STUDIES ON POWDERY SCAB ON POTATO IN SOUTH AFRICA

by

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DECLARATION

I, Jessica Wright, declare that the thesis, which I hereby submit for the degree Magister Scientiae (Agriculture) Plant Pathology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: _____

Date: _____

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Studies on powdery scab on potatoes in South Africa

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Abstract

Spongospora subterranea (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson (*Sss*) is the causal organism of potato powdery scab. This blemish disease is of economic concern to fresh and seed-tuber producers around the world, as it diminishes both tuber quality and marketability.

In this study, techniques for detecting and quantifying *Sss* were evaluated. Comparison of enzyme linked immunosorbent assay (ELISA), conventional PCR and real-time PCR showed that conventional PCR is more sensitive than ELISA, as conventional PCR detected *Sss* inoculum from both tuber and soil samples whereas ELISA only detected *Sss* inoculum from tuber samples. Real-time PCR not only detected *Sss* DNA in a variety of sample types, but real-time PCR could also be used to quantify *Sss* DNA. The high sensitivity of real-time PCR gave consistent detection of standard DNA quantities ranging from 10 000 to as few as 1 sporeballs per ml. The benefit of real-time PCR is that it can be used for the study of the different life stages of *Sss* in a range of sample types.

During 2008 to 2010 powdery scab samples were collected from Ceres, KwaZulu Natal, Mpumalanga and Sandveld and herbarium samples dated 1936 from the Sandveld were also obtained to investigate genetic variation in ITS1/2 sequences. Comparisons to known *Sss*

Group Type I/II sequences were made to determine the specific Group Type/s found in South Africa. All these South African samples were identified as belonging to *Sss* Group Type II.

A bioassay was developed to investigate the dormancy period of *Sss*. The results showed that *Sss* zoospores are released four days after inoculation and infection of tomato roots occurs six days after inoculation. Plasmodia in tomato root hairs develop eight days after inoculation, followed by zoosporangia development by day 12.

Several strategies are being used to minimize the risk of powdery scab as there is no single effective method for controlling the disease. One such strategy includes cultivar resistance. Cultivar susceptibility of six South African cultivars (Argos, BP1, Buffelspoort, Caren, Up-to-Date and Valor) was evaluated. Pot trials showed that all the evaluated cultivars are susceptible to infection by *Sss* and that there is a positive correlation between the severity of root galls and of tuber lesions.

Various crops (cabbage, mustard, soybean and tomato) were assessed to determine the host range of *Sss* in rotation crops in South African potato growing regions and to identify possible trap crops for *Sss*. Of the crops evaluated, the pathogen was unable to complete its life cycle in wheat and cabbage, whereas soybean is a non-host crop. Both mustard and tomato are hosts of *Sss*.

A severe outbreak of powdery scab occurred during 2006 in a potato mini-tuber production facility in Ceres, Western Cape, South Africa. A study was conducted in the production facility to detect *Sss* and to identify the source/s of contamination, so that corrective measures could be taken to eradicate the pathogen. Swab samples specified areas in the production facilities that were infested with *Sss* sporeballs. Following eradication efforts to improve facility hygiene, a second set of swab samples was taken in 2009 to determine the efficacy of the eradication methods. From 2009 onwards, disease-free mini-tubers have been harvested.

This comprehensive study will lead to a better understanding of *Spongospora subterranea* f.sp. *subterranea* and has emphasised the need for further research which will focus on strategies that will help to reduce the impact that powdery scab has on potato production, not only in South Africa, but globally too.

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CHAPTER 1
General Introduction

Spongospora subterranea (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson (*Sss*), which belongs to the plasmodiophorids (Braselton, 1995) is the causal organism of potato powdery scab. This blemish disease was initially described in Germany in 1841 (Morse, 1913) and is now a major economical concern for fresh and seed-tuber producers around the world, as it diminishes both tuber quality and marketability.

The disfiguring lesions that form on tubers contain masses brown of resting structures known as sporeballs or cystosori. These structures survive in the soil for many years, even in the absence of host plants. Under favourable environmental conditions, high soil moisture and cool soil temperatures, the sporeballs release primary zoospores and infect the potato host tissue. Once the primary zoospores enter the host's tissues, plasmodia form in the root hairs and then develop into zoosporangia, which release secondary zoospores when mature (van de Graaf *et al.*, 2003). Powdery scab infections can arise from inoculum that is seed-borne or soil-borne (De Boer *et al.*, 1982; Letham *et al.*, 1988).

There are a number of methods for detecting and quantifying *Sss* including enzyme linked immunosorbent assay (ELISA), conventional PCR and real-time PCR. Comparison of the three techniques showed that conventional PCR was more sensitive than ELISA at detecting *Sss* inoculum from both tuber and soil samples. Real-time PCR, however not only detected *Sss* DNA in a variety of sample types, but was also able to quantify *Sss* DNA (Chapter 3).

During 2008 to 2010 a number of samples were collected from four potato growing regions in South Africa (Ceres, KwaZulu Natal, Mpumalanga and Sandveld) and two powdery scab samples dated 1936 from the Sandveld were also obtained. All these samples were sequenced and identified as *Sss* Group Type II (Chapter 4).

Factors that control dormancy and germination are currently unknown. Thus a bioassay was developed to investigate dormancy period of *Sss*, which indicated that zoospores are released four days post inoculation and root infection occurs six days after inoculation. Plasmodia and zoosporangia development require 12 and 14 days, respectively from the time of inoculation (Chapter 5).

The only reliable control of powdery scab disease is to plant disease-free seeds in uncontaminated fields (Harrison *et al.*, 1993; Walsh *et al.*, 1996; Genet *et al.*, 2005; Merz, 2008), yet several strategies are being used to minimize the risk of powdery scab disease as there is no single effective method for controlling powdery scab (Merz, 1993; Iftikhar *et al.*, 2007). The different strategies include cultivar resistance, cultural practices, disease-free seed or mini-tubers, fungicides, antagonists and legislation (Gudmestad *et al.*, 2007; Falloon, 2008).

There is very little information regarding the resistance of South African potato cultivars to powdery scab. The cultivar susceptibility pot trial indicated that none of the tested cultivars (Argos, BP1, Buffelspoort, Caren, Up-to-Date (UTD) and Valor) are immune to infection by the pathogen. There were no significant differences in disease incidence and severity of powdery scab (tuber lesions and root galling) between the cultivars (Chapter 6).

In the absence of effective control measures, integrated crop management with long crop rotations that include trap or decoy crops may be a feasible control method for powdery scab (Elad *et al.*, 1980; Thurston, 1990; Honeycutt *et al.*, 1996; Katan, 2000). Thus, it is important to determine the host range of *Sss* in rotation crops in South African potato growing regions and identify possible trap crops for *Sss*. Of the crops evaluated, *Sss* was unable to complete its life cycle in wheat and cabbage indicating that these hosts might help in reducing *Sss* inoculum in the soil (Chapter 7).

Infected mini-tubers play an important part in the dissemination of *Sss*, transmitting it to areas where the disease was not previously present. Mini-tubers infected with the *Sss* are commercially unacceptable and are not recommended for the production of new crops (van de Graaf *et al.*, 2005). During 2006, a potato mini-tuber production facility in Ceres, Western Cape, South Africa had a severe outbreak of powdery scab in one of their production tunnels. A study was done in the production facility to detect *Sss* and to identify the source/s of contamination, so that corrective measures could be taken to eradicate the pathogen. This was the first study in which swabs were used for the detection of *Sss* (Chapter 8).

The objective of this study was to understand the various techniques used to detect and quantify *Sss*, so that various aspects such as host range, cultivar resistance and pathogen studies could be carried out. This will lead to a better understanding of *Spongospora*

subterranea f.sp. *subterranea*, so that the negative effects that it poses, both environmentally and economically can be reduced and managed effectively.

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CHAPTER 2

A Review of Powdery Scab on Potatoes

Abstract

Spongospora subterranea f.sp. *subterranea* (*Sss*) is the causal agent of powdery scab of potatoes. This disease causes a reduction in both tuber quality and marketability. Powdery scab lesions contain sporeballs that have the ability to survive in the soil for many years. Under cool, moist environmental conditions, sporeballs will release zoospores, which infect the potato crop as well as a range of other host species. Seed-tubers and naturally infested soils both contribute to the level of inoculum in a potato field. Techniques used to detect and quantify *Sss* inoculum include bioassays, ELISA, conventional PCR and real-time PCR. Conventional PCR can detect as few as one sporeball per gram of soil. There are a number of control practices for powdery scab, yet none is singularly effective thus, an integrated management approach should be used to manage this disease. Therefore, the ability to detect and quantify the inoculum within the field as well as identify the conditions under which powdery scab develops, will allow for more accurate risk assessments, which ultimately will assist the growers to make more informed management decisions.

2.1 Introduction

Powdery scab of potato (*Solanum tuberosum* L.) is caused by the pathogen *Spongospora subterranea* (Wallroth) Lagerheim f.sp. *subterranea* Tomlinson (*Sss*), which belongs to the plasmodiophorids (Braselton, 1995). Powdery scab was first described in Germany in 1841 (Morse, 1913) and is now a major problem for fresh and seed-tuber producers around the world, as it diminishes tuber quality and marketability (Wale, 2000). The occurrence of powdery scab and its severity varies from season to season as well as from field to field. The scabs that form on tubers contain masses of resting structures known as sporeballs or cystosori. These structures have the ability to survive in the soil for many years (Harrison *et al.,* 1997; Merz & Falloon, 2009). Under favourable environmental conditions of high moisture and cool temperatures, these sporeballs release primary zoospores, which infect the potato host plants (Hughes, 1980; Christ & Weidner, 1988). Once the primary zoospores enter the host's root tissues, plasmodia form in the root hairs and then develop into zoosporangia, which release secondary zoospores when mature (Merz, 1992). Powdery scab infections can arise from inoculum that is seed-borne and/or soil-borne.

To date, there are no effective control measures for powdery scab, which further elicits the need to study this obligate pathogen. Detecting and quantifying the obligate *Sss* from soils and seed-tubers has in previous years proved to be rather difficult (Bell *et al.,* 1999). Thus the development and implementation of reliable techniques that detect and quantify inoculum in both the field and seed-tubers will form an essential part of disease risk assessment, which will thus, allow growers to make more informed management decisions.

The aim of this literature study is to document the conditions which favour disease development and to identify techniques that are currently used for the detection and quantification of *Sss* from potato tubers, soils and water. Since there are no effective control measures for powdery scab of potatoes, growers need to consider the level of risk of the disease occurring in the fields. The ability to detect and quantify the inoculum from seed-tubers and from fields as well as to identify the environmental conditions that will promote disease development will assist in providing more precise risk assessments. Risk assessments will support decision making for powdery scab management strategies and may include one or more of the following: disease-free seed tubers, cultivar resistance, fungicides, cultural practices, antagonists and/or legislation.

