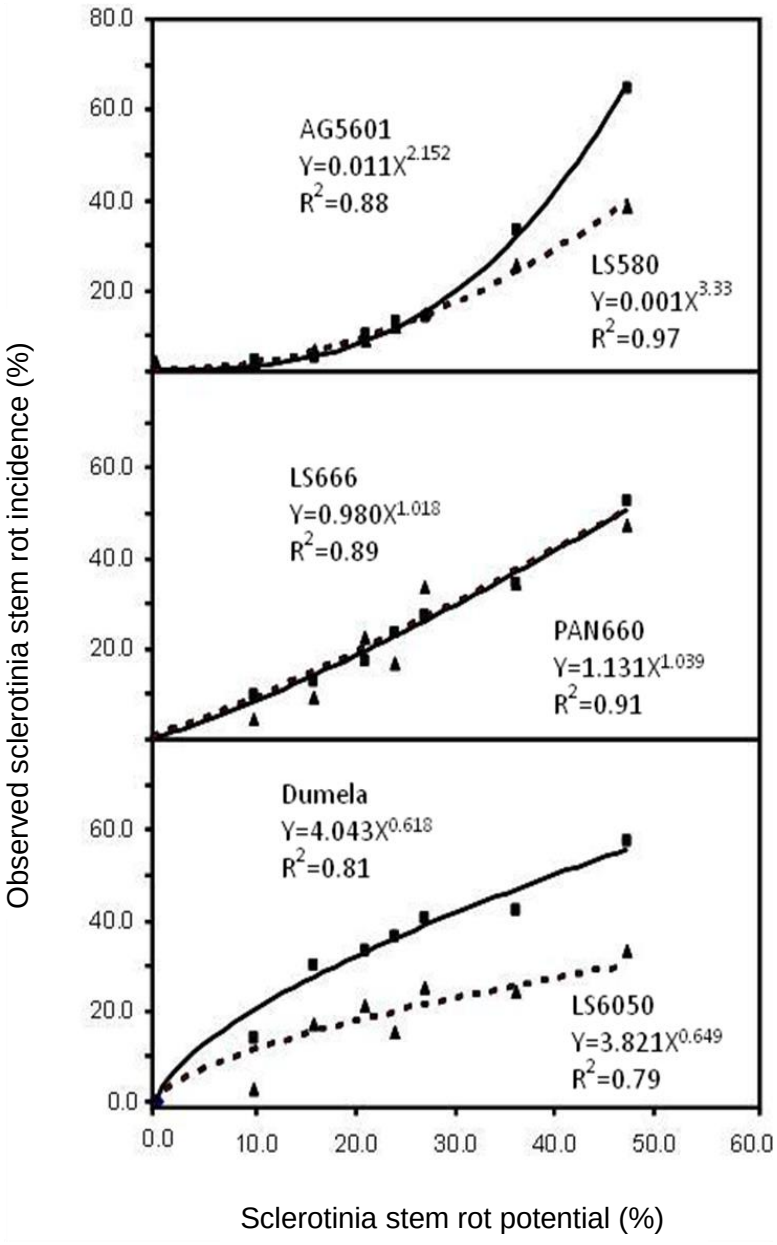

Figure 5.1. Sclerotinia stem rot potential and observed Sclerotinia stem rot incidence in evaluated cultivars indicating the three most distinct disease responses to changing disease potential.

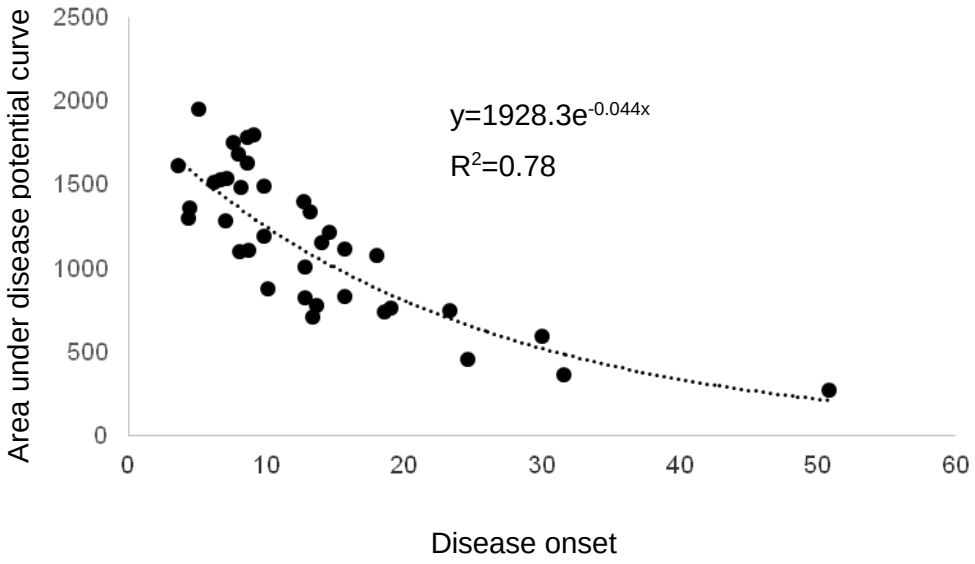

Figure 5.2. Relationship between disease onset and area under disease potential curve for field trials.

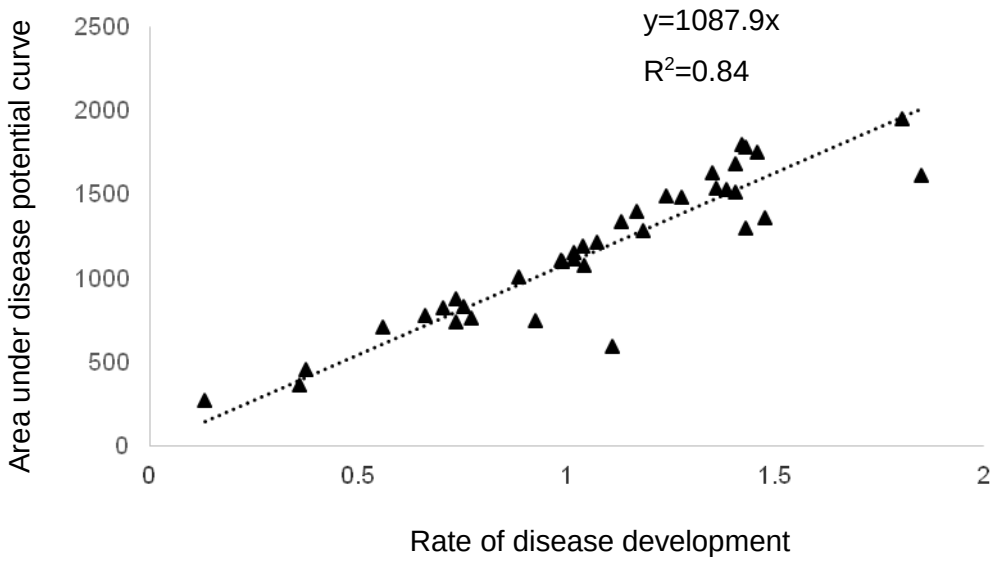

Figure 5.3. Relationship between rate of disease development and area under disease potential curve for field trials.

CHAPTER 6

MOLECULAR EVALUATION OF *SCLEROTINIA SCLEROTIORUM* ISOLATES IN SOUTH AFRICA

6.1 Abstract

Global production of economically important crops such as soybean is threatened by the cosmopolitan devastating pathogen, *Sclerotinia sclerotiorum.* Understanding the etiology of the disease is crucial for development of control strategies. Assessment of genetic variation within the pathogen population is crucial for resistance breeding strategies. The aim of this study was to determine genetic variation of *S. sclerotiorum* isolates collected from commercial production fields in South Africa. Seventy seven isolates from commercial fields were collected across five of the nine South African provinces. Isolates were grown at 10°C, 15°C, 20°C, 25°C and 30°C on PDA to determine differences in growth requirements. Molecular characterisation was done by means of AFLP analysis using 4 different primer pair combinations. Scoring of polymorphic bands was done between 300 and 800 base pairs. Results indicated that isolates grew optimally at 20°C and 25°C and growth is significantly impaired at 10°C, 15°C and 30°C. Furthermore, a relatively uniform distribution with evidence of genetic diversity within and amongst isolates were observed. The low recombination frequency suggests that the population in South Africa is young and developing. The minimal spanning network confirmed these findings as evidence was found that *S. sclerotiorum* is spreading to new areas. The dendogram showed high similarity between isolates from within province as isolates from Mpumalanga showed similarity ranging between 80% and 90% and isolates 15 and 18 from KwaZulu Natal showed 77% similarity. Significant similarity between isolates from different provinces was also seen as with isolates 14 and 15, from Mpumalanga and KwaZulu-Natal respectively which showed 86% similarity and isolates 17, from Mpumalanga and 18 from KwaZulu-Natal that showed 94% similarity. A possible explanation might be that Mpumulanga was identified as the region of origin and from there the disease spread to KwaZulu-Natal. AFLP is a useful molecular tool in the characterisation of *S. sclerotiorum* isolates.

6.2 Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a homothallic ascomycete that causes destructive diseases on numerous plant hosts globally, affecting seedlings, mature plants and their harvested products (Agrios, 1997). This devastating and cosmopolitan pathogen (Bolton *et al.,* 2006) has a broad host range of over 400 species enabling populations to persist and spread easily (Boland & Hall, 1994) especially on agronomically important crops such as soybean, sunflower and dry beans. This makes the implementation of a crop rotation system for disease control difficult. The increase in the importance of *Sclerotinia* diseases is mainly due to a lack of genetic resistance, efficacious chemical fungicides and biological control agents, hence difficulty in disease control. Identifying the modes of pathogenicity of *Sclerotinia* is important in the identification of effective alternate control strategies among agronomically important crops since previous control measures have failed (Cessna *et al*., 2000).

Temperature and moisture are the two most important factors influencing growth and development of *S. sclerotiorum*. Optimum temperatures for development have been reported in a number of studies to be mostly within the range of 16°C to 25°C (Abawi & Grogan, 1979; Phillips & Botha, 1990; Harikrishnan & Del Rio, 2006), while Botha *et al*. (2011) reported the optimum temperature for disease development in greenhouse conditions to be 21°C. *Sclerotinia sclerotiorum* has been shown to be sensitive to extreme temperatures and no disease development was reported at 30°C and 5°C (Abawi & Grogan, 1979). According to Wu *et al*. (2008) growth rate of *S. sclerotiorum* was much greater at 15°C and 20°C in culture (8.2 mm and 11.1 mm respectively) after 12h when compared to growth of *Verticillum dahlia* and *Cladosporium* sp. indicating that *S. sclerotiorum* is favoured by cool temperatures.

Factors that determine the genetic structure of a population include mutations, selection, gene flow between populations and genetic recombination within a population (Petrofeza & Nasser, 2012). An understanding of the genetic structure of the pathogen population is especially important when devising disease management and resistance-screening strategies (Sexton & Howlett, 2004; Li *et al*., 2009) as well as the documentation of changes occurring in a population and the environment (Sharma *et al*., 2013). Information on the genetic structure of *S. sclerotiorum* populations could assist in the breeding of resistant and improved cultivars with durable resistance (Zhao & Meng, 2003). It is especially important to know if new pathogen genotypes have evolved that are better adapted to, and more pathogenic to crops. If the potential for new genotypes to become

widely dispersed is taken into consideration, it would be wise to use clones known to the area of deployment in breeding programmes and apply these to screening for resistance in new lines (Hambleton *et al*., 2002).

