STUDIES ON *PHAKOPSORA PACHYRHIZI*, THE CAUSAL ORGANISM OF SOYBEAN RUST

by

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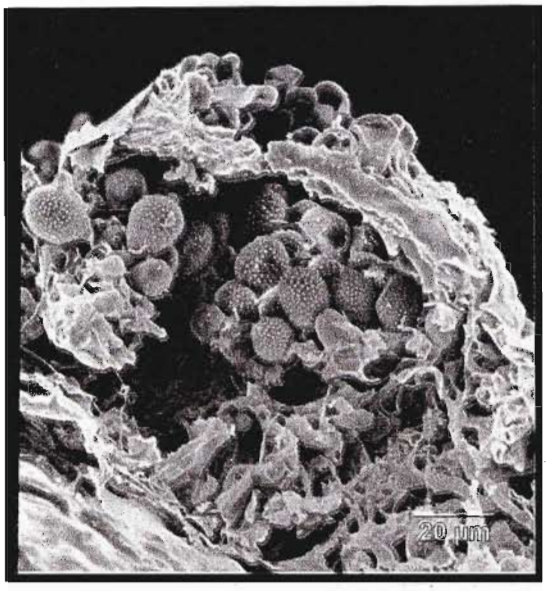
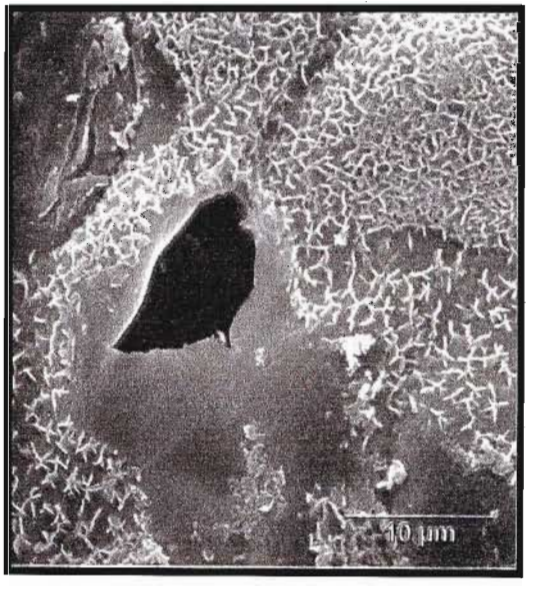


Electron micrograph showing a uredium with uredospores.



***Phakopsora pachyrhizi* is one of the few fungi that use direct penetration through epidermal cells.**

Rust never sleeps
Neil Young

ABSTRACT

Phakopsora pachyrhizi H. Syd and P. Syd, the causal organism of soybean rust (SBR) was first reported in Japan in 1902. In 1934 the pathogen was found in several other Asian countries and as far south as Australia. In India, SBR was first reported on soybeans in 1951. There have been several early reports of SBR in equatorial Africa but the first confirmed report of *P. pachyrhizi* on the African continent was in 1996 from Kenya, Rwanda and Uganda. Since then, the pathogen has spread south with reports from Zambia and Zimbabwe in 1998 and in Mozambique in 2000.

In February 2001, *P. pachyrhizi* was first detected on soybeans near Vryheid, in Northern KwaZulu-Natal, South Africa (SA). As the season progressed, the disease was observed in other parts of the province, and epidemic levels were found in the Cedara, Greytown, Howick and Karkloof production regions. Soybean rust subsequently spread to Amsterdam and Ermelo in the Highveld region of SA. The disease reappeared in SA in March 2002. It is now established that the pathogen is a threat to soybean production in the country with yield losses in the region of 10-80%.

A literature review on SBR investigating the taxonomy of the pathogen, its morphology, symptoms, host range, infection process, epidemiology, control options and the economic importance of *P. pachyrhizi* was complied to provide the necessary background information to conduct research under local conditions and to assist in interpretation of results of experiments.

Epidemiological trials were conducted at the University of KwaZulu-Natal under controlled environmental conditions in a dew chamber and conviron. Development of *P. pachyrhizi* on the susceptible cultivar (LS5995) was quantified in combinations of seven temperatures (15, 19, 21, 24, 26, 28 and 30°C) and five leaf wetness durations (LWD) (6, 9, 12, 14 and 16hrs) at three relative humidities (RH) (75%, 85% and 95%). Studies indicate that optimum temperature for uredospore infection is 21-24°C with a LWD greater than 12hrs and RH 85-95%. The number of pustules as well as lesion

size on the abaxial and adaxial leaf surface increased with increasing LWD at all the RH values tested. Infection did not occur on plants incubated at 15°C and 30°C at 85% or 95%RH whereas at 75%RH infection did not occur on plants incubated at 15°C, 19°C and 30°C regardless of LWD. Number of pustules per lesion produced at 75%, 85% and 95%RH was highest at 24°C and showed a gradual increase with increasing LWD. Lesion size on both leaf surfaces increased after 12hrs LWD at 24°C at 75% and 85%RH whereas at 95%RH lesion size increased after 14hrs LWD at 24°C.

Exposure of uredospores to ultraviolet light which is equivalent to ultraviolet C (sunlight) which is < 280nm, shows a decrease in germination (7%). Under continuous darkness, the germination percentage was found to range from 58% after 48 hrs. Germination was found to peak at 16hrs in darkness with a gradual decrease as time increased whereas germination under ultraviolet light was highest after 6hrs with a gradual decrease with increased exposure to light. Germ tube lengths were found to be shorter when exposed to ultraviolet light (107µm) compared to controls kept in the dark (181µm). Results obtained clearly show a negative effect of ultraviolet light on the germination and germ tube length of uredospores. A 0.1ml suspension of uredospores on 1.25% water agar Petri dishes was exposed to cycles of 14h ultraviolet light and 10h darkness for 48h. Results indicate an increase in germination percentage of uredospores when exposed to 10h of darkness following a 14h period under ultraviolet light.

Controlled environmental studies were conducted to determine alternative hosts of *P. pachyrhizi* in SA. The control used in this experiment was Prima 2000, a susceptible cultivar to soybean rust. Seven legume plants [*Cajanus cajan* (L.) Huth, *Glycine max* (L.) Merr, *Lablab purpureus* (L.) Sweet, *Lupinus angustifolius* (L.) Finnish, *Phaseolus vulgaris* (L.), *Pueraria lobata* (M&S) Willd and *Vigna unguiculata* (L.) Walp] and three dry bean lines (Bonus; OPS-RS2 and PAN 159) showed typical SBR symptoms when rated after 21 days post inoculation with uredospores for percentage disease severity. Disease severity was significantly different within the alternative hosts, but *G. max*,

P. vulgaris and *P. lobata* were not significantly different from Prima 2000 (control). A uredospore suspension of 2.5 x 10^5 uredospores ml^{-1} from plants that showed typical SBR symptoms was made and inoculated onto Prima 2000, a susceptible soybean cultivar. Uredospores from pustules on *G. max*, *L. purpureus*, *L. angustifolius*, *P. vulgaris*, *P. lobata*, *V. unguiculata*, Bonus and PAN 159 produced viable uredospores on PRIMA 2000. These plants are considered alternative hosts of *P. pachyrhizi*.

Effect of leaf age on susceptibility of soybean to SBR was tested under controlled environmental conditions. Mean number of lesions as well as lesion size were greater on younger leaves than on older leaves of plants at the same physiological age. Plants at the early vegetative and reproductive stages had a significantly lower number of lesions as well as a smaller lesion size. Plants at the V6 and R1 growth stages were significantly more susceptible to *P. pachyrhizi* than plants at other developmental stages.

Trichoderma harzianum Rifai, Eco-77® a commercial biological control product, was evaluated for its efficacy as a biological control agent of *P. pachyrhizi*. *Trichoderma harzianum* sprayed at the standard concentration on infected soybean plants was significantly more effective in controlling *P. pachyrhizi* than plants sprayed at 1/2X and 2x the standard concentration. This was noted in both Trial 1 and 2. Data indicate that spraying the filtrate two days after inoculation produces less disease.

DECLARATION

I, Archana Nunkumar, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. This thesis has not been submitted for any degree or examination at any other university.

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Archana Nunkumar (Candidate)

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Dr P.M. Caldwell (Supervisor)

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Finally, I would like to thank God for seeing me through this study.

DEDICATION

To my mother, Kocellia Nunkumar,
for her support, encouragement
and understanding
during my years of study

FOREWORD

The research presented in this thesis was undertaken in the Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg under the supervision of Dr P.M. Caldwell.

Since the first report of *Phakopsora pachyrhizi* Syd., the causal organism of soybean rust (SBR) on soybeans, in South Africa (SA) during March 2001 much research has been undertaken in the study of this unique fungal pathogen. Soybean rust is a new disease in SA and it has been established that *P. pachyrhizi* is now endemic to SA. Very little knowledge about this pathogen in SA is available.

Research on SBR pathogen has been carried out by institutes including the University of KwaZulu-Natal, University of the Free State, Protein Research Foundation, Department of Agriculture and Environmental Affairs, Cedara and Agricultural Research Council to find effective solutions to what has become a serious yield reducing pathogen before it causes irreversible damage to the soybean industry in SA.

The extent of this research is broad, traversing seven chapters. The main objectives of the research in this thesis were: a review of the literature on the history and geographic distribution of *P. pachyrhizi*, the economic importance and pathogen taxonomy and morphology, the symptoms, host range, infection process and epidemiology, and control options available for the control of *P. pachyrhizi* (Chapter One); the epidemiology of *P. pachyrhizi* under controlled environmental conditions (Chapter Two); *in vitro* screening of *P. pachyrhizi* uredospores to the effects of ultraviolet light (Chapter Three); an alternative host study (Chapter Four); an evaluation of soybean plants at different developmental stages to SBR (Chapter Five); an evaluation of a commercial biological control product to control SBR (Chapter Six) and a review of the experimental results, conclusions and recommendations for future research on SBR in SA (Chapter Seven).

Chapter Two and Three will aid in the production of a disease prediction model for SBR. Chapter Four gives an indication of which legume plants are alternative hosts of this pathogen in SA. Chapter Five gives an indication at which time plants are more susceptible to the pathogen, hence, providing a more accurate time to apply chemical sprays for control. Chapter Six will help determine if *P. pachyrhizi* can be controlled by alternative methods, as at present chemical applications are the only effective means for control.

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Soybean, *Glycine max* (L.) Merril. is an ancient crop with numerous food, feed and industrial uses. This crop is native to eastern Asia and has been used for thousands of years for human and animal food as well as medicine to treat many human diseases (Hartman *et al.*, 1999). Soybeans provide protein and are the primary source of vegetable oil, which constitutes 40% of the world's edible vegetable oil. Soybean products are economically important due to their ability to produce new, low-cost, nutritionally balanced, high- protein foods, fit for human consumption (Sinclair, 1982).

Soybeans are grown from temperate to tropical regions of the world, with production being highest in Brazil, China and the United States of America (U.S.A). Emphasis in research has been on breeding of soybeans, appropriate for tropical environments (Hartman *et al.*, 1999). Due to an increase in soybean production throughout the world, diseases that affect this crop have also, therefore, increased in number and severity.

In Africa, soybean cultivation has increased in the last four decades from 72 000 tonnes on 191 000ha in 1961 to 989 000 tonnes on 1 090 000ha in 2002. However, it accounts for only 0.5% of the annual global production of 179 917000 tonnes (Singh *et al.*, 2004). In South Africa (SA) soybean is a strategically important crop and is grown under natural rainfall and irrigated conditions, usually in summer rainfall areas (Bell *et al.*, 1990). In KwaZulu-Natal (KZN) SA, approximately 30 000-35 000ha of soybeans are grown annually. Due to an ever-increasing demand for soybeans, expansion of production is still possible in the northern and midland areas of KZN (Ward, 2003).

Soybeans are affected by more than 100 pathogens, with approximately 35 of economic importance (Earthington *et al.*, 1993). All parts of the soybean plant are susceptible to numerous pathogens, resulting in a reduction in quality and quantity of seed yields (Sinclair and Backman, 1989).

Phakopsora pachyrhizi Sydow, the causal organism of soybean rust (SBR) is one of the major disease problems limiting soybean yield. In February 2001, *P. pachyrhizi* was detected for the first time on soybeans near Vryheid in Northern KZN, SA (Pretorius *et al.*, 2001). As the season progressed, the disease was observed in other parts of the province, and epidemic levels were found in the Cedara, Greytown, Howick and Karkloof production regions. Soybean rust subsequently spread to Amsterdam and Ermelo in the Highveld region of SA (Caldwell *et al.*, 2002).

The disease reappeared in SA in March 2002. It is clear that the pathogen is now an established threat to soybean production in the country. Yield losses in SA are reported to be in the region of 10-80% (Caldwell and Laing, 2002).

Soybean rust has spread around the globe causing extensive damage to soybean crops throughout the Southern hemisphere. Apparently it is able to travel great distances via wind-borne spores. Also known as Asian rust, this fungal infection can defoliate soybean fields rapidly, often resulting in severe and sometimes total loss (Stewart *et al.*, 2005).

1.2 BACKGROUND INFORMATION

1.2.1 HISTORY

Soybean rust has been known in the Orient for many decades (Vakili and Bromfield, 1976). The fungus was identified as (Bresadola, 1881 cited by Bromfield, 1984), *Uredo vignae*, and this was the first record of this fungus in the Western hemisphere. In 1903, Henning (1903 cited by Bromfield, 1984) identified the fungus as *Uredo sojae* from a specimen on *Glycine usssuriensis* Rgl et Moach or *G. soja*. In 1914 H. and P. Sydow gave the name *P. pachyrhizi* to the fungus on *Pachyrhizus erosus* (L.) Urban. *Phakopsora pachyrhizi* is now generally accepted as the name for the pathogen inciting SBR (Bromfield, 1984).

The late 1940s marked the beginning of scientific research on *P. pachyrhizi*. It is believed that research on this disease began well after it was first identified due to a lack of trained researchers with the ability to conduct scientific research on the disease in areas where it was epidemic. In the 1970s, research on the disease increased due to an increase in soybean production in traditional soybean growing areas and other areas where soybeans were not previously grown. In 1971 the United States Department of Agriculture (USDA) began research on this unique pathogen (Bromfield, 1984).

1.2.2 GEOGRAPHIC DISTRIBUTION

The first report of the disease was from Japan in 1902 (Figure 1.1). By 1934 the pathogen had been found in several Asian countries and as far south as Australia (Bromfield and Hartwig, 1980). In India, SBR was first reported on soybeans in 1951 (Sharma and Mehta, 1996). There have been several early reports of SBR in equatorial Africa (Javaid and Ashraf, 1978; Bromfield, 1980), but the first confirmed report of *P. pachyrhizi* on the African continent was in 1996 from Kenya, Rwanda, and Uganda. Since then, the pathogen has spread south with reports from Zambia and Zimbabwe in 1998, Mozambique in 2000 and SA in 2001 (Pretorius *et al.*, 2001; Levy *et al.*, 2002). The westward movement of the pathogen on the African continent was reported from Nigeria in 1999 (Akinsanmi *et al.*, 2001).

In South America the first report of *P. pachyrhizi* was from Paraguay in March 2001 (Morel *et al.*, 2004). It was subsequently reported in the state of Paranå, Brazil in 2001 (Yorinori, 2004). The disease was found in Hawaii in 1994 on cultivated soybeans on the islands of Hilo, Kakaha, Kauai and Oahu. (Kilgore and Heu, 1994).

By 2002, SBR was widespread throughout Paraguay and in limited areas of Brazil bordering Paraguay, with reports of severe disease in some fields in both countries (Morel and Yorinori, 2002). The pathogen also was found in a limited area in northern Argentina (Rossi, 2003).

In August 2004, the USDA and the Animal Plant Health Inspection Service (APHIS) confirmed a report of SBR in Colombia (Caspers-Simmet, 2004). On the 10 November 2004, the USDA issued a press release on the first report of SBR on the USA mainland (Rogers and Redding, 2004). It is now established that SBR occurs in all major soybean producing areas around the world.

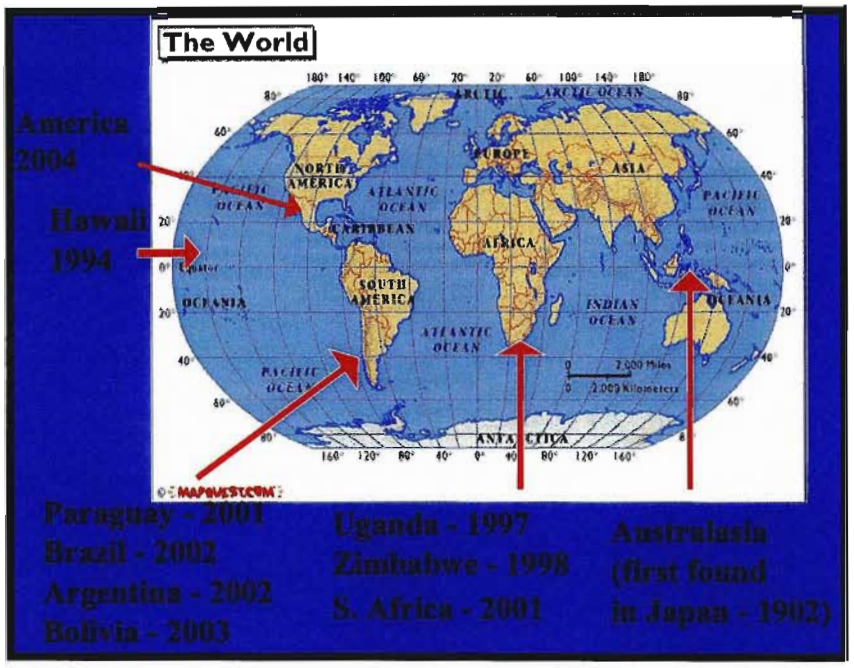

Figure 1.1 Worldwide distribution of SBR caused by *Phakopsora pachyrhizi* (Miles *et al.*, 2003).

1.2.3 ECONOMIC IMPORTANCE

Soybean rust causes severe economic losses in many parts of the world where soybeans are grown on a large commercial scale and is considered the most destructive foliar disease of soybeans (Miles *et al.*, 2003). In 1973 APHIS declared SBR to be one of the hundred most dangerous exotic pests and diseases and a number one threat to soybeans (Hershman, 2003).

Soybean rust reduces yield through premature defoliation, decreasing the number of filled pods and by reducing the weight of seeds per plant. It also lowers the quality of seed produced. The severity of loss and the particular components of yield affected depend primarily on the time of disease onset and the intensity of disease at particular growth stages of the soybean crop (Bromfield, 1984). When early infection and unfavourable environmental conditions exist, yield losses of 50-60% can be experienced (Kloppers, 2002).

Yield losses as high as 10-50% have been reported in Southern China, 40% in Japan,10-40% in Thailand, and 25-90% in Taiwan (Sinclair and Backman, 1989). Nearly complete yield losses can occur in limited areas in most of these countries. Yield losses in Uganda were estimated to be 22.9% in 2003 (Kawuki *et al.*, 2003).

South Africa produces 208 000 tonnes of soybean seed on 193 000ha of land. Farmers in KZN plant about 35 000ha, i.e., about 18% of arable land is planted to soybeans in SA. In SA yield losses of 10-80% were reported, with losses of up to 100% where monocropping was practiced (Caldwell and Laing, 2002).

Yang *et al.* (1991) reported that the number of pods per plant at growth stage R6 was reduced by as much as 40%, but the number of seeds per pod was not affected, i.e., the disease affected the attainable yield by reducing pod set. From growth stage R6 to R7, percentage of pod abortion was high for severely diseased plants. Seed growth rate (grams per day) from R4 to R7 was reduced by 40-80% in diseased

plants. The time for diseased plants to grow from R4 to R7 was reduced by as many as 16 days compared to protected plants (Yang *et al.*, 1991).

In 1984, an economic risk analysis projected that the potential losses in the U.S.A would be $7.1 billion per year, once SBR was established in the main soybean growing area of the U.S.A. (Kuchler *et al.*, 1984). A conservative prediction indicated yield losses greater than 10% in nearly all the U.S.A growing areas with losses of 50% in the Mississippi delta and the southeastern coastal states (Yang, 1995).

1.3 THE PATHOGEN

1.3.1 TAXONOMY AND MORPHOLOGY

Phakopsora pachyrhizi belongs to the Phylum Basidiomycota (Alexopoulos and Mims, 1979), the Class Uredinomycetes, the Order Uredinales, the Family Melampsoraceae and the Genus *Phakopsora* (Agrios, 1997). A related rust fungus, *Phakopsora meibomiae* Arthur, also infects soybeans but is considered less virulent than *P. pachyrhizi* (Caldwell *et al.*, 2002).

Phakopsora pachyrhizi and *P. meibomiae* may only be differentiated based upon the morphological characteristics of telia. However, primers have been developed specifically for the two species to facilitate a polymerase chain reaction (PCR) so that the two species can be accurately and quickly identified (Frederick *et al.*, 2002).

Uredia are globose, subepidermal, and erumpent and light cinnamon to reddish brown. They form abundantly on the abaxial leaf surface, where they range from 100 to 200µm in diameter (Sinclair and Backman, 1989). Pycnia and aecia are unknown. Uredospores are globose, subglobose, ovate or ellipsoidal and are essentially hyaline to light yellow-brown and open through a central pore to form a germ tube. The size of the spores is highly variable, in the range of 18-45 x 13-28µm, depending on the host and environmental conditions (Figure 1.2). Paraphyses, which are found surrounding the inner wall of the uredia, unite at the base, forming a domelike

covering over the sporophores. The paraphyses are inward curving, hyaline to subhyaline, prominently capitate at the apex, with a narrow lumen, and measure about 7-15µm toward the apex (Figure 1.3) (Sinclair and Backman, 1989).

Phakopsora pachyrhizi is one of five rust fungi that can infect without the formation of an appressorium. Direct penetration is usually observed. Telia are rare but occasionally form subepidermally, mostly on the abaxial leaf surface, among the uredia and at the edges of lesions. They are orange-brown or light brown when young, and become dark-brown to black with age. They are crustose, irregular to round, sparse to aggregate, and about 150-250µm in diameter, with 3-5µm irregular layers of teliospores (Sinclair and Backman, 1989).

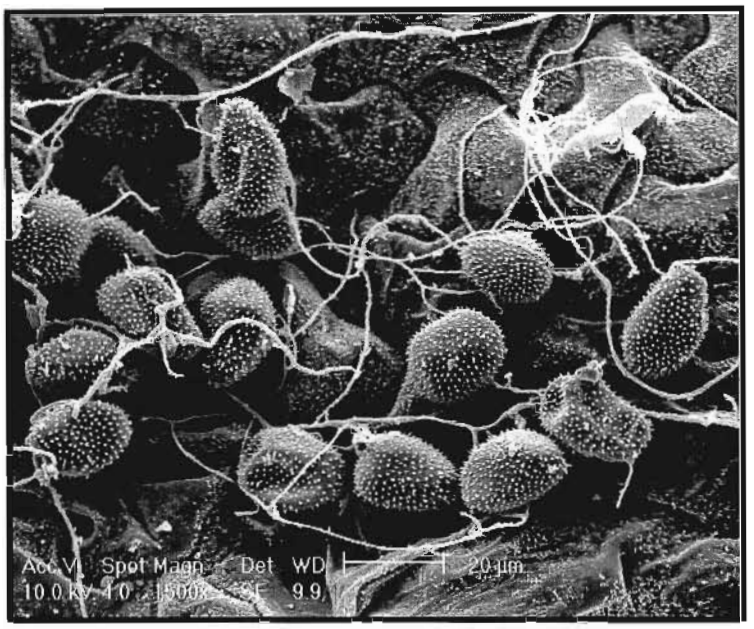

Figure 1.2 Electron micrograph showing soybean rust uredospores and germ tubes (Nunkumar, A).