2.2 Epidemiology of Potato Powdery Scab

Powdery scab was first described in local crops in Braunschweig, Germany in 1841 (Wallroth, 1842). Since then, this disease has been reported in many countries around the world (Figure 2.1). S*pongospora subterranea* f. sp. *subterranea* is thought to have originated from the Andes, where it spread to the rest of the world, presumably through trade and contaminated seed tubers (Cook, 1932; Jeger *et al.,* 1996; Harrison *et al.,* 1997). The increased use of certain agricultural practices has lead to the development of micro-climates that favour powdery scab (Jeger *et al.,* 1996; Walsh *et al.,* 1996; Harrison *et al.,* 1997; Bulman & Marshall, 1998; Nakayama *et al.,* 2007; Merz, 2008), thus leading to the current powdery scab situation worldwide.

Figure 2.1: Countries with a record of *Spongospora*. (●) Possible centre of origin of *Spongospora subterranea* f.sp. *subterranea*; (•) the disease powdery scab has been recorded; (▲) long history of powdery scab research; (■) powdery scab research started more recently; (○) potato mop top virus research (Scandinavia); (◘) centre of *Spongospora subterranea* f.sp. *nasturtii* research (Merz, 2008).

2.2.1 Pathogen

There are three species of *Spongospora* (*Spongospora subterranea* which is parasitic in Solanaceae, *Spongospora capanulae* which is parasitic in Campanula and *Spongospora cotulae* which is parasitic in Cotula) of which *Spongospora subterranea* is the only species of economic concern (Harrison *et al.*, 1997; Down *et al.*, 2002; Merz & Falloon, 2009). Powdery scab of potato is caused by the pathogen, *Spongospora subterranea* (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson, whereas crook root of watercress is caused by *Spongospora subterranea* f. sp. *nasturtii* (*Ssn*) (Tomlinson, 1958). The classification of *Spongospora* is complex as it was previously regarded as a distinct class of fungi, the Plasmodiophoromycetes, within the division Myxomycota (Clay & Walsh, 1997). Currently *Spongospora* species are considered to be protozoans, within the order *Plasmodiophoridae* (Braselton, 1995). *Spongospora subterranea* f.sp. *subterranea*; as well as *Plasmodiophora brassicae* Woronin, the cause of club root disease of brassicas; *Polymyxa* species, the vectors of several plant pathogens; and *Spongospora subterranea* f.sp. *nasturtii* Tomlinson, the cause of crook root disease of watercress, are considered as economically important members of the order *Plasmodiophorida* (Karling, 1968).

Both *Sss* and *Ssn* are important pathogens not only for the diseases that they cause but also

because they are vectors of the viruses, *Potato mop-top virus* and *Watercress leaf-spot virus* respectively (Cooper & Harrison, 1973; Walsh *et al.,* 1989). *Potato mop-top virus* (PMTV) vectored by *Sss*, leads to internal necrotic arc and fleck tuber systems, thus lowering the market potential of the tubers (Harrison *et al.,* 1993; Merz & Falloon, 2009). PMTV currently occurs in many of the potato producing regions around the world, including Northern Europe, Japan and Australia (Merz & Falloon, 2009).

2.2.2 Symptoms

Spongospora subterranea f.sp. *subterranea* causes powdery lesions on the surfaces of tubers, and thus the name powdery scab was derived for this disease (Falloon *et al.,* 1996; Harrison *et al.,* 1997; Falloon, 2008; Nitzan *et al.,* 2009). The lesions are scab-like in appearance and contain sporeballs (Harrison *et al.,* 1993; Montero-Astua *et al.,* 2005; Qu *et al.,* 2006). Sporeballs, also known as cystosori, are sponge-like and are between 19-85 µm in size (Bell *et al.,* 1999; Qu & Christ, 2006; van de Graaf *et al.,* 2007).

The initial visible symptom of powdery scab is the development of purple-brown pimple-like swellings at the rose end of the tubers (Cook, 1932; Christ & Weidner, 1988; Harrison *et al.,* 1997). The individual circular scab lesions can develop to approximately 10 mm in size and the shapes may become irregular when the lesions become large and merge together (Genet *et al.,* 2005; Harrison *et al.,* 1997). Mature lesions become hollow and filled with a powdery mass of sporeballs (Figure 2.2). Other symptoms may include galls and cankers which develop on the roots and tubers resulting in their gross deformities (Harrison *et al.,* 1997). Galls may also develop on potato stolons and roots becoming dark brown as they mature (Figure 2.3) (Walsh *et al.,* 1996; Harrison *et al.,* 1997; Bulman & Marshall, 1998; van de Graaf *et al.,* 2003).

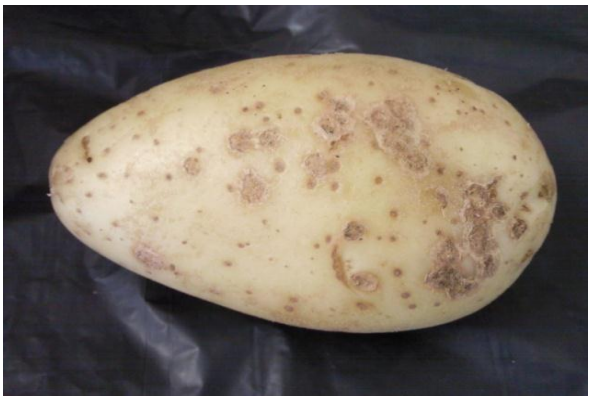

Figure 2.2: Powdery scab lesions on tubers (cultivar: Mondial).

Figure 2.3: Powdery scab galls on potato roots.

Powdery scab symptoms are often confused with the symptoms of common scab (*Streptomyces scabies* (Thaxt.) Waksman and Henrici) as they both cause quality limiting scab-like lesions on tubers (de Haan & van den Bovenkamp, 2005). Therefore it is necessary to correctly identify the causal pathogen (McCartney *et al.*, 2003). To differentiate between the two diseases, microscopic examination can be done, which relies on the experience and skill of the person making the diagnosis (McCartney *et al.*, 2003; de Haan & van den Bovenkamp, 2005). The presence or absence of sporeballs in tuber lesions indicates powdery scab or common scab respectively. Current methods such as ELISA or PCR require little skill and can be used to accurately identify powdery scab within a few days, even within a few hours (Harrison *et al.*, 1993; McCartney *et al.*, 2003; de Haan & van den Bovenkamp, 2005; Qu *et al.*, 2006).

Consumers as well as processors have high quality standards for tubers (Genet *et al.*, 2005; Brierley *et al.*, 2009). Powdery scab of potatoes is a blemish disease (Montero-Austua *et al.*, 2005); thus tubers with powdery scab lesions are most likely to be rejected by the fresh, processing or seed markets (Walsh *et al.*, 1996; Harrison *et al.*, 1997; van de Graaf *et al.*, 2007; Falloon, 2008; Merz, 2008). Severe and deep lesions will also have negative implications for processing (de Nazareno & Boschetto, 2002; Falloon *et al.*, 1996). Plant tissues infected with *Sss* have also been found to be more susceptible to other diseases, such as late blight (*Phytophthora infestans* (Mont.) de Bary), pink rot (*Phytopthora erythroseptica* Pethybr.) and dry rot (*Fusarium coeruleum* (Lib.) Sacc.) (Harrison *et al.*, 1997; Merz & Falloon, 2009).

Spongospora subterranea f.sp. *subterranea*, as previously stated, is the vector of *Potato mop-*

top virus (PMTV) (Calvert & Harrison, 1966). Infection with this virus leads to the poor growth and development of the tubers, as well as spraing symptoms (Harrison *et al.*, 1997), which in turn, reduces the marketability (Merz, 2008).

2.2.3 Life Cycle

Spongospora subterranea f.sp. *subterranea* is able to survive for up to 18 years as resting spores (de Haan & van den Bovenkamp, 2005) and these spores are so resistant that they can survive the passage through the gastrointestinal tract of animals (Harrison *et al.*, 1997; Merz & Falloon, 2009). Individual resting spores, 4 µm in diameter, are found clumped together within the sporeballs (Harrison *et al.*, 1997; Merz, 1997). When environmental conditions are favourable (high soil moisture and low soil temperature) (van de Graaf *et al.*, 2003), and only in the presence of free water, the resting spores germinate and release a single uninucleate zoospore (primary zoospores) (Figure 2.4). The length of time (approximately two hours) that the primary zoospores will swim before they infect host tissue is dependent on the free water in the soil and the temperature (Merz, 1992; Wallace *et al.*, 1995; Qu & Christ, 2006; Merz, 2008). According to Merz & Falloon (2009) zoospore release occurs at five to 25˚C and zoospores are most active at temperatures of 12-13˚C.

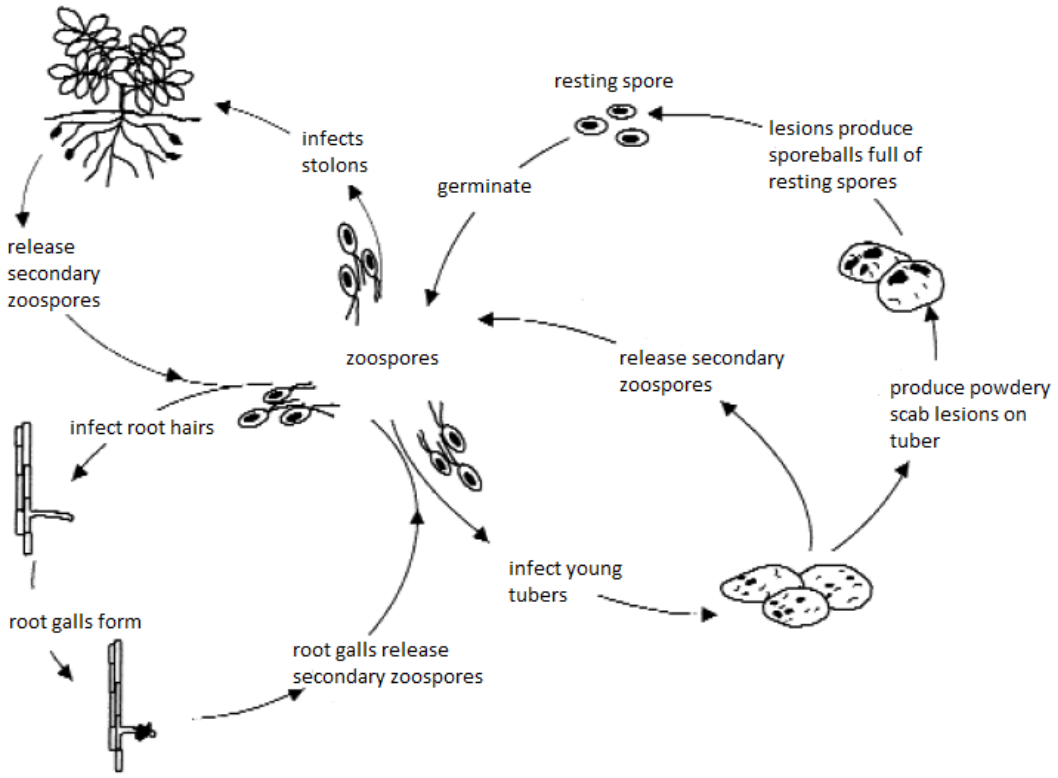

Figure 2.4: Life cycle of *Spongospora subterranea* f. sp. *subterranea* (Johnson, 2002).