Several studies have focused on the characterisation of *S. sclerotiorum* isolates in specific areas across the world for a better understanding to manage the diseases associated with this pathogen. *Sclerotinia sclerotiorum* isolates appear to be closely related with limited genetic variability which suggests that the pathogen has been newly introduced into a specific area or that it has had limited time to undergo genetic change (Meinhart *et al*., 2002).

Other reports, however, had indicated more genetic variation among isolates. Sexton & Howlett (2004) reported moderate to high levels of differentiation between populations from Australian canola fields. Wide variation among *S. sclerotiorum* isolates was reported by Akram *et al*. (2008) in Pakistan. Carpenter *et al*. (1999) examined genetic variation of *S. sclerotiorum* isolates in New Zealand using DNA fingerprinting and a high level of genetic variation was observed. In Brazil, studies have indicated high levels of genetic diversity and also the potential for sexual recombination (Litholdo Junior *et al*., 2011). The impact of high levels of genetic diversity on breeding strategies is important as the expression of disease resistance may be dependent on the strain of the pathogen used (Li *et al*., 2009).

Differentiation between isolates may be influenced by geographical separation or climatic conditions which in turn influence survival, severity of infection, the number of sclerotia produced and the timing of apothecial germination (Sexton & Howlett, 2002). The prevailing environmental conditions and crop rotation practices may also favour sexual recombination of populations and therefore, explain the variation among isolates (Gomes *et al*., 2011). Kohn *et al*. (1990) suggested however, that correlation between host specificity and mycelia compatibility should be done among local populations to exclude the geographical separation factor.

The aim of this study was to determine the genetic variation in *S. sclerotiorum* isolates collected from commercial production fields in South Africa using amplified fragment length polymorphism analysis. The hypothesis is that *S. sclerotiorum* isolates in South Africa are genetically different from each other based on locality and host plant.

6.3.1 Sampling and culturing of *S. sclerotiorum* isolates

Nineteen isolates were collected from infected plants in commercial soybean and sunflower fields. A further fifty eight samples were received from Dr. Bradley Flett, Agricultural Research Council - Grain Crops Institute, Potchefstroom (Table 6.1). These were collected from infected fields in Potchefstroom and surrounding areas. Sclerotia selected for this study were washed in running tap water, surface sterilised in 3% sodium hypochlorite and rinsed twice in sterile distilled water. The sclerotia were aseptically cut into smaller pieces (± 3 mm lengths) plated onto potato dextrose agar (PDA, Biolab) and incubated at 22^{o}C for three days. These cultures were transferred to PDA once more to obtain a pure culture for subsequent experiments. Pure growing colonies were sub-cultured after 2 days to water agar. Hyphal tips were then isolated after 1 to 2 days, using a dissecting microscope and transferred to PDA containing streptomycin (3ml/ℓ). Pure colonies were stored on agar slants in the cold room at 5°C for preservation.

6.3.2 Temperature Growth Study

Petri dishes (90 cm in diameter) were filled with 20 ml of PDA containing streptomycin (3ml/ℓ). Agar plugs (5 mm in diameter) were cut from an actively growing 3 day old culture of each of the test isolates and placed in the center of each petri dish. Five temperatures were used ie. 10°C, 15°C, 20°C, 25°C and 30°C. Each treatment was repeated three times. Colony diameter (mm) was determined as the average of the two perpendicular measurements and was measured after 48h using a digital caliper.

6.3.3 AFLP analysis

6.3.3.1. Sampled material

Stock cultures were sub-cultured in potato dextrose broth for 7 days and the resultant mycelia were harvested and freeze-dried for three days at -60^{o}C in a Virtis Advantage Freezemobile II. Samples were stored in a -80°C freezer until further use.

6.3.3.2 DNA extraction

Freeze dried mycelia were ground to a fine powder using a Qiagen TissueLyser for 30 seconds at 30 rs^{-1}. Total genomic DNA was isolated using the CTAB (hexadecyktrimethylammonium bromide) DNA isolation method (Saghai-Maroof *et al*., 1984). Approximately 250 µl of finely grounded mycelia was mixed with 750 µl CTAB buffer consisting of 100 mM Tris-HCl , 20 mM EDTA (ethylenedriamineteracetate), 1.4 M NaCl, 2% (w/v) CTAB and 0.2% (v/v) β-mercapto-ethanol was added. The mixture was shaken and incubated at 65°C for 1h.

A volume of 500 µl chloroform:isoamylalcohol [24:1 (v/v)] was added and mixed well. Samples were centrifuged at 12000 *g* for 5 min at 5ºC. DNA from the aqueous phase was precipitated with 0.66 volumes isopropanol and incubated at room temperature (20-25ºC) for 20 min. Tubes were centrifuged for 5 min at 12 000 g, the supernatant was discarded and tubes drained upside down. The DNA was washed by adding 500 µl ice-cold 70% (v/v) ethanol and incubated for 20 min at room temperature.

The suspension was centrifuged at 12 000 g for 5 min and supernatant was discarded. The DNA pellet was air-dried for 1 h at room temperature. Air-dried DNA was resuspended in 200 µl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C overnight RNase A (0.1 mg/ml) was added and incubated at 37ºC for 1-2 h.. DNA was precipitated with 7.5 M ammonium acetate and 200 µl choloroform:isoamyl alcohol [24:1 (v/v)] and centrifuged for 5 min at 12 000 g. DNA was precipitated from the aqueous phase overnight in 500 µl ice-cold 100% ethanol.

Samples were centrifuged for 15 min at 12 000 g and the supernatant was discarded. DNA was washed twice with 500 µl ice-cold 70% ethanol by centrifugation for 10 min each time and supernatant was discarded. Tubes were drained and placed upside down to air-dry. The pellet was resuspended in 50 µl TE-buffer (pH 8.0) and held overnight at 4ºC before the DNA concentration was determined.

6.3.3.3 DNA concentration

DNA concentrations were determined using a spectrophotometer and measuring the absorbances at 260 nm and 280 nm. The DNA concentration was calculated using the formula [DNA = optical density (OD260) x dilution factor x constant (50 µg ml^{-1}). DNA samples were diluted to a working concentration of 200 ng μl^{-1} in 1 x TE Buffer and stored

at 4°C. DNA quantity and quality were estimated from a 0.8% (w/v) agarose gel with electrophoresis at 80 V in UNTAN (40 mM Tris-Cl; 2 mM EDTA, pH adjusted to pH 7.4 with acetic acid) buffer. DNA was visualised with ethidium bromide staining under UV light.

6.3.3.4 AFLP procedure

AFLP analysis was performed according to Vos *et al*. (1995) with minor modifications as described by Herselman (2003). Primers and adapters were synthesised by Integrated DNA Technologies Inc, Coralville, IA, USA. Oligonucleotides used as adapters for AFLP analysis were purified by the manufactures using polyacrylamide gel electrophoresis (PAGE). Adapters were prepared by mixing equimolar amounts of the single-stranded oligonucleotides, heating for 10 min at 65°C in a water bath and then allowing the mixture to cool down to room temperature (20-25°C).

6.3.3.4.1 Restriction digestion and adapter ligation

Two restriction enzymes, *Eco*RI (six bp recognition site) and *Mse*I (four bp recognition site) were used to digest genomic DNA from the *Sclerotinia sclerotiorum* isolates. The 50 µl reaction mixture consisted of approximately 1 µg DNA, 4 U *Mse*I (New England Biolabs, Ipswich, MA, USA) and 1x *Mse*I buffer [50 mM NaCl, 10 mM Tris-Cl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. DNA was digested at 37°C for 5 h. This digestion was followed by a second digestion overnight at 37°C using 5 U *Eco*RI (Roche Diagnostics, Mannheim, Germany) and NaCl to a final concentration of 100 mM. A 10 µl reaction mixture containing 50 pmol *Mse*I-adapter, 5 pmol *Eco*RI-adapter, 1 U T4 DNA Ligase (USB Corporation, Cleveland, Ohio, USA), 0.4 mM adenosine 5'-triphosphate (ATP) and 1x T4 DNA Ligase buffer (66 mM Tris-Cl pH 7.6, 6.6 mM $MgCl_2$,10 mM DTT, 66 µM ATP) was added to the 50 µl restriction digestion mix to ligate the adapters. The ligation reaction mixture was incubated overnight at 16°C.

6.3.3.4.2 Pre-selective amplification

The digested/ligated DNA was amplified in a 20 µl reaction mixture consisting of 5.0 µl DNA, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 30 ng of *Mse*I primer+C, 30 ng of *Eco*RI primer+A and 1 U Go*Taq*® Flexi DNA polymerase (Promega). The conditions used for the pre-selective amplifications were: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s, a final 10 min

elongation 72°C and then held at 10°C. Amplified fragments were separated in a 1.5% (w/v) agarose gel at 80 V for one hour and visualised using ethidium bromide under UV light, after which DNA dilutions (1:15 to 1:50) were made to prepare samples for selective amplification.

6.3.3.4.3 Selective amplification

The pre-selective amplified DNA was used as the template for the AFLP analysis. The selective PCR reactions were performed using four primer combinations, namely *Eco*RI-AGC/*Mse*I-CTA, *Eco*RI-ACT/*Mse*I-CTG, *Eco*RI-AGC/*Mse*I-CTC and *Eco*RI-ACC/*Mse*I-CAG (Table 6.2). Reactions contained 5 µl pre-amplified DNA, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 100 µg/ml bovine serum albumin, 30 ng of *Mse*I primer+3, 30 ng of *Eco*RI primer+3 and 0.75 U Go*Taq*® Flexi DNA polymerase (Promega) in a final volume of 20 µl. The AFLP-PCR amplification programme was: 94°C for 5 min followed by 94°C for 30 s, 65°C for 30 s, decreasing by 1°C every cycle and 72°C for 60 s for 9 cycles followed by 94°C for 30 s, 56°C for 30 s and 72°C for 60 s for 25 cycles and 1 cycle of 72°C for 2 min followed by a 10°C hold.

6.3.3.4.4 Visualisation of amplified fragments

The AFLP-PCR products were separated on a 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide:bis-acrylamide, 7 M urea, 1x TBE Buffer (89 mM Tris-CI, 89 mM boric acid, 2.0 mM EDTA)]. The amplified reactions were mixed with equal volumes formamide loading dye [98% (v/v) de-ionised formamide, 10 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol]. These reactions were denatured by incubation for 5 min at 94°C. After denaturation, mixtures were immediately placed on ice prior to loading. Each PAGE gel contained at least sixty individuals, which represented one primer pair combination. Samples were compared to the *Sclerotinia* reference isolates, which were used as standards in this experiment, and fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Samples were set up and loaded arbitrarily to enable unbiased scoring and to compensate for the sample size. Electrophoresis was performed at constant power of 80 W for approximately 2 h.

The separated AFLP-PCR products were visualised by silver staining by following the Silver Sequence™ DNA Sequencing System manual (Promega). After the final wash step, stained gels were left overnight in an upright position to air-dry and then photographed.

The dried stained PAGE gel was positioned on photographic paper (Ilford Multigrade IV RC) the same size as the gel and exposed to a dim white light for approximately 20 s. The fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Scorable polymorphic AFLP bands, ranging from 300 bp to 800 bp were scored manually as 1 when present and 0 when absent in each individual and used to construct a binary data matrix. Scored data were used to construct a data matrix for statistical analysis. Homologous AFLP-PCR fragments in different individuals were assumed to represent the same allele.