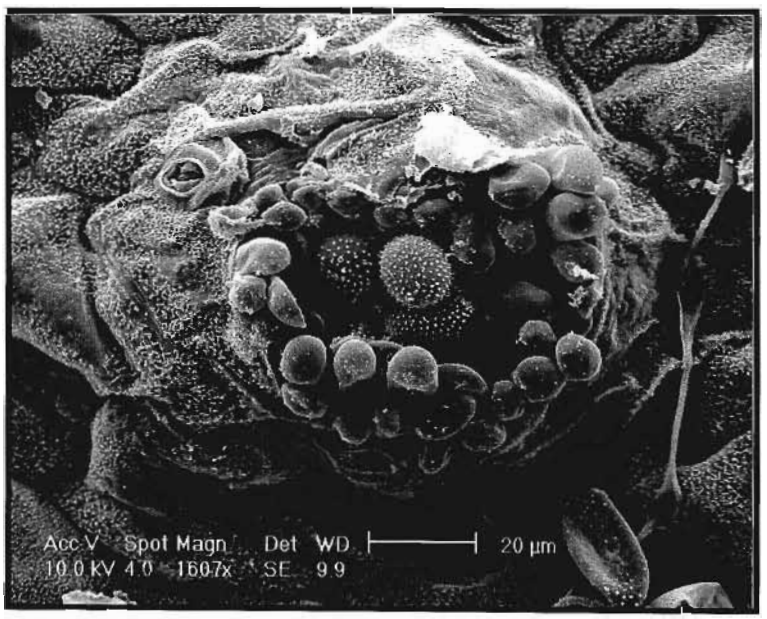

Figure 1.3 Paraphyses surrounding the inner wall of a uredium (Nunkumar, A).

1.3.2 SYMPTOMS

Soybean rust is an obligate parasite and a low sugar disease (Caldwell *et al.*, 2002). This is indicated by the fact that infection usually begins on the older, lower leaves of plants at, or after the flowering stage, but is generally not noticed until the pods are set. Early symptoms appear as small water-soaked lesions, which gradually increase in size, turning from grey to tan or brown and are restricted by leaf veins. The release of visible clouds of rust spores is another identifying characteristic of SBR (Caldwell and Laing, 2002). Initially SBR symptoms may be confused with bacterial pustule (*Xanthomonas axonopodis* pv. *glycines* Nakano). However, bacterial pustule has water-soaked lesions containing mucilaginous or sticky bacteria (Caldwell and Laing, 2002). This bacterial pathogen also causes defoliation of plants, but no pustules are visible.

Lesions are found mainly on the leaves, where they are common on the abaxial leaf surface exuding clumps of brownish spores called uredospores (Bromfield *et al.*, 1980; Sinclair and Backman, 1989) (Figure 1.4). However, in severe cases, lesions can also be found on pods, stems and petioles (Caldwell *et al.*, 2002). Within each of the lesions is one to many erumpent, globose uredia. Reddish-brown lesions appear to indicate a semi-compatible reaction, while those with a tan coloration, without extensive necrosis indicate a compatible interaction (Caldwell *et al.*, 2002).

Once lesions appear, premature yellowing occurs and defoliation is rapid, resulting in fewer pods and seeds, lower seed weight and early maturity (Caldwell *et al.*, 2002). The infected leaves turn bronze or yellow and these patches in the field are known as "hot spots" (Figure 1.5). Once these hot spots are observed in the field, it is usually too late to apply fungicides.

Although quantitative data are lacking, it is generally thought that leaf yellowing and defoliation are correlated with the number of lesions per leaflet. As the number of lesions per unit area increases, yellowing and defoliation becomes more pronounced.

The rate of severity of these processes may be influenced by the host variety and the pathogen isolate involved (Bromfield, 1984).

Figure 1.4 Lesions on the abaxial leaf surface exuding clumps of uredospores (Nunkumar, A).

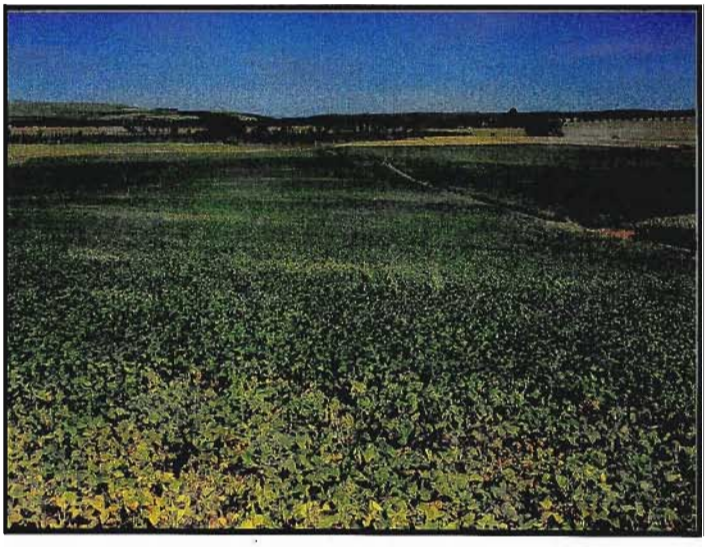

Figure 1.5 Infected leaves in the field indicating "hot spots (Kloppers, 2002[1]).

[1] Kloppers, R. 2002. Pannar®, Greytown, KwaZulu-Natal, South Africa.

1.3.3 HOST RANGE

Phakopsora pachyrhizi is an obligate parasite and cannot survive independently of its hosts or on debris. It must, therefore, find alternate hosts on which to survive under host-free conditions.

This pathogen has an unusually wide host range. *Phakopsora pachyrhizi* has been reported to produce natural infections on 31 plant species in 17 genera of legumes and 60 species of plants in 26 additional genera when inoculated (Chu and Chuang, 1961).

Many researchers have proposed lists of alternate hosts, but some lists require cautious interpretation. This is because many researchers do not state the criteria used to determine a "host'. The host in question should only be considered as an alternate host if the fungus sporulates on it. In some instances, hosts, which do not support sporulation, have been included in lists of alternate hosts (Bromfield, 1984).

A full host range has, therefore, not been clearly identified (Miles *et al.*, 2003) and is complicated by pathotypes or races of the fungus and strains or varieties of the host. The same legume species may support sporulation of the fungus in one region but not in another due to differences in races of the pathogen (Bromfield, 1984).

Rytter *et al.* (1984) tested 35 species within 23 genera of legumes for reactions to three races of *P. pachyrhizi*. Twelve species were found to be new alternative hosts, including *Coronilla varia* (L.) Koch, *Lespedeza striata* (H&A) Thunb, *Lupinus luteus* (L.) Finnish, *Sesbania sericea* (Willd.) Link and *Trifolium repens* (L.).

Shinde and Thakare (2000) tested various leguminous and pulse crops under glasshouse conditions during 1997-1999 to determine possible hosts of *P. pachyrhizi*. It was found that *Vigna unguiculata* (L.) Walp. (cowpea); *Phaseolus vulgaris* (L.) (French bean), *Phaseolus aureus* Roxb. (blackgram), *Cajanus cajan* (L.) Huth.

(pigeon pea) and *Cicer arietinum* (L.) (chickpea) and *Glycine wightii* (Wight & Arn.) Verdc. (perennial soybean) were infected by the pathogen.

Some common hosts include *Melilotus indica* Color. (yellow sweet clover), *Lupinus angustifolius* (L.) Finnish (lupin), *Phaseolus vulgaris* (L.) (green/kidney bean), *Phaseolus lunatus* (L.) (lima/butter bean. One of the rather important alternate host is *Pueraria lobata* (M&S) Willd. (kudzu vine), which is widespread in the U.S.A and South America (Miles *et al*., 2003).

1.4 INFECTION PROCESS AND EPIDEMIOLOGY

1.4.1 LIFE CYCLE AND INFECTION PROCESS

Successful infection of a host by a pathogen is the culmination of a series of events that must occur in sequence, i.e., spore germination, appressorium formation, and penetration. Each of these steps and the subsequent ones of colonization and sporulation are influenced by biotic factors of the pathogen and host, and abiotic factors of the environment.

Two spore types are known in *P. pachyrhizi*. The uredospore is the common spore type found throughout the season. Uredospores are readily dispersed by wind and multiple spore cycles occur throughout the season. Telia and teliospores have been found on infected plants late in the season (Miles *et al.*, 2003). Since no alternate host has been identified, there has been no further characterization of the life cycle (Miles *et al.*, 2003). The infection process starts when uredospores germinate to produce a single germ tube that grows across the leaf surface, 5 to 400 µm, until an appressorium forms. Appressoria form over anticlinal walls or over the center of epidermal cells, but rarely over stomata. Penetration of epidermal cells is by direct penetration through the cuticle by an appressorial peg. When appressoria form over stomata, the hyphae penetrate one of the guard cells rather than entering the leaf through the stomatal opening.

This rust and related species are unique in their ability to directly penetrate the epidermis; most rust pathogens enter the leaf through stomatal openings and penetrate cells once inside the leaf. The direct penetration of the epidermal cells and the non-specific induction of appressoria in the infection process of *P. pachyrhizi* may aid in understanding the broad host range of the pathogen. Under dry conditions this extended sporulation capacity allows the pathogen to persist and remain a threat. If conditions for re-infection are sporadic throughout the season, significant inoculum potential still remains from the initial infection to reestablish an epidemic. A general life cycle of the heterocious rust is presented in Figure 1.6.

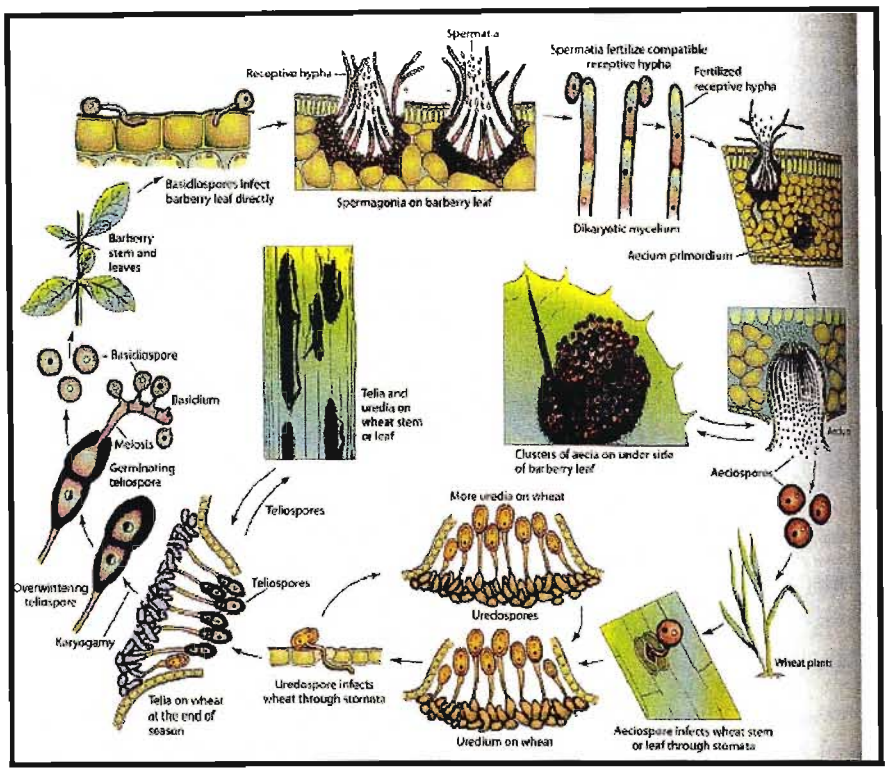

Figure 1.6 A general life cycle of the heterocious rust (Agrios, 1997).

Successful infection is dependant on the availability of moisture on plant surfaces. At least 6 hours of free moisture is needed for infection with maximum infections occurring with 10 to 12 hours of free moisture. Temperatures between 15 and 28°C are ideal for infection (Miles *et al.*, 2003). The infection process of *P. pachyrhizi* and the developmental stages of SBR are given in Table 1.1 (Marchetti *et al.*, 1975; Bonde *et al.*, 1976; McLean, 1979; Koch *et al.*, 1983; Miles *et al.*, 2003).

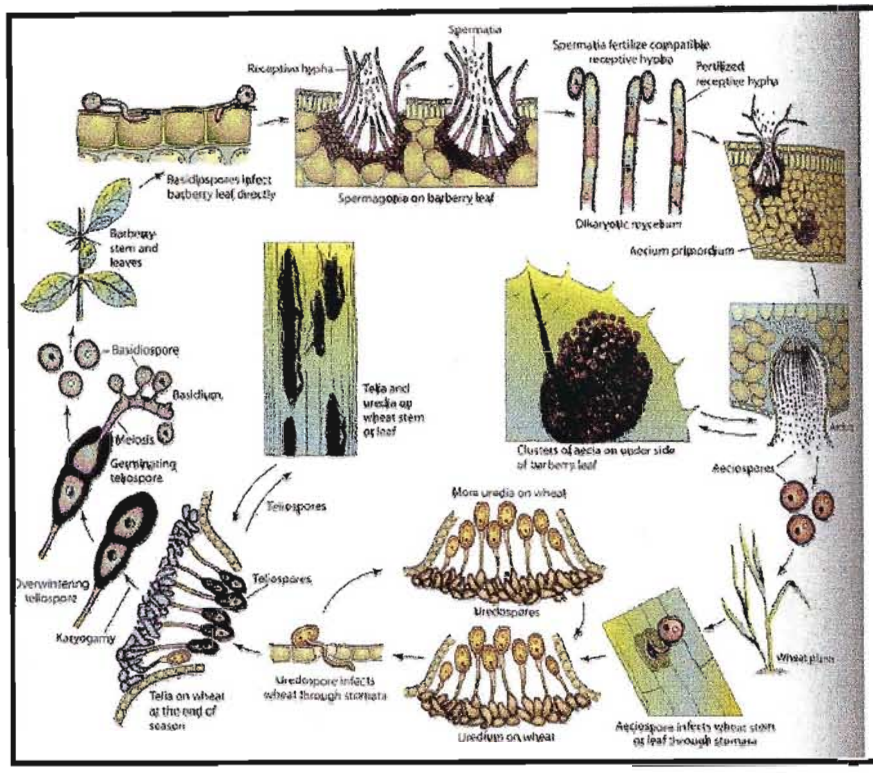

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Table 1.1 Sequence of events over time in the development of *Phakopsora pachyrhizi* (Marchetti *et al.*, 1975; Bonde *et al.*, 1976; McLean, 1979; Koch *et al.*, 1983; Miles *et al.*, 2003).

Sequence of events	Time
1. A uredospore lands on soybean leaf surface over epidermal cell	0 hpi
2. Germ-tube development (5-400µm)	12 hpi
3. An appressorium-cone formed	16 hpi
4. Penetration hyphae formed	16 hpi
5. First hyphal septum formed	18-20 hpi
6. Primary hyphae produced	18-20 hpi
7. Collapse of epidermal cell	24 hpi
8. Haustorium formed	24-48 hpi
9. Branching into secondary hyphae	48-72 hpi
10. Mycelial development inside spongy mesophyll and intercellular space	3 dpi
11. Collapse of appressorium and penetration hyphae	4 dpi
12. Necrotic lesions appear on leaf	6 dpi
13. Runner hyphae passing through mesophylls	7 dpi
14. Hyphae aggregate, uredial primordia formed	9 dpi
15 Uredospore mature	11-12 dpi

hpi- hours post infection

dpi- days post infection

1.4.2 EPIDEMIOLOGY OF SOYBEAN RUST

The ability of *P. pachyrhizi* to cause an epidemic in soybeans depends on a number of factors. Two of these factors are temperature and leaf wetness duration, which together determine the suitability of infection periods. Another factor affecting epidemic development is the timing of the first rain on the crop and the amount of rain (Tschanz *et al.*, 1984).

Studies have shown that the rate of SBR development is closely associated with the development and maturation of the soybean plant. Delayed rust onset, therefore results in less serve infection levels. Therefore, the effect of soybean development and maturation on rust development has to be accounted for in epidemiological and host resistance studies (Tschanz and Tsai, 1982; Tschanz *et al.*, 1984).

Rust epidemics are most severe during extended periods of leaf wetness when the average daily temperature is less than 28°C (Hartman *et al.*, 1999) with relative humidities of 75-80% (Caldwell *et al.*, 2002). Dry conditions, excessive precipitation or daily mean temperatures greater than 30°C or less than 15°C inhibit rust development (Sinclair and Backman, 1989). Moisture on plant surfaces is crucial for germination to occur (Caldwell *et al.*, 2002), Hence areas where prolonged periods of leaf wetness due to dew, mist and light rain occurs provide optimum conditions for germination (Kloppers, 2002). Temperatures above 27°C for extended periods retard rust development even with adequate free moisture on the leaf surface (Casey, 1979).

In areas where rainfall occurs more evenly throughout the season, SBR develops more rapidly as opposed to areas where rainfall occurs in uneven patterns. Hence rust development varies according to prevailing rainfall patterns. To determine the effect of precipitation and irrigation on rust development, field soybeans were watered with overhead irrigation and furrow irrigation (Wang and Hartman, 1992). Results indicate that rust was more prevalent in overhead-irrigated plots.

Field studies by Casey (1979) demonstrated that the development of a severe rust epidemic requires about 10 h d^{-1} of leaf wetness and a daily mean temperature of 18-

26°C. In another field study, mean night temperatures consistently below 14°C prevented or greatly inhibited rust development, while mean night temperatures above 25.5°C had little effect on rust development when they occurred in conjunction with frequent, long leaf wetness periods (Tschanz *et al.*, 1984).

At optimum temperatures between 20°C and 25°C, infection of a susceptible host can occur during 6hrs of leaf wetness. Within this temperature range, maximum infection occurs within 1-12hr of leaf wetness. Increased periods of leaf wetness are necessary for infection when temperatures fall outside the optimum temperature range (Marchetti *et al.*, 1976).

Phakopsora pachyrhizi telia and teliospores are induced by low temperatures and occur when night temperatures are between 5 and 15°C and day temperatures between 20 and 25°C (Kitani and Inoue, 1960; Hsu and Wu, 1968; Bromfield, 1980). Yeh *et al.* (1981) also induced formation of telia and teliospores on a number of other leguminous hosts. So far teliospore germination has not been observed, and its role in the epidemiology of the disease is unknown.

1.5 DISEASE MODELING

The extent to which mathematical models are used in biological sciences varies greatly over the range of the disciplines. In some disciplines, such as population genetics, the need for mathematics is recognized and permeates teaching and research. In other disciplines, such as plant pathology, the need for mathematics appears only in rather esoteric applications (Jeger, 2004).

A model is an abstraction of the real world, a simplified approximation to reality (or parts of it), but by no means the reality itself, nor its replica. This implies that a model is rarely complete, final and an objective in itself. Effective modeling is only achieved when striking an appropriate balance between realism and abstraction for the purpose in hand (Putter, 1982).

Mathematical expressions in models are functions obtained from multivariate regression analyses, differential equations for more general facets of populations, transfer functions, stochastic sub- models, or combinations of them with non-linear differential equations for randomly varying phenomena, e.g., spore dispersal, infection processes, or incubation periods (Kranz, 1974).

Whatever form a model eventually attains when developed from the conceptual model, at the start to the final version at the end of the research project, it should have the following desirable characteristics, i.e., it must be as simple as possible, logical, mathematically correct but it must be goal-orientated, validated and reliable and able to predict in the mathematical sense. (Kranz and Royle, 1978).

A disease model for SBR has been developed by Yang (2004) at Iowa State University, U.S.A. The basic model used for risk assessment is a stimulation model. The model is a simple disease model but includes the most important factors influencing disease epidemics. It was defined to determinatively stimulate daily development of SBR on two susceptible soybean cultivars, i.e., TK-5 and G-8587.

The model consists of a main program, an input and an initiation program, and a graphic and statistic output program. The main program has five subroutines with 10 state variables, and some constants. Models of infection rate, latent period, and senescence have been developed. The model of latent period explains up to 98.7% of the variation, with no uredia present until 6.35 physiological days after inoculation. Sixteen physiological days after inoculation, up to 95% of the lesions became infectious (Kranz and Royle, 1978).

The rationale for modeling and analysis of disease progress data derives from the desire to compare epidemics. The comparison may be years, locations or environments, management practices or pathosystems. The goals of such comparisons are to identify environmental factors that influence epidemic development, to decide upon the efficacy of specific management practices. The ultimate goal, of course, is to manage diseases the best way possible to minimize the impact of the disease on the plants (Sall, 1980).

Previous soybean rust risk assessments with an assumption of availability of spores early in a season showed that weather conditions (dew and temperature) during a growing season, in general, are suitable for disease development in U.S.A soybean-growing regions. Predicting the time of rust appearance in a field is critical to determining the destructive potential of rusts, including soybean rust. Epidemiology is most likely used to assess rust incipient time (Pivonia and Yang, 2006).

Few biologically based models to assess the risk of soybean rust have been developed because of difficulty in estimating variables related to infection rate of the disease. A fuzzy logic system, however, can estimate apparent infection rate by combining meteorological variables and biological criteria pertinent to SBR severity. A fuzzy logic apparent infection rate (FLAIR) model was developed to simulate severity of SBR and validated using data from field experiments. The FLAIR model estimated daily apparent infection rates of SBR and simulated disease severity based on population dynamics. In weekly simulation, the FLAIR model explained >85% of variation in disease severity. In simulation of an entire epidemic period, the FLAIR

model was able to predict disease severity accurately once initial values of disease severity were predicted accurately. Results suggest that a model could be developed to determine apparent infection rate and an initial value of disease severity in advance using forecasted weather data, which would provide accurate prediction of severity of SBR before the start of a season (Kim *et al.*, 2005).

1.6 DISEASE MANAGEMENT

Successful SBR management can be expected to result from the skillful utilization of appropriate fungicides applied when necessary, the establishment of effective biological control agents and deployment of disease resistant and tolerant varieties. In each of these areas, additional research is required to provide the "manager" with more powerful tools to accomplish the job (Bromfield, 1984).

1.6.1 CULTURAL CONTROL

Modifications to present day cultural practices, or adoption of new ones, frequently prevent or reduce the incidence or progress of a disease. Modification of planting dates, ulitilization of early maturing varieties, utilization of varieties with a short pod-filling stage, control of weed hosts and selection of planting sites may be used to effectively control or reduce SBR (Bromfield, 1984).

It is recommended that soybeans be grown far from pastures containing *Glycine wightii* (Wight & Arn.) Verdc., a common pasture legume known to be an alternative host of SBR. Production of cultivated crops such as *Phaseolus* spp., which are also alternative hosts of *P. pachyrhizi*, should be limited in soybean growing areas. Although the destruction of weed hosts may reduce the level of inoculum, weed hosts are extensive in range and the pathogen has the ability to travel long distances. Crops should be irrigated in the middle of the day, allowing leaves to dry before dew sets in, or at night, thereby preventing extension of the dew period (Caldwell and Laing, 2002).

Field observations at the Asian Vegetable Research and Development Center (AVRDC), Taiwan, demonstrated that the physiological age of the soybean plant plays a role in SBR development. It was observed that later maturing cultivars are less affected by SBR on the same day than earlier maturing, susceptible cultivars. This indicates that development is slower on late maturing cultivars on a calendar scale and is affected by the physiological growth stage of the host (Tschanz and Tsai, 1982). Dadke and Kachapur (1997) observed that 30 and 45 day

old plants were highly susceptible whereas 15-day-old plants were less susceptible to SBR. The transport of susceptible wild or crop host material from known areas of infestation should be limited (Sinclair, 1978).

1.6.2 CHEMICAL CONTROL

The first report of chemical control of SBR was during the 1960s. Since the pioneering work of Kitane and colleagues on the effectiveness of lime-sulphur, Bordeaux mixture, mercurials and zineb for the control of SBR in Japan, numerous protectant and eradicant fungicides have been tested (Bromfield, 1984). In the 1970s systemic fungicides in the form of Plantvax® (oxycarboxin) and Benlate® (benomyl) were tested (du Preez and Caldwell, 2004).

At present, fungicides remain the most effective means of control of SBR. Mancozeb is widely used as a protectant spray. However, frequent applications (4 applications per season) are necessary for highly effective control, and the spray schedule has to be initiated before symptoms appear. Triadimefon also gives good control and can be applied less frequently than mancozeb. Preventative spraying is said to be far more effective than curative spraying and, if possible, is recommended especially and specifically for areas where disease occurred the previous season (Hartman *et al.*, 1999).

In Zimbabwe, chemicals have been used to effectively control SBR. The Chemical Registration Authority has approved various chemicals for the control of SBR in Zimbabwe (Table 1.2) (Anonymous, 2003a).

Since the first occurrence of soybeans in SA during 2000/01 growing season, several fungicides have received emergency registration (Table 1.3), thereby providing an initial measure of control (Anonymous, 2003b). These fungicides received emergency registration due to the strength of their use in Zimbabwe as well as their use on other crops, such as beans (Caldwell and Laing, 2002).

Table 1.2 Fungicides and rates registered for *Phakopsora pachyrhizi* control in Zimbabwe (Anonymous, 2003a).

Trade Name	Active Ingredient	Application Rate (ml ha^{-1})
Alto®	cyproconazole	300
Folicur®	tebuconazole	1000
Funginex®	triforine	1500
Impact®	flutriafol	800
Punch Xtra®	carbendazim/flusilazole	350/500*
Score®	difenoconazole	300/500**
Shavit®	triadimenol	500
Tilt®	propiconazole	500

*Lower rate for ground application, **Higher rate for aerial application

Table 1.3 Emergency fungicides and rates registered for *Phakopsora pachyrhizi* control in South Africa for the 2002/03 growing season (Anonymous, 2003b).