When a primary zoospore comes in contact with a susceptible root hair, the flagellae withdraw and the zoospore encysts (Merz, 1992; Jeger *et al.*, 1996; Harrison *et al.*, 1997). A tube that contains a bullet-shaped stylet (also called a 'rohr' and 'stachel') develops within the zoospore cyst and the stylet is rapidly forced through the root hair cell wall (Merz, 1997; Merz, 2008). The pathogen is injected into the host cell and becomes an unwalled protoplast separated from the host by a single unit membrane (Cook, 1932; Harrison *et al.*, 1997). This is known as a multinucleate plasmodium (Figure 2.5). The plasmodium develops and enlarges to form zoosporangia in the root hair (Qu & Christ, 2006; Falloon, 2008), which subsequently gives rise to between four and eight secondary zoospores (Merz, 1992; de Haan & van den Bovenkamp, 2005; van de Graaf *et al.*, 2005; Merz, 2008).

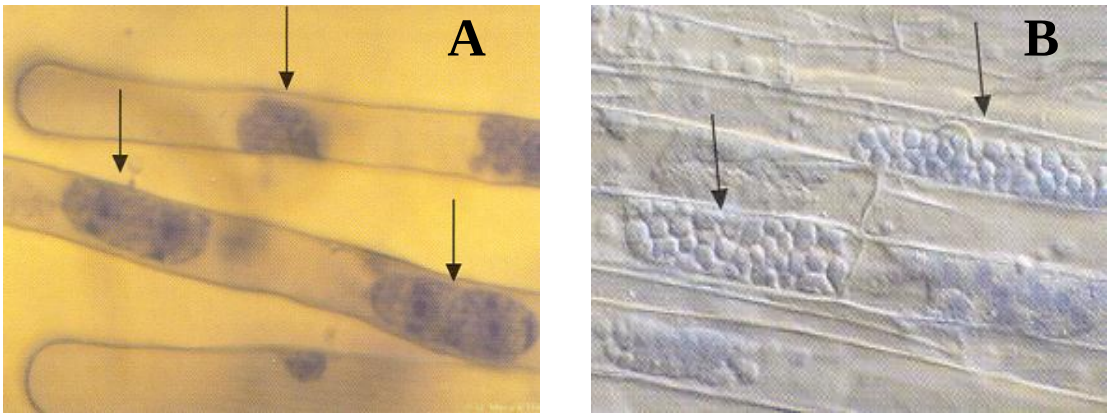

Figure 2.5: A) Plasmodia of *Spongospora subterranea* f.sp. *subterranea* in root hairs B) Zoosporangia of *Spongospora subterranea* f.sp. *subterranea* in roots (Nitzan *et al.*, 2007).

Secondary zoospores are indistinguishable from primary zoospores and have the ability to re-infect roots so that more zoospores can be produced and released into the soil and therefore, increasing inoculum levels (Merz, 1992; Qu & Christ, 2006; Falloon, 2008).

Primary and secondary zoospores can infect tubers, roots and young shoots (Jeger *et al.*, 1996; Qu & Christ, 2006). This means that different plant tissues can become infected at the same time (Falloon, 2008). The infection of unsuberized lenticels leads to tuber scab development, thus indicating that the tubers will be most susceptible to infection during the first two to three weeks after their initiation (Harrison *et al.*, 1997; van de Graaf *et al.*, 2005).

2.2.4 Environmental Factors

Ideal conditions for powdery scab development include high soil moisture (free water) and

low soil temperatures (Ebbels, 1983; Christ, 1989; Wallace *et al.,* 1995; Iftikhar *et al.,* 2000; van de Graaf *et al.,* 2005; Merz, 2008). Powdery scab is a polycyclic disease with multiple infection cycles per season (Merz *et al.,* 2004; Merz, 2008; Nitzan *et al.,* 2009). This means that while environmental conditions are favourable for disease development, zoospores continually infect susceptible roots and new sporeballs will develop within root and tuber tissues. (Harrison *et al.,* 1997; Montero-Astua *et al.,* 2005; Nitzan *et al.,* 2007).

2.2.4.1 Temperature

The occurrence of severe powdery scab has often been associated with plants grown at temperatures below 20˚C (Ebbels, 1983; Harrison *et al.,* 1997). Generally, crops that are planted early in the growing season develop more powdery scab than crops planted later in the growing season. This is due to the cool temperatures that occur during the early periods of the growing season (Harrison *et al.,* 1997). It has been found that the optimum temperatures for disease development range between 12 and 17°C, with the maximum temperature being 22 to 25°C and the minimum temperature less than 11°C (Hughes, 1980; Christ, 1989; Baldwin *et al.,* 2008). Certain stages of infection and symptom development occur at different temperatures. For example five to 25˚C will promote zoospore release, 12 to 13˚C influences zoospore activity and tuber infection and 17˚C promotes root galling (van de Graaf *et al.,* 2007; Merz & Falloon, 2009).

2.2.4.2 Soil Moisture

Soil that has a high water content (most soil pore spaces are filled with water) is associated with higher incidence of powdery scab (Harrison *et al.,* 1997; van de Graaf *et al.,* 2005; Baldwin *et al.,* 2008). High soil moisture content encourages zoospore release, and facilitates the movement of the zoospores towards susceptible roots or other susceptible host tissues (Merz, 1992; Baldwin *et al.,* 2008; Merz, 2008; Merz & Falloon, 2009).

The soil moisture content also has an indirect effect on disease development, as the oxygen concentration in the soil is reduced with higher soil moisture levels (Harrison *et al.,* 1997; Baldwin *et al.,* 2008). Tuber development is thus slower and this increases the period of susceptibility to infection by *Sss* (van de Graaf *et al.,* 2005). Therefore, tubers grown in soils that are poorly drained or in soil types that have large pore spaces and high water holding capacities often have a higher incidence of powdery scab, whereas soils that have small pore spaces and lower oxygen levels will result in reduced infection and disease.

Growing regions that have higher annual rainfall tend to favour powdery scab development, as rainfall influences the water content in the soil. The severity of powdery scab is determined by the intensity and duration of the rainfall (precipitation), as well as the rate at which water drains away from the zone of soil containing the developing tubers (Harrison *et al.*, 1997).

2.2.4.3 pH of the Soil

Spongospora subterranea f.sp. *subterranea* can be infectious across a wide pH range, 4.7 to 7.6. Hughes (1980) and Wale (2004), state that powdery scab incidence is slightly less in acid soil conditions. The different stages of the disease cycle may be affected differently by pH. For example, zoospore release from sporeballs was not affected by a pH range of five to eight, but was reduced by pH below five and above eight. van de Graaf *et al.* (2005) suggests that acid soils may prevent disease by inducing the release of zinc and manganese ions, which are toxic to *Sss.*

2.2.5 Host

Spongospora subterranea f.sp. *subterranea* is an obligate parasite that can only multiply within the living tissues of a susceptible host plant (Walsh *et al.*, 1996; Merz *et al.*, 2005; Qu & Christ, 2006; Christ, 2008). There is a wide host range for *Sss*, which includes potato, oilseed rape, sugar beet and spinach as well as a large number of common weed species, one being hairy nightshade (Andersen *et al.*, 2002; Nitzan *et al.*, 2009; Shah *et al.*, 2010). The host of the greatest economic concern is the potato (*Solanum tuberosum*). The most susceptible stage of potato growth is one week before tuber set. This is defined as the period where more than 50% of the stolons have tips swollen to at least 0.6 cm in diameter.

In some hosts the pathogen completes its life cycle and produces sporeballs (tomato, yellow mustard, oats and hairy nightshade), whereas in other hosts the pathogen produces zoosporangia, without sporeball production (rye, buckwheat and yellow nutsedge) (Qu & Christ, 2006; Merz & Falloon, 2009; Nitzan *et al.*, 2009). This knowledge can be of great importance for powdery scab management as hosts in which sporeball production occurs will contribute to an increase in inoculum, whereas those where no sporeball development occurs will lead to a decrease in inoculum (Qu & Christ, 2006). Hosts where no sporeballs develop are known as alternate hosts and can be considered as trap crops. Planting alternate hosts just

before planting a potato crop can greatly reduce the amount of inoculum that can infect the potato crop (Agrios, 2005; Qu & Christ, 2006; Merz & Falloon, 2009).

2.2.6 Other factors that affect the development of Powdery Scab

2.2.6.1 Cultivar Resistance

All potato cultivars are to some degree susceptible to powdery scab infection (Harrison *et al.*, 1997; Merz *et al.*, 2004; Baldwin *et al.*, 2008) and develop one or more of the following symptoms: sponge-like galls on the roots and/or powdery lesions on the surfaces of tubers. If the tubers become infected, they will develop scabby-like lesions, thus the most reliable method to manage this disease will be by planting potato cultivars that show resistance to tuber symptom development (Merz *et al.*, 2004; Nitzan *et al.*, 2007).

2.2.6.2 Inoculum Level

The quantity of inoculum present in the field cannot be used to directly determine the severity of powdery scab that will occur, unless the environmental factors are taken into account (Harrison *et al.*, 1997; Merz, 2008). This is because low numbers of sporeballs can rapidly give rise to high numbers of zoospores under favourable conditions (Iftikhar *et al.*, 2000; van de Graaf *et al.*, 2005; Baldwin *et al.*, 2008; Merz & Falloon, 2009). Yet, if the conditions are less favourable for disease development, then the level of inoculum can be better correlated with the disease severity (Harrison *et al.*, 1997). The initial amount of inoculum results from a number of factors, for example soil contaminated with sporeballs from the previous season, infected seed tubers, contaminated equipment and contaminated manure (Merz, 1993; Iftikhar *et al.*, 2000).

2.2.6.3 Crop Rotation

Spongospora subterranea f.sp. *subterranea* can survive for many years in the soil (Merz *et al.*, 2005; Merz, 2008). Falloon (2008) states that the pathogen is able to survive in the soil for over 50 years in the absence of a host. To prevent the amount of inoculum increasing in the soil before planting a potato crop, it is advisable to follow a crop rotation program (Agrios, 2005; Qu & Christ, 2006). A general crop rotation of three to five years with non-*Solanum* species has been suggested (Harrison *et al.*, 1997; Qu & Christ, 2006) and in Japan four year crop intervals are practiced due to powdery scab (Nakayama *et al.*, 2007). It is impossible to make an informed decision regarding how long a crop rotation program must be, due to the uncertainty of the length of time that the pathogen survives in the soil.

2.3 Detection Techniques

A number of techniques may be used to detect *Sss* from seed-tubers, plant material and soil, which include microscopy, bioassays, enzyme linked immunosorbent assay (ELISA) systems, conventional PCR techniques and real-time PCR techniques (Falloon, 2008). Techniques, such as PCR and real-time PCR increase the rate at which analysis can be done and achieves a high degree of sensitivity without the need for complex cultivation (Cloete & Atlas, 2006) and thus, obligate organisms such as *Sss* can be detected within hours instead of days, which may be required by traditional methods such as bioassays.

Quantitative data on inoculum levels is an important component for disease risk assessments as it will assist in decision making on cultural and control issues such as the application of biocides, avoiding high risk fields, matching cultivars to fields according to their respective resistance ratings and disease risk, crop rotation strategy, irrigation regime, and prioritizing land according to the crop end use (Bell *et al.,* 1999).