6.3.4 Data analysis

Data of the temperature growth study were analysed using Number Cruncher Statistical Systems (NCSS) (Hintze, 2007). An analysis of variance was use to distinguish between isolates, temperatures as well as the interaction between these components. Fishers Least Significant Difference (LSD) at P = 0.05 was used to compare means.

Two different statistical programmes were used for the analysis of genetic variance. Allelic polymorphic information content (PIC) was determined using the formula $PIC=1-\Sigma (P_{ij})^2$, with P_{ij} representing the i^{th} allele in the j^{th} population for each location (Botstein *et al*., 1980). PIC evaluates polymorphism of a marker by characterising the efficiency of each primer for detecting polymorphic loci (Shete *et al.,* 2000).

A cluster analysis based on unweighted pair-group method using arithimetic averages (UPGMA) was done using DARwin 5.0.155 software and a hierarchical rooted neighbour-joining dendrogram with 500 bootstrap repetitions. The objective of the analysis was to define the components that could influence overall genetic differentiation and significant structures of the isolates as well as to assess the robustness of the nodes. The ordination of the structure of the genetic diversity within and among isolates and was analysed based on principal component analysis (PCA) using the same software. PCA was chosen to balance the cluster analysis information due to its informative nature regarding distances among larger groups. This data is then projected on two or three axes to enable visualisation of differences between isolates and the detection of clusters formed by related isolates. Isolates in the PCA functions as the uncorrelated variables (principal components) measured against the original variables (the molecular markers).

Network 4.6.1.1 software was used to elucidate a minimal spanning network with median joining (MJ) to assess the genetic structure of the sclerotinia isolates collected across five

of the nine South African provinces from different hosts. The result can be used to infer ancestral, evolutionary occurrences by calculating genetic distances between individuals and identifying population expansion events (Bandelt et al., 1995; 1999). A minimum-spanning tree was used for network simplification where pairs of sequence types are listed in increasing order of their genetic distances. The MJ network algorithm enables the construction of higher-resolution networks form multi-state data. The ancestral node of a network can be determined by comparing the network nodes with suitable outgroups. This process often referred to as "rooting the network" (Bandelt *et al.,* 1999).

6.4 Results

6.4.1 Temperature growth study

Significant differences were detected between isolates, temperature and the interaction between these two variables. Mean colony diameter of isolates ranged from 16.47 mm to 51.32 mm (Table 6.3). Mean isolate diameter was the lowest at 10°C (17.15 mm) followed by 30°C (26.86 mm). Maximum growth occurred at 20°C and 25°C with mean colony diameters of 64.92 mm and 66.74 mm respectively.

Isolate 60 was the most sensitive to warm temperatures as no growth was recorded at 30°C. Isolate 52 was the most sensitive to cold temperatures as it showed the lowest growth at 10°C (10.28 mm), however isolate 28 was the least sensitive to cold temperatures and the colony diameter was 45.19 mm. The largest colony diameter was measured with isolate 41 at 25°C (88.19 mm) followed by isolate 34 (88.17 mm). Isolate 61 was the least sensitive to high temperatures as the largest colony diameter (49.46 mm) was recorded with this isolate at 30°C.

6.4.2 Evaluation of *S. sclerotiorum* isolates with AFLPs

AFLP is a highly informative and reproducible technique and was used to identify genetic variation within and amongst *S.sclerotiorum* isolates. Results from the current study indicated definite genetic differences between isolates. A total of 205 fragments were identified of which 199 were polymorphic and 6 were monomorphic. These fragments were between 300 and 800 base pairs in length. Each primer pair generated an informative fingerprint across all isolates and produced between 41 and 61 distinguishable polymorphic AFLP fragments. Primer combination 2, E-ACT/M-CTG yielded the largest

number of fragments (61 fragments) and primer combination 3 (E-AGC/M-CTC) the smallest number of fragments (41 fragments).

The PIC values for the four primer combinations ranged from 0.40 to 0.77 (Table 6.4). Primer pair combination 2 that yielded the most polymorphic fragments, also had the highest PIC. This indicates that this primer pair is the most informative primer combination to identify the genetic distance between and amongst samples.

6.4.3 Rooted cluster analysis

A dendogram was constructed to serve as an indicator for the genetic diversity in the *S. sclerotiorum* isolates. Three major groups could be distinguished amongst the 77 isolates (Figure 6.1). Dissimilarity values obtained with DARwin ranged between 6% and 80% indicating a significant level of genetic diversity between the three groups as well as between the isolates. Outgroups indicated high dissimilarity with *S. sclerotiorum* isolates as was expected. Isolates from KwaZulu-Natal, Limpopo and Free State provinces were visible throughout the dendogram in each of the three identified groups. Only isolates from Mpumalanga tend to cluster together in groups 1 and 3. Sporadic clustering of isolates from different locations was observed and no relationship between *S. sclerotiorum* isolates and geographic origin or host plant could be detected.

6.4.4 Principal component analysis

Principal component analysis is based on the classification of the structure of the genetic diversity between the *S. sclerotiorum* isolates and can be divided into four quadrants (Figure 6.2). PCA indicated that isolates were distributed among all four quadrants with most of the isolates in quadrants 2 and 3. Mpumalanga was the only location where isolates formed a distinct group in quadrants 2 and 3 with most in quadrant 3. Isolates from Free State and KwaZulu-Natal were mostly visible in quadrant 3 and 4 with no isolates in quadrant 1. The isolates from the North West province are scattered across all four quadrants. Limpopo isolates are present in quadrants 1-3 with no isolates in quadrant 4.

6.4.5 Network analysis

A minimum-spanning network containing the 77 *S. sclerotiorum* isolates from five of the nine South African provinces and 3 reference isolates contained groups similar to those seen in the PCA and NJ cluster analysis.

No correlation was visible between host plant and locality using the network analysis. Isolates collected from sunflowers tended to cluster together, however integrated clusters with other groups could also be observed (Figure 6.3). A low recombination frequency of the isolates from the five provinces was also visible using the minimum spanning network. Isolates collected from fields in the North West appear to be at the end of the nodes while isolates from Free State province and Limpopo province were scattered throughout the network. Isolates from Mpumalanga province tend to cluster together and were usually closely associated with isolates from KwaZulu-Natal (Figure 6.4). The first mutation was observed between isolates from Mpumalanga and KwaZulu-Natal province (Figure 6.5) indicating Mpumalanga province to be the region of origin.

6.5 Discussion

Understanding the population genetic structure of a pathogen and how the underlying mechanisms are involved in the history of a pathogen and its distribution across different geographic areas and wide host range has been emphasised as crucial research (Petrofeza & Nasser, 2012).

Temperature and moisture are two crucial factors that affect growth and survival of *S. sclerotiorum* mycelia (Huang & Kozub, 1993). The current temperature study indicates that *S. sclerotiorum* is sensitive to temperature and that the optimum temperature for growth was determined as between 20°C and 25°C which was also reported in previous studies. (Abawi & Grogan, 1979; Boland & Hall, 1987; Philips & Botha, 1990; Botha *et al*., 2011). It was clearly seen that pathogen growth is not favoured by excessive cold or hot temperatures as temperatures in the current study of 10°C and 30°C yielded poor growth and development. This suggests that disease development in the field can be suppressed by temperatures not favourable for growth. The pathogen however, still survives under these conditions and subsequently becomes a problem when temperatures favouring growth and development arise. This was confirmed by Harikrishnan & Del Rio (2006) who found that mycelia remained viable for 144h after drying, indicating that they are tolerant

to desiccation and unfavourable conditions. Some isolates had the ability to withstand less favourable temperatures better ie. isolates 61 and 28 and significant pathogen activity can be expected and yield losses due to disease may occur with these isolates. These data suggest that ecotypes of the pathogen may occur.

Sclerotinia sclerotiorum isolates from at least 14 countries across the world including, Canada, North America, South America, Asia, parts of Europe and Australia have been characterised to determine the level of genetic diversity within and between field populations (Kohli & Kohn, 1998; Hambleton *et al.*, 2002; Kull *et al.*, 2004; Malvarez *et al.*, 2007; Li *et al.*, 2009; Ekins *et* al., 2011; Karimi *et al.*, 2011). The genetic diversity observed in *S. sclerotiorum* populations depends on the techniques used and the size of the population evaluated. AFLP analysis, a highly reproducible and polymorphic fingerprinting technique, has been used in numerous population genetic studies of other pathogens to evaluate genetic differences across large parts of the pathogen's genome in a single assay, with no prior sequence information. Most investigations of the genetic variability of *S. sclerotiorum* populations documented moderate to high levels of genetic diversity within field populations sampled across large geographic regions (Sun *et al.*, 2005; Sexton *et al*., 2006; Barari *et al*., 2010; Karimi *et al.,* 2011; Litholdo Junior *et al.,*2011). Limited studies of the genetic structure of populations of this pathogen reported low levels of genetic diversity (Osofee *et* al., 2005; Li *et al*., 2009; Mandal & Dubey, 2012) with some evidence of recombination (Kohn *et al*., 1991; Chen *et al*., 2010), where some showed evidence of clonal populations (Carbone *et al.,* 1999). AFLPs were used in the current study to evaluate and characterise the genetic variation of *S. sclerotiorum* isolates from five of the nine provinces in South Africa. To date, no study has been conducted to evaluate *S. sclerotiorum* isolates for genetic differences in South Africa. In the current study *S. sclerotiorum* isolates in South Africa showed significant genetic differences even though some isolates appear to be closely related.

The diversity of 77 isolates of *S. sclerotiorum* on different hosts representing five of the nine South African provinces can be observed from the three groups in the dendrogram. Although genotypic diversity between clusters was observed, some isolates within a cluster shared similarities. The dendogram confirms similarity between isolates from the same provinces ie. similarity between isolates 7, 8, 11, 12, 14, 16 and 17 from Mpumalanga ranging between 80% and 90% while the similarity between isolates 15 and 18 from KwaZulu-Natal was 77%. *Sclerotinia sclerotiorum* is homothallic and has the ability reproduce sexually by self-fertilization, thus the formation of apothecia and ascospores, suggesting a limited amount of gene flow which accounted for the high

similarities and low levels of genetic diversity. According to Sexton & Howlett (2004) high similarities, suggesting low genetic diversity, present in *S. sclerotiorum* can be attributed to its homothallic nature, resulting in a large amount of self-crossing and asexual propagation that occurs via myceliogenic germination of sclerotia. According to Meinhart *et al*. (2002) low genetic variability may also suggest that the pathogen has been newly introduced into the area or that it has had limited time to undergo genetic change. This in particular could account for the high similarities between isolates in the same province.

Similarities between isolates from different provinces were also observed within the clusters of the dendrogram, ie. isolates 14 and 15, from Mpumalanga and KwaZulu-Natal respectively showed 86% similarity and isolates 17, from Mpumalanga and 18 from KwaZulu-Natal showed 94% similarity. Even though the physical distance between these two provinces is big, high levels of similarity were observed especially between isolates from these two provinces. Soybeans are planted annually across these two provinces and this might provide a pathway of cross infection for the pathogen from these two regions. In these provinces similar growth seasons, weather conditions and agricultural practices occur which means that no barrier exists in ecological adaptation of the pathogen (Li *et al*., 2009). Mandal & Dubey (2012) also suggest that high levels of genetic similarity might be due to clonal development as environmental conditions prevailing in an area might not be favourable for sexual reproduction. Barari *et al.* (2013) suggested that fungal inoculum can spread by means of irrigation, humans and airborne ascospores over long distances which may also contribute to the high similarities between isolates from different provinces.