Trade Name	Active Ingredient	Dosage Rate (ml ha^{-1})	
		Ground	Aerial
Bayfidan®	triadimenol	500	625
Capitan®	flusilazole	400	500
Denanin®	triforine	1500	-
Folicur®	tebuconazol	750	1000
Impact®	flutriafol	1000	1250
Punch C®	carbendazium/flusilazole	400	500
Punch Xtra®	carbendazium/flusilazole	600	750
Score®	difenoconazole	325	-
Shavit®	triadimenol	500	625

The registered fungicides in SA all belong to the same chemical group, i.e., the sterol biosynthetic inhibitors (SBI's) (Caldwell *et al.*, 2002). Within the SBI's the registered fungicides mostly belong to the triazole sub-group (Anonymous, 2003b). Thus, if resistance developed to one of these fungicides due to use at lower rates than recommended (Caldwell *et al.*, 2002), *P. pachyrhizi* resistance would easily be conferred to the other fungicides. Therefore, these fungicides should be used cautiously to prevent fungicide-resistance problems emerging.

1.6.3 BIOLOGICAL CONTROL

More than 30 genera of fungi have been found inhabiting pustules on rust infected plants (Littlefield, 1981), but it is uncertain as to how many of these are truly parasitic on the rust fungus. *Eudarluca caricis* (Fr.) OE Erikss., *Tuberculina vinosa* (Saac.) and *Lecanicillium lecanii* (Zimm.) Gams and Zare were listed as the most important hyperparasitic fungi on rust by Blakeman and Fokkema (1982). *Eudarluca caricis* has not been reported on *P. pachyrhizi*, but Naidu (1978) has reported its parasitism of *P. elettariae* (Racib) Cummins, the causal agent of cardamom rust in India.

Pon *et al.* (1954) described a soilborne bacterium, *Xanthomonas parasitica* Dastur, disseminated by rain splash, which parasitizes uredia of various cereal rust fungi and causes uredospore lysis. The genus *Bacillus* has also been implicated in uredospore lysis and in the inhibition of uredospore germination (Littlefield, 1981).

Urocladium spp. and *Sphaerolopsis* spp. may be effective as biological control agents of SBR. *Verticillium psalliotae* Treschow, a mycoparasite, has the ability to infect and colonize uredospores of SBR. *Verticillium psalliotae* forms appressorium-like structures at infection sites. Uredospores are not penetrated by *Verticillium psalliotae*, but appear degraded and eventually burst to produce lytic enzymes (Saksirirat and Hoppe, 1990).

1.6.4 RESISTANCE

Host plant resistance was first reported in the 1960s from field evaluations in Taiwan. Physiological races of *P. pachyrhizi* were first described in 1966 when a set of nine single uredospore isolates was inoculated onto six soybean and five legume accessions (Lin, 1966). Reactions of the nine isolates were similar on all six of the soybean genotypes, but six pathotypes were identified based upon their reactions on the legume accessions. The first example of virulence diversity on soybean cultivars was described in Queensland, Australia (McLean *et al.*, 1976) where one rust isolate was found to be virulent on the cultivar 'Wills' but avirulent on the accession PI200492. Another isolate was virulent on both soybean genotypes. Several other

studies have also shown considerable variation in virulence among isolates from the same field as well as isolates collected from wide geographical areas (Anonymous, 1983; Poonpolgul and Surin, 1985; Shin and Tschanz, 1986).

Specific resistance to *P. pachyrhizi* is known, and four single dominant genes have been identified as Rpp_1, Rpp_2, Rpp_3 and Rpp_4. These four genes condition resistance to a limited set of rust isolates (Table 1.4). Rpp_1 was described as having an immune reaction when inoculated with a few isolates, including India 731. Inoculation of most rust isolates on plants containing Rpp_1 produces a resistant red-brown (RB) lesion with no or sparsely sporulating uredia. The RB lesion type is considered to be a resistant lesion type when compared to a fully susceptible TAN lesion (Miles *et al.*, 2003).

Single gene resistance has not been durable, and the usefulness of the single genes was lost soon after the sources were identified (Kochman, 1977). For example, the accession PI230970 was identified as resistant in field evaluations in 1971-1973. In 1976, a few susceptible lesions were observed on plants in the field and by 1978, most of the lesions were of the susceptible TAN type (Bromfield, 1984). By 1966, susceptible lesions were found on plants of Komata in field trials, and by the mid 1970s the line was not considered to be a useful source of resistance (Kochman, 1977). Resistance in Ankur, identified in the early 1970s (Singh *et al.*, 1975) was overcome in the early 1980s (Bromfield, 1984), and provided another example where a single gene for resistance was not useful. Only Bing Nang, the source of the Rpp_4 gene, has not been reported to be defeated in the field.

Table 1.4 Named single genes, original sources and *Phakopsora pachyrhizi* isolates used in studies of inheritance of resistance to soybean rust (Miles *et al.*, 2003)

		Phakopsora	***pachyrhizi*** **isolates**
Named single gene	**Accession number and cultivar name of original source**	**Resistant reaction**	**Susceptible reaction**
Rpp1	PI200492 Komata	IN 73-1[bc]	TW 72-1 TW 80-2
Rpp2	PI230970	AU 72-1[c] IN 73-1 [c] PH 77-1 [c] TW 72-1 [c]	TW 80-2
Rpp3	PI462312 Ankur	IN 73-1 [c]	TW 72-1 TW 80-2
Rpp4	PI459025 Bing Nang	IN 73-1 [c] TW 72-1 [c] TW 80-2 [c]	

[a] AU=Australia, IN=India, PH=Philippines, TW=Taiwan

[b] Immune reaction type

[c] Isolates used in original inheritance studies to examine segregation patterns

Partial resistance, or rate reducing resistance, is also known in soybeans (Wang and Hartman, 1992). Lines with partial resistance in field evaluations were rated as moderately resistant, since fewer lesions developed on plants throughout the season. In greenhouse studies, host-pathogen combinations that resulted in RB reaction types tended to have longer latent periods, lower rates of increase in pustule number over time, and smaller lesions compared to susceptible interactions that resulted in a TAN reaction type (Marchetti *et al.*, 1975; Bromfield *et al.*, 1980).

Identification and utilization of partial resistance in breeding programmes has been limited. Evaluation methods are time consuming and difficult to incorporate into breeding programmes and have been limited to use with advanced generations. These difficulties, at least in part, lead to the development of a strategy to select genotypes with tolerance (Singh *et al.*, 1975, Anonymous, 1992, Wang and Hartman, 1992, Hartman, 1995).

Tolerance is the strategy to select genotypes with high yield potential that have less yield loss from SBR. Screening for tolerance to SBR was started at the AVRDC (Anonymous, 1992; Hartman, 1995), where yields from paired plots, with and without the fungicide Dithane M-45® applied every 2 weeks, were compared to determine losses due to rust. High yielding cultivars with less yield loss under severe rust conditions were considered to be tolerant. Rust development rates and estimates of rust severity on foliage were not correlated with yield loss in tolerant material. Using fungicide protected plots as yield checks, tolerant lines from breeding populations were identified as early as the F5 generation without having to take detailed notes on rust severity (Anonymous, 1983; Hartman, 1995).

1.7 SUMMARY

Soybean rust caused by *P. pachyrhizi* is an important disease in many parts of the world where soybeans are grown on a large commercial scale. SBR is a devastating disease that causes large economic losses worldwide. It is for this reason that major research programs on SBR are conducted yearly in many countries.

Soybean producing countries now free from SBR are understandably concerned about the possible introduction and establishment of the causal pathogen within their borders. Similarly, countries contemplating production expansion or the initiation of soybean production must consider the possibility of SBR as one of the many variables impinging on decisions (Bromfield, 1984). For now, the U.S.A is relying on the application of fungicides as a control measure. Incorporation of resistance or tolerance into commercial germplasm may also occur (Miles *et al.*, 2003).

In SA, a number of organizations, including the Department of Agriculture and Environmental Affairs, the Protein Research Foundation, the Agricultural Research Council and private companies, together with the University of KwaZulu-Natal and the University of the Free State, are all involved in collaborative research programmes to find solutions to this devastating disease on soybeans (Caldwell and McLaren, 2004). The development of resistant varieties will take several years. Fungicides provide a short-to-medium term solution to the problem (Caldwell *et al.*, 2002). Early recognition of the disease is imperative for implementation of successful control. Fungicides at the recommended rates should be applied as soon as the disease is observed (Kloppers, 2002), thereby ensuring a high-yield (Caldwell *et al.*, 2002).

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CHAPTER TWO

DEVELOPMENT OF *PHAKOPSORA PACHYRHIZI* AT DIFFERENT TEMPERATURES, RELATIVE HUMIDITIES AND LEAF WETNESS DURATIONS

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ABSTRACT

In order to successfully control a plant pathogen, its epidemiology must be well understood. To investigate the interaction between temperature, relative humidity (RH) and leaf wetness duration (LWD), infection studies of rust (*Phakopsora pachyrhizi* Syd.) on soybean plants (*Glycine max* (L.) Merr.) were carried out under controlled environmental conditions in a dew chamber and conviron™. Development of *P. pachyrhizi* on the susceptible cultivar (LS5995) was quantified in combinations of seven temperatures (15, 19, 21, 24, 26, 28 and 30°C) and five LWD (6, 9, 12, 14 and 16h) at three relative humidities (RH) (75%, 85% and 95%RH). Following the temperature, RH and LWD treatments, plants were removed from the dew chamber and placed in a conviron™ (21-22°C, 80%RH, 14h photoperiod and a photosynthetic active radiation (PAR) of 260µmol/m^2sec^{-1}). Studies indicated that the optimum temperature for uredospore germination was 21-24°C, a LWD greater than 12h and RH 85-95%. Number of pustules per lesion (abaxial leaf surface) and lesion size (abaxial and adaxial leaf surfaces) were calculated at 21 days post-inoculation. Infection did not occur on plants incubated at 15°C and 30°C at 85% or 95%RH whereas at 75% infection did not occur on plants incubated at 15°C, 19°C and 30°C regardless of LWD. Number of pustules per lesion on the abaxial leaf surface as well as lesion size on both leaf surfaces increased with increasing LWD at all RH tested. At 75% and 85%RH, lesion size on the abaxial leaf surfaces increased after 12h LWD at 24°C. At 75%RH lesion

size on the adaxial leaf surface increased significantly after 14h LWD with the highest lesion size developing at 24°C whereas at 85%RH and 95%RH lesion size increased significantly after 14h LWD with the highest lesion size developing at 21°C. Number of pustules per lesion on the abaxial leaf surface produced at 75%, 85% and 95%RH was highest at 24°C and showed a gradual increase with increasing LWD.

2.1 INTRODUCTION

Successful infection of a host by a pathogen is the culmination of a series of events that must occur in sequence. These events are spore germination, appressorium formation and penetration of the fungus into the host. Each of these steps and subsequent ones of colonization and sporulation are influenced by biotic factors of the host and pathogen, and abiotic factors of the environment. Therefore, it is essential that these factors and their complex inter-relationships be understood to provide the rationale for ultimate management of soybean rust (SBR), *Phakopsora pachyrhizi* Syd. (Bromfield, 1984).

SBR epidemics are most severe during long periods of leaf wetness when the average daily temperature is between 15-28°C with 75-80%RH (Hartman *et al.*, 1999 and Caldwell *et al.*, 2002). Dry conditions, excessive precipitation or daily mean temperatures > 30°C or < 15°C inhibit rust development (Sinclair and Backman, 1989). Moisture on the plant surface is crucial for germination to occur (Caldwell *et al.*, 2002). Hence areas where prolonged periods of leaf wetness due to dew, mist and light rain occur provide optimum conditions for germination (Kloppers, 2002).

Epidemiology is essential to obtain a better knowledge of all aspects of plant disease epidemics. Through plant epidemiological studies the process of pathogen dispersal, infection, colonization, reproduction and disease spread can be better understood, providing precise information on which disease models can be developed, disease forecasting schemes devised, diversification strategies formulated and an integrated approach to plant disease management put into practice (Wang and Hartman, 1992).

Most knowledge of the epidemiology of *P. pachyrhizi* is from field observations of the pathogen under natural infection. Studies conducted in controlled environments make it possible to isolate the effect of specific factors and remove confounding stresses imposed under field conditions.

The aim of this study was to investigate the optimum conditions under which SBR caused by *P. pachyrhizi* infects soybean plants (*Glycine max* (L.) Merr.). In this study, effects of temperature, RH and leaf wetness duration (LWD) on infection and lesion development were investigated under controlled environmental conditions.

2.2 MATERIALS AND METHODS

2.2.1 Host material

Single soybean plants (LS5995[1]) were grown in seedling containers (35mm x 95mm) (Clausen Plastics[2]) placed in plastic containers filled with water (Figure 2.1). Composted pine bark was the growth medium for the duration of the study. Due to the fact that pine bark is nutritionally poor and has a poor water holding capacity, plants were hand watered once a day and fertilized every two weeks with Nitrosol® (N:P:K) (8:2:5.8). Plants were grown in a growth room at 25°C, 60%RH, a photoperiod of 14h and a light intensity of 347.17µmol/sec/m^2.

[1] Link Seed® (Pty) Ltd, P.O. Box 755, Greytown, KwaZulu-Natal, Republic of South Africa
[2] Clausen Plastics®, Johannesburg, Republic of South Africa

Figure 2.1 Plants were grown in a growth room to avoid natural infection by wind-blown uredospores in 35mm x 95mm seedling containers in plastic dishes of water.

2.2.2 Inoculum production

Uredospores of *P. pachyrhizi* were obtained from naturally infected soybean plants grown in a tunnel (20-30°C, 50-100%RH with a 12-14h photoperiod) at the University of KwaZulu-Natal, Pietermartizburg, South Africa. Uredospores of *P. pachyrhizi* were collected from the uredia on the abaxial leaf surfaces of naturally infected soybean plants using a wet paintbrush and suspended in distilled water. Uredospore concentration was adjusted to 5.5 $x10^5$ spores ml^{-1} using a haemocytometer.

2.2.3 Spore concentration

Three spore concentrations, i.e., 1.37 x 10^5, 2.75 x 10^5 and 5.5 x 10^5 spores ml^{-1} were inoculated onto soybean plants to determine which spore concentration gave the best distribution of pustules on the leaf surface of the soybean cultivar LS5995, so that they could be easily counted and also to ensure there was no competition between pustules for spore germination. Plants were inoculated at the V3 growth stage. Plants were grown in a growth room at 25°C, 60%RH, a photoperiod of 14h and a light intensity of 347.17µmol/sec/m^2. Five plants with three replications were used.

2.2.4 Uredospore germination tests

Uredospores were plated onto 1.25% water agar at the beginning, middle and end of each inoculation period to determine any possible differences in spore germination during the course of the inoculation period which was 3h. Five plates with three replicates were used. At least 150 uredospores from each plate were counted. Petri dishes were incubated in the dark at 21°C for 16h and after this period germinating uredospores were counted using a compound microscope under X40 and the germination percentage determined.

2.2.5 Inoculation

Uredospores were applied to the abaxial leaf surface of the third trifoliate leaf from a distance of 5-10cm using a modified Andres and Wilcoxson (1984) inoculator. The suspension was deposited as a uniform layer of droplets at the centre of the leaf. Plants were left to dry for 15 minutes before placing them in a dew chamber. Leaves were sprayed with distilled water to ensure the start of the leaf wetness period. Plants were placed in a dew chamber at the required temperature, RH and LWD in continuous darkness for infection to take place. The dew chamber was set at the required temperature and RH and allowed to stabilize two hours before plants were placed inside. After this plants were transferred to a conviron™ (21-22°C, 80%RH, 14h photoperiod and a light intensity of 66.4µmol/sec/m^2) for 21 days.

2.2.6 Experimental design

Five plants with three replicates were used in each trial. Development of *P. pachyrhizi* on the susceptible soybean cultivar (LS5995) was quantified in combinations of seven temperatures (15, 19, 21, 24, 26, 28 and 30°C) and five LWD (6, 9, 12, 14 and 16h) at 75%, 85% and 95%RH in a dew chamber. The trial was repeated with replication.

2.2.7 Disease assessment

Number of pustules per lesion on the abaxial leaf surface and lesion size on the abaxial and adaxial leaf surface was determined weekly for 21 days post inoculation (dpi). A template was designed for counting number of pustules as well as lesion size at six sites on the leaf surface. These results were then averaged

and used in the analyses. The number of pustules per lesion was counted using a compound microscope under 40X magnification. Lesion size was calculated according to the following equation: Area = length x width x 0.76 (Melching *et al.*, 1988).

2.2.8 Statistical analyses

Experiments were treated as a randomized complete block design. All data were subjected to a three-way analysis of variance test using GenStat® Executable Release 7.1 Statistical Analysis (Lawes Agricultural Trust, 2003) software to determine the difference between treatment means. All least significant differences were determined at $P<0.05$. Means from the ANOVA results were used to plot the 3-D graphs.

2.3 RESULTS

Similar trends and patterns were noticed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled.

2.3.1 Spore concentration

The best spore concentration was found to be 5.5×10^5 spore's ml^{-1}. This concentration was significantly different from the other concentrations used. At this concentration pustules produced on the leaves were evenly distributed, and easy to count. Therefore this was the concentration used to inoculate plants in all experiments carried out in these trials.

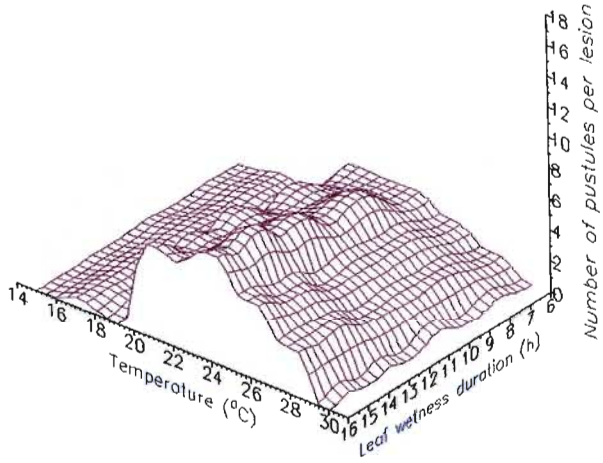
2.3.2 Uredospore germination tests

The germination percentages determined on agar plates at the start of inoculation, halfway through inoculation, and at the finish of inoculation were not significantly different from one another within the study in both Trial 1 and 2. This indicates that the inoculum germination remained constant throughout the inoculation period.

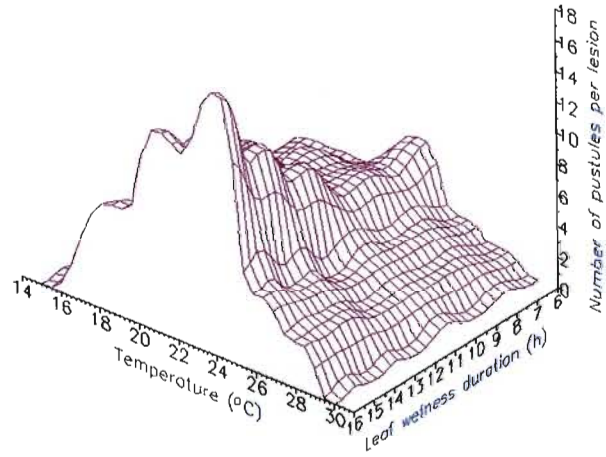
2.3.3 Number of pustules per lesion

The interaction of temperature, RH and LWD had a significantly different interaction. No infection occurred on plants subjected to temperatures of 15°C and 30°C at 85%RH and 95%RH, whereas at 75%RH no infection occurred at 15°C, 19°C and 30°C regardless of the LWD (Appendix 1a). At 75%RH and 85%RH number of pustules per lesion increased significantly after 12h LWD with the optimum number of pustules produced at 24°C (Figures 2.2i and ii). At 95%RH an increase in the number of pustules was found after 14h LWD at 24°C (Figure 2.2iii). However, the number of pustules per lesion was significantly higher at 85%RH and 95%RH than 75%RH (Appendix 1a).

(i) 75%RH



(ii) 85%RH



(iii) 95%RH

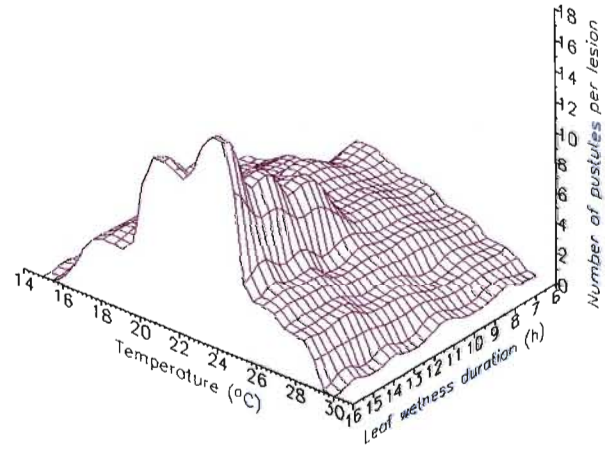
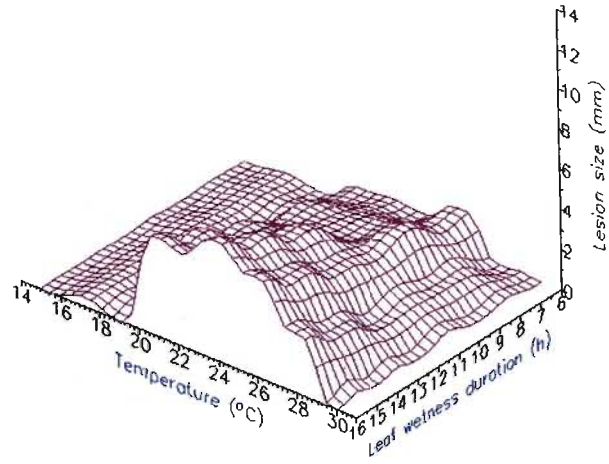


Figure 2.2 Effect of temperature (°C), relative humidity and leaf wetness duration on number of pustules/lesion on abaxial leaf surface at 75% (i), 85% (ii) and 95% (iii) relative humidity.

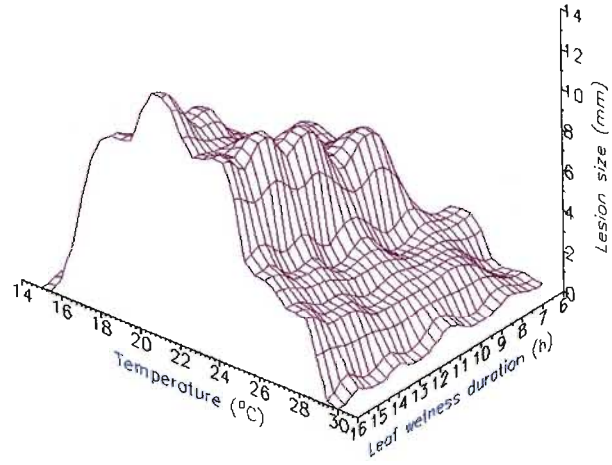
2.3.4 Lesion size (mm) on the abaxial leaf surface

Results obtained for both trials showed similar trends (Appendix 1b). The interaction of temperature, RH and LWD had a significantly different interaction (Appendix 1b). No infection occurred on plants subjected to temperatures of 15°C and 30°C at 85%RH and 95%RH, whereas at 75%RH no infection occurred at 15°C, 19°C and 30°C regardless of the LWD (Appendix 1b). At 75%RH and 85%RH lesion size increased significantly after 12h leaf wetness duration with the highest lesion size produced at 24°C (Figures 2.3i and ii). At 95%RH an increase in lesion size was found after 12h leaf wetness at 24°C (Figure 2.3iii).