2.3.1 Microscopy

Spongospora subterranea f.sp. *subterranea* is an obligate parasite that cannot be cultured on any known media (Qu & Christ, 2007). It has a resistant resting stage that enables it to survive for many years in the soil (Merz *et al.*, 2005). One method of identifying *Sss* is by observation of the zoospore flagella using a microscope. Biflagellate zoospores develop from the resting spores of *Sss* (Merz, 1992). These flagella are unequal in length, apically attached and oppositely directed (Figure 2.6) (Merz, 1992). The size of the individual zoospore is between 3 to 4 µm in diameter (Harrison *et al.,* 1997).

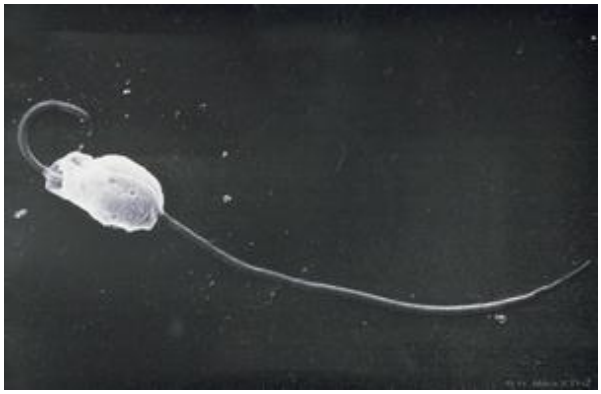

Figure 2.6: Biflagellate zoospore with flagella at about 180˚ to each other (Merz, 1992).

2.3.2 Bioassay

Bioassay is a baiting technique used to assess plant tissues and soils contaminated with *Sss* by using tomato seedlings as bait plants (Flett, 1983; Merz, 1989; Wallace *et al*., 1995; Jeger *et al.,* 1996; Montero-Astua *et al.,* 2005). Root hair infections of the tomato seedlings are estimated visually by assessing the root hairs under a microscope after growing the seedlings in a test soil (Flett, 1983; Merz, 1989). This technique is time consuming as it can take several weeks to complete (Walsh *et al.,* 1996; Harrison *et al*., 1997; Qu *et al.,* 2006; Nakayama *et al.,* 2007). Although the visual assessment of the bait roots requires experience (Montero-Astua *et al.,* 2005), it can however, be a sensitive technique for detecting and quantifying viable *Sss* levels in the soil (Merz, 1989; Harrison *et al.,* 1997; Merz *et al.,* 2004).

Only infections arising from resting spores that release zoospores are detected (Merz *et al.,* 2004; Nakayama *et al.,* 2007). This means that caution must be taken if the results are going to be used for estimating risk because factors controlling dormancy and germination are currently unknown (Merz, 1989; Harrison *et al.,* 1997; Merz & Falloon, 2009). Bioassays with potato plants have been used successfully for pre-screening potato cultivars for powdery scab resistance (Merz *et al.,* 2004).

2.3.3 ELISA

ELISA is a serological method that is used to detect and quantify powdery scab sporeballs using specific antibodies (Clark & Adams, 1977; McCartney *et al.,* 2003; de Haan & van den Bovenkamp, 2005). This technique is rapid, but it does not distinguish between living and dead resting spores. The quantification of *Sss,* with ELISA using monoclonal antibodies can only be determined when there are 100 or more sporeballs per gram of soil (Harrison *et al.,* 1993).

ELISA has also been used to distinguish between powdery scab and common scab lesions (Harrison *et al.,* 1993; Montero-Astua *et al.,* 2005; Merz *et al.,* 2005) as the antibodies are pathogen-specific.

2.3.4 Conventional PCR

Polymerase chain reaction (PCR) with *Sss*-specific primers is used for direct detection of *Sss* in samples such as plant tissues, roots and soil as it is more sensitive than ELISA (Bulman & Marshall, 1998; Bell *et al.,* 1999).

PCR is able to detect small quantities of DNA (Okubara *et al.*, 2005). It is extremely important to ensure that the DNA extracted from the soil is pure (Bulman & Marshall, 1998; Brierley *et al.*, 2009). DNA extraction may be problematic due to inhibitory compounds in the soil, such as humic acids and phenolic compounds, as well as the small sample sizes that are usually processed (Bulman & Marshall, 1998; Qu *et al.*, 2006; Brierley *et al.*, 2009).

PCR-amplified products (391 bp, according to Bell *et al.*, 1999) are detected by techniques such as electrophoresis on agarose gels and after staining of the amplification products by a fluorochrome dye or by hybridization with a labelled probe (Bell *et al.*, 1999; Cloete & Atlas, 2006). Bell *et al.* (1999) reported that one to ten sporeballs per gram of soil can be detected using PCR.

Quantitative PCR is a variation of conventional PCR assays. This assay was developed using an internal control (competitor) based on a DNA fragment of a smaller size than the target DNA, but which has the same primer binding sites (Bell *et al.*, 1999). A fixed concentration of competitor DNA is co-amplified with DNA extracted from a dilution series of *Sss* sporeballs (McCartney *et al.*, 2003). The intensity of the bands on a gel is used to estimate the ratio of the amount of both products (Bell *et al.*, 1999; Okubara *et al.*, 2005). This data is used to generate a standard curve to estimate the DNA concentration from unknown numbers of sporeballs amplified under the same conditions, and hence the numbers of target organisms can be determined in a test sample (McCartney *et al.*, 2003).

Conventional PCR is currently used to detect and quantify *Sss* in naturally infested potato tubers, in infected symptomless host plants and in naturally infested field soils as they are highly specific, relatively fast and reliable (Bell *et al.*, 1999; McCartney *et al.*, 2003; Qu *et al.*, 2006; Nakayama *et al.*, 2007). These results are of immense value in powdery scab disease management because they are related to final disease severity on the tubers (Nakayama *et al.*, 2007).

2.3.5 Real-time PCR

Real-time PCR (qPCR) is a technique in which DNA of a specific target organism, in this case *Sss*, is quantified by measuring the intensity of fluorescence with time (Lees *et al.*, 2008). The source of DNA may be water, plant tissues or soil (Lees *et al.*, 2008). The measurement is done during the exponential phase of DNA amplification (McCartney *et al.*,

2003; van de Graaf *et al.,* 2003). Fluorescence is generated due to the cleavage of a TaqMan® probe by *Taq* DNA polymerase, which is specific to *Sss,* and released during amplification of the target DNA by specific primers (van de Graaf *et al.,* 2003). The fluorescence is measured by specialized equipment, and can be translated into DNA quantities. This requires the use of a standard dilution series of target DNA, which is used to create a standard curve (van de Graaf *et al.,* 2003). van de Graaf *et al.* (2003), were able to detect 0.1 sporeballs per ml of soil water for both artificially and naturally contaminated soil samples, whereas Lees *et al.* (2008) were able to detect as few as 0.025 sporeballs using this qPCR technique.

Real-time PCR is specific, sensitive and reliable for detecting and quantifying the different life stages (sporeballs, zoospores, plasmodia and zoosporangia) of *Sss* in a range of sample types (van de Graaf *et al.,* 2003; Ward *et al.,* 2004; de Haan & van den Bovenkamp, 2005). This technique allows a large number of samples to be processed relatively quickly and easily. Real-time PCR techniques are less labour-intensive (van de Graaf *et al.,* 2005) and reduce the probability of contamination by soil humic compounds compared to conventional PCR techniques (van de Graaf *et al.,* 2003). Real-time PCR quantification can be used for samples with varying levels of *Sss* DNA, either after DNA extraction directly from field samples or can be used in combination with bait plant tests (van de Graaf *et al.,* 2003).

Real-time PCR is of great use in studying the epidemiology of powdery scab as it can be used to determine the level of *Sss* in field soils, allowing the grower to estimate the risk for that specific potato field (McCartney *et al.,* 2003; van de Graaf *et al.,* 2003). Therefore qPCR assists the grower in choosing appropriate management practices to maintain low powdery scab levels or to reduce high *Sss* levels in the soil.

2.4 Risk Assessment, Incidence and Severity

2.4.1 Risk Assessment

Risk assessment is the process where one determines the probability that a certain intensity of incidence or severity of a disease will be reached (Agrios, 2005). For a powdery scab risk assessment numerous host, pathogen and environmental factors must be taken into account. When all aspects of disease development are known or estimated, the risk of the disease developing up to a certain level of severity can be calculated. This may require monitoring of the disease development by using incidence and severity keys (Wale, 2004). Disease

measurement is important because plant disease recordings or reporting will be qualitative as well as quantitative (Falloon *et al.*, 1995). The level of risk is usually reported as low, moderate or high.

2.4.2 Incidence and Severity

Agrios (2005) defines incidence of disease as the number or proportion of plant units that are diseased, whereas Madigan & Martinko (2006) state that incidence is the number of plants diseased (when the leaves, stems and/or fruit show any symptoms) in a population. Severity of a disease is described as the proportion of the plant tissue that is affected (Agrios, 2005).

Disease incidence is relatively quick and easy to measure. It can be used to study the epidemiological spread of a disease (eg. powdery scab) through a field, region, or country. The level of powdery scab incidence is influenced by the effects of temperature and moisture, but the extent to which these factors influence incidence is currently unknown (van de Graaf *et al.*, 2005).

Nakayama *et al.* (2007) determined tuber powdery scab severity by using the following disease index: 0 = no symptoms, 1 = < 3 scabby lesions or < 3% of the total surface covered with lesions, 2 = four to ten scabby lesions or 3 – 13% of the total surface covered with lesions, 3 = 11 – 20 scabby lesions or 14 - 25% of the total surface covered with lesions and 4 = > 21 scabby lesions or > 25% of the total surface covered with lesions.

van de Graaf *et al.* (2007) determined the severity of powdery scab root galling on infected plants, using a descriptive index as agreed by European powdery scab researchers: 0 = no root galls, 1 = one or two root galls, 2 = several galls, mostly small (< 2 mm in diameter), 3 = many galls, some > 2 mm in diameter and 4 = most major roots with galls, some or all > 4 mm in diameter.

Falloon *et al.* (1995) used a pictorial scale to determine the severity of powdery scab (Figure 2.7).