The minimal spanning network indicated a low frequency of recombination which emphasizes that the *S. sclerotiorum* population in South Africa is still young and in a developing stage. Isolates collected from the North West province appeared to be at the end of the nodes in most clusters, indicating that the isolates from this region could be regarded as part of a young field population, compared to the orientation of the isolates from the other regions. The integration of these isolates with isolates from other provinces suggests the possibility of gene flow between provinces. This may also explain the recent outbreaks of this disease in the sunflower production areas of this province. A possible explanation for this movement can be the adaptation of *S. sclerotiorum* isolates to new geographic and climatic regions of South Africa. Mpumulanga is regarded as the region of origin and from there disease spread to KwaZulu-Natal as observed from the first mutation recorded between isolates 17 and 18 in the minimal spanning network (Figure 6.5). Principal component analysis also indicated the clustering of the Mpumalanga

isolates, which suggests this province to be the region of origin from where the disease spread to other geographic and climatic regions. This may also vindicate the similarity between isolates from Mpumalanga and other provinces.

The first report of *S. sclerotiorum* in South Africa on soybeans was in 1979 in the Lydenburg area, Mpumalanga (Thompson & Van der Westhuizen, 1979) which supports the data of the current study that Mpumalanga may be the region of origin as indicated by both the minimum-spanning network and the PCA from where it then spread to other provinces as the cultivation of crop such as soybean and sunflower spread and increased. No correlation between host plant or locality and genetic similarity could be made in this study and more isolates are needed in relation to host and location with equal ratio's to make an accurate correlation. This was also observed by Litholdo Junior *et al*. (2011) who reported that no tendency of isolates towards geographical origin or host was observed. Carpenter *et al*. (2013) also reported that *S. sclerotiorum* shows no evidence of host-specificity and emphasized that the wide host range provides frequent opportunities for movement between host species.

Thus far, the total South African *S. sclerotiorum* population consisting of 77 isolates can be considered a mixture of isolates due to the diversity unraveled between isolates. The high genotypic diversity within the South African population suggests that recombination occurs relatively frequently under field conditions. The observation of high genetic variation amongst the *S.sclerotiorum* isolates can be explained by the possibility of sexual reproduction by outcrossing as only a small amount of sexual recombination is necessary to contribute to high genetic diversity (Atallah *et al*., 2004). These data illustrated that recombination has an influence on the genetic diversity of *S. sclerotiorum* isolates, therefore recombination can be regarded as a mechanism to achieve and maintain high genetic diversity and gene flow within larger *S.sclerotiorum* populations. Although we did not test for outcrossing, favourable environmental conditions may facilitate outcrossing. The analyses of the PCA, NJ and minimum-spanning network all concluded that the current *S.sclerotiorum* population is a mixture of different isolates, because of the integration of isolates from all locations. These results indicated that little sexual reproduction occurred between isolates across all locations

The hypothesis tested with the current study, stated that *S. sclerotiorum* isolates in South Africa is genetically different from each other based on locality and host plant. Results from this study partially reject the hypothesis as the genetic variation could not be linked to either location or host; however genetic diversity between the *S. sclerotiorum* isolates were

recorded. Even though the proportion of the genetic variation across all locations and amongst the isolates was high in the three major groups illustrated by the dendrogram, the genetic diversity of isolates within some clusters showed similar genetic backgrounds. These results indicated that geographical distance could not be related to increase and/or decrease in genetic diversity among these isolates. Isolates from different provinces did not cluster together as would be expected to geographic adaption had occurred ie. similar temperature requirements needed. This may indicate that isolates are not specific to temperatures prevailing in provinces but rather to environmental factors favouring disease development.

Most previous studies found high levels of genetic diversity in field populations of *S. sclerotiorum* or populations sampled across a broad geographical scale (Sun *et al.*, 2005; Sexton *et al*., 2006; Barari *et al*., 2010; Karimi *et al.,* 2011; Litholdo Junior *et al.,*2011). Petrofeza & Nasser (2012) reviewed these studies spanning across more than 14 countries worldwide and found that the proportion of the total genetic diversity within individual populations ranged from 44% to 98.4%. The high genetic diversity within populations can be explained by the presence of recombination due to ability of *S. sclerotiorum* to reproduce sexually by self-fertilization.

In conclusion, relatively uniform distributions with evidence of genetic diversity within and amongst isolates across the five provinces were visible. The significant amount of genetic diversity suggest that this population in South Africa is still young and in a developing stage. In the current study no correlation could be made with growth pattern of the isolates in the growth study and data from the genetic evaluation. Resolving the population dynamics of *S. sclerotiorum* populations in South Africa could benefit from adding more isolates and to include mycelial compatibility analysis.

6.6 References

Abawi, G.S. & Grogan, R.G. 1979. Epidemiology of diseases caused by Sclerotinia species. Phytopathology 69: 899-903.

Agrios, G.N. 1997. Plant Pathology. Fouth Edition. Academic press. New York.

Akram, A., Iqbal, S.M., Ahmed, N., Iqbal, U., & Ghafoor, A. 2008. Morphological variability and mycelia compatibility among the isolates of *Sclerotinia sclerotiorum* associated with stem rot of chickpea. Pakistan Journal of Botany 40: 2663-2668.

Atallah, Z.K.., Larget, B., Chen, X. & Johnson, D.A. 2004. High genetic diversity, phenotypic uniformity, and evidence of outcrossing in *Sclerotinia sclerotiorum* in the Columbia Basin of Washington State. Phytopathology 94: 737-742.

Bandelt, H-J., Forster, P., Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16: 37-48.

Bandelt, J.H., Forster, P., Sykes, B.C. & Richards, M.B. 1995. Mitochondrial portraits of human populations using median networks. Genetics 141: 743-753.

Boland, G.J. & Hall, R. 1987. Evaluation of Soybean cultivars for resistance to *Sclerotinia sclerotiorum* under field conditions. Plant Disease 71: 934-936.

Boland, G.J. & Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum.* Canadian Journal of Plant Pathology 16: 93-108.

Bolton., M.D., Thomma, B.P.H. & Nelson, B.D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molecular Plant Pathology 7: 1-16.

Barari, H., Alavi, V. & Badalyan, S.M. 2010. Genetic and morphological diversities in *Sclerotinia sclerotiorum* isolates in northern parts of Iran. World Applied Sciences Journal 8: 326–333.

Barari, H., Dalili, S.A., Rezall, S.A.A. & Badalian, S.M. 2013. Genetic Structure Analysis of *Sclerotinia Sclerotiorum* (Lib.) de Bary Population from different host plant species in North of Iran. Romanian Biotechnological Letters 18: 8197-8205.

Botha, C., McLaren, N.W. & Swart, W.J. 2011. The effect of temperature and period of high humidity on the development of Sclerotinia stem rot on soybeans in the greenhouse. South African Journal of Plant and Soil 28: 208-211.

Carbone, I., Anderson, J.B. & Kohn, L.M. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. Evolution 53: 11-21.

Carpenter, M.A., Frampton, C. & Stewart, A. 1999, Genetic variation in New Zealand populations of the plant pathogen *Sclerotinia sclerotiorum*. New Zealand Journal of Crop and Horticultural Science 27: 13-21.

Cessna, S.G., Sears, V.E., Dickman, M.B. & Low, P.S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. The Plant Cell 12: 2191-2199.

Chen, X.J., Cao, M.J. & Kong, W.W. 2010. Physiological and biochemical features of rocymidone-resistant mutants to *Sclerotinia sclerotiorum* and preliminary study of resistance mechanism. Chinese Journal of Oil Crop Science 32: 534-539.

Ekins, M., Hayden, H., Aitken, E. & Goulter, K. 2011. Population structure of *Sclerotinia sclerotiorum* on sunflower in Australia. Australasian Plant Pathology 40: 99-108.

Gomes, E.V., Do Nascimento, L.B., De Freitas, M.A., Nasser, L.C.B. & Petrofeza, S. 2011. Microsatellite markers reveal genetic variation within *Sclerotinia sclerotiorum* populations in irrigated dry bean crops in Brazil. Journal of Phytopathology 159: 94-99.

Hambleton, S., Walker, C. & Kohn, L.M. 2002. Clonal lineages of *Sclerotinia sclerotiorum* previously known from other crops predominate in 1999-2000 samples from Ontario and Quebec soybean. Canadian Journal of Plant Pathology 24: 309-315.

Harikrishnan, R. & Del Rio, L.E. 2006. Influence of temperature, relative humidity, ascospore concentration and length of dry bean flowers on white mold development. Plant Disease 90: 946-950.

Herselman, L. 2003. Genetic variation among Southern African cultivated peanut (*Arachis hypogaea* L.) genotypes as revealed by AFLP analysis. Euphytica 133: 319-327.

Huang, H.C. & Kozub, G.C. *1993*. Influence of inoculum production temperature on carpogenic germination of sclerotia of *Sclerotinia sclerotiorum*. Canadian Journal of Microbiology 39*:*548–*550.*

Karimi, E., Safaie, N. & Shams-bakhsh, M. 2011. Assessment of genetic diversity among *Sclerotinia sclerotiorum* populations in canola fields by Rep-PCR. Trakia Journal of Sciences 9: 62-68.

Kohli, Y. & Kohn, L. 1998. Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. Fungal Genetic Biology 23: 139-149.

Kohn, L.M., Carbone, I. & Anderson, J.B. 1990. Mycelial interactions in *Sclerotinia sclerotiorum*. Experimental Mycology 14: 255-267.

Kohn, L.M., Stasovski E., Carbone I., Royer J. & Anderson J.B., 1991. Mycelial incompatibility and molecular markers identify genetic variability in field populations of *Sclerotinia sclerotiorum*. Phytopathology 81: 480-485.

Kull, L.S., Pedersen, W.L., Palmquist, D. & Hartman, G.L. 2004. Mycelial compatibility grouping and aggressiveness of *Sclerotinia sclerotiorum*. Plant Disease 88: 325-332.

Li, Z., Wang, Y., Chen, Y., Zhang, J. & Dilantha Fernando, W.G. 2009. Genetic diversity and differentiation of *Sclerotinia sclerotiorum* populations in sunflower. Phytoparasitica 37: 77-85.

Litholdo Junior, C.G., Gomes, E.V., Lobo Junior, M., Nasser, L.C.B. & Petrofeza, S. 2011. Genetic diversity and mycelial compatibility groups of the plant-pathogenic fungus *Sclerotinia sclerotiorum* in Brazil. Genetics and Molecular Research 10: 868-877.

Malvarez, M., Carbone, I., Grünwald, N.J., Subbarao, K.V., Schafer, M. & Kohn, L.M. 2007. New populations of *Sclerotinia sclerotiorum* from lettuce in California and peas and lentils in Washington. Phytopathology 97: 470-483.

Mandal, A.K. & Dubey, S.C. 2012. Genetic diversity analysis of *Sclerotinia sclerotiorum* causing stem rot in chickpea using RAPD, ITS-RFLP, ITS sequencing and mycelia

compatibility grouping. World Journal of Microbiological Biotechnology 28: 1849-1855.