(i) 75%RH



(ii) 85%RH



(iii) 95%RH

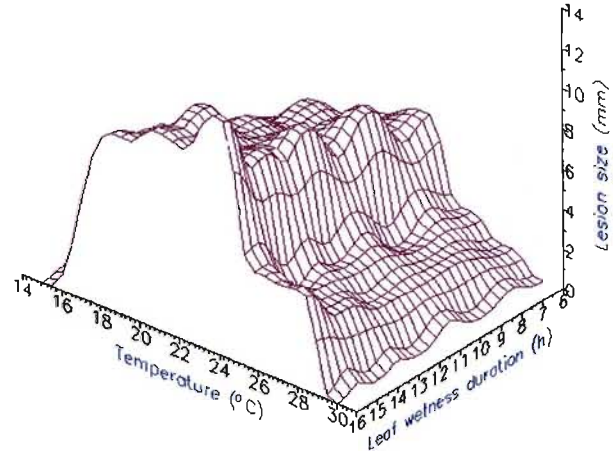
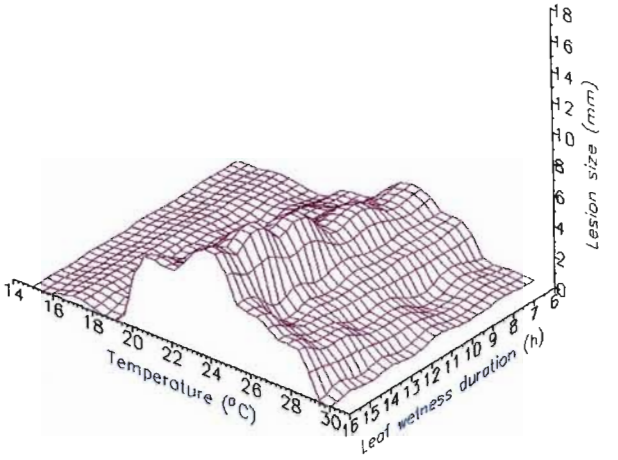


Figure 2.3 Effect of temperature (°C), relative humidity and leaf wetness duration on lesion size (mm) on abaxial leaf surface at 75% (i), 85% (ii) and 95% (iii) relative humidity.

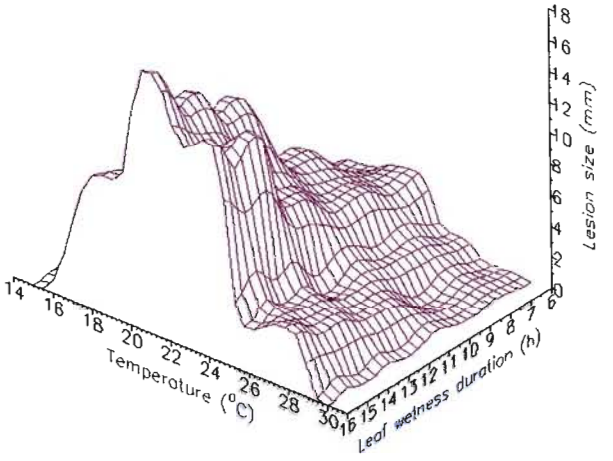
2.3.5 Lesion size (mm) on the adaxial leaf surface

The interaction of temperature, RH and LWD had a significantly different interaction (Appendix 1c). No infection occurred on plants subjected to temperatures of 15°C and 30°C at 85%RH and 95%RH, whereas at 75%RH no infection occurred at 15°C, 19°C and 30°C regardless of the LWD. Hence no lesion development was seen on plants at these temperatures (Figures 2.4i, ii and iii). At 75%RH lesion size increased significantly after 14h LWD with the highest lesion size developing at 24°C whereas at 85%RH and 95%RH lesion size increased significantly after 14h LWD with the highest lesion size developing at 21°C (Figures 2.4i, ii and iii). Lesion size on the adaxial leaf surface was lower than the lesion size found on the abaxial leaf surface.

(i) 75% RH



(ii) 85% RH



(iii) 95%RH

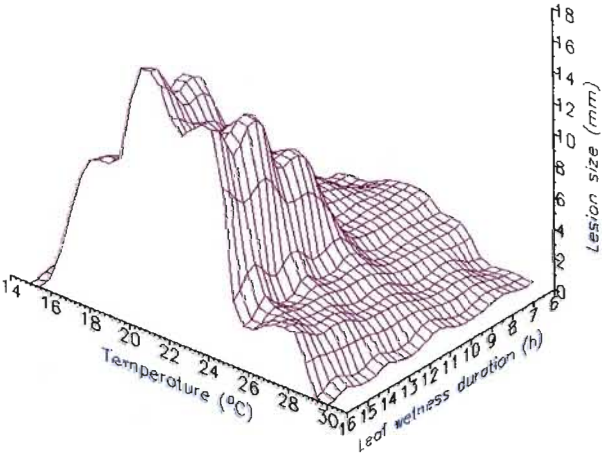


Figure 2.4 Effect of temperature (°C), relative humidity and leaf wetness duration on lesion size (mm) on adaxial leaf surface developed at 75% (i), 85% (ii) and 95% (iii) relative humidity.

2.4 DISCUSSION

Germination of uredospores used as inoculum was statistically unchanged during the inoculation period, as estimated from the germination trials on agar plates. These results correlate with the results obtained by Melching *et al.* (1988), which also indicated no change in germination of uredospores during the inoculation period.

The ability of *P. pachyrhizi* to cause an epidemic in soybeans is dependent on a number of factors. In these trials temperature, RH and LWD indicate that these factors play an important role in the development of *P. pachyrhizi.* Although the frequency and duration of infection periods appear to be useful parameters in predicting SBR development, sufficient data are unavailable to accurately predict the effects of temperature, RH and LWD on the infection efficiency of SBR (Marchetti *et al.*, 1976). It has been shown in the field that extended periods of LWD of approximately 10h per day and moderate temperatures (18-26°C) are necessary for severe epidemics (Casey, 1979).

Successful infections were obtained within the temperature range of 19-24°C when leaves were wet for more than 9h. This was also noticed by Gottwald (1985). From the data obtained it can be suggested that infection of soybeans by *P. pachyrhizi* occurs at night during cooler temperatures when leaf wetness is present. However, when extrapolating results from controlled environmental conditions to field conditions for which experimental data still remain to be collected, caution should be observed.

Marchetti *et al.* (1976) observed that at 20°C-25°C a minimum LWD of 6h was required for infection to occur. Increasing the LWD results in an increase in disease. Marchetti *et al.* (1976), as well as Wang and Hartman (1992), found this to be true. Temperatures above 27°C inhibited infection with no infection occurring at 30°C. These results are similar to those found by Marchetti *et al.* (1976), and Wang and Hartman (1992).

In the trails conducted in this thesis, the humidifier produced fine droplets of mist that could be seen on the leaf surface at 85%RH and 95%RH. At 75% RH, the humidifier still produced the mist droplets which produced a thin film of water on the leaf surface throughout the trial. According to Stromberg[3] (pers. comm.), because the leaf is always transpiring, it always has a thin film of water on its surface.

Wang and Hartman (1992) found that temperatures that were favourable for growth and development of soybean plants, in general, favoured infection in the field. Therefore environmental conditions in KwaZulu-Natal should be conducive to promoting SBR. Wang and Hartman (1992) found that an increase in disease in the field was influenced by temperature and leaf wetness. Fewer infection periods were associated with low temperatures; low night temperatures < 15°C reduced disease and adversely affected the growth and development of soybean plants.

Results obtained in these trials indicate that no infection occurred at 15°C. Wang and Hartman (1992) found that extended periods of leaf wetness >10h and moderate temperatures (18-26°C) increased disease. Results obtained in this study showed that LWD > 12h as well as temperatures of 21-24°C increased disease.

This study will contribute accurate data to develop a model for early prediction and subsequent fungicide applications for SBR. Spraying at the correct time, rather than on a calendar-based schedule, will improve yields and profit margins for farmers as well as optimize labour usage.

The epidemiology of SBR has been a major focus of research in South Africa. A number of important discoveries related to the interaction of soybean, *P. pachyrhizi* and the environment have been made. However, there are still some questions to answer with regard to the origin of the initial inoculum that starts the epidemic, the races that predominate and the utilization of environmental parameters to forecast disease outbreaks.

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CHAPTER THREE

EFFECT OF ULTRAVIOLET LIGHT ON THE GERMINATION OF UREDOSPORES OF *PHAKOPSORA PACHYRHIZI*

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ABSTRACT

The effect of ultraviolet light (<280nm) on uredospore germination of *Phakopsora pachyrhizi* Syd. the causal organism of soybean rust on soybeans [*Glycine max* (L.) Merr.] was studied. A 0.1ml uredospore suspension was plated onto 1.25% water agar Petri dishes. Control plates were covered in black plastic bags and also placed under ultraviolet light. Percentage germination and germ tube length were measured after 6, 9, 12, 14, 16, 20, 24, 36 and 48h. Uredospore germination under ultraviolet light was 7% after 48h. However, under continuous darkness, the germination percentage was 58% after 48 h. Germination was found to peak at 16h in darkness with a gradual decrease as time increased whereas germination under ultraviolet light was highest at 6h with a gradual decrease with increased exposure to light. Germ tube lengths were shorter when exposed to ultraviolet light (107µm) compared to controls kept in the dark (181µm). Germ tube lengths were longest at 6h when exposed to ultraviolet light with a gradual decrease with increased exposure to light whereas germ tube lengths were highest at 16-20h in darkness. Results obtained clearly show a negative effect of ultraviolet light on the germination and germ tube length of uredospores. In a second experiment a 0.1ml suspension of uredospores on 1.25% water agar Petri dishes was exposed to cycles of 14h ultraviolet light and 10h darkness for 48h. Results indicate an increase in germination percentage of uredospores when exposed to 10h of darkness following a 14h period under ultraviolet light. A hypothetical explanation of this phenomenon is outlined in this chapter.

3.1 INTRODUCTION

Fungi are greatly influenced by light, as seen in the formation of reproductive structures, pigment biosynthesis, phototaxis, and phototropism (Tan, 1978; Kumagai, 1988). Light can have a stimulatory or inhibitory effect on many processes of the growth, development, reproductive and behavioral processes of fungi (Koch and Hoppe, 1987).

Phototropism of germ tubes of plant pathogenic fungi is important in the plant infection process (Yarwood, 1932; Koch and Hoppe, 1987; Honda *et al.*, 1992 and Islam and Honda, 1996). Among these are positive or negative phototropic reactions, which are significant in relation to spore dispersal and spore germination (Koch and Hoppe, 1987).

Phycomyces blakesleeanus Burgeff, when induced for germination by blue light, shows positive phototropism of sporangiophores and is one of the best-studied examples. The side of the sporangiophores nearest to the light source grows more slowly than the far side. This results in positive phototropism during exposure to unilateral blue light (Banbury, 1959, Delbrück, 1963 and Gressel and Rau, 1983).

Compared to positive phototropism, negative phototropism is rare in nature (Koch and Hoppe, 1987). Germ tubes of several rust species and *Botrytis cinerea* Pers., Fr show negative phototropism to light (Gettkandt, 1954; Jaffe and Etzold, 1962 and Carlile, 1965). Negative phototropism has been observed in uredospore germ tubes of several rust species. The most intensively studied examples include *Puccinia graminis* f. sp. *tritici* Pers, *P. recondita* f. sp. *tritici* Rob. ex Des. and *P. recondita* f. sp. *secalis* Rob. ex Des (Koch and Hoppe, 1987).

A decrease in germination of uredospores of *P. tritici* and *P. recondita* when exposed to ultraviolet light and an increase when exposed to darkness was shown by Givan and Bromfield (1964a and b) and Koch and Hoppe (1987). Light inhibition of uredospore germination has previously been reported for *Puccinia* spp. (Subrahmanyam *et al.*, 1988; Tapsoba and Wilson, 1997 and Mueller and Buck, 2003). These studies have evaluated shorter incubation periods and

suggest that the first 2h of incubation may be the most important (Tapsoba and Wilson, 1997). High intensity light inhibited uredospore germination of *P. graminis* f. sp. *tritici* (Weston, 1932; Givan and Bromfield, 1964a and Lucas *et al.*, 1975).

The aim of this trial was to determine the effect of ultraviolet light on germination and germ tube length of uredospores of *Phakopsora pachyrhizi* H. Syd and P. Syd, the causal organism of soybean rust (SBR) on soybeans [*Glycine max* (L.) Merr.]. Results from this experiment will facilitate our understanding of uredospore germination when exposed to conditions that favour germination under natural environmental conditions. These results will help with the production of a disease prediction model, which is the ultimate aim of this research.

3.2 MATERIALS AND METHODS

3.2.1 Fungal inoculum

Uredospores of *P. pachyrhizi* were collected from naturally infected leaves of soybean plants which were grown in a tunnel (20-25°C, 80-90%RH and a photoperiod of 14h) at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. Uredospores of *P. pachyrhizi* were collected from the abaxial leaf surfaces using a wet paintbrush and suspended in distilled water. The uredospore concentration was adjusted to 5.5 x 10^5spores ml^{-1} using a haemocytometer.

3.2.2 Uredospore exposure to ultraviolet light

A 0.1ml uredospore suspension was plated onto 1.25% water agar in Petri dishes. Plates were exposed to ultraviolet light for 6, 9, 12, 14, 16, 20, 24, 36 and 48h. Control plates were covered in black plastic bags and also placed under ultraviolet light (<280nm). Petri dishes were placed directly below (27cm) the ultraviolet light source. For each treatment, five plates with four replicates were used. The number of germinated and non-germinated uredospores from each plate was counted with the aid of a compound microscope at 40X magnification. Uredospores with germ tube lengths greater than or equal to the diameter of the uredospore were considered germinated. At least 150 uredospores from each plate were counted. Data were converted to percentage germination. A thermometer was placed in the

ultraviolet light cabinet 48h prior to the experiment to determine if the temperature was constant. The trial was repeated once.

3.2.3 Uredospore exposure to cycles of ultraviolet light and darkness

A 0.1ml uredospore suspension was pipetted onto 1.25% water agar in Petri dishes. Plates were exposed to ultraviolet light for 14h followed by 10h of darkness for a 48h period. Plates exposed to darkness were covered in black plastic. For each treatment, five plates with four replicates were used. The number of germinated uredospores from each plate was counted after each light and darkness treatment with the aid of a compound microscope at 40X magnification. Data were converted to percentage germination. The trial was repeated once.

3.2.4 Statistical analyses

Treatments were arranged in a Randomized Complete Block Design (RCBD). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 7.1 Statistical Analysis Software (Lawes Agricultural Trust, 2003) to determine differences between treatment means. All least significant differences were determined at $P<0.05$.

3.3 RESULTS

3.3.1 Uredospore exposure to ultraviolet light

Similar trends and patterns were noticed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled. Exposure of uredospores to ultraviolet light and darkness did significantly influence the germination and germ tube lengths of SBR uredospores.

Germination of uredospores under darkness was found to increase steadily with optimum germination occurring at 16h (Appendix 2a). Uredospores exposed to ultraviolet light showed a gradual decrease in germination percentage with the lowest germination percentage (7%) occurring at 48h (Figure 3.1). Uredospores that were exposed to a longer uv light period were shown to have germinated but these spores had germ tubes being shriveled and detached therefore it was regarded as non-viable spores.

No significant differences were found in germination of uredospores between 9 and 12h, 16 and 20h, 24 and 36h and 36 and 48h but the germination % at 48h was significantly different from 24h, when exposed to ultraviolet light (Appendix 2a). No significant differences in germination were found between 12, 14, 20, 24 and 36h exposure to darkness.

Uredospore germ tube length increased with prolonged to darkness increased from 169-181µm in 48h. The highest germ tube lengths (190-193µm) were found at 16 and 20h darkness (Figure 3.2). Germ tube lengths were highest at 6h (172µm) when exposed to ultraviolet light with a gradual decrease with increased exposure to light (Appendix 2a and Figure 3.2). At 48h exposure to ultraviolet light germ tube lengths were decreased from 172 to 107µm. Significant differences between germ tube lengths were found between the ultraviolet light and darkness treatments. When exposed to ultraviolet light germ tubes were found to have dehydrated and withered and were much shorter. However, when exposed to darkness they started growing normally again. Therefore dehydrated germinated uredospores and germ tubes were not counted with increased exposure to ultraviolet light.

3.3.2 Uredospore germination when exposed to cycles of ultraviolet light and darkness

Similar trends and patterns were noticed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled. Uredospore germination under darkness (51-55%) was significantly higher following exposure to ultraviolet light (37-40%) (Appendix 2b and Figure 3.3). Exposure to ultraviolet light decreased germination of uredospores while exposure to darkness increased germination.

It was observed that germinating uredospores exposed to ultraviolet light became dehydrated and appeared to be dying but once exposed to darkness they were subsequently rehydrated. Recovery from dehydration was much quicker in the dark. Therefore dehydrated germinated uredospores and germ tubes were not counted with increased exposure to ultraviolet light.

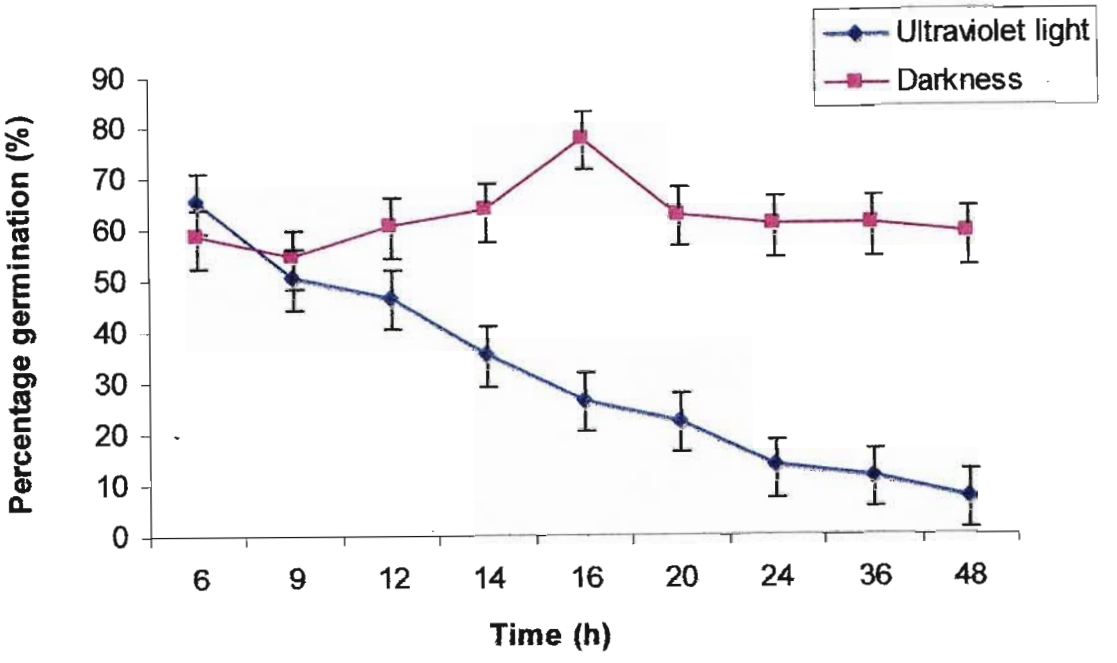

Figure 3.1 Effect of ultraviolet light on uredospore germination of *Phakopsora pachyrhizi* after 48h. Bars represent the standard deviation of the treatment mean of pooled data.

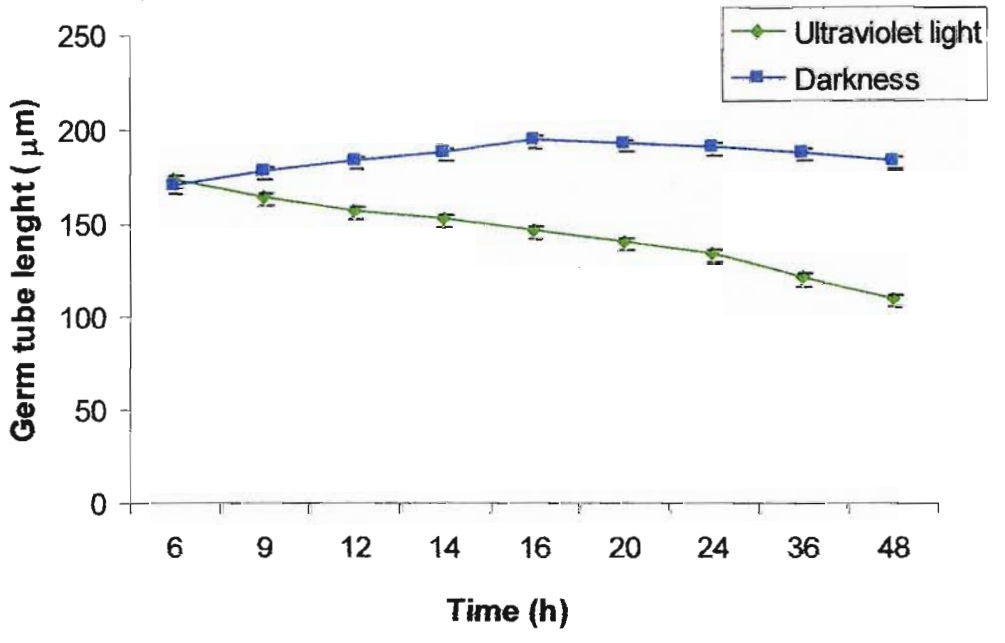

Figure 3.2 Effect of ultraviolet light on germ tube length (µm) of uredospores of *Phakopsora pachyrhizi* after 48h. Bars represent the standard deviation of the treatment mean of pooled data.

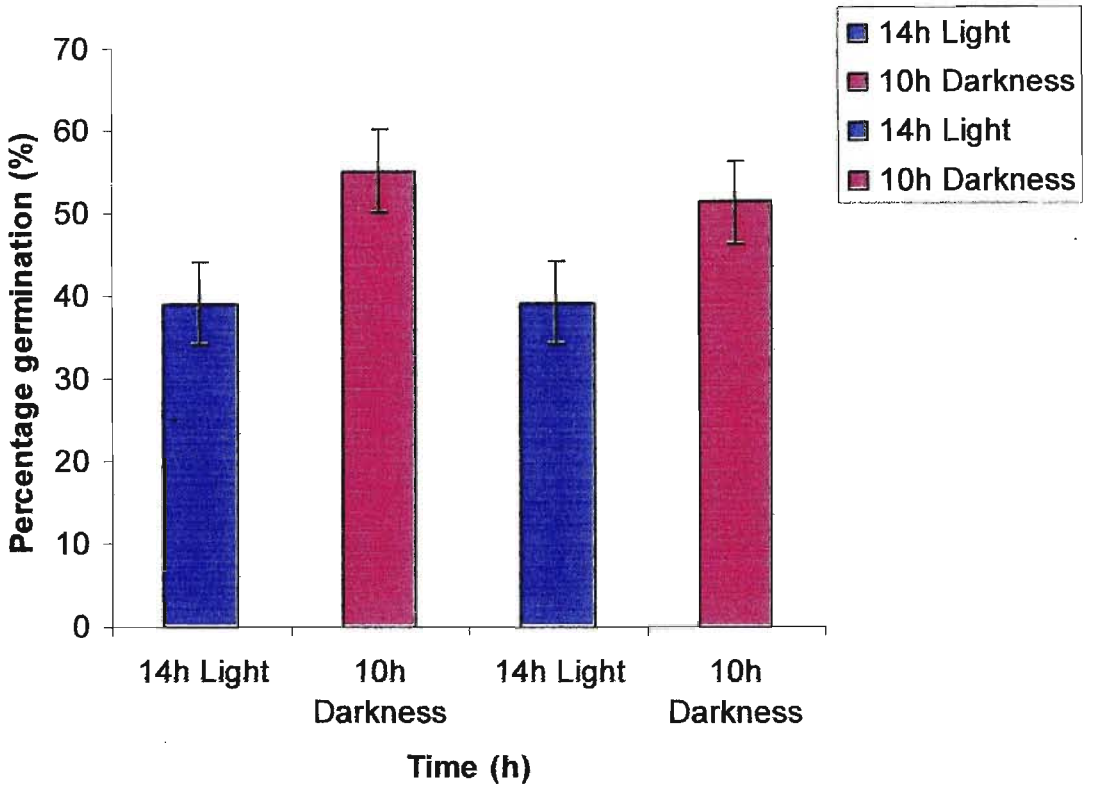

Figure 3.3 Germination of uredospores of *Phakopsora pachyrhizi* as affected by 14h cycles of ultraviolet light and 10h darkness for a 48h period. Bars represent the standard deviation of the treatment mean of pooled data.

3.4 DISCUSSION

Under natural conditions, illumination will always be variable both in intensity and duration. Therefore, the study of the interaction of time and light intensity is informative in the study of germination of uredospores (Tapsoba and Wilson, 1997).

Germination of uredospores of *P. pachyrhizi* is significantly affected by environmental factors acting on spores during the germination process (Tapsoba and Wilson, 1997), e.g., if temperature does not remain constant it would have an effect on uredospore germination. Inhibition of uredospore germination by visible light has been studied in detail with cereal rusts. Light inhibition of uredospore germination has previously been reported for *Puccinia* spp. (Subrahmanyam *et al.*, 1988; and Tapsoba and Wilson, 1997). High intensity light inhibited uredospore germination of *P. graminis* f. sp. *tritici* (Weston, 1932; Givan and Bromfield, 1964a and Lucas *et al.*, 1975).