Risk management is not a precise science as the knowledge is based on factors unrelated to plant pathology that impact on the decision making. Risk assessment however does provide a

timely warning to the grower who can respond with appropriate management measures in order to optimize production, quality and profit (Wale, 2004)

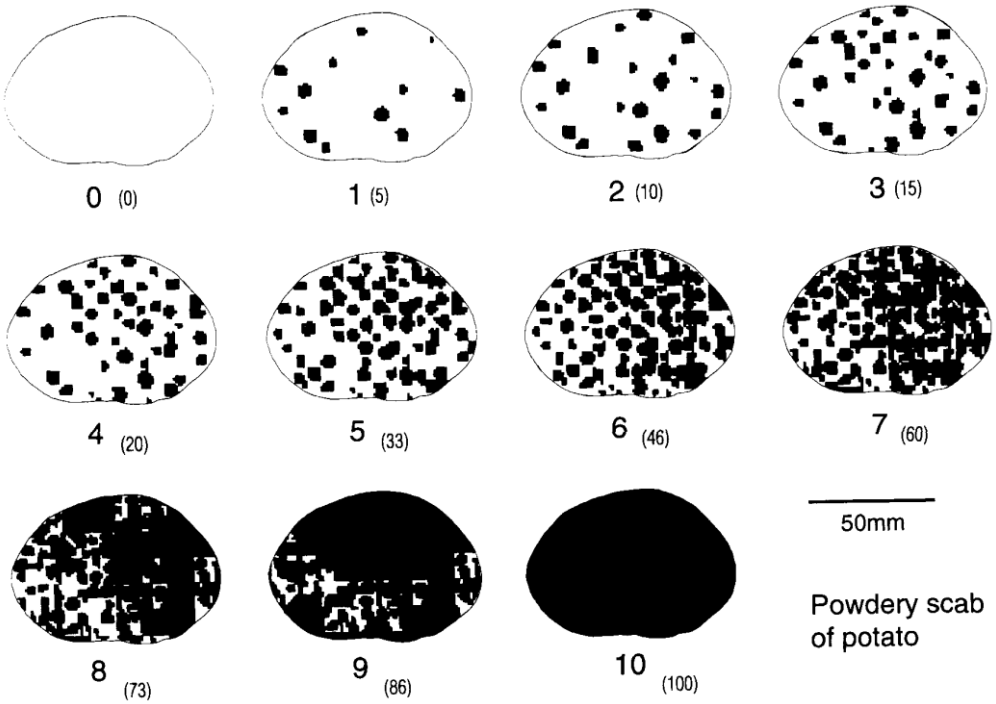

Figure 2.7: Illustrated disease severity key for powdery scab (Falloon *et al.*, 1995).

2.5 Control Practices

The only reliable control of powdery scab disease is to plant disease-free seeds in uncontaminated fields (Harrison *et al.*, 1993; Walsh *et al.*, 1996; Genet *et al.*, 2005; Qu *et al.*, 2006; Merz, 2008). Several strategies are used to minimize the risk of powdery scab disease as there is no single effective way of controlling powdery scab (Merz, 1993; Iftikhar *et al.*, 2007). The integration of management practices can reduce the potential of powdery scab becoming a serious problem (Harrison *et al.*, 1997; Iftikhar *et al.*, 2007). The different methods for managing powdery scab include disease-free seed tubers, cultivar resistance, fungicides, cultural practices, antagonists and legislation (Gudmestad *et al.*, 2007; Falloon, 2008).

2.5.1 Disease-free seed tubers

There is a need to produce seed tubers that have a minimum of phytopathogenic infections and they must be true-to-type (Ebbels, 1983; Rolot & Seutin, 1999). This is because seed tubers tend to accumulate disease to the next generation. Seed tubers are the main source of inoculum in areas where powdery scab was not previously established (Jeger *et al.*, 1996;

Tsror *et al.,* 1999). Seed potatoes with powdery scab are commercially unacceptable for establishment of new crops because the pathogen can be transmitted on infected and infested seed potatoes. Seed certification systems which allow low tolerance levels for powdery scab incidence lowers the market value of those seed tubers (Jeger *et al.,* 1996).

2.5.2 Cultivar Resistance

The potato cultivar that is grown in the field is usually chosen by the producers for its marketability and/or processing characteristics, not purely on its ability to resist infection by *Sss*. All potato cultivars are susceptible to powdery scab to some degree (Wallace *et al.,* 1995; Qu & Christ, 2004; Merz *et al.,* 2005; Iftikhar *et al.,* 2007; Falloon, 2008). Thus the most cost-effective and environmentally safe way of controlling powdery scab is to develop cultivars that are disease resistant (Jeger *et al.,* 1996; Merz *et al.,* 2005; Iftikhar *et al.,* 2007). Resistance may be achieved through genetic engineering. This means that the genes that convey resistance must be isolated from resistant species and introduced into the host plant's genome. These genes exist in several wild species such as *S. curtilobum* and *S. andigenum* (Harrison *et al.,* 1997).

Currently potato cultivars are being bred for resistance using conventional breeding methods. This process is lengthy as it may take more than three years to complete (Baldwin *et al.,* 2008).

2.5.3 Fungicides

Many fungicides can be used to control plant diseases satisfactorily (Harrison *et al.,* 1997). Yet, chemical control for soil-borne pathogens like *Sss* is difficult, due to the soil properties that reduce the efficiency of the fungicides, as well as the high inoculum levels in the soil (Wallace *et al.,* 1995). This means that soil fungicide applications have a limited use and may not be cost effective. Falloon *et al.* (1996) demonstrated that there are a number of chemicals [chlorophenol (dichlorophen-Na), dithiocarbamate (mancozeb), and pyridinamine (fluazinam) groups] that can be used to reduce the incidence and severity of powdery scab, yet these chemicals only form part of an integrated strategy for managing the disease. The soil application of selected chemicals may lead to the reduction of powdery scab. For example, applications of sulphur or zinc salts have shown to reduce disease (Jeger *et al.,* 1996; Wale, 2004; Falloon, 2008). However, soil applications are costly and may not be environmentally acceptable.

2.5.4 Reducing Seed Tuber-borne and Soil-borne Inoculum

There is no information regarding the level of seed-tuber infection necessary to induce powdery scab disease within a field (Merz & Falloon, 2009). The effect on disease levels, of disinfecting seed-tubers with chemicals is dependent on the extent of soil infestation levels with *Sss*, other soil factors that determine disease development and cultivar resistance (Falloon *et al.*, 1996; Harrison *et al.*, 1997). Under conditions that favour powdery scab development in heavily infested soils, the potential benefits of using tuber disinfection would be minimal as the chemical control will only be effective in the soil-zone that is in direct contact with the seed-tubers. Once the plant grows beyond this zone, infection can occur (Falloon, 2008).

Alternatively, heat has been used for the reduction of seed tuber-borne inoculum (Harrison *et al.*, 1997). This was achieved by immersing the tubers in water with a temperature of 55°C for ten minutes. This method is not favoured as it results in less sprouting of the tubers (Harrison *et al.*, 1997; Merz, 2008).

The minimum level of *Sss* in the soil necessary to start an epidemic is unknown (Merz, 1993). The total removal of all the *Sss* inoculum in the infested soils will be the ultimate approach to reduce soil inoculum. There are two main approaches to reducing soil inoculum. The first is soil sterilization, which aims to eliminate a wide range living organisms, and the second is applying fungicides. The first approach destroys the ecological balance in the soil as it kills the organisms, including the beneficial organisms (Harrison *et al.*, 1997). The second approach, fungicides, is more environmentally friendly as they are usually more selective in their effects, provided the fungicides are correctly applied.

2.5.5 Antagonists

The use of antagonistic microbes to control powdery scab is attractive because antagonists will act specifically against *Sss* and this approach is more environmentally friendly than chemical and certain cultural practices. It has been shown that *Trichoderma harzianum* has the ability to parasitize sporeballs of *Sss* under controlled experimental growing conditions (Merz & Falloon, 2009) yet further testing is required for the antagonist to be effective in the field.

2.5.6 Cultural Practices

There are a number of factors that must be considered when determining the suitability of a field for potato production (Table 2.1). Most commonly, fields are selected for economic reasons and to ensure a good skin finish (Wale, 2004). Growers should consider avoiding fields that have a history of powdery scab (Harrison *et al.,* 1997; Larkin & Griffin, 2007).

Table 2.1: Factors that must be considered when determining the suitability of a field for potato production in order to avoid powdery scab (Wale, 2004)

Factor	**Comment**
Moisture retention	Influences irrigation scheduling and risk of disease favoured by free water
Drainage category	Poorer draining fields increase the risk of disease development
High water table	Fields with a high water table have a higher risk of disease development
Soil zinc level	Evidence suggests powdery scab is a much lower risk where soil zinc levels are > 6mg/kg
Soil pH	Only has a small impact on disease development
The use of slurry or manure	Sporeballs are unaffected by passage through livestock and may contaminate the soil

The use of plough pans and irrigating with smaller quantities of water more frequently will reduce the chances of creating soil conditions that are conducive to disease development (Harrison *et al.,* 1997; Nakayama *et al.,* 2007; Lees *et al.,* 2008). It is possible to control powdery scab severity by reducing irrigation during the most susceptible stage of crop growth (during tuber initiation) (Merz, 2008; Merz & Falloon. 2009).

The most important methods for reducing the risk of contamination of powdery scab-free soils are by using pathogen-free seed-tubers, cleaning and disinfecting all equipment and containers that come in contact with the tubers (Walsh *et al.,* 1996; Harrison *et al.,* 1997; van de Graaf *et al.,* 2005). Equipment can be disinfected with the use of chemicals such as formaldehyde and copper sulphate.

Powdery scab appears to be more of a problem in fields with little or no break between

successive potato crops (Merz, 1992; Harrison *et al.*, 1993; Falloon, 2008; Merz, 2008). In Italy and Russia it is recommended that potatoes be rotated with non-host crops every three years, whereas in Scotland, crop rotations must be between five and eight years (Harrison *et al.*, 1997; Falloon, 2008). When determining the length of rotations, the grower must consider the soil type and the varying climatic conditions that will influence the quantity of inoculum in the soil for the following growing season (Falloon, 2008).

Growing trap crops to reduce the soil-borne inoculum directly before the potato crop is planted, offers an alternative approach to managing *Sss* (Jeger *et al.*, 1996; Qu & Christ, 2006). For example, planting *Datura stramonium* (Jimsonweed) or *Raphanus sativus* (leafy daikon) in heavily infested soil before planting the potato crop can reduce the severity of powdery scab (Harrison *et al.*, 1997; Qu & Christ, 2006; Larkin & Griffin, 2007).

2.5.7 Legislation

Seed-tuber inspections, strict quarantine and compulsory crop rotations have been implemented to reduce the problems caused by powdery scab in certain parts of the world (Harrison *et al.*, 1997). These practices do not have a large effect on powdery scab control because of the widespread distribution of *Sss* in soils, its ability to multiply rapidly in favourable conditions and the likelihood that it can survive for many years in the absence of potato crops.

2.5.8 Integrated Control

Singularly, management practices have minor effects in controlling powdery scab (Larkin & Griffin, 2007; Christ, 2008; Falloon, 2008). Combining the various management practices (crop rotation, cultural practices, host resistance and fungicide use) is the basis of integrated control of powdery scab (Gudmestad *et al.*, 2007; Falloon, 2008). This will allow manipulation of various factors affecting the severity of powdery scab. The benefits resulting from optimizing each control practice are additive and therefore a reduction in powdery scab can be achieved.

2.6 Summary

Powdery scab of potato is caused by *Sss*. This disease is a problem for potato producers worldwide, as it reduces tuber quality and tuber marketability (Harrison *et al.*, 1997; Gudmestad *et al.*, 2007; Merz, 2008). The scab lesions that develop on the tubers contain

masses of sporeballs, which are also known as cystosori (Montero-Astua *et al.,* 2005; Qu *et al.,* 2006). Sporeballs are able to survive in the soil for many years (Jeger *et al.,* 1996; Nitzan *et al.,* 2009). Under favourable environmental conditions, sporeballs release primary zoospores, which infect and colonize the potato host plants (van de Graaf *et al.,* 2003; Falloon, 2008). After infection, plasmodia form in the roots which mature into zoosporangia releasing secondary zoospores (Merz, 1992; Qu & Christ, 2006). *Spongospora subterranea* f.sp. *subterranea* infections can initiate from seed-borne and/or soil-borne inoculum (Falloon, 2008).