Meinhardt, L.W., Wulff, N.A., Bellato, C.M. & Tsai, S.M. 2002. Telomere and microsatellite primers reveal diversity among *Sclerotinia sclerotiorum* isolates from Brazil. Fitopatologia Brasileira 27: 211-215.

Hintze, J.L. 2007. Number cruncher statistical systems (NCSS). Kaysville, UT: Number Cruncher Statistical Systems.

Osofee H., Hameed K.H. & Mahasneh A. 2005. Relatedness among indigenous members of *Sclerotinia sclerotiorum* by mycelial compatibility and RAPD analysis in the Jordan Valley. Plant Pathology Journal 21: 106-110.

Petrofeza, S. & Nasser, L.C.B. 2012. Case Study: *Sclerotinia sclerotiorum*: Genetic diversity and disease control, the molecular basis of plant genetic diversity. Prof. M. Caliskan (Ed.), ISBN: 978-953-51-0157-4, InTech. http://www.intechopen.com/books.the-molecular-basis-of-plant-genetic-diversity/case-study-sclerotinia-sclerotiorum-genetic-diverstiy-and-disease-control

Phillips, A.J.L & Botha, W.J. Sclerotinia stem rot of soybeans. Farming in South Africa, Soybeans F.1/1990.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. & Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal; location and population dynamic. Proceedings of National Academy of Science, USA 81: 8014-8019.

Sexton, A.C. & Howlett, B.J. 2004. Microsatellite markers reveal genetic differentiation among populations of *Sclerotinia sclerotiorum* form Australian canola fields. Current Genetics 46: 357-365.

Sexton, A.C., Whitten, A.R. & Howlett, B.J. 2006. Population structure of *Sclerotinia sclerotiorum* in an Australian canola field at flowering and stem-infected stages of the disease cycle. Genome 49: 1408-1415.

Sharma, P., Meena, P.D., Kuman, S & Chauhan, J.S. 2013. Genetic diversity and morphological variability of *Sclerotinia sclerotiorum* isolates of oilseed Brassica in India. African Journal of Microbiology Research 7: 1827-1833.

Shete, S., Tiwari, H. & Elston, R.C. 2000. On estimating the heterozygosity and polymorphism information content value. Theoretical Population Biology 57: 265-271.

Sun, J.M., Irzykowski, W., Jedryczka, M. & Han, F.X. 2005. Analysis of the structure of *Sclerotinia sclerotiorum* (Lib.) de Bary populations from different regions and host plants by random amplified polymorphic DNA markers. Journal of Integrative Plant Biology 47: 385-395.

Thompson, A.H. & van der Westhuizen, G.C.A. 1979. *Sclerotinia sclerotiorum* (Lib.) de Bary on soybean in South Africa. *Phytophylactica* 11: 145-148.

Vos, P., Hogers, R., Bleeter, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: a new concept for DNA fingerprinting. Nucleic Acids Research 23: 4407- 4414.

Wu, B.M., Subbarao, K.V. & Qin, Q-M. 2008. Nonlinear colony extension of *Sclerotinia minor* and *S. sclerotiorum*. Mycologia 100: 902-910.

Zhao, J. & Meng, J. 2003. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.) Theoretical and Applied Genetics 106: 759-764.

Table 6.1. Sources of isolates of *Sclerotinia sclerotiorum* collected in South Africa.

Isolate number	Province	Host plant
1	KwaZulu-Natal	Sunflower (*Helianthus annuus*)
2	Free State	Soybeans (*Glycine max*)
3	North West	Sunflower (*Helianthus annuus*)
4	North West	Sunflower (*Helianthus annuus*)
5	North West	Sunflower (*Helianthus annuus*)
6	North West	Sunflower (*Helianthus annuus*)
7	Mpumalanga	Soybeans (*Glycine max*)
8	Mpumalanga	Soybeans (*Glycine max*)
9	Mpumalanga	Soybeans (*Glycine max*)
10	Limpopo	Soybeans (*Glycine max*)
11	Mpumalanga	Sugar bean (*Phaseolus vulgaris*)
12	Mpumalanga	Soybeans (*Glycine max*)
13	Mpumalanga	Sunflower (*Helianthus annuus*)
14	Mpumalanga	Soybeans (*Glycine max*)
15	KwaZulu-Natal	Soybeans (*Glycine max*)
16	Mpumalanga	Sunflower (*Helianthus annuus*)
17	Mpumalanga	Soybeans (*Glycine max*)
18	KwaZulu-Natal	Soybeans (*Glycine max*)
19	North West	Sunflower (*Helianthus annuus*)
20	North West	Sunflower (*Helianthus annuus*)
21	North West	Sunflower (*Helianthus annuus*)
22	North West	Sunflower (*Helianthus annuus*)
23	North West	Sunflower (*Helianthus annuus*)
24	North West	Soybeans (*Glycine max*)
25	North West	Sunflower (*Helianthus annuus*)
26	North West	Sunflower (*Helianthus annuus*)
27	North West	Sunflower (*Helianthus annuus*)
28	North West	Sunflower (*Helianthus annuus*)
29	North West	Sunflower (*Helianthus annuus*)
30	North West	Sunflower (*Helianthus annuus*)
31	North West	Sunflower (*Helianthus annuus*)
32	North West	Sunflower (*Helianthus annuus*)
33	North West	Sunflower (*Helianthus annuus*)
34	North West	Sunflower (*Helianthus annuus*)
35	North West	Sunflower (*Helianthus annuus*)
36	North West	Sunflower (*Helianthus annuus*)
37	North West	Sunflower (*Helianthus annuus*)
38	North West	Sunflower (*Helianthus annuus*)
39	North West	Sunflower (*Helianthus annuus*)
40	North West	Sunflower (*Helianthus annuus*)
41	North West	Sunflower (*Helianthus annuus*)
42	North West	Sunflower (*Helianthus annuus*)
43	North West	Sunflower (*Helianthus annuus*)
44	North West	Sunflower (*Helianthus annuus*)
45	North West	Sunflower (*Helianthus annuus*)
46	Free State	Soybeans (*Glycine max*)
47	North West	Sunflower (*Helianthus annuus*)
48	North West	Sunflower (*Helianthus annuus*)
49	North West	Sunflower (*Helianthus annuus*)
50	North West	Sunflower (*Helianthus annuus*)
51	North West	Sunflower (*Helianthus annuus*)
52	North West	Sunflower (*Helianthus annuus*)
53	North West	Sunflower (*Helianthus annuus*)
54	North West	Sunflower (*Helianthus annuus*)
55	North West	Sunflower (*Helianthus annuus*)
56	North West	Sunflower (*Helianthus annuus*)
57	North West	Sunflower (*Helianthus annuus*)
58	North West	Sunflower (*Helianthus annuus*)
59	North West	Sunflower (*Helianthus annuus*)
60	North West	Sunflower (*Helianthus annuus*)
61	North West	Sunflower (*Helianthus annuus*)
62	North West	Sunflower (*Helianthus annuus*)
63	North West	Sunflower (*Helianthus annuus*)
64	North West	Soybeans (*Glycine max*)
65	North West	Sunflower (*Helianthus annuus*)
66	North West	Sunflower (*Helianthus annuus*)
67	North West	Sunflower (*Helianthus annuus*)
68	North West	Sunflower (*Helianthus annuus*)
69	North West	Sunflower (*Helianthus annuus*)
70	North West	Sunflower (*Helianthus annuus*)
71	North West	Sunflower (*Helianthus annuus*)
72	North West	Sunflower (*Helianthus annuus*)
73	North West	Sunflower (*Helianthus annuus*)
74	Free State	Soybeans (*Glycine max*)
75	North West	Sunflower (*Helianthus annuus*)
76	North West	Sunflower (*Helianthus annuus*)
77	Limpopo	Hubbard Squash (*Curcubita maxima)*

Table 6.2. Adapters and primers used in AFLP analyses of *Sclerotinia sclerotiorum* populations.

Adapters/primers	**Sequences (5'-3')**
Eco RI - adapters	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
Mse I - adapters	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
Pre-selective primers	
Eco-A	GACTGCGTACCAATTCA
Mse-C	GATGAGTCCTGAGTAAC
Selective primers	
E-AGC : M-CTA	GAGTGCGTACCAATTCAGC
	GATGAGTCCTGAGTAACTA
E-ACT : M-CTG	GAGTGCGTACCAATTCACT
	GATGAGTCCTGAGTAACTG
E-AGC : M-CTC	GAGTGCGTACCAATTAGC
	GATGAGTCCTGAGTAACTC
E-ACC : M-CAG	GAGTGCGTACCAATTCACC
	GATGAGTCCTGAGTAACAG

Table 6.3. Mean colony diameter of *Sclerotinia sclerotiorum* isolates on PDA at different temperatures.

Isolate	Temperature (Colony diameter mm)					Mean
	10°C	15°C	20°C	25°C	30°C	
1	16.10	33.63	63.47	53.13	34.74	40.21
2	13.69	41.44	55.80	60.77	29.25	40.19
3	14.21	38.06	62.06	65.43	29.55	41.86
4	17.15	41.57	73.61	80.16	30.92	48.68
5	15.28	42.67	66.50	68.22	28.52	44.24
6	14.74	46.81	73.03	82.90	20.42	47.58
7	19.11	41.35	64.33	70.46	14.67	41.98
8	14.59	43.20	66.67	83.35	12.80	44.12
9	22.06	38.00	67.37	72.80	25.91	45.23
10	22.98	46.53	69.14	76.15	16.25	46.21
11	12.50	39.83	65.26	65.33	26.98	41.98
12	16.54	45.70	65.57	70.75	24.75	44.66
13	12.87	37.38	67.82	61.61	28.14	41.56
14	17.20	33.30	70.30	71.64	16.27	41.74
15	19.35	40.79	73.04	71.43	40.55	49.03
16	12.49	27.99	56.95	65.08	26.54	37.81
17	20.34	48.42	60.20	79.02	15.63	44.72
18	25.97	47.39	67.76	85.57	13.34	48.01
19	27.88	45.84	56.89	79.82	21.16	46.32
20	19.60	30.24	37.64	34.49	19.88	28.37
21	16.48	27.94	52.52	55.44	35.66	37.61
22	14.82	35.68	64.84	65.86	29.86	42.21
23	13.85	25.19	49.97	47.58	30.87	33.49
24	16.06	40.58	68.48	56.74	41.42	44.66
25	12.77	31.27	55.34	49.03	36.18	36.92
26	17.12	49.20	71.92	74.54	33.87	49.33
27	13.88	40.62	72.52	71.84	29.73	45.72
28	45.19	38.65	74.49	81.28	17.01	51.32
29	31.87	41.30	78.39	66.28	27.02	48.97
30	22.97	40.19	72.55	67.05	18.28	44.21
31	18.28	35.40	62.33	42.41	27.09	37.10
32	15.04	28.40	56.61	55.30	42.94	39.66
33	13.62	37.43	71.94	61.69	36.44	44.22
34	24.70	41.11	71.73	88.17	12.96	47.74
35	11.98	25.55	57.62	53.21	32.68	36.21
36	14.45	38.56	52.28	87.31	19.38	42.40
37	12.61	33.73	63.01	73.34	36.48	43.83