Germination of uredospores shows a negative response to ultraviolet light and a positive response to darkness. Complete suppression of uredospore germination when exposed to ultraviolet light was not noted. However, at 48h exposure to ultraviolet light percentage germination was low (Appendix 2a and Figure 3.1). A decrease in germination when exposed to ultraviolet light and an increase when exposed to darkness is similar to the results found by Givan and Bromfield (1964a and b) and Koch and Hoppe (1987) when working on *P. tritici* , *P. recondita* and *P. pachyrhizi.*

Germ tube length decreased when exposed to increased durations of ultraviolet light and increased when exposed to darkness. These results are similar for those obtained by Koch and Hoppe (1987). They hypothesized that the reduced germ tube length could be due to a delay in germination or a reduction in growth rates. From observations it was noted that germ tubes usually grew away from the light source. These results are similar to that found by Koch and Hoppe (1987).

Givan and Bromfield (1964a and b) observed that uredospores of *P. graminis* var. *tritici* and *P. graminis* var. *recondita* incubated under 1h of light followed by 1h of darkness showed considerably better germination than those incubated in continuous light for 2h. Recovery is clearly much more rapid in the dark than in the light. This was observed in the trial conducted. These studies have evaluated shorter incubation periods and suggest that the first 2h of incubation may be the most important as found by Tapsoba and Wilson (1997). In this trial it was noted that uredospores exposed to ultraviolet light are dehydrated but once exposed to darkness these spores are subsequently rehydrated. Germination of uredospores was not checked < 6h as literature indicates that uredospore germination is more prevalent at >6h (Marchetti *et al.*, 1976).

Givan and Bromfield (1964a and b) suggested the following hypothesis to account for the difference in recovery rates in darkness and in light: formation of an inhibitory germination substance proceeds rapidly when spores are placed in the light, with the rate of inhibitor formation remaining constant throughout the incubation period for any given light intensity. There is a delay, however, in initiation of reactions breaking down the inhibitor, so that initially there is accumulation of the inhibitor within the spores. After several hours, reactions breaking down the inhibitor move rapidly enough to deplete the endogenous concentration, so that germination can proceed. Transfer of spores to darkness terminates production of the inhibitor, so that the concentration decreases rapidly with a concomitant increase in germination rate. However, it must be emphasized that there is no direct physiological or biochemical evidence for occurrence of such an inhibitor and inhibitory effects of light may not involve inhibitor formation (Givan and Bromfield, 1964).

These results indicate that uredospores of *P. pachyrhizi* in the air/field during the day only have a small germination potential, implying that more uredospores germinate and infect at night. Caution should be observed, however, when extrapolating the results of germination on an artificial moist medium to field conditions. The highly variable levels of light, temperature and moisture in the field may impose different constraints on germination than the relatively constant

conditions provided by artificial light thus, moisture during daytime is not epidemiologically important as moisture at night.

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CHAPTER FOUR

ALTERNATIVE HOST STUDY OF *PHAKOPSORA PACHYRHIZI* IN SOUTH AFRICA

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ABSTRACT

Controlled environmental studies were conducted to determine alternative hosts of *Phakopsora pachyrhizi* Syd., the causal agent of soybean rust (SBR). Twenty legume plants and kudzu vine from the Department of Agriculture and Environmental Affairs at Cedara, South Africa (SA) and 15 experimental dry bean lines from the Agricultural Research Council, SA, were tested for susceptibility to SBR. Plants at the V3 leaf stage were inoculated with a 5.5 x 10^5ml^{-1} suspension of uredospores and placed under continuous darkness in a dew chamber set at 24°C, 85%RH and 16h leaf wetness duration (LWD). Following incubation in the dew chamber, plants were placed in a Conviron™ (21-22°C, 80%RH, 14h photoperiod and a photosynthetic active radiation of 260µmol/m^{2}sec^{-1}) for 21 days. Host reaction was recorded 21 days post inoculation (dpi). Seven legume species [*Cajanus cajan* (L.) Huth, *Glycine max* (L.) Merr, *Lablab purpureus* (L.) Sweet, *Lupinus angustifolius* (L.) Finnish, *Phaseolus vulgaris* (L.), *Pueraria lobata* (M&S) Willd and *Vigna unguiculata* (L.) Walp] and three dry bean lines [Bonus; OPS-RS2 and PAN 159] showed typical SBR symptoms. Disease severity was significantly different within the alternative hosts, with *Glycine max*, *Phaseolus vulgaris*, *Lupinus angustifolius* and *Pueraria lobata* not being significantly different from Prima 2000 (control). A uredospore suspension of 2.5 x 10^5 uredospores ml^{-1} from plants that showed typical SBR symptoms was made and inoculated onto Prima 2000, a susceptible soybean cultivar. Prima 2000 was placed in a dew chamber at

24°C, 85%RH and 16h LWD under continuous darkness. Following incubation in the dew chamber, plants were placed in a Conviron™. Uredospores from species that infected Prima 2000 were considered alternative hosts of *P. pachyrhizi*. Uredospores from pustules on *G. max*, *L. purpureus*, *L. angustifolius*, *P. vulgaris*, *P. lobata*, *V. unguiculata*, Bonus and PAN 159 produced viable uredospores on Prima 2000. These plants are considered alternative hosts of *P. pachyrhizi*.

4.1 INTRODUCTION

Phakopsora pachyrhizi Syd., the causal organism of soybean rust (SBR) is an obligate parasite and cannot survive independently of its hosts or on debris. It must, therefore, find alternate ways in which to survive unfavourable conditions and over season between soybean cropping cycles. *Phakopsora pachyrhizi* does this by living on alternative hosts (Caldwell *et al.*, 2002). Host range studies have been conducted by many researchers. Reviews and additions to the host range of *P. pachyrhizi* have most notably been made by Sinclair (1982), Tschanz (1982), Bromfield (1984) and Rytter *et al.* (1984). Complications in identifying these alternative hosts have risen through the renaming of host plant species, host species tested and rust pathotypes. A full host range has therefore not been clearly identified (Miles *et al.*, 2003) and is complicated by pathotypes or races of the fungus and strains or varieties of the hosts.

Many researchers have proposed possible alternative hosts, but some references require cautious interpretation. The host in question should only be considered an alternative host if the fungus sporulates on it. In some instances, hosts, which do not support sporulation, have been included in lists of alternative hosts (Bromfield, 1984).

Phakopsora pachyrhizi has an unusually wide host range. This pathogen has been reported to produce infections on 31 plant species in 17 genera of legumes and 60 species of plants in 26 additional genera (Chu and Chuang, 1961).

The purpose of this research was to identify alternative hosts of this pathogen that could provide primary inoculum for soybean crops in South Africa (SA) or serve as overwintering sources for the pathogen. Legumes chosen for screening have been reported to be alternative hosts in other areas of the world.

4.2 MATERIALS AND METHODS

4.2.1 Inoculum sources

Phakopsora pachyrhizi was initially established from uredospores on leaves from naturally infected plants which were grown in a tunnel (20-25°C, 80-90%RH and a photoperiod of 14h) at the University of KwaZulu-Natal, Pietermaritzburg, SA. Uredospores of *P. pachyrhizi* were collected from the abaxial leaf surfaces using a wet paintbrush and suspended in distilled water and the concentration was adjusted to 5.5 x 10^5 spores ml^{-1} using a haemocytometer.

4.2.2 Plant production

Twenty legume seeds and young kudzu vine plants were obtained from the Department of Agriculture and Environmental Affairs at Cedara[1] (Table 4.1) and 15 experimental dry bean lines, either susceptible or resistant to *Uromyces appendiculatus* Pers.:Pers., were obtained from the Agricultural Research Council[2] (Table 4.2). Plants were grown in seedling containers (Clausen Plastics[3]) in a growth room at 21-22°C, 60%RH, a photoperiod of 14h and a light intensity of 347.17µmol/sec/m^2 (Figure 2.1). Plants were fertilised every two weeks with Nitrosol® (8:2:5.8) (N: P: K). Once plants reached the third trifoliate stage (V3) they were inoculated with soybean rust (SBR) uredospores. Five plants with three replications were used.

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Table 4.1 Potential alternative hosts obtained from the Department of Agriculture and Environmental Affairs, Cedara, South Africa

Latin name	Common name
Cajanus cajan (L) Huth	Pigeon pea
Cajanus cajan (L) Huth	Pigeon pea Line MN5
Cajanus cajan (L) Huth	Pigeon pea ICPL 85010
Cajanus cajan (L) Huth	Pigeon pea Line 87
Canavalia ensiformis (L) DC	Jack bean
Coronilla varia (L) DC	Crown vetch
Glycine max (L) Merr	Vegetable soybean
Lablab purpureus (L) Sweet	Lablab
Lespedeza cuneata (Dum.-Cours)	Sericea lespedeza
Lupinus angustifolius (L) Finnish	Lupin
Lupinus angustifolius (L) Finnish	Lupin (Cedara cultivar)
Medicago sativa (L) DC	Lucerne
Mucuna pruriens (L) DC	Macuna velvet bean
Mucuna pruriens (L) DC	Macuna velvet bean
Phaseolus vulgaris (L) DC	Dry bean
Pueraria lobata (M&S) Willd	Kudzu vine
Trifolium repens (L) DC	Clover (crimson)
Vigna unguiculata (L) Walp	Cowpea

Table 4.2 Experimental dry bean cultivars and lines obtained from the Agricultural Research Council, South Africa

Cultivar	Seed type	Resistance to dry bean rust
Teebus	Small white bean	Susceptible
PAN 159	Red speckled sugar bean	Susceptible
Bonus	Red speckled sugar bean	Susceptible
Teebus-RR1	Small white bean	Resistant
OPS-KW1	Small white bean	Resistant
PAN 185	Small white bean	Resistant
Mkuzi	Carioca bean	Resistant
PAN 150	Carioca bean	Resistant
PAN 116	Red speckled sugar bean	Resistant
Kranskop	Red speckled sugar bean	Moderately resistant
Jenny	Red speckled sugar bean	Moderately resistant
OPS-RS 1	Red speckled sugar bean	Moderately resistant
OPS-RS 2	Red speckled sugar bean	Moderately resistant
PAN 148	Red speckled sugar bean	Moderately resistant
PAN 128	Red speckled sugar bean	Moderately resistant

4.2.3 Inoculation and incubation

A concentration 5.5 x 10^5 spores ml^{-1} was used to inoculate the plants. A drop of Tween 20 was added to the uredospore suspension to ensure uredospores adhered to the leaf surface. The abaxial leaf surface of the V3 growth stage was inoculated using an Andres and Wilcoxson inoculator (1984). The suspension was deposited as a uniform layer of droplets on the centre of the leaf. Plants were left to dry for 15 minutes before placing them in a dew chamber. Leaves were sprayed with distilled water to ensure the start of the leaf wetness period. Plants were placed in a dew chamber under continuous darkness at 24°C, 85%RH and 16h LWD. The dew chamber was set at the required temperatures and RH and allowed to stabilize 2h before plants were placed inside. After completion of the LWD, plants were transferred to a Conviron™ (21-22°C, 80%RH, a photoperiod of

14h and a light intensity of 66.4µmol/sec/m^2) for 21 days. Prima 2000, a susceptible soybean cultivar were used as control plants.

4.2.4 Uredospore germination tests

Uredospores were plated onto 1.25% water agar at the beginning, middle and end of each inoculation period (3h) to determine any possible differences in spore germination during the course of the inoculation period. Five plates with three replicates were used. Petri dishes were incubated in the dark at 21°C for 16h and after this period germinating uredospores were counted using a compound microscope at 40X magnification. At least 150 uredospores from each plate were counted.

4.2.5 Rating scale used to differentiate between a host and non-host

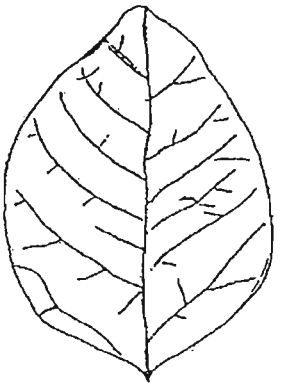
Host reaction was recorded 21 days post inoculation. Plants that produced no infection were left for a further seven days. If no infection was found after this time, then these plants were classified as resistant. The modification of a scheme proposed by Vakili and Bromfield (1976) was adopted to record the following reactions: NI = no infection, R = resistant, necrotic flecks, light brown to dark brown or purple, no uredia, S = susceptible, uredia found on the leaf. Plants that produced typical SBR symptoms were rated for disease severity as proposed by the Asian Vegetable Research Development Centre, Tainan, Taiwan (Figure 4.1).

4.2.6 Re-inoculation and re-infection studies

Uredospores from alternative host plants that produced uredia and showed sporulation, were inoculated onto Prima 2000. Five plants with three replications were used. Soybean plants were placed in a dew chamber at the required temperature, RH and LWD under continuous darkness for infection to take place (24°C, 85%RH and 16h LWD). After 16h LWD, plants were transferred to a Conviron™ (21-22°C, 80%RH, a photoperiod of 14h and a light intensity of 66.4µmol/sec/m^2) for 21 days. Uredospores from species that were able to infect Prima 2000 were considered alternative hosts.

4.2.7 Statistical analyses

Treatments were arranged in a Randomized Complete Block Design (RCBD). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 7.1 Statistical Analysis Software (Lawes Agricultural Trust, 2003) to determine differences between treatment means. All least significant differences were determined at $P<0.05$. The experiment was repeated once.



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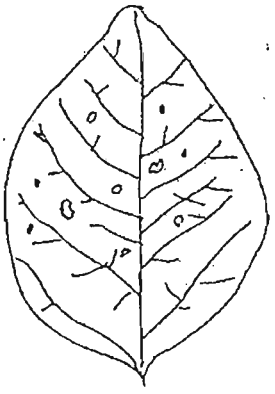

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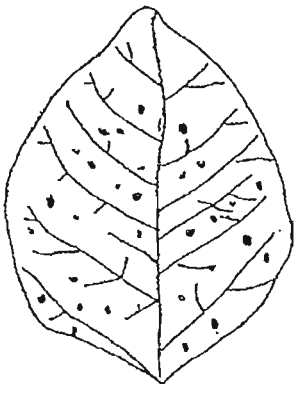

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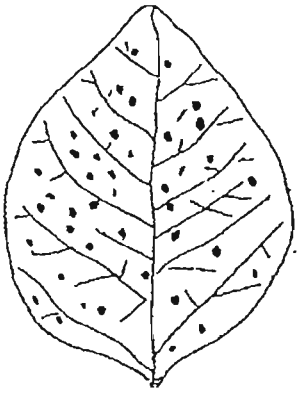
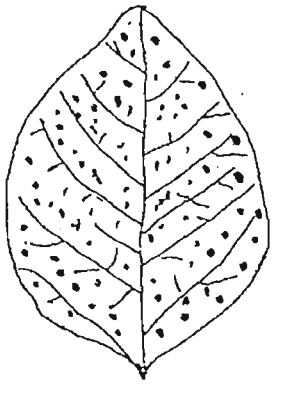
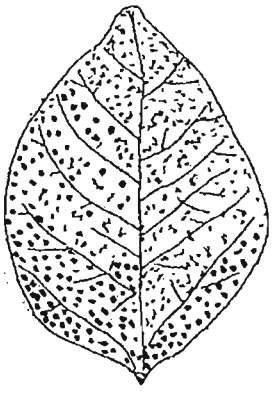


60%: MEDIUM INFECTION



80%: HEAVY INFECTION



100%: LEAF AREA COVERED WITH PUSTULES

Figure 4.1 Rating scale used to determine percentage leaf area infected with uredia of *Phakopsora pachyrhizi* (Asian Vegetable Research Development Centre).

4.3 RESULTS

Trial 2 confirmed results that were obtained in Trial 1. According to the ANOVA, experiments did not differ, and data were therefore pooled.

4.3.1 Uredospore germination tests

Germination percentages determined on agar plates at the start of inoculation, halfway through inoculation, and at the end of inoculation were not significantly different from one another. This indicates that the germination percentage of the inoculum remained constant throughout the inoculation period.

4.3.2 Initial host reactions observed

Table 4.3 shows the initial reaction of the host plants to *P. pachyrhizi* after 21dpi. *Trifolium repens* (clover), *M. sativa* (lucerne), *Lespedeza cuneata* (*Sericea lespedeza*), OPS-RS 1, PAN 128 ,PAN 148 and Teebus-RR1 did not show any reactions on the leaf surface. After a further seven days in the Conviron™, these plants still did not show symptoms of infection and were classified as resistant. *Canavalia ensiformis* (jack bean), *C. varia* (crown vetch), *M. pruriens* (macuna velvet bean) and the experimental dry bean lines Jenny, Kranskop, Mkuzi, OPS-KW1, PAN 116, Pan 150, PAN 185 and Teebus were also classified as resistant to SBR as the leaf surfaces of these plants showed necrotic flecks, light brown to dark brown or purple lesions with no uredia on the leaf surface. The remainder of the plants had a susceptible reaction to SBR inoculations (Table 4.3). Table 4.4 shows the disease severity ratings obtained on the alternative hosts. Disease severity was significantly different within the alternative hosts, with vegetable soybean, kudzu vine and dry beans not being significantly different from PRIMA 2000 (control) (Table 4.4). These alternative hosts and PRIMA 2000 had the highest disease severity compared to the other plants (Appendix 3). Pigeon pea (Line MN5) had the lowest disease severity (Appendix 3) and was significantly different from the rest of the plants.

Table 4.3 Host reactions produced after inoculation with uredospores of *Phakopsora pachyrhizi*

Host plant common name	Host plant Latin name	Host reaction
LEGUME PLANTS		
Cowpea	*Vigna unguiculata*	Susceptible
Dry bean	*Phaseolus vulgaris*	Susceptible
Kudzu vine	*Pueraria lobata*	Susceptible
Lablab	*Lablab purpureus*	Susceptible
Lupin	*Lupinus angustifolius*	Susceptible
Lupin (Cedara cultivar)	*Lupinus angustifolius*	Susceptible
Pigeon pea MN5	*Cajanus cajan*	Susceptible
Pigeon pea ICPL 85010	*Cajanus cajan*	Susceptible
Pigeon pea 87	*Cajanus cajan*	Susceptible
Pigeon pea	*Cajanus cajan*	Susceptible
Vegetable soybean	*Glycine max*	Susceptible
Crown vetch	*Coronilla varia*	Resistant
Jack bean	*Canavalia ensiformis*	Resistant
Macuna velvet bean	*Mucuna pruriens*	Resistant
Clover (crimson)	*Trifolium repens*	No reaction/ resistant
Lucerne	*Medicago sativa*	No reaction/ resistant
Sericea lespedeza	*Lespedeza cuneata*	No reaction/ resistant
DRY BEAN LINES		
Bonus	*Phaseolus vulgaris*	Susceptible
OPS-RS 2	*Phaseolus vulgaris*	Susceptible
PAN 159	*Phaseolus vulgaris*	Susceptible
Jenny	*Phaseolus vulgaris*	Resistant
Kranskop	*Phaseolus vulgaris*	Resistant
Mkuzi	*Phaseolus vulgaris*	Resistant
OPS-KW1	*Phaseolus vulgaris*	Resistant
PAN 116	*Phaseolus vulgaris*	Resistant
PAN 150	*Phaseolus vulgaris*	Resistant
PAN 185	*Phaseolus vulgaris*	Resistant
Teebus	*Phaseolus vulgaris*	Resistant
OPS-RS 1	*Phaseolus vulgaris*	No reaction/ resistant
PAN 128	*Phaseolus vulgaris*	No reaction/ resistant
PAN 148	*Phaseolus vulgaris*	No reaction/ resistant
Teebus-RR1	*Phaseolus vulgaris*	No reaction/ resistant
CONTROL-SOYBEAN		
PRIMA 2000	*Glycine max*	Susceptible

Table 4.4 Disease severity ratings of alternative hosts that showed susceptibility to soybean rust

Host plant	Disease severity %
Lupin (Cedara)	37^{gh}
Cowpea	26^{c}
Pigeon pea MN5	17^{a}
Pigeon pea ICPL 85010	22^{b}
Pigeon pea ICPL 87	23^{bc}
Pigeon pea	20^{ab}
Lablab	36^{fg}
Lupin	23^{bc}
Vegetable soybean	40^{h}
Dry beans	39^{gh}
Kudzu vine	37^{gh}
Bonus	20^{ab}
OPS-RS 2	22^{b}
PAN 159	31^{e}
Prima 2000	41^{h}
F. test	(0.001)
l.s.d	3.244
s.e.d	1.584
cv%	6.5

Means with same letter are not significantly different at P<0.005.

4.3.3 Re-inoculation and re-infection studies

Cowpea, dry beans, kudzu vine, lablab, lupin, vegetable soybeans, Bonus and PAN 159 produced an infection on PRIMA 2000 with uredospores being produced when they were inoculated with uredospores produced from the initial inoculation trials. These plants were defined as alternative hosts of *P. pachyrhizi* in this trial.

4.4 DISCUSSION

Six legume species and 12 dry bean lines were classified as resistant to *P. pachyrhizi* (Table 4.3). However, these plants may possibly be hosts of *P. pachyrhizi* in other areas in SA which provide a longer LWD or higher temperatures. The possibility of these plants being alternative hosts must not be ruled out before further field trials are conducted. The physiological age or maturity of soybean plants also affects the rate of SBR development (Melching *et al.*, 1988). While the physiological age of some host plants seems to determine when lesions and uredospores appear, as well as how fast they develop, it is not known how widespread these phenomena are among alternative host plants (Melching *et al.*, 1988). Experimental and field observations by Vakili and Bromfield (1976) indicated that sporulation and appearance of lesions and uredial development might be predetermined by the physiological age of at least some hosts. As availability of space was a problem, plants were inoculated at the V3 growth stage.

Lesions with uredia were observed on eleven legume species and three dry bean lines (Table 4.3). However, not all uredospores from these lesions infected PRIMA 2000. Eight of these, i.e., cowpea, dry beans, kudzu vine, lablab, lupin, vegetable soybeans, Bonus and PAN 159, caused infection on PRIMA 2000 and have been identified as possible alternative hosts of *P. pachyrhizi* (Table 4.3). du Preez *et al.* (2005), found dry beans to be alternative host of *P. pachyrhizi* in KwaZulu-Natal. Kudzu vine is regarded as the prime alternative host in South America (Miles *et al.*, 2004). In South Africa, SBR symptoms on kudzu vine growing naturally in the Mpumalanga province have been found (Pretorius pers. comm.[4]). It is speculated that the uredospores on the kudzu vine are blown into KZN every season. However, the kudzu vine at Cedara is grown in a small area which is used for research. It does not grow naturally in this province and may only be providing a little inoculum for the next season. The alternative hosts grown widely in KZN, need to be followed up with field observations in summer.

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CHAPTER FIVE

EFFECT OF LEAF AGE ON SUSCEPTIBILITY OF SOYBEAN TO *PHAKOPSORA PACHYRHIZI*

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ABSTRACT

Soybeans (Prima 2000) were grown in a growth room (25°C, a photoperiod of 14h at 60%RH). At different growth stages (V1, V3, V6, R1, R3 and R6) soybean plants (*Glycine max* (L.) Merr.) were inoculated with a concentration of 5.5 x 10^5 uredospores ml^{-1} of *Phakopsora pachyrhizi* Syd and placed under continuous darkness in a dew chamber set at 24°C, 85%RH and 16h leaf wetness duration (LWD). Following incubation in the dew chamber, plants were placed in a Conviron™ (21-22°C, 80%RH, a photoperiod of 14h and a photosynthetic active radiation of 260µmol/m^2sec^{-1}) for 21 days. Numbers of lesions as well as lesion size were assessed on the abaxial leaf surface at 8, 12, 16 and 20 days post-inoculation. Mean number of lesions and lesion sizes were greater on younger leaves than on older leaves of plants at the same physiological age. This was noticed at all growth stages of the soybean plants. Plants at the early vegetative (V1) and late reproductive stages (R6) had a significantly lower number of lesions as well as lesion size compared to plants at the other growth stages. Plants at the V6 and R 1 growth stages were significantly more susceptible to *P. pachyrhizi* Syd., than plants at other developmental stages.

5.1 INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) is considered a "wonder crop" due to its dual qualities, i.e., high protein (40%) and oil (20%) content. This two-in-one crop has gained considerable importance in agricultural economy (Dadke *et al.*, 1997).