Spongospora subterranea f.sp. *subterranea* can infect more than one host species (Andersen *et al.,* 2002; Nakayama *et al.,* 2007). The use of an alternate host as a trap crop can be used to reduce the level of inoculum in the soil (Qu & Christ, 2006; Merz & Falloon, 2009). Environmental conditions that favour powdery scab development in a potato crop include cool temperatures, high soil moisture, and a pH range of 4.7 to 7.6 (Harrison *et al.,* 1997; Merz *et al.,* 2004; Nitzan *et al.,* 2009).

There are a number of techniques that are used for the detection and quantification of *Sss* which include enzyme linked immunosorbent assay (ELISA) systems, bioassays, conventional PCR techniques and Real-time PCR techniques (Falloon, 2008). The use of PCR techniques has allowed for rapid quantification of *Sss*. Techniques to detect and quantify inoculum in both the field and seed-tubers form an essential part of disease risk assessment.

There are no singularly effective control measures for powdery scab. An integrated control approach is the most effective way to control the disease. Integrated control makes use of a combination of control practices (resistant cultivars, fungicides, and cultural practices) (Gudmestad *et al.,* 2007; Falloon, 2008). The benefits resulting from optimizing each control practice is additive, therefore a reduction in powdery scab can be achieved.

Spongospora subterranea f.sp. *subterranea* is a pathogen that needs to be studied further, so that the negative effects that it poses, both environmentally and economically can be reduced and controlled effectively.

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CHAPTER 3

Comparison of Techniques for the Detection of *Spongospora subterranea* f.sp. *subterranea* from Potatoes and Soil in South Africa

Abstract

Spongospora subterranea f.sp. *subterranea* (*Sss*) is the causal agent of powdery scab, a blemish disease of potato tubers. Bioassays, using tomato seedlings as bait plants have been used to detect *Sss* in soil whereas ELISA and PCR were adopted to detect *Sss* from soil and tubers. Real-time PCR (qPCR) is able to detect and quantify DNA from water, plant tissues or soil. The aim of this study was to detect and quantify *Sss* from tuber lesions, contaminated water and soil. Sensitivities of ELISA, conventional PCR and qPCR were compared. ELISA detected sporeballs from tuber lesions, yet the accuracy improves with standards of 100 sporeballs/ml or greater. ELISA did not detect sporeballs from soil samples in this experiment. DNA was extracted from powdery scab lesions for conventional PCR and qPCR. Both conventional PCR and qPCR detected *Sss* DNA from tuber, soil and water samples. Real-time PCR was very sensitive with consistent detection of standard DNA quantities of 1, 10, 100, 1 000 and 10 000 sporeballs/ml. Therefore, when comparing the three techniques, conventional PCR was more sensitive than ELISA at detecting *Sss* inoculum, as it was able to detect *Sss* DNA from both tuber and soil samples. Real-time PCR had the advantage over conventional PCR in that it not only detected *Sss* DNA in a variety of sample types, but was able to quantify *Sss* DNA as well. Since *Sss* is an obligate parasite, detection and quantification techniques are valuable tools for future epidemiological and powdery scab management studies on *Sss.*

3.1 Introduction

Spongospora subterranea (Wallroth) Lagerheim f. sp. *subterranea* (*Sss*) Tomlinson is the causal agent of powdery scab, a blemish disease of potato tubers (*Solanum tuberosum* L.). Powdery scab was first reported on potatoes in 1841, yet the understanding of its pathology remains poor, mainly due to its biotrophic nature (Harrison *et al.*, 1997; Bell *et al.*, 1999; Merz *et al.*, 2005). Powdery scab can lead to serious yield losses of marketable tubers and it is a major disease problem for growers in many areas of the world, particularly where the climate favours disease development (Harrison *et al.*, 1993; van de Graaf *et al.*, 2005; Qu *et al.*, 2006). Powdery scab infections are manifested as disfiguring lesions, each containing

masses of brown powdery sporeballs (cystosori) (de Haan & van den Bovenkamp, 2005). Sporeballs are sponge-like aggregations of many thick walled cysts that are 19 - 85 µm in diameter (Wallace *et al.,* 1995; Bell *et al.,* 1999). Sporeballs can survive for many years in soil, in the absence of host plants, before germinating in cool moist conditions and releasing zoospores (Merz *et al.,* 2005; Montero-Astua *et al.,* 2005; van de Graaf *et al.,* 2005).

Control of powdery scab is difficult as there is no effective chemical treatment and all potato cultivars are susceptible to some degree to this disease (Wallace *et al.,* 1995; Qu *et al.,* 2006). The most reliable management measure is to minimise soil-borne and seed tuber-borne inoculum, thus reducing the risk of disease (Harrison *et al.,* 1997; Bell *et al.,* 1999). Visual seed-tuber inspections often confuse powdery scab symptoms with those of disorders and other diseases such as common scab, caused by *Streptomyces scabies* (Thaxt.) Waksman and Henrici (Bulman & Marshall, 1998; de Haan & van den Bovenkamp, 2005; Montero-Astua *et al.,* 2005). In addition, infections in symptomless tubers go undetected (Merz *et al.,* 2005; Qu *et al.,* 2006). Therefore, methods to detect *Sss* are essential to allow for detailed studies into the epidemiology and management of powdery scab.

Bioassays using tomato or potato seedlings as bait plants have been used successfully to detect *Sss* from infested soil (Flett, 1983; Merz, 1989). Seedling roots are then microscopically examined for the presence/absence of *Sss* plasmodia and/or *Sss* zoosporangia (Flett, 1983; Merz, 1989). Although bioassays are sensitive (detecting as few as 100 sporeballs per gram of soil), they are slow, laborious, require experience to recognize infection and may be unable to differentiate inoculum levels (Walsh *et al.,* 1996). Thus, a more rapid enzyme-linked immunosorbent assay (ELISA) was adopted, which was able to detect *Sss* from soil and tubers, including symptomless tubers (Harrison *et al.,* 1993; Wallace *et al.,* 1995; Walsh *et al.,* 1996; Merz *et al.,* 2005). According to Qu *et al.* (2006) ELISA-based methods are not always specific for pathogen recognition due to cross reactions of polyclonal antisera with proteins in potato sap extracts.

Species-specific polymerase chain reaction (PCR) tests are available for a number of plant pathogens, including *Sss* (Bulman & Marshall, 1998; Bell *et al.,* 1999; Qu *et al.,* 2006). PCR is used for direct detection of *Sss* DNA in environmental samples such as naturally infested potato tubers, in latently infected host plants and in naturally infested field soils (Qu et al., 2006).

Real-time PCR (qPCR) is a technique in which DNA of a specific target organism, in this case *Sss*, is quantified from water, plant tissues or soil. This is achieved by measuring the intensity of fluorescence with time, where fluorescence is generated due to the cleavage of a TaqMan® Sss-specific probe by *Taq* DNA polymerase, and release during amplification of the target DNA by specific primers (McCartney *et al.*, 2003; van de Graaf *et al.*, 2003; Ward *et al.*, 2004), which is measured and translated into DNA quantities. Quantitative PCR allows for a large number of samples to be processed relatively quickly and easily.

The detection of *Sss* in tubers and soil is the initial step in developing a management programme for powdery scab. The purpose of this study was to detect and quantify *Sss* from tuber lesions, infested water and soil. The sensitivity of ELISA, conventional PCR and qPCR detection methods were compared.

3.2 Materials and Methods

3.2.1 Inoculum Preparation

Powdery scab lesions were cut from infected tubers (cv. Mondial) using a sterile scalpel and were ground using a pestle and mortar. The powdery mass was passed through a series of mesh sieves (200, 100, 75 and 53 µm) and then suspended in 10 ml of sterile distilled water (SDW) and centrifuged at 4000 *g* for one minute. The supernatant was discarded and the pellet was resuspended in 10 ml SDW and centrifuged at 4000 *g* for 1 minute. This process was repeated five times. The final pellet was suspended in 5 ml SDW and transferred to a Petri dish. Using a dissection microscope and a pipette, single sporeballs were transferred to microcentrifuge tubes to make up sporeball concentrations of 1, 10 and 100 sporeballs per ml of SDW. Sporeball concentrations of 1 000 and 10 000 sporeballs per ml SDW were determined using a haemocytometer. These known concentrations were then used to determine the sensitivity of ELISA, conventional PCR and qPCR.

Samples of unknown sporeball concentrations from tuber lesions, soil and water were included in the assays (Table 3.1). Sporeballs from the lesions were scraped off using a scalpel and collected in a microcentrifuge tube. Water samples were collected from a water bath after washing infected tubers. Each sample was tested using three methods (ELISA, conventional PCR and qPCR) and each experiment was repeated twice.

Table 3.1: Sample with unknown sporeball concentrations from tuber lesions, soil and water

Sample type	**Obtained from**
Soil A	Sandveld
Soil B	Sandveld
Tuber lesion A	Tuber (cv. Mondial)
Tuber lesion B	Tuber (cv. Mondial)
Water A	Prepared in the laboratory
Water B	Prepared in the laboratory

3.2.2 ELISA

A complete ELISA kit containing the required reagents, buffers, substrate and microtitre plates was used (Art. No. 111172, BIOREBA). ELISA was carried out as described by Merz *et al.* (2005). A working volume of 200 µl for all the steps was used. The procedure followed was the same for both tuber and soil samples.

3.2.3 Conventional PCR

ZR Soil Microbe DNA kit™ (Zymo Research Corp, USA) was used, according to the manufacturers instruction, to extract DNA from each of the prepared sporeball samples. PCR was performed in a final volume of 25 µl containing 0.5 µl of dNTPs (250 µM of each dATP, dTTP, dGTP and dCTP) (Bioline, London, UK), 2.5 µl PCR reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL at pH 8.8) (Bioline), 0.25 µl *Taq* polymerase (1 U) (Bioline), 1.25 µl $MgCl_2$ (2.5 mM) (Bioline), and 0.25 µl (0.5 µM) *Spongospora subterranea*-specific primers, *Sps*1 (5'-CCT GGG TGC GAT TGT CTG TT-3') and *Sps*2 (5'-CAC GCC AAT GGT TAG AGA CG-3') (Bulman & Marshall, 1998). Five µl of DNA template was added to each reaction.

A thermocycler and 200 µl thin walled PCR tubes were used in the PCR process. The PCR reaction conditions were: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of melting (95°C for 20 seconds), annealing (64°C for 25 seconds) and extension (72°C for 50 seconds) with a final cycle of 72°C for 10 min. Amplified DNA, a positive and a negative control were stained with ethidium bromide and were subjected to electrophoresis on 1% agarose gel alongside standard size markers (HyperLadder II, Bioline, London, UK). The agrose gels were viewed under ultraviolet illumination.

3.2.4 Real-time PCR (qPCR)

The LightCycler®480 (Roche Diagnostics GmbH, Germany) was used for real-time PCR amplification and detection. Real-time quantification (qPCR) was carried out as described by van de Graaf *et al.* (2003). Standards were prepared using DNA extracted from suspensions with known sporeball concentrations (1, 10, 100, 1 000 and 10 000 sporeballs per ml, respectively) and diluted with TE buffer to obtain a dilution series of DNA equivalent to 0.01, 0.1, 1, 10 and 100 sporeballs per µl. The diluted DNA was then used to obtain a standard curve. By comparing the *C*t values of the unknown samples with the *C*t values of the standard curve the amount of *Sss* DNA in each unknown sample (Table 3.1) was determined.