38	13.50	42.66	77.10	69.92	28.22	46.28
39	21.60	41.62	69.41	83.81	12.41	45.77
40	15.37	42.03	76.84	68.71	23.76	45.35
41	12.00	42.57	66.82	88.19	20.04	45.93
42	42.8	43.37	47.46	49.01	11.99	38.93
43	12.61	33.54	65.84	64.43	34.85	42.25
44	14.18	38.20	70.16	62.77	23.84	41.83
45	12.53	32.05	51.66	50.23	30.53	35.40
46	16.93	32.41	64.49	56.24	26.74	39.36
47	15.76	40.22	72.72	78.71	18.04	45.09
48	23.35	41.78	61.45	74.83	41.76	48.63
49	20.93	25.58	66.80	76.08	23.26	42.53
50	15.54	46.14	70.10	67.20	27.65	45.32
51	16.21	37.30	64.18	67.78	38.27	44.75
52	10.28	34.45	62.54	56.26	33.39	39.38
53	12.97	26.08	53.58	46.89	26.53	33.21
54	15.22	39.09	69.06	66.67	32.17	44.44
55	15.33	49.44	76.06	76.55	23.29	48.13
56	12.65	31.96	62.13	66.55	42.54	43.16
57	12.59	37.23	68.11	57.81	32.74	41.70
58	16.63	46.16	75.80	79.70	28.02	49.26
59	11.40	29.22	49.83	48.28	31.85	34.11
60	16.64	21.41	22.00	22.29	0.00	16.47
61	18.22	37.17	55.33	58.84	49.46	43.80
62	11.07	35.99	28.91	46.90	29.72	30.52
63	11.86	30.57	73.88	69.82	14.55	40.14
64	14.85	41.93	70.85	73.44	22.98	44.81
65	18.12	45.13	74.00	62.36	23.34	44.59
66	13.06	44.93	67.35	52.66	30.76	41.75
67	21.13	46.65	77.10	74.55	24.49	48.78
68	25.23	40.41	73.49	80.78	21.48	48.28
69	11.99	43.44	73.29	71.29	21.15	44.23
70	18.60	42.80	82.43	77.51	31.34	50.53
71	21.34	33.07	66.67	73.25	37.68	46.40
72	11.24	41.67	78.21	77.54	28.26	47.38
73	16.47	43.11	73.31	71.63	20.59	45.02
74	14.75	44.73	70.36	73.62	19.27	44.55
75	19.67	35.89	73.21	77.58	27.35	46.74
76	11.14	37.07	57.57	56.64	40.95	40.68
77	14.10	33.73	58.86	66.78	28.44	40.38
Mean	**17.15**	**38.36**	**64.92**	**66.74**	**26.86**	**42.81**
	LSD (P0.05)=3.70					

Table 6.4. Selective primer combinations and polymorphic information content values for primers used in this study.

Primer combination	Polymorphic information content (PIC)
*Eco*RI-AGC/*Mse*I-CTA	0.56
*Eco*RI-ACT/*Mse*I-CTG	0.77
*Eco*RI-AGC/*Mse*I-CTC	0.40
*Eco*RI-ACC/*Mse*I-CAG	0.46

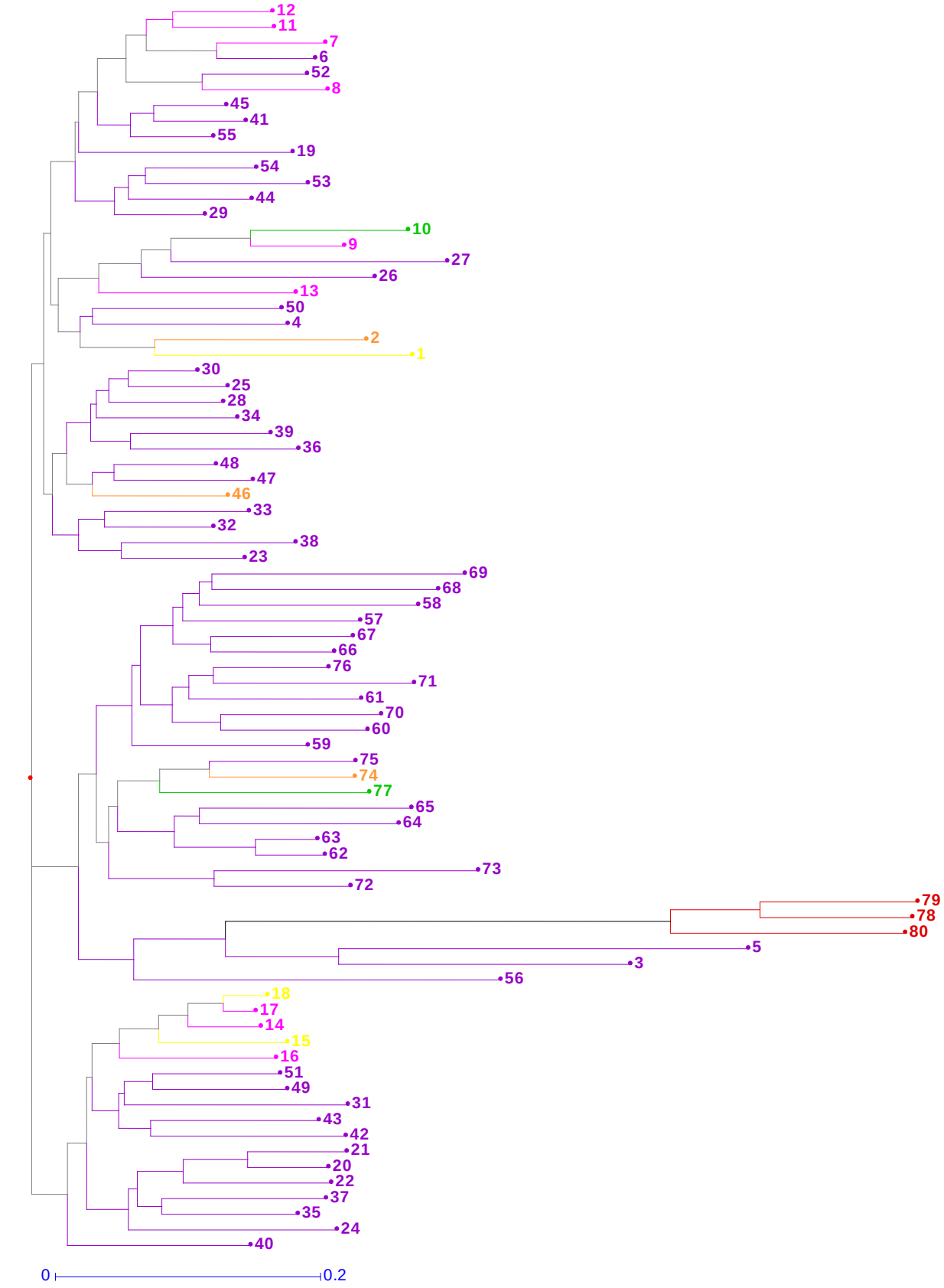

Figure 6.1. Dendogram based on AFLP data constructed for 77 *Sclerotinia sclerotiorum* isolates using Jaccard's dissimilarity coefficient.

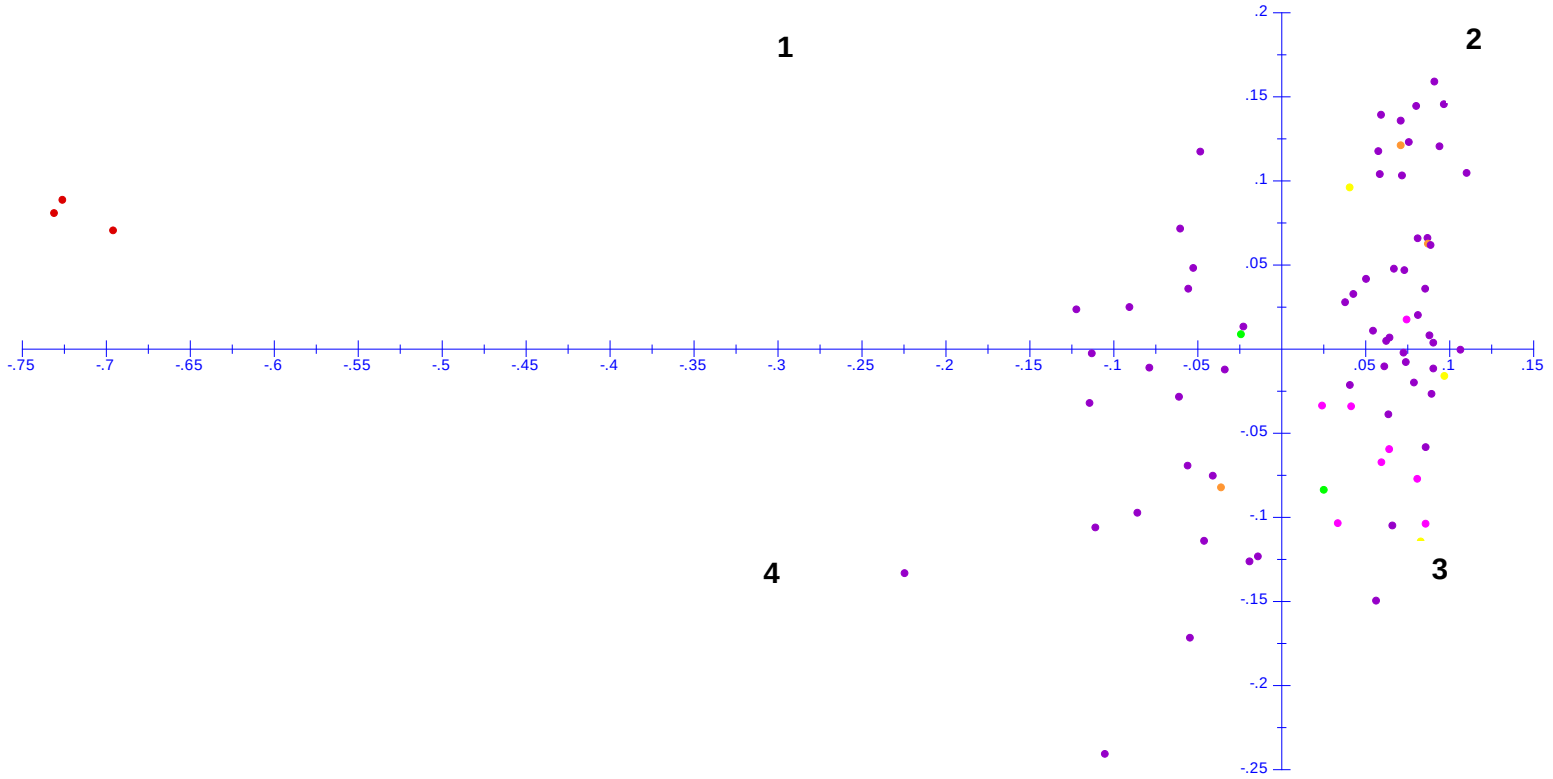

Figure 6.2. Principal component analysis biplot (axes 1 and 5) of 77 *Sclerotinia sclerotiorum* isolates and 3 reference isolates.