In Africa, soybean cultivation has increased in the last four decades from 72 000 tonnes on 191 000ha in 1961 to 989 000 tonnes on 1 090 000ha in 2002. However, it accounts for only 0.5% of the annual global production of 179 917 000 tonnes (Singh *et al.*, 2004). South Africa (SA) produces 208 000 tonnes of soybean seed on 193 000ha of land tonnes (Singh *et al.*, 2004).

In SA soybean is a strategically important crop and is grown under natural rainfall and irrigated conditions, usually in summer rainfall areas (Bell *et al.*, 1990). More than 100 pathogens are known to affect soybean, of which 35 are economically important (Earthington *et al.*, 1993). All parts of the soybean plant are susceptible to numerous pathogens, resulting in a reduction in quality and quantity of seed yields (Sinclair and Backman, 1989). *Phakopsora pachyrhizi* H. Syd and P. Syd, the causal organism of soybean bean rust (SBR) is one of the major disease problems limiting soybean yield. Yield losses of 50-60% are common, as well as complete crop losses where early infection and favourable environmental conditions exist (Kloppers, 2002). Yield losses of up to 40% have been reported in Japan, 10-50% in southern China, 10-40% in Thailand and 23-90% in Taiwan (Sinclair and Backman, 1989). Zimbabwe has experienced yield loss of 60-80% in commercial crops (Caldwell *et al.*, 2002). In SA yield losses of 10-80% were reported, with losses of up to 100% where monocropping with no rotation was practiced (Caldwell and Laing, 2002).

The soybean plant is highly sensitive to its environment. The total growth and yield of the crop may vary widely depending upon location, soil, planting date, variety selection, weed competition, diseases, pest injury, and fertility levels. This level of environmental sensitivity often varies according to the particular growth stage of the crop. Therefore, careful manipulation of these factors and good troubleshooting techniques are necessary to get top yields and profits (Naeve,

2006). Development of the soybean plant begins at germination and ends when the mature seed is ready for harvest (Figure 5.1). The plant's total growth and lifespan, including length of vegetative growth, time of flowering, and maturity, are greatly influenced by photoperiod and day-night temperatures. Most varieties have a "critical threshold" night length requirement for floral initiation and development. Plants grow vegetatively during periods with shorter nights, and start reproductive development when nights exceed the critical threshold length. Thus, a variety with a short night length requirement flowers and matures earlier than varieties with long night length requirements. The amount of vegetative growth before flowering depends on the variety and environment. The amount of vegetative growth occurring after flower initiation depends on either environment and growth habit, which may be determinate, or indeterminate (Bell *et al.*, 1990).

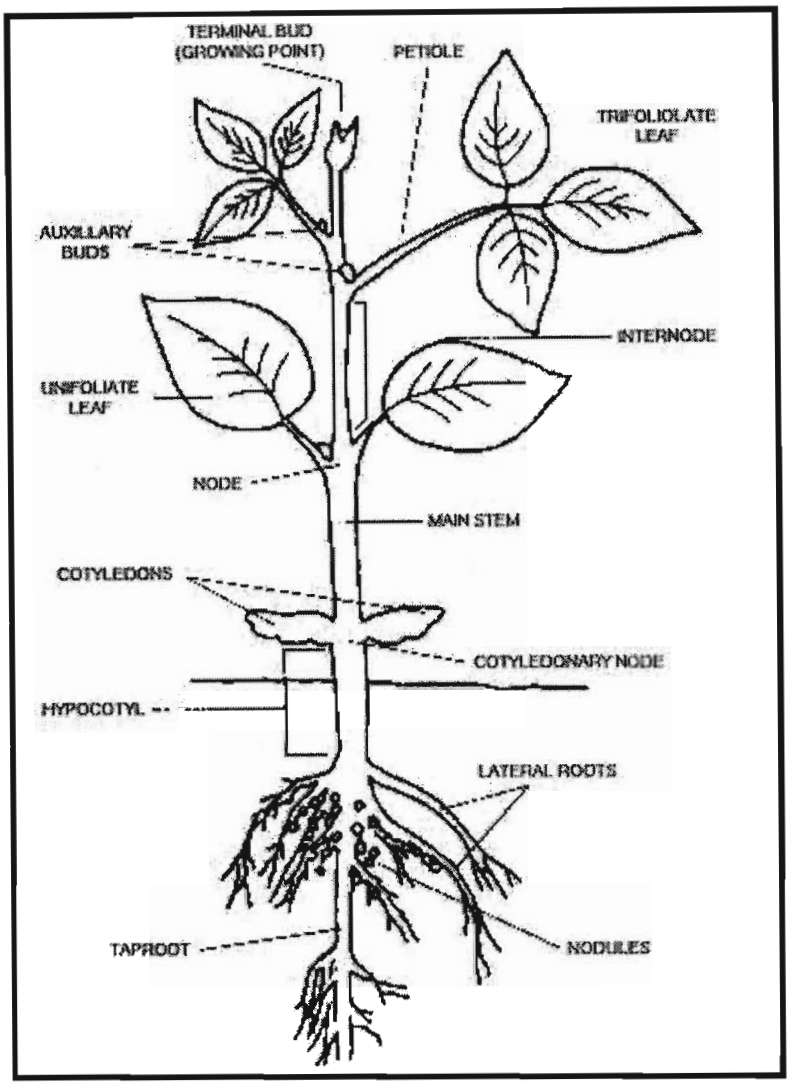

Figure 5.1 Diagram showing soybean growth and development (Naeve, 2006).

The present investigation was carried out on the soybean variety PRIMA 2000 at different growth stages of the crop to determine the relationship between plant development and leaf age and susceptibility *to P. pachyrhizi.*

5.2 MATERIALS AND METHODS

5.2.1 Test plants

Soybean plants (Prima 2000[1]) were grown singly in seedling containers (3 x 3 x 5) (Clausen Plastics[2]) placed in plastic containers filled with water in a growth room (25°C, 60%RH, a photoperiod of 14h and a light intensity of 347.17µmol/sec/m^2) (Figure 2.1). Plants were fertilized twice a week with Nitrosol® (8:2:5.8) (N: P: K). Prima 2000 was selected because it is a cultivar of commercial importance and is susceptible to SBR. Plantings were made at 5 to 7 day intervals to provide plant populations of different ages and stages of development. Five plants with three replications were used for each physiological age group.

5.2.2 Inoculum

Uredospores of *P. pachyrhizi* were obtained from naturally infected soybean plants grown in a tunnel (20-30°C, 50-100%RH and a photoperiod of 12-14h) at the University of KwaZulu-Natal, Pietermartizburg, SA. Uredospores of *P. pachyrhizi* were collected from the uredia on the abaxial leaf surfaces of naturally infected soybean plants using a wet paintbrush and suspended in distilled water. Uredospore concentration was adjusted to 5.5 x10^5 uredospores ml^{-1} using a haemocytometer.

5.2.3 Inoculation

Selected plants were inoculated with uredospores suspended in distilled water containing Tween 20 (polyoxyethlene sorbitan monlaurate, 0.25% v/v) to facilitate the adherence of the uredospores to the leaf surface. Plants at the V1, V3, V6, R1, R3 and R6 growth stages were inoculated using an Andres and Wilcoxson (1984) inoculator.

[1] Pannar Seed (Pty) P.O, Box 19, Greytown 3250, Republic of South Africa.
[2] Clausen Plastics®, Johannesburg, Republic of South Africa

5.2.4 Uredospore germination tests

Uredospores were plated onto 1.25% water agar at the beginning, middle and end of each inoculation period to determine any possible differences in spore germination during the course of the inoculation period which was 3h. Five plates with three replicates were used. Petri dishes were incubated in the dark at 21°C for 16h and after this period germinating uredospores were counted using a compound microscope at 40X magnification and the germination percentage determined. At least 150 uredospores from each plate were counted.

5.2.5 Post-inoculation treatment

Following inoculation, plants were left to dry for 15 minutes before placing them in a dew chamber [22-24°C, 85%RH and 16h leaf wetness duration (LWD)] under continuous darkness. Leaves were sprayed with distilled water to ensure the start of the leaf wetness period. The dew chamber was set at the required temperatures and RH and allowed to stabilize 2h before plants were placed inside. Following the 16h LWD plants were transferred to a Conviron™ (21-22°C, 80%RH, a photoperiod of 14h and a light intensity of 66.4µmol/sec/m^2) for 21 days.

5.2.6 Disease rating

A template was designed for counting number of lesions and lesion size at six sites on the abaxial leaf surface. These results were then averaged and used in the analyses. Area of leaflets was measured and lesion size was estimated by use of the equation: area = length x width x 0.76 (Melching *et al.*, 1988). Number of lesions was calculated at 8, 12, 16 and 20 days post inoculation (dpi). Ten lesions on the abaxial leaf surface of each leaf were measured and the average lesion size (µm) and number of lesions determined. Lesion size and number of lesions were determined using a compound microscope at 40X magnification for each age group.

5.2.7 Statistical analyses

The trial was repeated once with treatments arranged in a Randomized Complete Block Design (RCBD). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 7.1 Statistical Analysis Software (Lawes

Agricultural Trust, 2003) to determine differences between treatment means. All least significant differences were determined at $P<0.05$.

5.3 RESULTS

Trial 2 confirmed results that were obtained in Trial 1. According to the ANOVA, experiments did not differ, and data were therefore pooled.

5.3.1 Viability of inoculum

Germination percentages determined on agar plates at the start of inoculation, halfway through inoculation, and at the finish of inoculation were not significantly different from one another. This indicates that the germination percentage of the inoculum remained constant throughout the inoculation period.

5.3.2 Number of lesions per leaf produced by *Phakopsora pachyrhizi* at the different developmental stages of soybean plants

ANOVA indicated significant differences between the different plant stages and time. Number of lesions that developed at 8 dpi was significantly higher on plants at the V6 and R1 growth stages (Appendix 4a). Number of lesions was found to be significantly higher on plants at the V6 and R1 growth stages at 20 dpi (Figure 5.2).

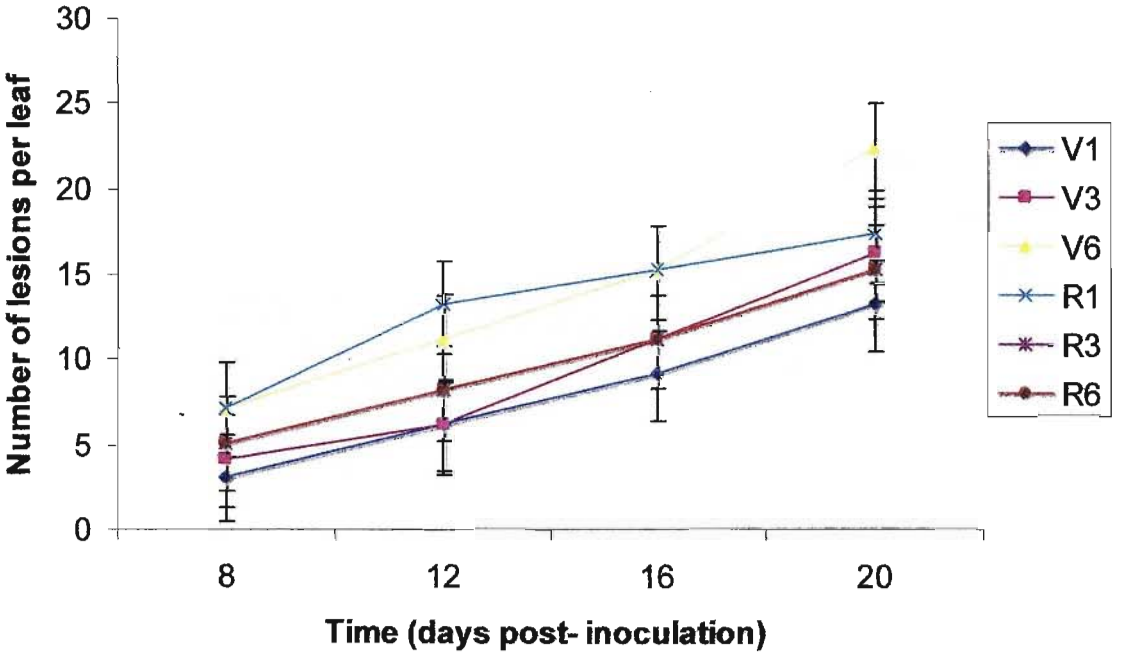

Figure 5.2 Effect of growth stages on number of lesions per leaf of *Phakopsora pachyrhizi*. Bars represent the standard deviation of the treatment mean of pooled data.

5.3.3 Lesion size (mm) of *Phakopsora pachyrhizi* produced at the different developmental stages of soybean plants

ANOVA indicated significant differences between the different plant stages and time. Lesion sizes produced by *P. pachyrhizi* at 16 dpi and 20 dpi were consistently higher on plants at the V6 and R1 growth stages. (Appendix 4b and Figure 5.3). Lesion sizes were significantly larger on the younger than on the older leaves. Lesion sizes were found to be highest on plants at the V6 and R1 growth stages at 20 dpi (Figure 5.3). At growth stages V1 and R6 lesion size was relatively smaller compared to the V6 and R1 growth stages, i.e., plants are most susceptible to infection at the V6 and R1 growth stages.

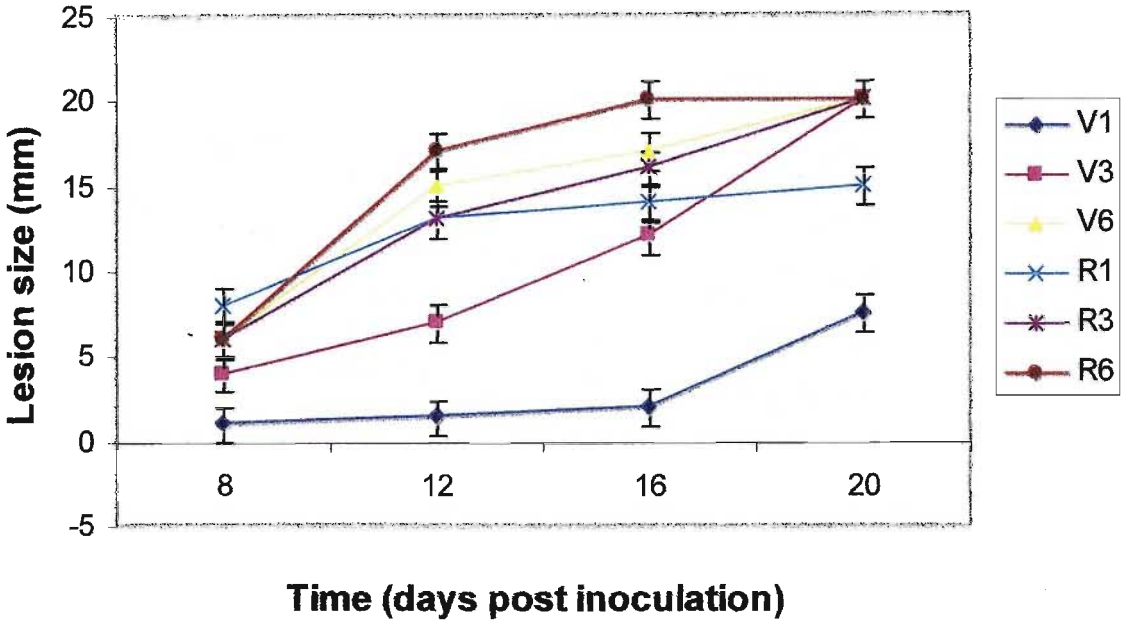

Figure 5.3 Effect of plant growth stages on lesion size (mm) per leaf of *Phakopsora pachyrhizi*. Bars represent the standard deviation of the treatment mean of pooled data.

5.4 DISCUSSION

Adequate standardization of plant age, inoculum density and quality, and environmental conditions is required to recognize true differences in susceptibility to pathogens (Yarwood, 1959; Schein, 1965; Populer, 1978 and Melching *et al.*, 1988). In the research conducted, environmental conditions during plant propagation prior to inoculation and during post-dew development were sufficiently defined and controlled to provide an acceptable level of variation in disease development that attributed solely to environmental factors.

For fungal pathogens, susceptibility of a host to a disease may be caused by any one of the following four components of susceptibility: larger number of infections resulting from a given amount of inoculum, larger lesions or lesions expanding more rapidly, a shorter latent period and a higher yield of spores per unit area of tissue per lesion (Yarwood, 1959; Populer, 1978; Parlevliet, 1979, Zadoks and Schein, 1979 and Melching *et al.*, 1988).

Results obtained from the current study indicate that the number of lesions and lesion size showed significantly different levels of susceptibility to SBR associated with differences in plant age. Once again these results are similar with those obtained by Melching *et al.* (1988). Yang *et al.* (1991) reported that *P. pachyrhizi* causes the number of soybean pods per plant at growth stage R6 to be reduced by as much as 40%, but the number of seeds per pod was not affected, indicating that disease affected the attainable yield by reducing pod set. The time for diseased plants to grow from R4 to R7 was reduced by as many as 16 days compared to protected plants (Yang *et al.*, 1991). From growth stage R6 to R7, percentage pod abortion was high for severely diseased plants. Seed growth rate (grams per day) from R4 to R7 was reduced by 40-80% in diseased plants. The study of susceptible stages of the crop helps producers to take precautionary measures to avoid disease incidence. It also helps in the screening of genotypes for resistance.

Susceptibility of soybean plants to SBR appears to change with the growth stage of the plants. Low disease severity was observed on plants that were at the V1

and R6 developmental stage. Overall, plants at the V6 and R1 growth stages were more susceptible to SBR than plants at the other growth stages tested in terms of number of lesions and lesion size, i.e., plant age has an influence on susceptibility and resistance to SBR.

Environment may have some effect on the susceptibility of plants in the field. Attack by *P. pachyrhizi* may occur at any stage of growth but older leaves appear have a defence mechanism that inhibits disease development. Data obtained emphasize the importance of considering the age of soybean plants in screening for resistance as well as in determining the most effective timing for use of chemicals.

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CHAPTER SIX

EVALUATION OF *TRICHODERMA HARZIANUM* AS A POSSIBLE BIOLOGICAL CONTROL AGENT FOR *PHAKOPSORA PACHYRHIZI*

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ABSTRACT

Trichoderma harzianum Rifai, Eco-77® a commercial biological control product, was evaluated for efficacy as a biological control agent of *Phakopsora pachyrhizi* (P. and H. Syd.) the causal organism of soybean rust (SBR). Eco-77® was evaluated at three concentrations [standard (1g in 2L water), ½ standard (0.5g in 2L water) and 2x standard (2g in 2L water] as well as in a filtrate form. To evaluate which concentration of Eco-77® controlled *P. pachyrhizi*, uredospores (5.5 x 10^5 uredospores ml^{-1}) were inoculated onto soybean plants (*Glycine max* (L.) Merril) at the V3 growth stage two days before spraying with the biological control agent. Five plants with five replicates were used for each trial. Plants were placed in a dew chamber (21-23°C, 16h leaf wetness duration and 85%RH) and transferred to a Conviron™ (21-22°C, 80%RH, with a photoperiod of 14h and a light intensity of 66.4µmol/sec/m^2). Liquid paraffin and distilled water were applied as controls. To evaluate if Eco-77® was effective in the filtrate form, *T. harzianum* was grown in potato dextrose broth for seven days. It was then centrifuged and the filtrate sprayed onto soybean plants two days before and two days after inoculation with uredospores of *P. pachyrhizi*. Potato dextrose broth and distilled water were applied as controls. Soybean plants were evaluated weekly for leaf area infected on a rating scale of 0-100%. Data indicated that plants sprayed with the standard concentration of Eco-77® after inoculation with SBR uredospores had the least leaf area infected by *P. pachyrhizi*. No statistical differences were found between

paraffin and distilled water treatments but were significantly higher from plants sprayed with Eco-77®. The area under disease progress curve shows that plants sprayed with 2x the standard concentration had significantly higher disease compared to the standard concentration treatments. Data indicate that spraying the filtrate two days after inoculation results in lower disease severity. No statistical differences were found between potato dextrose broth and distilled water but there were statistical differences between the potato dextrose broth, distilled water and the biological control agent, Eco-77®.

6.1 INTRODUCTION

Microorganisms, as naturally occurring resident antagonists, play an important role in plant disease control and therefore can be managed or exploited to achieve the desired results (Mathre *et al.*, 1999). In intensive agricultural production systems it is significant to protect plants from adverse biotic factors which affect the efficiency and microbiological quality of crops as raw materials. Available plant protection methods have been reviewed in the past decade due to the renewed emergence on sustainable agricultural production systems. Therefore, the importance of using environmentally-friendly and food-hygienically safe plant protection methods, and plant-protecting agents of biological origin, has been greatly emphasized in recent years (Földes *et al.*, 2000).

Fungi belonging to the Basidiomycetes, particularly the rusts, have been frequently noted as hosts of other parasites. The control of rust diseases is usually carried out using resistant varieties (Johnson, 1992 and Kolmer, 1995) and application of synthetic fungicides (Dalal and Singh, 1994; Harko *et al.*, 1994; Hofle *et al.*, 1995). Since rusts produce external structures as secondary inoculum aiding disease spread, they are liable to be controlled more effectively by hyperparasites than other diseases such as leaf spots. Both primary and secondary inoculum can be parasitized, thereby affecting disease at the time of infection, and later, its subsequent spread (Sharma and Sankaran, 1988).

Hyperparasites present an attractive alternative to fungicides in control of biotrophic plant pathogens. There are probably no environmental hazards involved in using these widespread enemies of powdery mildew and rusts to reduce disease losses (Sundheim, 1986).

More than 30 genera of fungi have been found inhabiting pustules on rust infected plants (Littlefield, 1981), but it is uncertain as to how many of these are truly parasitic on the rust fungus. *Eudarluca caricis* (Fr.) O.E. Erikss. and *Lecanicillium lecanii* (Zimm.) Gams and Zare were listed as the most important hyperparasitic fungi on rust by Blakeman and Fokkema (1982). *Eudarluca caricis* has not been reported on *Phakopsora pachyrhizi* Syd., but Naidu (1978) has reported its parasitism of *P. elettariae* (Racib) Cummins, the causal organism of cardamomn rust in India.

Pon *et al.* (1954) described a soilborne bacterium, *Xanthomonas parasitica*, disseminated by rain splash, which parasitizes uredia of various cereal rust fungi and causes uredospore lysing. The genus *Bacillus* has also been implicated in uredospore lysing and in the inhibition of uredospore germination (Littlefield, 1981).

Urocladium and *Sphaerolopsis* may be effective as biological control agents of soybean rust (SBR). *Verticillium psalliotae*, a mycoparasite, has the ability to infect and colonize uredospores of SBR. *Verticillium psalliotae* forms appressorium-like structures at infection sites. Uredospores are not penetrated by *V. psalliotae*, but appear degraded and eventually burst to form lytic enzymes (Saksirirat and Hoppe, 1990).

Trichoderma spp. are fungi that are present in nearly all agricultural soils and in other environments such as decaying wood. The antifungal abilities of these beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then. These fungi grow tropically toward hyphae of other fungi, coil about them in a lectin-mediated reaction, and degrade cell walls of the target fungi by the secretion of different lytic enzymes. This process (mycoparasitism) limits growth and activity of plant pathogenic fungi. Specific strains of fungi in the genus *Trichoderma* colonize and penetrate plant root tissues and initiate a series of morphological and biochemical

changes in the plant, considered to be part of the plant defense response, which in the end leads to induced systemic resistance (ISR) in the entire plant (Yedidia *et al.*, 1999).

Pesticide hazards and resistance problems, as well as effects on non-target plants and pests, have produced renewed interest in naturally occurring pesticides and biological control agents. These natural compounds are often less toxic and less persistent and are assumed to be environmentally more acceptable and less hazardous to humans and animals (Eldoksch *et al.*, 2001).

The present investigation aimed to study the antifungal activity of the formulated product Eco-77®[1] (*Trichoderma harzianum* Rifai) against SBR under controlled environmental conditions.

6.2 MATERIALS AND METHODS

6.2.1 Test plants

Single soybean plants (Prima 2000[2]) were grown in seedling containers (3 x 3 5c) (Clausen Plastics[3]) placed in plastic containers, filled with water, in a growth room (25°C, 60%RH, a photoperiod of 14h and a light intensity of 347.17µmol/sec/m^2) (Figure 2.1). PRIMA 2000 was selected because it is a cultivar of commercial importance, and it exhibits a susceptible reaction to SBR.

6.2.2 Inoculum

Uredospores of *P. pachyrhizi* were obtained from naturally infected soybean plants grown in a tunnel (20-30°C, 50-100%RH and with a photoperiod of 12-14h) at the University of KwaZulu-Natal, Pietermartizburg, South Africa. Uredospores of *P. pachyrhizi* were collected from the uredia from the abaxial leaf surfaces of naturally infected soybean plants using a wet paintbrush and suspended in distilled water. The uredospore concentration was adjusted to 5.5 x 10^5 uredospores ml^{-1} using a haemocytometer.