3.3 Results

3.3.1 ELISA

The negative control, 1 sporeball per ml and 10 sporeballs per ml gave average A_{405} values of 0.196, 0.203 and 0.24, respectively. Sporeball concentrations greater than 100 sporeballs per ml were most easily detected. Therefore only sporeball concentrations of 100 per ml and greater could be detected. Results show that the relationship between sporeball concentration and A_{405} values is exponential (equation on Figure 3.1). ELISA was not sensitive enough to detect sporeballs in soil samples at any concentration.

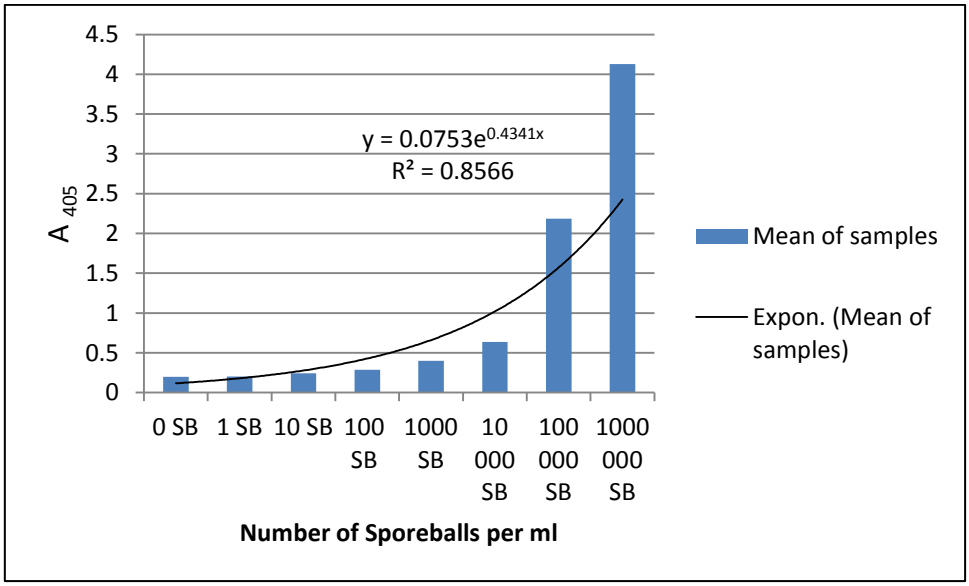

Figure 3.1: Exponential relationship of the concentration of sporeballs of *Sss* prepared from tuber lesions.

3.3.2 Conventional PCR

Sss-specific primers *Sps*1 and *Sps*2 (Bell *et al.*, 1999) gave a visible 391 base pair (bp) amplification product from *Sss* DNA. Positive results were obtained with DNA extracted from 1, 10, 100, 1 000 and 10 000 sporeballs per ml (Figure 3.2). Conventional PCR also amplified *Sss* DNA from samples of soil, lesions and water with unknown sporeball concentrations.

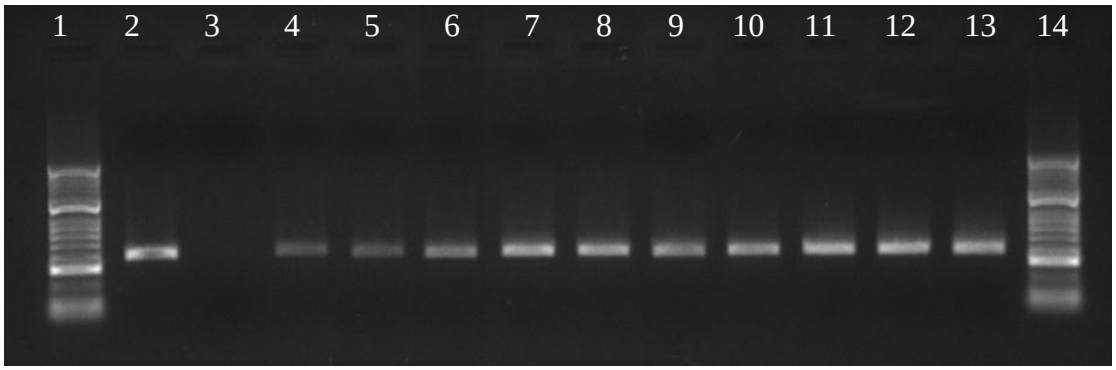

Figure 3.2: EtBr-stained agarose gel of PCR products (using *Spongospora subterranea*-specific primers) obtained from DNA extracted from solutions containing different concentrations of sporeballs/ml (Lane 1: Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4: 1 Sporeball/ml, Lane 5: 10 Sporeballs/ml, Lane 6: 100 Sporeballs/ml, Lane 7: 1 000 Sporeballs/ml, Lane 8: 10 000 Sporeballs/ml, Lane 9: Soil A, Lane 10: Soil B, Lane 11: Lesion A, Lane 12: Lesion B, Lane 13: Water A and Lane 14: Ladder).

3.3.3 Real-time PCR (qPCR)

The qPCR standard curve was obtained using 0.01, 0.1, 1, 10 and 100 sporeballs per µl of TE buffer (Figure 3.3). Based on three sample replications (each 45 cycles), the LightCycler®480 detection system automatically calculated the starting concentration of *Sss* DNA by a comparison of the Ct values from unknown samples with those of the standard curve (Figure 3.3). The linear correlation coefficient of the standard curve was r^2 = 0.9909, demonstrating the accuracy of PCR-based quantification. Conventional PCR amplified *Sss* DNA from the samples with unknown sporeball concentrations whereas qPCR was not only able to detect sporeball DNA concentrations from the soil, lesion and water samples, but also quantified the sporeball DNA of the samples. Table 3.2 shows *Sss* DNA concentrations per sample.

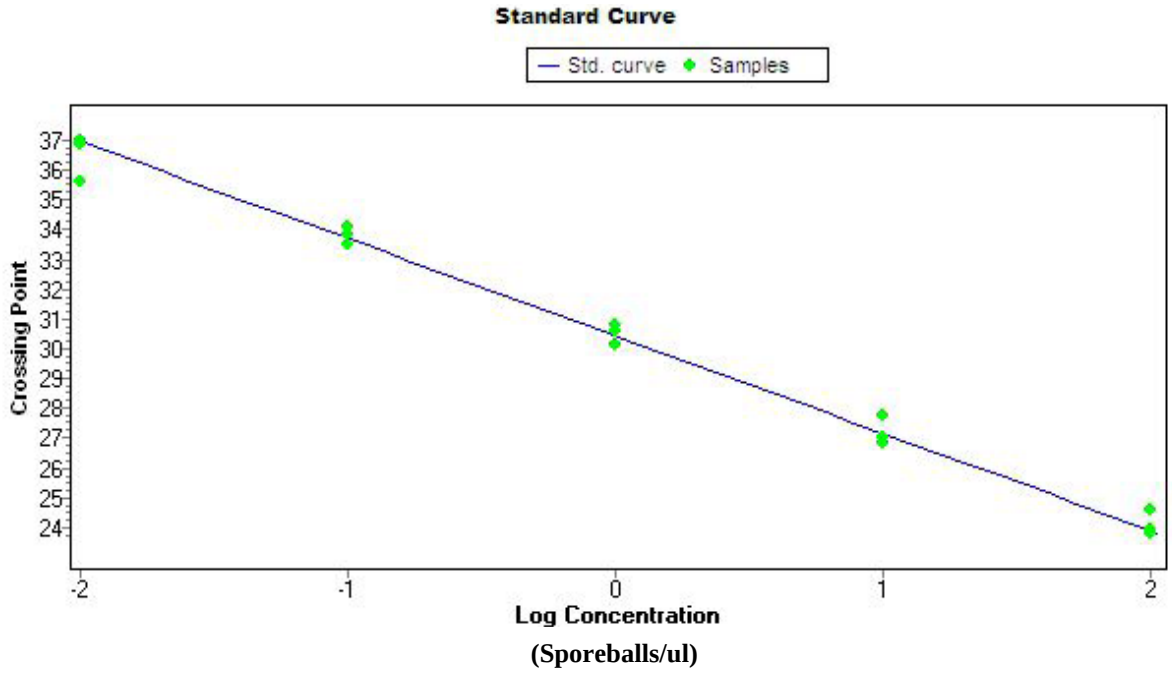

Figure 3.3: Standard curve (y = -3.269x + 30.43) used in qPCR assay for the quantification of *Sss* sporeball DNA samples extracted from soil, water and plant tissue (error = 0.0491 and efficiency = 2.023) was analysed with the LightCycler®480 software 1.5.0.

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Table 3.2: Quantification of *Sss* in unknown samples using real-time PCR (values are given as *C*t value and sporeball equivalents per ml)

Sample	**Ct value**	***Sss* sporeball per µl**
Soil A	32.88	8.38
Soil B	30.36	87.5
Lesion A	20.14	66 800
Lesion B	21.38	32 200
Water A	18.55	213 000
Water B	19.64	93 400

3.4 Discussion and Conclusion

Sporeballs of *Sss* were detectable in tuber samples by ELISA. The A_{405} values did not differ at inoculum levels between 0 - 100 sporeballs per ml. However, when more than 100 sporeballs per ml were present in the tuber sample an exponential relationship between sporeball concentration and A_{405} value was observed, indicating that detection is more consistent at levels of 100 sporeballs per ml or greater. These results were similar to those of Merz *et al.* (2005) for water and tuber lesion samples. ELISA was unable to detect sporeballs

in the soil samples in this experiment. Harrison *et al.* (1993) and Wallace *et al.* (1995) were able to detect *Sss* from tuber samples but not soil samples and concluded that this was due to a low level of cross-reaction of antibody with other organisms. This may have been due to the matrix effect of soil components interfering with the antigen-antibody reaction, thus masking the detection of the sporeballs. ELISA tests are, however, relatively cost-effective and can be used for seed-tuber certification programmes. This technique is also used to distinguish between powdery scab and common scab, as the antiserum is specific for sporeballs of *Sss* (Harrison *et al.*, 1993).

Conventional PCR detected *Sss* DNA that was extracted from tuber, soil and water samples in this study. Bell *et al.* (1999) used this technique to demonstrate the sensitive detection of *Sss* sporeballs in soil and tuber washings as well as peel. Qu *et al.* (2006) and Nakayama *et al.* (2007) emphasised that it is essential to have a DNA extraction method that ensures that the resulting DNA is free from PCR inhibitors usually present in soils as they interfere with *Taq* polymerase activity, primer annealing, recovery of microbes from agricultural samples, and amplicon detection. The ZR Soil Microbe DNA kit™ used in this experiment yielded DNA with minimal soil inhibitors as spin columns were used.

This experiment demonstrated that the real-time PCR assay developed for the detection and quantification of *Sss* DNA (van de Graaf *et al.*, 2003) can detect the pathogen in water, soil and plant tissue. The qPCR assay was very sensitive with consistent detection of standard DNA quantities of 0.01, 0.1 1, 10 and 100 sporeballs per µl of TE buffer. qPCR is specific and sensitive (van de Graaf *et al.* (2003), as it was able to detect 0.1 sporeball per ml of soil water for both artificially and naturally contaminated soil samples.