Free State Limpopo Mpumalanga KwaZulu-Natal North West Outgroups

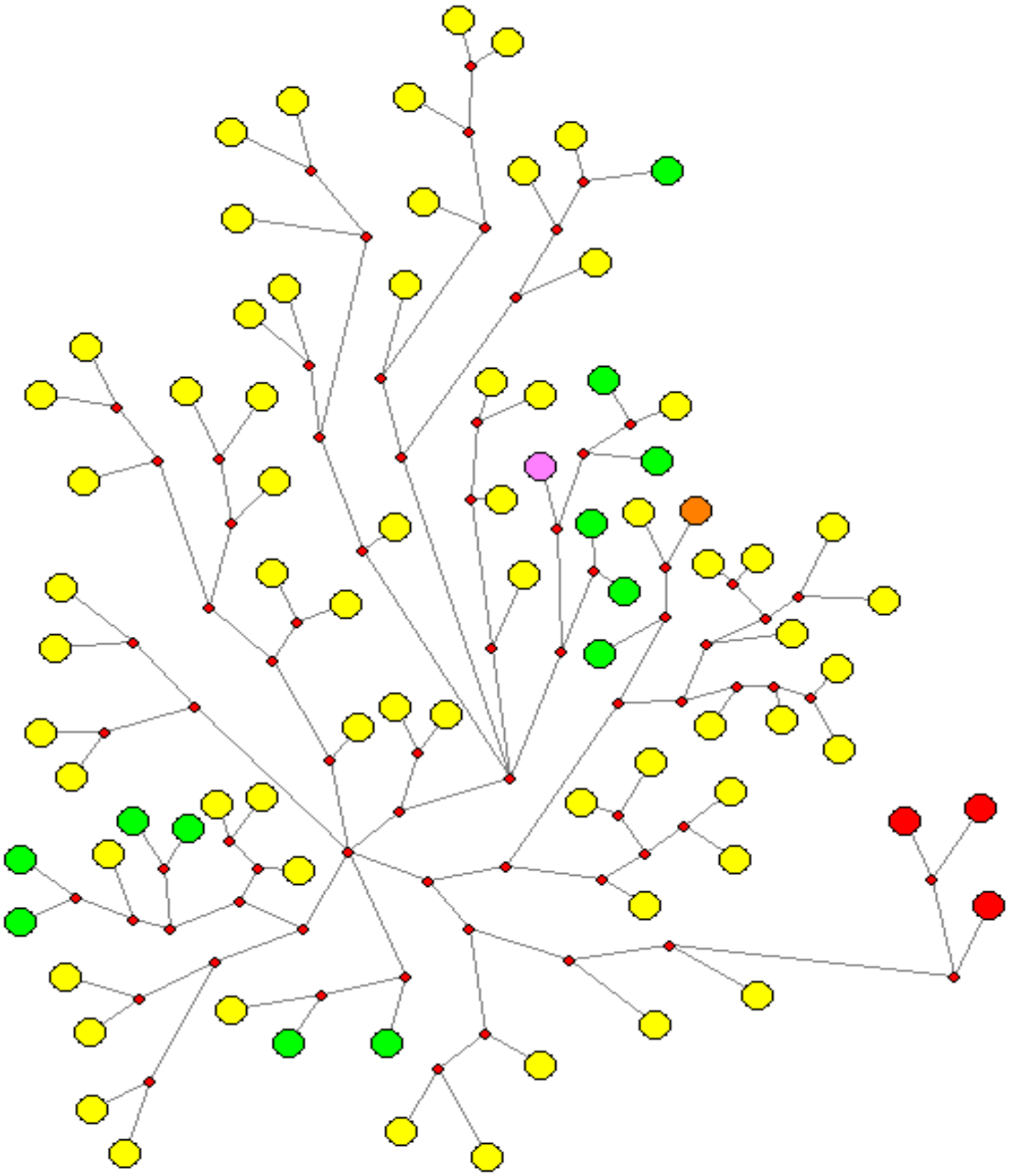

Figure 6.3. Minimum spanning network of 77 South African *Sclerotinia sclerotiorum* isolates based on amplified fragment length polymorphism analysis (AFLP) data generated with 4 primer combinations. The circle at each end of the node indicates an isolate linked to a host plant while the red dots connecting lines indicate a mutation.

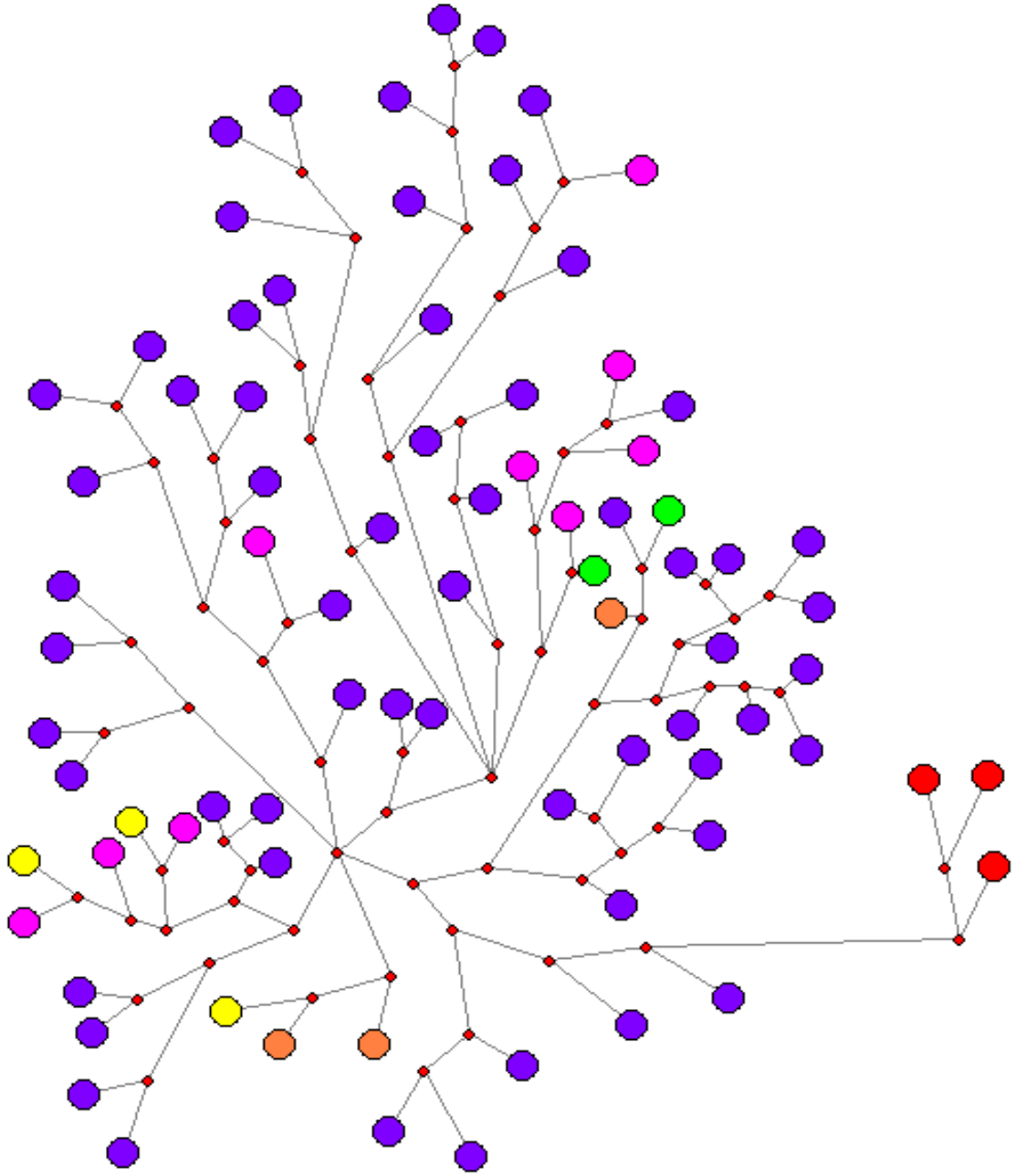

Figure 6.4. Minimum spanning network of 77 South African *Sclerotinia sclerotiorum* isolates based on amplified fragment length polymorphism analysis (AFLP) data generated with 4 primer combinations. The circle at each end of the node indicates an isolate linked to a province while the red dots connecting lines indicate a mutation.

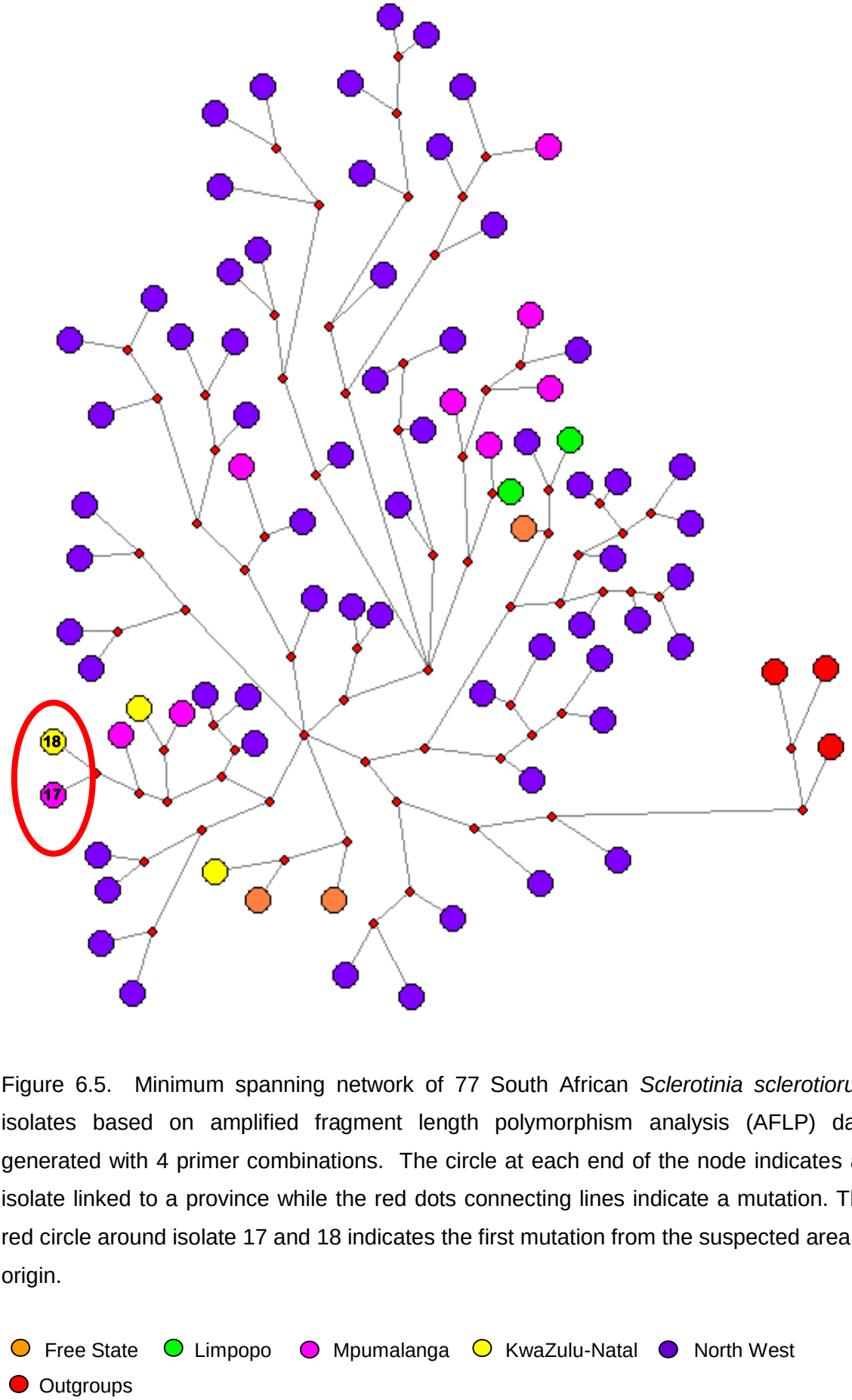

Figure 6.5. Minimum spanning network of 77 South African *Sclerotinia sclerotiorum* isolates based on amplified fragment length polymorphism analysis (AFLP) data generated with 4 primer combinations. The circle at each end of the node indicates an isolate linked to a province while the red dots connecting lines indicate a mutation. The red circle around isolate 17 and 18 indicates the first mutation from the suspected area of origin.