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[1] Plant Health Products (Pty) Ltd, P.O. Box 207, Nottingham Road, Republic of South Africa
[2] Pannar Seed (Pty), P.O. Box 19, Greytown 3250, Repbulic of South Africa
[3] Clausen Plastics®, Johannesburg, Republic of South Africa

6.2.3 Inoculation

Five plants with five replications were used for each trial. Eight tagged leaves of plants in each replicate were inoculated with SBR uredospores in distilled water containing Tween 20 (polyoxyethlene sorbitan monlaurate, 0.25% v/v). Tween 20 was used to allow the uredospores to adhere to the abaxial leaf surface. Plants were inoculated with an Andres and Wilcoxson (1984) inoculator.

6.2.4 Uredospore germination tests

Uredospores were plated onto 1.25% water agar at the beginning, middle and end of each inoculation period to determine any possible differences in spore germination during the course of the inoculation period of 3h. Five plates with three replicates were used. Petri dishes were incubated in the dark at 21°C for 16h and after this period germinating uredospores were counted using a compound microscope at 40X magnification and the germination percentage determined. At least 150 uredospores from each plate were counted.

6.2.5 Eco-77® germination tests

Before the start of the experiment Eco-77®, (*Trichoderma harzianum*), at the standard concentration, was suspended in distilled water and plated onto potato dextrose agar (PDA). This was done to determine the viability of the biological control agent. Petri dishes were incubated at room temperature and spores were counted after 16h using a compound microscope at 40X magnification. Five plates with three replicates were used.

6.2.6 Determination of the optimum concentration of Eco-77® for the control of soybean rust

Eco-77® was evaluated at the following concentrations: standard (1g in 2L water), ½ standard (0.5g in 2L water) and 2x standard (2g in 2L water). Liquid paraffin was mixed (4ml in 1L water) with Eco-77® before suspending the mixture in distilled water. Eco-77® was sprayed onto the plants in the form of a spore suspension. 2 x 10^9 spores per gram were applied to the leaf surface. This was done to help Eco-77® adhere to the leaf surface. Two days after inoculation plants were sprayed with the different concentrations of Eco-77®. The volume of Eco-77® sprayed onto the plants was 2ml. Leaves at the V3 growth stage were

inoculated with uredospores at 5.5 x 10^5spores ml^{-1} and placed in a dew chamber [21-23°C, 85% RH and 16h leaf wetness duration (LWD)]. Once inoculated plants were left to dry for 15 minutes before placing them in a dew chamber. Leaves were sprayed with distilled water to ensure the start of the leaf wetness period and were placed in a dew chamber at the required temperature, RH and LWD in continuous darkness for infection to take place. The dew chamber was set at the required temperature and RH and allowed to stabilize 2h before plants were placed inside. After this, plants were transferred to a Conviron™, (21-22°C, 80%RH, 14h photoperiod and a light intensity of 66.4µmol/sec/m^2) for 21 days. Liquid paraffin and distilled water were used as controls and were sprayed onto the plant two days after inoculation.

6.2.7 Determination of the correct time of application of Eco-77® filtrates

An Eco-77® suspension at the standard concentration was spread over PDA in Petri dishes. Plates were incubated at 25°C in an incubator for a period of 14 days. Mycelial plugs (3mmx 3mm) were cut and inoculated in potato dextrose broth (PDB) and incubated at 25°C in a water-bath shaker[4] at 120 oscillations min^{-1} for seven days. The PDB containing Eco-77® was filtered through cheesecloth to remove the mycelial mat that had grown. The liquid content was centrifuged using a Beckman J2 HS Centrifuge at a speed of 9000xg for 25 minutes at 4°C. Plants at the V3 growth stage were sprayed with the filtrate two days before inoculation (2DB) and two days after inoculation (2DA) with uredospores at 5.5 x 10^5 spores ml^{-1}. Following inoculation, plants were left to dry for 15 minutes before placing them in a dew chamber at 22-24°C, 85%RH and 16h LWD under continuous darkness for infection to take place. Leaves were sprayed with distilled water to ensure the start of the leaf wetness period. The dew chamber was allowed to stabilize 2h before plants were placed inside. Following the 16h LWD plants were transferred to a conviron (21-22°C, 80%RH, with a photoperiod of 14h and a light intensity of 66.4µmol/sec/m^2) for 21 days. Distilled water and PDB were used as controls and sprayed onto the plants 2DB and 2DA inoculation. Plants that were sprayed with the filtrate 2DA were also exposed to conditions in the dew chamber.

[4] Gesellschaft für Labortechnik, mbH,D-30938, Burgwedel, Germany

6.2.7 Ratings

Rating for leaf area infected (Figure 4.1) was carried out on the plants weekly for a period of 21 days. The rating scale is used by the Asian Vegetable Research Development Centre, Tainan, Taiwan but has been modified for the purposes of this work. A rating scale of 0-100% of percentage leaf area infected with uredospore pustules was used.

6.2.8 Statistical analyses

The trial was repeated once with treatments arranged in a Randomized Complete Block Design (RCBD). Area under disease progress curve (AUDPC) was calculated for the different concentrations of Eco-77® (6.2.6) and filtrate trials (6.2.7). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 7.1 Statistical Analysis Software (Lawes Agricultural Trust, 2003) to determine differences between treatment means. All least significant differences were determined at $P<0.05$.

6.3 RESULTS

Trial 2 confirmed results that were obtained in Trial 1. According to the ANOVA, experiments did not differ, and data were therefore pooled.

6.3.1 Viability of biological control agent and inoculum

Germination of Eco-77® was checked on PDA and it was found that there was 100% germination of the biological control agent used.

Germination percentages of uredospores determined on agar plates at the start of inoculation, halfway through inoculation, and at the end of inoculation were not significantly different from one another within the study. This indicates that germination remained constant throughout the inoculation period. These results were constant in both Trials.

6.3.2 Final percentage disease severity and area under disease progress curve (AUDPC) of infected plants subjected to different concentrations of Eco-77®

Results show significant differences between each treatment for final percentage disease severity and AUDPC (Tables 6.1). Plants sprayed with the standard concentration had a significantly lower final percentage disease severity and AUDPC than the other treatments. Plants treated with liquid paraffin (control) were not significantly different from plants sprayed with distilled water only (control) but were significantly different from plants sprayed with Eco-77® at all concentrations (Appendix 5a). Therefore, it appears that Eco-77® is responsible for the control of *P. pachyrhizi*. Plants sprayed at ½ the standard concentration had a significantly lower percentage disease severity than plants sprayed at 2x the standard concentration (Table 6.1). AUDPC results indicate that plants sprayed at the standard concentration had a significantly lower AUDPC than other treatments (Table 6.1 and Appendix 5b).

Table 6.1 Final percentage disease severity and Area under disease progress curve (AUDPC) of plants inoculated with uredospores of *Phakopsora pachyrhizi* and sprayed with different concentrations of Eco-77®

Concentration of Eco-77®	Final disease severity (%)	Area under disease progress curve
Standard	26[a]	368[a]
½ Standard	31[b]	430[b]
2x Standard	41[c]	578[c]
Liquid paraffin	49[d]	681[d]
Distilled water	49[d]	693[d]
F test	<0.001	<0.001
l.s.d.	0.746	15.44
s.e.d.	0.358	7.4
cv%	1.4	2.1

Means with same letter are not significantly different at P<0.005

6.3.3 Final percentage disease severity and area under disease progress curve (AUDPC) of infected plants subjected to the filtrate of Eco-77® at different times

Results show significant differences between each treatment for final percentage disease severity and AUDPC (Tables 6.2). Plants sprayed with Eco-77® filtrate 2DA inoculation had a significantly lower final percentage disease severity and AUDPC values than all other treatments. This indicates that plants sprayed with the filtrate 2DA inoculation can control SBR. Plants sprayed 2DB inoculation with Eco-77® filtrate had a significantly higher final percentage disease severity than plants sprayed 2DA inoculation (Table 6.2). Plants sprayed with PDB (control) 2DB and 2DA inoculation were not significantly different from plants sprayed with distilled water (control) 2DB and 2DA inoculation (Appendix 5c and d).

Table 6.2 Final percentage disease severity and Area under disease progress curve of plants inoculated with uredospores *Phakopsora pachyrhizi* and spayed with Eco-77®, at the standard concentration, at different times

Intervals of spraying Eco-77®	Final disease severity (%)	Area under disease progress curve
2 Days before inoculation	27[b]	335[b]
2 Days after inoculation	23[a]	317[a]
2 Days before inoculation-potato dextrose broth	48[c]	676[c]
2 Days after inoculation-potato dextrose broth	48[c]	671[c]
2 Days before inoculation-distilled water	48[c]	670[c]
2 Days after inoculation-distilled water	47[fc]	662[c]
F test	<0.001	<0.001
l.s.d.	1.261	59.44
s.e.d.	0.611	28.80
cv%	2.4	8.2

Means with same letter are not significantly different at P<0.005.

6.4 DISCUSSION

Natural products and non-pathogenic fungi, bacteria and yeasts have proved to be potential sources of environmentally safe antimicrobial agents useful in plant protection (Biles and Hills, 1988; Bar-Nun and Mayer, 1990; Abdel-Moity *et al.*, 1993; Eldoksch and Abdel-Moity, 1997; Hassanein and Eldoksch, 1997 and Hammouda *et al.*, 1999). Therefore, the importance of using environmentally-friendly and hygienically safe plant protection methods, and plant protecting agents of biological origin, have been greatly emphasized in recent years (Földes *et al.*, 2000).

Eco-77® (*T. harzianum*) was evaluated for its efficacy to reduce SBR under greenhouse conditions. Percentage disease severity, area under disease progress curve (AUDPC) and yield have been used to evaluate the effect of biological treatments on crops (Hassanein and Eldoksch, 1997). In this study percentage final disease severity and AUDPC were used to evaluate the biocontrol (Eco-77®) treatments on soybeans infected with *P. pachyrhizi.*

Data in Tables 6.2 and 6.3, generally, showed that soybean treatments with Eco-77® at the standard concentration were more effective in controlling the pathogen than spraying the plants with Eco-77® at ½ standard and 2x standard concentrations. Plants that were sprayed with the filtrate of Eco-77® 2DA inoculation had a significantly lower percentage disease than plants sprayed with the filtrate 2DB inoculation with uredospores of *P. pachyrhizi.* This indicates the plants need to be infected with *P. pachyrhizi* before spraying with the filtrate of Eco-77®. Neither the PDB nor the distilled water had any effect on controlling the disease and therefore Eco-77® was responsible for the control of this pathogen.

Under greenhouse conditions Eldoksch *et al.* (2001) found *T. harzianum* to be effective in controlling leaf wheat rust compared to the fungicide Sumi-8 diniconazole. The inhibition of rust severity by antagonistic species when they are applied to the leaves may be due to inhibitory substances produced by these biocontrol agents or competition for nutrients and space (Eldoksch *et al.*, 2001).

Many soybean pathogens are present in SA; most occur every season but are seldom of economic importance. Soybean rust has resulted in significant economic losses in commercial soybean production since 2001 and has emerged as a major threat to the soybean industry. Fungicides are the general control measure used at present. However, Eco-77® has shown initial control of this pathogen. A further study is needed to evaluate the economic impact of Eco-77® compared to various fungicide treatments to establish differences in gross margins between the two control measures.

It can be concluded that the use of Eco-77® as a non-chemical approach to managing SBR exhibited effective control in reducing disease severity under greenhouse conditions. Field evaluations need to be conducted before this product can be registered as an effective biological control agent against *P. pachyrhizi*.

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CHAPTER SEVEN

GENERAL OVERVIEW

In Africa, soybean cultivation has increased in the last four decades from 72 000 tonnes on 191 000ha in 1961 to 989 000 tonnes on 1 090 000ha in 2002. However, it accounts for only 0.5% of the annual global production of 179 917000 tonnes (Singh *et al.*, 2004). In South Africa (SA) soybeans are a strategically important crop and are grown under natural rainfall and irrigated conditions, usually in summer rainfall areas (Bell *et al.*, 1990). In KwaZulu-Natal (KZN) SA approximately 30 000-35 000ha of soybeans are grown annually. Due to an ever-increasing demand for soybeans, expansion of soybean production is still possible in the northern and midland areas of KZN (Ward, 2003).

Soybeans are affected by more than 100 pathogens, with approximately 35 of economic importance (Earthington *et al.*, 1993). All parts of the soybean plant are susceptible to numerous pathogens, resulting in a reduction in quality and quantity of seed yields (Sinclair and Backman, 1989). *Phakopsora pachyrhizi* Sydow, the causal organism of soybean rust (SBR) is one of the major disease problems limiting soybean yield. Soybean rust is not unique as it mirrors outbreaks of other plant diseases like those of barley yellows, wheat stripe rust and karnal bunt.

The first report of the disease was from Japan in 1902 (Bromfield, 1984). By 1914, the pathogen appeared in numerous eastern countries, with an epidemic occurring in south-east Asia (Caldwell and Laing, 2002). By 1934, the pathogen was reported as far east as Australia (Miles *et al.*, 2003), but epidemic proportions were never reached (Caldwell and Laing, 2002). The first report of the disease in India was in 1951 (Miles *et al.*, 2003).

There have been several early reports of SBR in equatorial Africa (Javaid and Ashraf, 1978; Bromfield, 1980), but the first confirmed report of *P. pachyrhizi* on the African continent was in 1996 from Kenya, Rwanda, and Uganda (Levy *et al.*,

2000). Since then, the pathogen has spread south with reports from Zambia and Zimbabwe in 1998 and Mozambique in 2000 (Caldwell and Laing, 2002).

It is believed that the pathogen was wind-borne from Asia to Africa (Caldwell and Laing, 2002). In South Africa (SA) the first report of this disease was near Vryheid in Northern KwaZulu-Natal, (Pretorius *et al.*, 2001). As the season progressed, the disease was observed in other parts of the province, and epidemic levels were found in the Cedara, Greytown, Howick and Karkloof production regions. Soybean rust subsequently spread to Amsterdam and Ermelo in the Highveld region of SA (Caldwell and Laing, 2002). The disease reappeared in SA in March 2002. It is clear that the pathogen is now an established threat to soybean production in the country.

In South America the first report of *P. pachyrhizi* was from Paraguay in March 2001 (Morel *et al.*, 2004). It was subsequently reported in the state of Paranả, Brazil in 2001 (Yorinori, 2004). By 2002, SBR was widespread throughout Paraguay and in limited areas of Brazil bordering Paraguay, with reports of severe disease in some fields in both countries (Morel and Yorinori, 2002). The pathogen also was found in a limited area in northern Argentina (Rossi, 2003). In August 2004, the United States Department of Agriculture (USDA) and the Animal Plant Health Inspection Service (APHIS) confirmed a report of SBR in Colombia (Caspers-Simmet, 2004).

Soybean rust was not present in the United States until the USDA confirmed its presence in Louisiana in early November 2004. Within a matter of days the disease had been found in eight more states in the south (Anonymous a). The arrival of SBR in the US was not unexpected (Anonymous a). USDA researchers believe the fungus arrived from Brazil on the hurricane winds of September 2004. During October-November 2004 conditions became ideal for SBR spores. Wind conditions out of Colombia, South America, associated with the hurricane activity would have brought spores into the southern states of America in jet stream winds (Anonymous b). Yield loss of soybeans due to soybean rust in Brazil totalled some US$ 2 billion in 2003 (Wolfgang and Butzen, 2006).

The fungus attacks a broad range of host plants including kudzu, an invasive vine that covers much of South America and the southern states of the U.S. Kudzu vine could thus provide an overwintering source for the fungus to multiply and spread.

Efforts to control the disease around the world have focused on educating farmers to identify it accurately and early, thus enabling them to spray only when needed. Several fungicides can control *P. pachyrhizi* but they are expensive to apply and thus reduce profit (Wolfgang and Butzen, 2006). Even with the application of fungicides, the USDA states that loss due to SBR could be between US$ 240 million and US$ 2 billion a year (Wolfgang and Butzen, 2006).

Long term, the solution for control must be some form of genetic resistance. Enhanced diversity would almost certainly offer some protection against SBR, especially to farmers who cannot afford to spray with fungicides (Wolfgang and Butzen, 2006). Genetic resistance has not yet been found, but will almost certainly be present in existing soybean genebanks (Wolfgang and Butzen, 2006). Resistance will eventually be found among commercial varieties or wild relatives and will be bred into commercial varieties, saving the harvest and reducing the cost of plant protection (Anonymous a and Wolfgang and Butzen, 2006).

Apart from genetic breeding, researchers are trying to implement disease models. The disease model will capture the dynamic nature of SBR which will provide disease warning to guide fungicide applications (Yang, 2004).

Current disease prediction models are computer-based to cope with large amounts of data collection, analysis and prediction. Disease prediction models have been developed for soybean rust from results collected in China and Taiwan during the 1990s (Yang, 2004). Two types of prediction models have been developed:

1. Simulation models - these models require daily input of weather information for accurate disease prediction
2. Neutral network models - this type of model also requires daily input of weather information

The establishment of a disease prediction model will allow for correct timing of application of fungicides thus improving yields and profit margins for farmers as well as optimizing labour usage.

Much of the research in this thesis focused on the epidemiology of this pathogen, determining the sustainability of uredospores when exposed to ultraviolet light, determining possible alternative hosts of SBR in SA, finding the correlation between susceptibility and growth stage of the soybean plant and to determine if *Trichoderma harzianum* Rifai, Eco-77®, a commercial biological control product is effective in controlling SBR. The research has confirmed the following:

1. *Phakopsora pachyrhizi* requires temperatures between 21-24°C with a leaf wetness duration greater than 12h and RH 85%-95% for infection to occur.
2. Temperatures > 26°C will result in a low infection percentage regardless of the leaf wetness duration and RH.
3. Exposure of uredospores to ultraviolet light has a negative effect on the germination percentage.
4. Seven legume plants [*Cajanus cajan* (L.) Huth, *Glycine max* (L.) Merr, *Lablab purpureus* (L.) Sweet, *Lupinus angustifolius* (L.) Finnish, *Phaseolus vulgaris* (L.), *Pueraria lobata* (M&S) Willd and *Vigna unguiculata* (L.) Walp] and three dry bean lines (Bonus; OPS-RS2 and PAN 159) showed typical, SBR symptoms. When inoculated with uredospores from pustules on *G. max*, *L. purpureus*, *L. angustifolius*, *P. vulgaris, P. lobata, V. unguiculata*, Bonus and PAN 159 produced viable uredospores on PRIMA 2000. These plants are considered alternative hosts of *P. pachyrhizi.*
5. Soybean plants at the V6 and R1 growth stages were significantly more susceptible to *P. pachyrhizi* than plants at other developmental stages.
6. *Trichoderma harzianium* sprayed at the standard concentration on infected soybean plants was significantly more effective in controlling *P. pachyrhizi* than plants sprayed at 1/2X and 2X the standard concentration.
7. *Trichoderma harzianium* sprayed in the form of a filtrate two days after inoculation with *P. pachyrhizi* resulted in a decrease in disease severity compared to the filtrate sprayed two days before inoculation with *P. pachyrhizi.*

Data from the epidemiology and exposure of uredospores to ultraviolet light will aid in a future disease prediction model. Simulation models can be developed to determine the effect of SBR on soybean yield; but these models are based on the assumption that rust uredospores are available early in a growing season. The earlier the disease occurs in a soybean field, the greater the potential impact on yield (Pivonia and Yang, 2006).

Assessment of epidemiological factors (e.g. infection efficiency, latent period, spore production and infection period) are needed for the prediction of epidemic progress (Pivonia and Yang, 2006). Measured disease data may contain considerable errors even under well-designed and executed experiments (Kim *et al.*, 2005). Fuzzy logic can be applied to develop a model without relying on precise and quantitative measurements (Kim *et al.*, 2005). Fuzzy logic is an extension of classic logic. Classic logic deals with exact logical reasoning using binary values whereas fuzzy logic handles imprecise representation of knowledge using a degree of truth (Kim *et al.*, 2005).

When environmental conditions are favourable for airborne disease development on a susceptible host, a delay in disease onset is probably due to the lack of inoculum. This means that spore concentration is below a threshold for disease onset. The threshold level should not be a constant value but a variable depending on environmental conditions and plant growth stage. Future studies needs to be conducted to include more factors into a disease stimulation model might reduce difference between model predictions and actual disease onset (Pivonia and Yang, 2006).

Despite its simplicity the general disease model should provide a tool to compare suitability of environmental conditions to the development of rust in time over soybean production regions in SA.

More extensive research needs to be conducted on the alternative hosts present in SA. The present study laid the foundation as to determine which alternative plants may be possible hosts. Results obtained in this work were from controlled environmental studies therefore field evaluations need to be conducted as different

plants could react differently under different environmental conditions. These alternative hosts will indicate the inoculum source of *P. pachyrhizi* in SA.

Field trials need to be conducted to determine if SBR can be controlled by Eco-77®. More isolation and intensive screening methods needs to be conducted on other possible biological control agents. This screening will be undergone in a greenhouse and then taken out into the field. A combination of these biological agents as well as adjuvants needs to be screened onto infected soybean plants.

7.1 Research Conducted, But Not Yet Reported

Trials that have been conducted at the University of KwaZulu-Natal, but not yet reported include:

- Application of potassium silicate to control SBR. A 50-60% reduction in SBR was found in these initial pot trials.

7.2 Proposed Future Research Priorities

- Results from the epidemiology and exposure to ultraviolet light will be used to develop a disease prediction model.
- The alternative host study has provided some information on the alternative hosts used by *P. pachyrhizi* in SA. This will help in determining the source of inoculum during the winter months and will enable one to determine which province is a high inoculum carrier and source of *P. pachyrhizi*. Field results and molecular techniques need to be conducted to determine more alternative hosts present in SA.
- Data from the biological control trial has shown some potential. Therefore more greenhouse and field trials need to be conducted to evaluate the efficacy of this biological control agent. Greenhouse and field trials using integrating cultural practices, biocontrol agents, resistant cultivars and chemical fungicides are necessary.
- Breeding lines for tolerance against SBR

- Mapping disease incidence.

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APPENDIX 1

Appendix 1a

Number of pustules per lesion on the abaxial leaf surface produced at different temperatures and leaf wetness durations at 75%, 85% and 95%RH

	75% RH					85% RH					95% RH				
	Leaf wetness duration (h)					Leaf wetness duration (h)					Leaf wetness duration (h)				
	6	9	12	14	16	6	9	12	14	16	6	9	12	14	16
Temperature (°C)															
15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
19	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	3^{c}	2^{bc}	3^{c}	5^{de}	7^{f}	1^{a}	1^{a}	3^{bc}	4^{c}	4^{c}
21	3^{bc}	4^{c}	5^{de}	5^{de}	6^{e}	3^{c}	6^{e}	9^{g}	11^{i}	13^{ji}	4^{c}	5^{d}	7^{e}	9^{g}	11^{i}
24	3^{bc}	5^{de}	6^{ef}	7^{f}	7^{f}	6^{e}	6^{e}	9^{g}	12^{i}	17^{m}	3^{bc}	4^{c}	8^{e}	10^{h}	14^{k}
26	2^{b}	3^{bc}	3^{bc}	4^{c}	5^{de}	2^{bc}	3^{c}	3^{c}	4^{d}	5^{de}	2^{b}	3^{bc}	3^{bc}	4^{c}	4^{c}
28	1^{a}	2^{b}	2^{b}	3^{bc}	3^{bc}	1^{ab}	2^{bc}	2^{bc}	3^{c}	3^{c}	1^{a}	1^{a}	2^{b}	3^{bc}	3^{bc}
30	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
F test			<0.001					<0.001					<0.001		
l.s.d.			1.3713					1.3713					1.3713		
s.e.d.			0.6956					0.6956					0.6956		
cv%			27					27					27		

Means with the same letter are not significantly different at P<0.005.