SUMMARY

- The annual increase in soybean production to meet the increasing demands is limited by environmental constraints, fungal diseases and insect pests. Sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*) is increasing in importance both locally and globally and the wide host range challenges disease management strategies. Soybean rust (caused by *Phakopsora pachyrhizi*) is a serious yield limiting foliar disease that has increased in importance due to rapid spread over large geographic areas. Reliable and repeatable screening methods have limited the quest for stable resistance to these diseases. Genetic variability in *S. sclerotiorum* isolates need elucidation since this could assist in the identification of stable resistance. Similalry, understanding the physiological processes associated with host response to to the respective pathogens could be useful in identifying new mechanisms of resistance or the timely implementing of appropriate control strategies. The absence of disease resistant cultivars, in particular to soybean rust, has necessitated extensive use of fungicides as a control method to ensure acceptable yields.

- A study was conducted to compare the reaction of experimental soybean lines to soybean rust in the greenhouse and evaluate epidemiological factors that need to be considered in field evaluations of germplasm. Greenhouse trials indicated significant differences in rust severity between lines while field data illustrated the importance of differences in relative life time at disease onset, apparent infection rate, AUDPC and relative life time at which 100% disease severity was reached. The importance of multiple evaluation criteria was emphasized. Constraints to achieving acceptable levels of disease resistance are the absence of a correlation between greenhouse and field results and repeatability of data over seasons and localities. Although lines differed in disease severity only Selection 43 showed a combination of late disease onset and slow apparent infection rate and hence a significant level of rust resistance. However, an extended study, which includes a variety of South African *P. pachyrhizi* races, is needed to determine the stability of the identified resistance.

- Yield losses associated with soybean rust in varieties from three maturity groups as well as the effects of row spacing and fungicide application on disease development were evaluated. Disease onset was closely related to host growth

stage and fungicides had little effect on delaying the disease. The apparent infection rate was higher in 45 cm, compared with 90 cm, row widths in the absence of fungicide applications while greater reductions in apparent infection rate due to fungicide applications were recorded in the wider rows. These responses were also reflected in AUDPC and yield. Differences in these variables between sprayed and unsprayed plots were more pronounced in the long maturity cultivar compared with the shorter maturity cultivars but the extent of these differences is dependent of spray efficiency. Differences in cultivar responses to the disease and row spacing do not provide adequate control and fungicide applications remain primary disease control strategy against soybean rust.

- Physiological changes associated with soybean growth stage in field trials indicated that soybean rust onset and development are determined by the activity of key pathogenesis-related enzymes. Environmental effects had a relatively small effect on disease severity once infection is initiated. Host responses to rust infection were directly related to the decline in chitinase and β-1,3-glucanase which coincided with flowering and pod formation and the subsequent rapid increase in rust severity. Plots that received fertilizer treatments indicated that chitinase, *β* -1,3- glucanase, peroxidase and phenol content were related to the form of nitrogen received. Higher chitinase activity, in particular, was also associated with lower disease severity.

- Evaluation of cultivars for resistance to Sclerotinia stem rot in the greenhouse and field failed to identify sources of resistance although lower risk cultivars were observed in field trials. Most cultivars were susceptible to Sclerotinia stem rot even when conditions were sub-optimal for disease development. A poor correlation between greenhouse and field data was observed. Field trials indicated that cultivars react differently to changing environmental conditions and disease potentials and high genotype x environment interactions were recorded. Data indicate that local cultivars still lack the desired level of resistance to Sclerotinia stem rot or stability to changing disease potentials.

- *In vitro* studies indicated that *S. sclerotiorum* is favoured by cool to moderate temperatures (20°C and 25°C) while extreme cold or hot temperatures impair growth. Molecular characterisation of isolates using AFLP analysis revealed a relatively uniform distribution with evidence of genetic diversity within and amongst isolates across the five provinces evaluated. A low, recombination frequency

suggests that the population is in a developing stage which is emphasized by isolates found on the end of the minimal spanning network. No correlation between host plant or locality and genetic similarity was observed but AFLP proved to be a valuable tool to characterize *S. sclerotiorum* isolates.

Keywords: *Sclerotinia sclerotiorum*, *Phakopsora pachyrhizi*, Sclerotinia stem rot, soybean rust, cultivar evaluation, yield loss, genetic variation, physiological changes, fungicides.

OPSOMMING

- Die jaarlikse toename in sojaboonproduksie om te voldoen aan die aanvraag word belemmer deur faktore soos omgewingsveranderinge, plantsiektes en insekplae. Sclerotinia stamvrot veroorsaak deur *Sclerotinia sclerotiorum* se belangrikheid neem toe en die wye gasheerreeks maak die beheer van hierdie patogeen 'n uitdaging. Sojaboonroes veroorsaak deur *Phakopsora pachyrhizi,* is 'n ernstige blaarsiekte en word aangetref in al die belangrike sojaboonproduksiegebiede wêreldwyd. 'n Beperking in evaluering van siekteweerstand is dat die metodes wat gebruik word nie herhaalbaar en betroubaar is nie. Die evaluering van genetiese variasie van *S. sclerotioum* isolate is belangrik aangesien dit gebruik kan word om moontlike weerstandsbronne te identifiseer. 'n Beter begrip van die fisiologiese prosesse in sojaboonplante tydens infeksie kan daartoe lei dat nuwe bronne van weerstand geïdentifiseer kan word of dat beter beheermetodes geïmplementeer kan word. Die afwesigheid van weerstandbiedende kultivars, spesifiek teen sojaboonroes, lei daartoe dat chemiese beheermetodes steeds grootskaals gebruik word om aanvaarbare oesopbrengste te kry.

- 'n Studie is gedoen om die reaksie van eksperimentele lyne teen sojaboonroes in die glashuis sowel as om die epidemiologiese faktore wat belangrik is vir veldevaluering van kiemplasma te ondersoek. Glashuisproewe het aangedui dat daar duidelike verskille in die vatbaarheid vir roesinfeksie tussen lyne is. Velddata het aangedui dat lyne verskil in die relatiewe leeftyd met die aanvang van siekte, die tempo van infeksie, die area onder die siekte-ontwikkelingskurwe en die relatiewe leeftyd van die gasheer wanneer 100% siekte aangeteken is. Alle lyne was vatbaar vir sojaboonroes. Die belangrikheid van veelvuldige evalueringskriteria by die evaluering van siekteweerstand is beklemtoon. Die swak korrelasie tussen glashuis- en velddata, asook die konsekwentheid van data oor seisoene en lokaliteite, is 'n bron van kommer. Alhoewel lyne verskil het in die graad van roesinfekies, het een inskrywing Selection 43 sekere nuttige eienskappe getoon soos latere aanvang van siekte en stadige tempo van siekte ontwikkeling. 'n Studie van *P. pachyrhizi* isolate in Suid Afrika sal egter eers gedoen moet word om 'n deeglike evaluering van kultivars of lyne te doen wat verdere navorsing regverdig.

- Opbrengsverliese veroorsaak deur roes op sojabone in drie kultivars van verskillende volwassenheidsgroeperings, sowel as die effek van ry-spasiëring en swamdoders op siekte-ontwikkeling, is geëvalueer. Die aanvang van siekte was nou verwant aan die gasheer-groeistadium en die toediening van swamdoders het ‘n minimale effek op die vertraging van siekte-aanvang gehad. In blokke waar geen swamdoders toegedien was nie, was die tempo van infeksie vinniger in die 45cm rye, in vergelyking met die 90 cm rywydtes. ‘n Groter afname in die tempo van infeksie is in die wyer rywydtes waargeneem, as gevolg van die toediening van swamdoder. Hierdie tendens is ook in die area onder die siekte-ontwikkelingskurwe en opbrengs waargeneem. Verskille in die veranderlikes wat gemeet is tussen die behandelde en onbehandelde blokke was die grootste vir die lang-groeiseisoen kultivar in vergelyking met die ander kultivars, maar die verskille is afhanklik van die spuitdoeltreffendheid. Data het aangedui dat verskille in die kultivars, asook rywydtes, nie afsonderlik voldoende is om siekte te beheer nie en dat die gebruik van swamdoders as beskerming teen sojaboonroes steeds gebruik moet word.

- Fisiologiese veranderinge, wat geassosieer is met spesifieke groeifases van sojaboonplante tydens veldproewe, het aangedui dat sojaboonroes ontwikkeling bepaal word deur die aktiwiteit van belangrike patogenese-verwante ensieme. Die effek van die omgewing was minimaal op die graad van siekte nadat infeksie plaasgevind het. Reaksie van die gasheerplant op roesinfeksie was direk verwant aan die afname in chitinase en β- 1,3 glukanase wat ooreengestem het met blom- en peulvorming en daaropvolgende toename in roesinfeksie. Blokke wat kunsmis toedienings ontvang het, het aangedui dat chitinase, β- 1,3-glukanase, peroksidase en fenool inhoud verwant was aan die vorm van stikstof wat toegedien is. ‘n Hoër vlak van chitinase is geassosieer met minder siekte.

- Evaluering van kultivars teen Sclerotinia stamvrot in die glashuis kon geen bronne van weerstand identifiseer nie, alhoewel laer risiko kultivars in die veld waargeneem is. Die meerderheid van kultivars was vatbaar vir siekte selfs onder minder optimale toestande. ‘n Swak korrelasie tussen glashuis- en velddata is waargeneem. Veldproewe het getoon dat kultivars verskillend reageer teenoor veranderende omgewingstoestande en siektepotensiaal. ‘n Hoë genotipe x omgewingsinteraksie is aangeteken. Data het aangetoon dat die gewenste vlak van weerstand steeds ontbreek in plaaslike kultivars, asook stabiliteit van die kultivars in toestande waar siektepotensiaal wissel.

- *In vitro* studies op die groei van *Sclerotinia sclerotiorum* isolate by verskillende temperature het aangedui dat koel tot matige temperature (20°C en 25°C) groei bevoordeel teenoor baie koue of warm temperature, wat ‘n inhiberende effek gehad het. Karakterisering van isolate met behulp van molekulêre tegnieke soos AFLP (geamplifiseerde fragment lengte polimorfisme) analise het getoon dat ‘n relatiewe uniforme verspreiding met bewyse van genetiese diversiteit in en tussen isolate wat in vyf provinsies versamel is voorkom. Die moontlikheid dat die populasie nog besig is om te ontwikkel is gestaaf deur ’n lae rekombinasie-frekwensie en word verder beklemtoon deurdat hierdie isolate op die punte van die minimum rykwydte netwerk analise voorkom. Geen korrelasie tussen gasheerplante of lokaliteite en die genetiese ooreenkoms is waargeneem nie, maar daar is gevind dat AFLP ‘n waardevolle tegniek is om *S. sclerotiorum* isolate te karakteriseer.

Sleutelwoorde: *Sclerotinia sclerotiorum*, *Phakopsora pachyrhizi*, Sclerotinia stam vrot, sojaboonroes, kultivar evaluering, opbrengs verlies, genetiese variasie, fisiologiese verandering, swamdoders