Appendix 1b

adaxial

Lesion sizes (mm) on the abaxial leaf surface produced at different temperatures and leaf wetness durations at 75%, 85% and 95% RH

	75% RH					85% RH					95% RH				
	Leaf wetness duration (h)					Leaf wetness duration (h)					Leaf wetness duration (h)				
	6	9	12	14	16	6	9	12	14	16	6	9	12	14	16
Temperature (°C)															
15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
19	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	2^{a}	4^{bc}	7^{c}	8^{d}	9^{d}	3^{b}	5^{c}	7^{c}	8^{d}	9^{d}
21	1^{a}	2^{ab}	3^{b}	4^{bc}	5^{c}	1^{a}	6^{c}	7^{c}	10^{e}	12^{i}	5^{c}	7^{c}	8^{d}	10^{e}	10^{e}
24	1^{a}	2^{ab}	4^{bc}	5^{c}	6^{c}	1^{a}	7^{c}	9^{d}	10^{e}	10^{e}	6^{c}	8^{c}	9^{d}	9^{d}	12^{i}
26	2^{ab}	3^{b}	3^{b}	4^{bc}	5^{c}	2^{a}	2^{a}	3^{b}	4^{bc}	5^{c}	2^{a}	3^{b}	3^{b}	3^{b}	5^{c}
28	0^{a}	1^{a}	1^{a}	2^{ab}	3^{b}	0^{a}	2^{a}	2^{a}	3^{b}	4^{bc}	1^{a}	2^{a}	2^{a}	3^{b}	5^{c}
30	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0.0^{a}	0^{a}	0^{a}
F test	<0.001					<0.001					<0.001				
l.s.d.	0.023974					0.023974					0.023974				
s.e.d.	0.012161					0.012161					0.012161				
cv%	34.9					34.9					34.9				

Means with the same letter are not significantly different at P<0.005

Appendix 1c

Lesion size (mm) on the adaxial leaf surface produced at different temperatures and leaf wetness durations at 75%, 85% and 95% RH

Temperature (°C)	75% RH Leaf wetness duration (h)					85% RH Leaf wetness duration (h)					95 RH Leaf wetness duration (h)				
	6	9	12	14	16	6	9	12	14	16	6	9	12	14	16
15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
19	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	1^{a}	2^{a}	5^{a}	7^{a}	9^{ba}	1^{a}	3^{a}	5^{a}	8^{b}	10^{b}
21	1^{a}	2^{a}	3^{a}	4^{a}	5^{a}	3^{a}	6^{a}	12^{b}	14^{f}	17^{j}	2^{a}	4^{a}	10^{b}	15^{g}	17^{i}
24	3^{a}	4^{a}	6^{a}	7^{a}	7^{a}	4^{a}	5^{a}	7^{a}	13^{e}	14^{f}	3^{a}	3^{a}	10^{b}	14^{f}	15^{g}
26	2^{a}	2^{a}	2^{a}	2^{a}	4^{a}	1^{a}	2^{a}	2^{a}	3^{a}	3^{a}	2^{a}	2^{a}	3^{a}	3^{a}	3^{a}
28	0^{a}	0^{a}	1^{a}	2^{a}	3^{a}	0^{a}	0^{a}	2^{a}	3^{a}	4^{a}	0^{a}	1^{a}	2^{a}	3^{a}	4^{a}
30	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
F test	<0.001					<0.001					<0.001				
l.s.d.	0.07979					0.07979					0.07979				
s.e.d.	0.04048					0.04048					0.04048				
cv%	18.3					18.3					18.3				

Means with the same letter are not significantly different at P<0.005

APPENDIX 2

Appendix 2a

Effect of ultraviolet light (<280nm) on uredospore germination and germ tube length

Germination (%)			Germ tube length (µm)		
Time (h)	**Light**	**Dark**	**Time (h)**	**Light**	**Dark**
6	65^{gh}	$58)^{i}$	6	172^{i}	169^{i}
9	50^{ef}	54^{fg}	9	163^{h}	177^{j}
12	46^{e}	60^{hi}	12	155^{g}	182^{k}
14	35^{d}	63^{hi}	14	151^{f}	186^{lm}
16	26^{c}	77^{j}	16	145^{e}	193^{n}
20	22^{c}	62^{hi}	20	138^{d}	190^{n}
24	13^{b}	60^{hi}	24	132^{c}	188^{mn}
36	11^{ab}	60^{hi}	36	119^{b}	185^{l}
48	7^{a}	58^{gh}	48	107^{a}	181^{k}
F test	(<0.001)	(<0.001	F test	(<0.001)	(<0.001)
l.s.d.	5.767		l.s.d	3.155	
s.e.d.	2.844		s.e.d	1.556	
cv%	7.5		cv%	1.2	

Means with the same letter are not significantly different at P<0.005

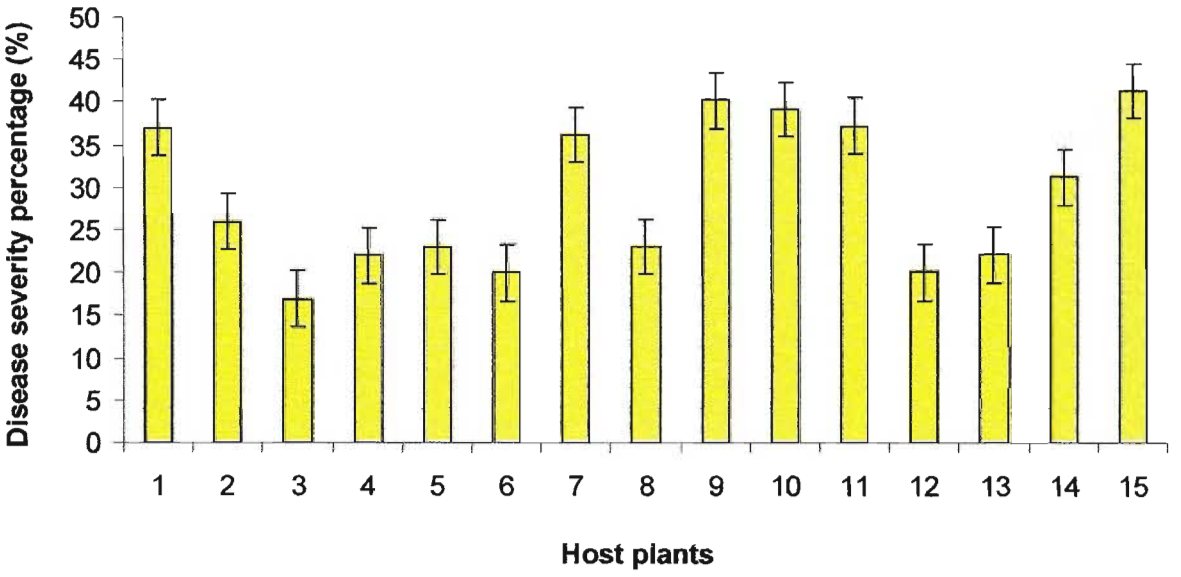
Appendix 2b

Uredospore germination of *Phakopsora pachyrhizi* as affected by cycles of ultraviolet light and darkness

Time (h)	Germination (%)
14h Light	39[a]
10h Darkness	55[dc]
14h Light	39[a]
10h Darkness	51[c]
F test	(<0.001)
l.s.d	4.939
s.e.d	2.018
cv%	5.4

Means with the same letter are not significantly different at P<0.005

APPENDIX 3



Disease severity percentages obtained when host plants were inoculated with soybean rust uredospores. Bars represent the standard deviation of the treatment mean of pooled data.

1 = Lupin (Cedara cultivar)
2 = Cowpea
3 = Pigeon pea MN5
4 = Pigeon pea ICPL 85010
5 = Pigeon pea ICPL 87
6 = Pigeon pea
7 = Lablab
8 = Lupin
9 = Vegetable soybean
10 = Dry beans
11 = Kudzu vine
12 = Bonus (Dry bean lines from Agricultural Research Council)
13 = OPS-RS 2 (Dry bean lines from Agricultural Research Council)
14 = PAN 159 (Dry bean lines from Agricultural Research Council)
15 = PRIMA 2000 (Control soybean plants)

APPENDIX 4

Appendix 4a

Effect of plant developmental stage on number of lesions produced by *Phakopsora pachyrhizi* over a 21 day period

Developmental stages	Number of lesions Time (dpi)			
	8	**12**	**16**	**20**
V1	3^{a}	6^{bc}	9^{cd}	13^{ef}
V3	5^{ab}	6^{bc}	11^{d}	16^{g}
V6	7^{c}	11^{d}	15^{fg}	22^{ij}
R1	7^{c}	13^{ef}	15^{fg}	17^{j}
R3	5^{ab}	8^{c}	11^{d}	15^{fg}
R6	5^{ab}	8^{c}	11^{d}	16^{g}
F test	(<0.001)			
l.s.d	2.575			
s.e.d	1.371			
cv%	15.4			

Means with the same letter are not significantly different at P<0.005

Appendix 4b

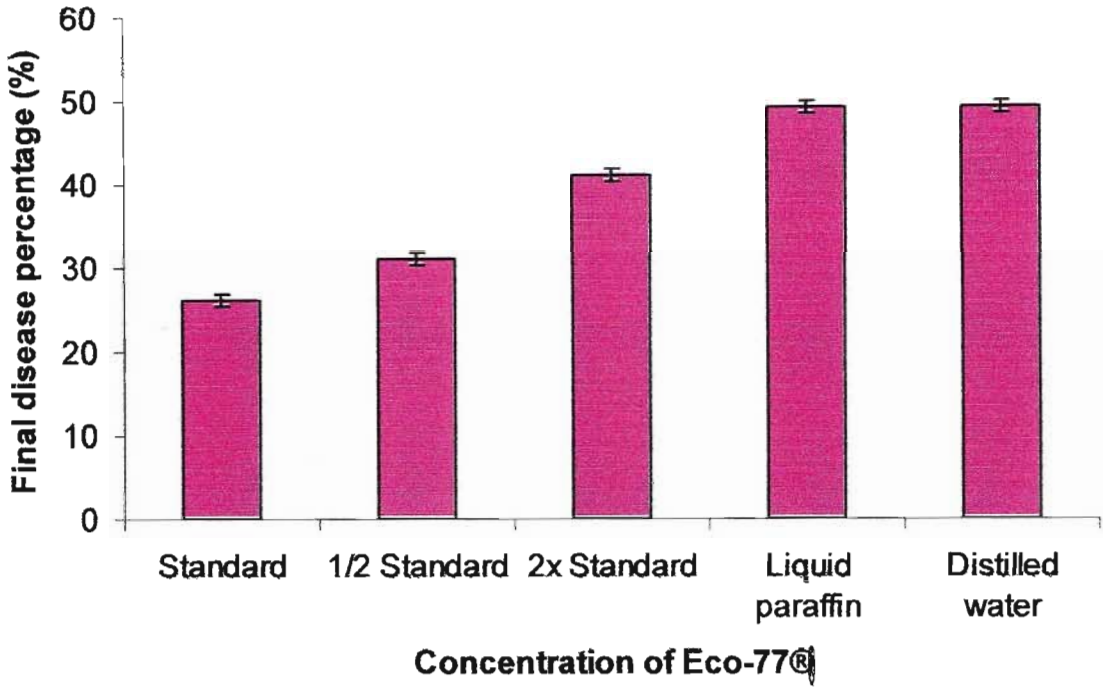
Effect of plant developmental stage on lesion size (mm) produced by *Phakopsora pachyrhizi* over a 21 day period

Developmental stages	Lesion size (mm) Time (dpi)			
	8	**12**	**16**	**20**
V1	1^{a}	1.4^{a}	2^{a}	7.5^{b}
V3	4^{a}	7^{b}	12^{ba}	20^{e}
V6	6^{a}	15^{ba}	17^{de}	20^{e}
R1	8^{b}	13^{ba}	14^{b}	15^{ba}
R3	6^{a}	13^{ba}	16^{d}	20^{e}
R6	6^{a}	17^{de}	20^{e}	20^{e}
F test	(<0.001)			
l.s.d	0.010824			
s.e.d	0.005383			
cv%	8.6			

Means with the same letter are not significantly different at P<0.005

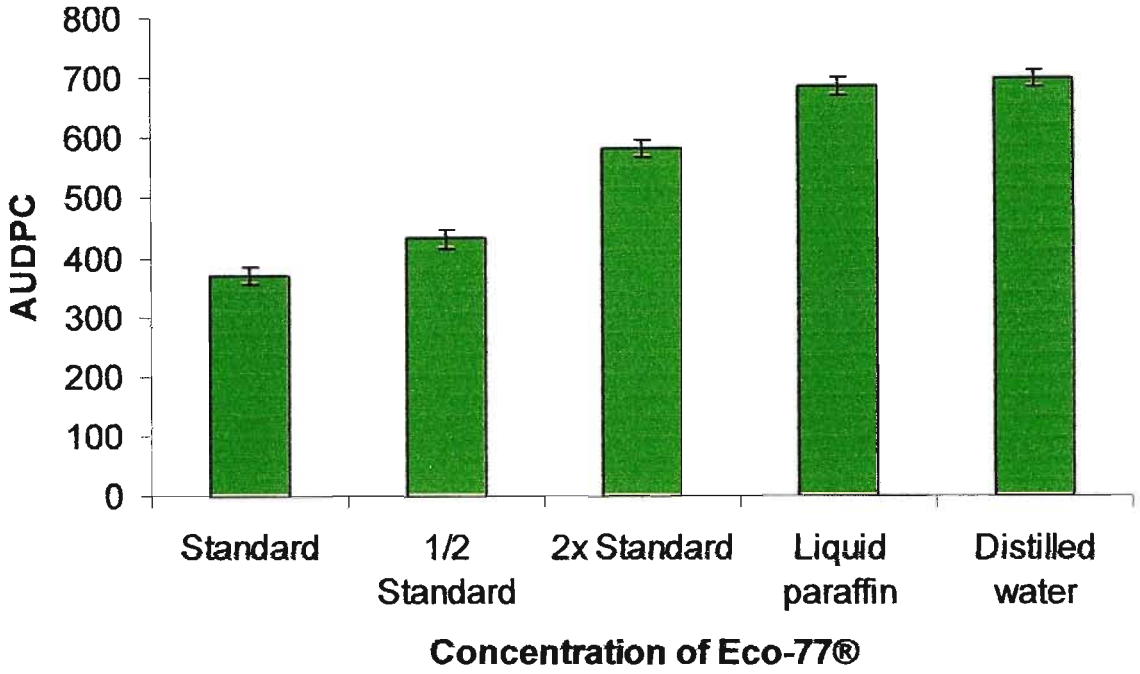
APPENDIX 5

Appendix 5a



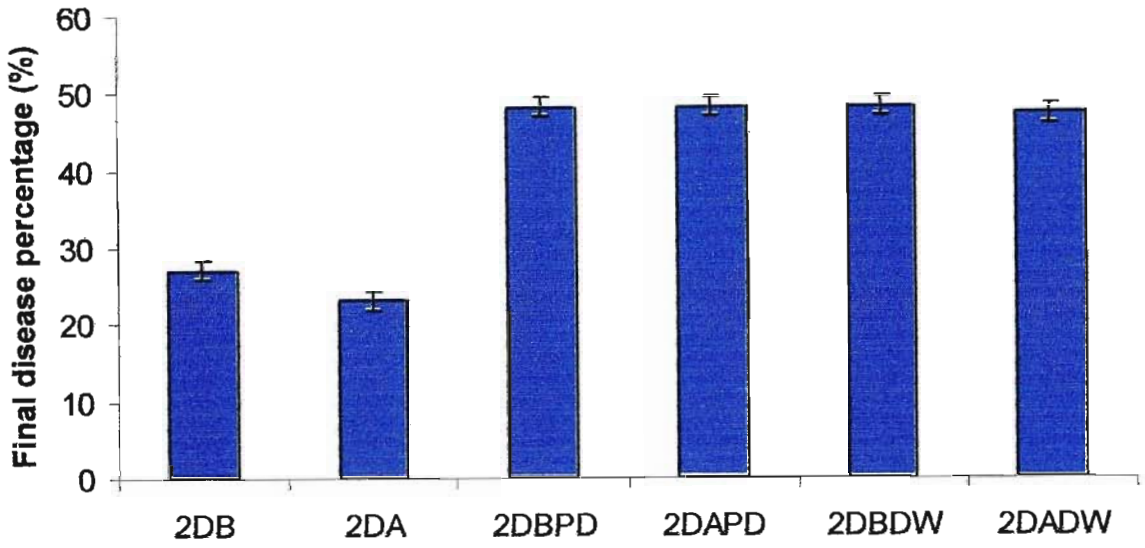
Final percentage disease severity of plants inoculated with uredospores of *Phakopsora pachyrhizi* and sprayed with different concentrations of Eco-77®. Bars represent the standard deviation of the treatment mean of pooled data.

Appendix 5b



Area under disease progress curve of plants inoculated with uredospores of *Phakopsora pachyrhizi* and sprayed with different concentrations of Eco-77®. Bars represent the standard deviation of the treatment mean of pooled data.

Appendix 5c



Intervals at which Eco-77® filtrate was sprayed

Final percentage disease severity of plants inoculated with uredospores of *Phakopsora pachyrhizi* and sprayed with Eco-77 ® filtrate at different times. Bars represent the standard deviation of the treatment mean of pooled data.

2DB = Sprayed with Eco-77® filtrate 2 days before inoculation

2DA = Sprayed with Eco-77® filtrate 2 days after inoculation

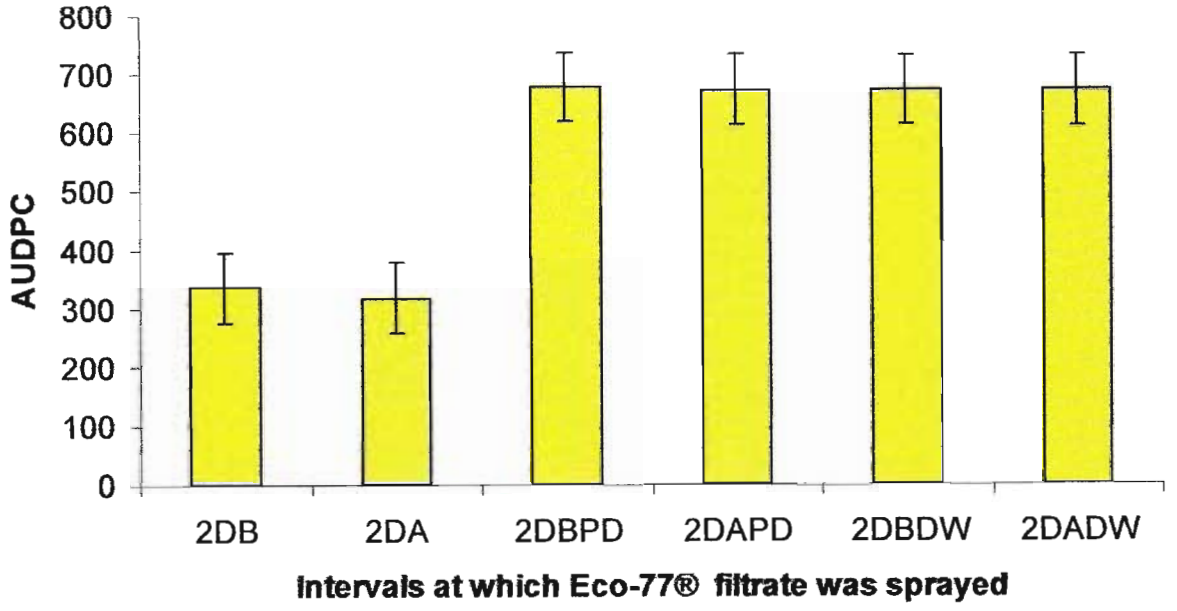
2DBPD = Sprayed with potato dextrose broth 2 days before inoculation

2DAPD = Sprayed with potato dextrose broth 2 days after inoculation

2DBDW = Sprayed with distilled water 2 days before inoculation

2DADW = Sprayed with distilled water 2 days after inoculation

Appendix 5d



Area under disease progress curve of plants inoculated with uredospores of *Phakopsora pachyrhizi* and sprayed with Eco-77 ® filtrate at different times. Bars represent the standard deviation of the treatment mean of pooled data.

2DB = Sprayed with Eco-77® filtrate 2 days before inoculation

2DA = Sprayed with Eco-77® filtrate 2 days after inoculation

2DBPD = Sprayed with potato dextrose broth 2 days before inoculation

2DAPD = Sprayed with potato dextrose broth 2 days after inoculation

2DBDW = Sprayed with distilled water 2 days before inoculation

2DADW = Sprayed with distilled water 2 days after inoculation

MICROSCOPY STUDIES OF *Phakopsora pachyrhizi* AND *Sclerotinia sclerotiorum*: TWO IMPORTANT YIELD LIMITING SOYBEAN DISEASES

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Soybeans, *Glycine max* (L.) Merrill. are a major source of vegetable oil and protein in the world[1]. Consumption trends in SA for soybean derived commodities far exceeds production trends, resulting in an annual import of 600 000-800 000 tonnes (almost $200 million) of oilcake in order to meet local demands[2]. Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum* (Lib.) de Bary has recently emerged from a previously minor pathogen of soybeans in SA, to a major pathogen, causing significant yield losses (0-100%)[3] particularly in the wetter production areas. Soybean rust (SBR) caused by *Phakopsora pachyrhizi* H. & P. Sydow was first identified in SA in 2001, causing yield losses of 10-60%. Together these pathogens threaten the viability of soybean production. Light and environmental scanning electron microscopy (ESEM) were used to study these two pathogens.

ESEM revealed that 5-day-old sclerotia of SSR consisted of a mass of interwoven mycelial strands. As sclerotia continued to develop, subsurface mycelial cells swelled to form bulbous rind cells, which darkened with age. Initially rind cells were rough in appearance, due to membranous material appressed to the rind cell surface, and later became smooth as sclerotia matured. *In vitro* dual culture bioassays of both hyphae and sclerotia of SSR were performed to identify possible bio-control mechanisms of EcoT® and Eco77®. ESEM studies showed that hyphae of EcoT® and Eco77® coiled around hyphae of SSR (Fig. 1), i.e. mycoparasitism occurred. ESEM also showed that both biocontrol agents colonized sclerotial surfaces by forming dense, branched mycelia in a thin mucilage, causing the sclerotia to disintegrate.

ESEM confirmed optimum conditions necessary for germination and illustrated the infection process of *P. pachyrhizi*. Uredospores were found to germinate on the leaf surface to form a short germ tube that terminated in the formation of an appressorium. The majority of germlings developed appressoria at the junction between epidermal cells. Appressoria were often sessile to the parent uredospore. Under optimal conditions, (21-24°C and 85% RH), germination commenced 6 hrs post inoculation (hpi), while appressoria formed 6-10 hpi. Results indicated that a minimum of 12 hrs leaf wetness at optimum temperature and relative humidity were required for penetration to occur. The pathogen was shown to penetrate the host leaf directly, through the cuticle (Fig. 2), as opposed to the more conventional stomatal penetration employed by many rust fungi in the uredial stage.

References
1. Singh, B.B. *et al.* (2004) Proc. of the VII World Soybean Res. Conf. Iguaçu Falls, Brazil, 29 Jan–05 Mar 2004, 56.
2. Joubert, J.S.G. (2004) http://www.proteinresearch.net/?dirname=html_docs/-550-protein%20 statistics.
3. Purdy, L.H. (1979) Phytopathology. 69, 875.

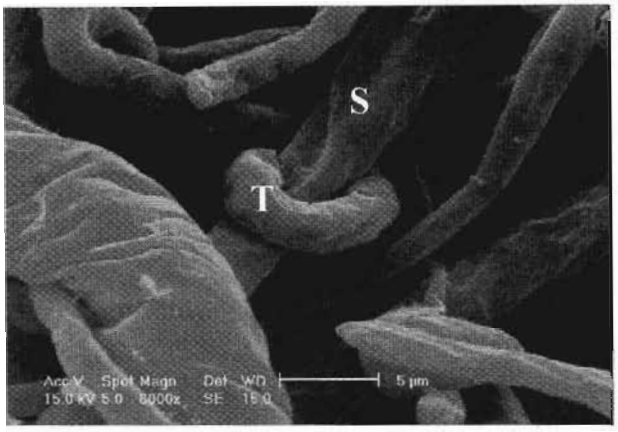

Figure 1. Mycoparasitism of *Sclerotinia sclerotiorum* hyphae (S) by hyphae of *Trichoderma* (T).

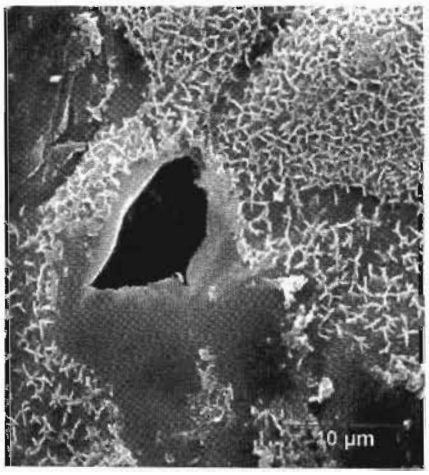

Figure 2. Penetration hole of *Phakopsora pachyrhizi* through soybean epidermal cells.

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