Conventional PCR is more sensitive than ELISA as conventional PCR detects sporeballs DNA from both tuber and soil samples. The sensitivity of ELISA, conventional PCR and qPCR are, however, similar at sporeball concentrations of 100 sporeballs per ml or greater but, qPCR offers an even lower limit of detection than the aforementioned methods. (van de Graaf *et al.*, 2003). The advantage of qPCR is that it reliably detects and quantifies the different life stages (sporeballs, zoospores, plasmodia and zoosporangia) of *Sss* in a range of sample types (van de Graaf *et al.*, 2003; Ward *et al.*, 2004; de Haan & van den Bovenkamp, 2005).

Detection as well as quantification techniques are valuable tools for future studies on *Spongospora subterranea* f.sp. *subterranea* in South Africa as they can be used detect the pathogen in the field before planting, allowing the grower to take appropriate management measures (eg. resistant cultivars) if the pathogen is present in the field.

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CHAPTER 4

Genetic Characterisation of *Spongospora subterranea* f.sp. *subterranea* in South Africa

Abstract

Powdery scab of potato, caused by *Spongospora subterranea* f. sp. *subterranea* (*Sss*) is an economically important disease; particularly as there are no effective control measures. *Sss* is a protozoan within the order *Plasmodiophorida*. *Sss* and *Spongospora subterranea* f.sp. *nasturtii* were previously grouped together as two special forms of *Spongospora subterranea*, only separated by their host range. Limited information regarding the genetic variation within *Sss* exists, as only two ITS Group Types, Group Type I and Group Type II, have been described. In this study, *Sss* samples from potato growing regions in South Africa were collected, sequenced and analysed to investigate genetic variation. Comparisons with known *Sss* ITS Group Type I/II sequences were made to determine the specific Group Type/s found in South Africa as well as their evolutionary position within the Plasmodiophorids. Tubers with *Sss* lesions were collected between 2008 and 2010 from four potato growing areas in South Africa. Dried tuber skins with powdery scab lesions from the Sandveld, dated 1936, were also obtained. Tuber lesions were removed and ground using a mortar and pestle and the powdery mass of sporeballs was used for DNA extraction and sequencing. Neighbour-joining (obtained using Kimura's 2-parameter distances) and Maximum parsimony were used to construct phylogenetic trees. South African samples grouped together along with known *Sss* Group Type II samples. Samples from South Africa formed a monophyletic group with other species from Plasmodiophorids. Long-term sampling from South African fields contaminated with *Sss* may indicate the presence of *Sss* subgroups which will be of importance for future resistance breeding programs.

4.1 Introduction

Powdery scab of potato is caused by the obligate biotroph, *Spongospora subterranea* (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson (*Sss*). This plant pathogen is economically significant as it produces scab-like lesions on potato tubers, which consequently reduces their marketability and carries infection to subsequent crops when the tubers are used as seed. Powdery scab infections can arise from inoculum that is seed-borne

or soil-borne (De Boer *et al.,* 1982; Letham *et al.,* 1988). Scabs that form on tubers contain masses of resting structures known as sporeballs or cystosori which have the ability to survive in the soil for a number of years (Bell *et al*., 1999; van de Graaf *et al.,* 2003). During favourable environmental conditions, high moisture and cool temperatures, primary zoospores are released from the sporeballs, which then infect the potato host plants. Within the infected host, plasmodia form in the root hairs and develop into zoosporangia, subsequently releasing secondary zoospores when mature (van de Graaf *et al*., 2003).

To date, there are no effective control measures for powdery scab and all potato cultivars are susceptible to powdery scab to some degree (Wallace *et al.,* 1995; Merz *et al.,* 2004; Qu & Christ, 2004; Iftikhar *et al.,* 2007; Falloon, 2008). Thus the most cost-effective and environmentally safe way to control powdery scab would be to develop cultivars that are resistant to infection (Jeger *et al.,* 1996; Harrison *et al.,* 1997; Merz *et al.,* 2004). This requires an understanding of the pathogen diversity and the ways virulence evolves in the pathogen population (Qu & Christ, 2004; Merz & Falloon, 2009).

Spongospora subterranea f.sp. *subterranea* as well as; *Plasmodiophora brassicae* Woronin, the cause of club root disease of brassicas; *Polymyxa* species, the vectors of several plant pathogens and *Spongospora subterranea* f.sp. *nasturtii* Tomlinson, the cause of crook root disease of watercress, are considered economically important members of the order *Plasmodiophorida* (Karling, 1968). The classification of these pathogens is complex as previously they were regarded as a distinct class of fungi, the Plasmodiophoromycetes, within the division Myxomycota, however, at present they are considered to be protozoans within the order *Plasmodiophorida* (Karling, 1968; Braselton, 1995; Down *et al.,* 2002). In a number of recent molecular studies small-subunit rRNA (SSU rRNA) was used to predict phylogeny for the species in the *Plasmodiophordia* (Castlebury & Domier, 1998; Ward & Adams, 1998; Qu & Christ, 2004). Based on phylogenetic analyses they concluded that Plasmodiophorids are not closely related to any other eukaryote, thus are a distinct group.

Ss f.sp. *subterranea* and *Ss* f.sp. *nasturtii* were previously grouped together as two special forms of *Spongospora subterranea* due to similarities in their resting spores, zoospores and zoosporangia (Harrison *et al.,* 1997; Down *et al.,* 2002). *Ss* f.sp. *subterranea* and *Ss* f.sp. *nasturtii* differ only in their primary host; potato and watercress respectively.

To date, two ITS Group Types of *Sss* have been described, Group Type I and Group Type II (Bulman & Marshall, 1998; Qu & Christ, 2004). Bulman & Marshall (1998) studied variation in ribosomal internal transcribed spacer (ITS1 and 2) sequences in 26 *Sss* samples from New Zealand, Australia, Scotland and Peru. They found that there was no sequence variation in the Australasian and European samples, with the exception of a single sample from Scotland which had a 5.1% divergence in the ITS spacer sequence. The samples from Peru were identical to the single sample from Scotland. Qu & Christ (2004) stated that the alignment of the ITS1 and ITS2 of 52 samples reveal that there are two distinct Group Types, I and II. They also noted that *Sss* samples from Australasia and North America were of Group Type II and the South American *Sss* samples were of only Group Type I. However, European samples consisted of Group Type I and II.

Qu & Christ (2004) suggested that that the occurrence of genetic groups may be cultivar related. This is because all 9 *Sss* samples from cv Saturna originating from Ireland and Scotland had Group Type I sequences and all 6 samples from cv Kerr's Pink from Ireland had Group Type II sequences. However, when approximately 100 samples of *Sss* collected from a range of cultivars and locations in Scotland, were recently assessed for ribotype using the method of Qu *et al.* (2004) both Groups were found and there was no association between ribotype, cultivar or location (Alison Lees, pers. comm.).

In this study *Sss* samples from potato growing regions in South Africa were collected, sequenced and analysed to investigate genetic variation in ITS1/2 sequences. Comparisons to known *Sss* Group Type I/II sequences were made to determine the specific Group Type/s found in South Africa.

4.2 Materials and Methods

4.2.1 *Spongospora subterranea* f.sp. *subterranea* Samples

Potatoes are produced in 16 main growing regions in South Africa, viz. Ceres, Eastern Cape, Eastern Free State, Gauteng, KwaZulu-Natal (KZN), Limpopo, Loskop Valley, Mpumalanga (Mpu), Northern Cape, North-Eastern Cape, North West, Sandveld, Southern Cape, South Western Cape, South Western Free State and Western Free State. Samples from powdery scab prevalent areas (Sandveld, Ceres, KZN and Mpu) were collected during 2008 to 2010 (Table 4.1). Two powdery scab samples dated 1936 from the Sandveld were also obtained

from the Biosystematics Division: Mycology ARC-Plant Protection Research Institute, Pretoria, South Africa.

Table 4.1: Origin of 28 South African *Spongospora subterranea* f.sp. *subterranea* samples from different fields within the regions and date of collection

Sample No.	Growing Region	Sampling Date
1	Sandveld	October 2008
2	Sandveld	March 2009
3	Sandveld	February 2010
4	Sandveld	October 2008
5	Sandveld	February 2010
6	Sandveld	October 2008
7	Sandveld	October 2008
8	Sandveld	March 2009
9	Sandveld	March 2009
10	Ceres	August 2009
11	Ceres	August 2009
12	Ceres	August 2009
13	Ceres	August 2009
14	Ceres	August 2009
15	Ceres	August 2009
16	Ceres	August 2009
17	Ceres	August 2002
18	KwaZulu-Natal	May 2009
19	KwaZulu-Natal	May 2009
20	KwaZulu-Natal	January 2010
21	Mpu	April 2010
22	Mpu	April 2010
23	Mpu	April 2010
24	Mpu	April 2010
25	Mpu	April 2010
26	Mpu	April 2010
27	Sandveld	1936
28	Sandveld	1936

Infected tubers were surface disinfected by spraying with 70% ethanol and allowed to air dry at room temperature. Individual powdery scab lesions were then cut from the various tuber

samples using a sterile scalpel and allowed to air dry. The tuber lesions were ground using a mortar and pestle. The powdery mass obtained was then used for DNA extraction.

4.2.2 PCR and DNA Sequencing

DNA was extracted from each of the sample using a ZR Soil Microbe DNA kit™ (Zymo Research Corp, USA) according to the manufacturer's instructions. PCR was carried out in a final volume of 25 µl consisting of 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20 (Fermentas Life Sciences, South Africa), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 250 nM of each *S. subterranea*-specific primer (*Sp01* (5'-ATT GTC TGT TGA AGG GTG-3') and *Sp02* (5'-GGT TAG AGA CGA ATC AGA A-3') (Bulman & Marshall, 1998), and 1U *Taq* polymerase (Fermentas Life Sciences, South Africa). One µl of DNA template was used in each reaction.

A thermocycler and 200 µl thin-walled PCR tubes were used in the PCR process. PCR reaction profiles included: Initial denaturation at 94°C for 2 minutes, followed by 35 cycles of melting (94°C for 30 seconds), annealing (53°C for 30 seconds) and extension (72°C for 45 seconds) with a final cycle of 72°C for 7 minutes. Amplified DNA, as well as a positive and negative control were subjected to electrophoresis on 1% agarose gel and stained with ethidium bromide beside standard size markers (HyperLadder II, Bioline, London, UK). The results were viewed under ultraviolet illumination. Amplified DNA products were purified using QIAquick PCR Purification Kit (Qiagen, Southern Cross Biotechnology).

The ITS fragment was sequenced in both directions with the forward primer *Sp01* and the reverse primer *Sp02* (Bulman and Marshall, 1998). Each 10 µl sequencing reaction contained 2 µl Big Dye Sequencing Reaction Mix, 1 µl Big Dye terminator 5 × sequencing buffer, 30 pmol primer and 2 µl purified DNA template. Sequencing PCR conditions included denaturation at 96°C for 60 seconds, 25 cycles of melting at 96°C for 10 seconds, annealing of primer at 50°C for 5 seconds and elongation at 60°C for 4 minutes. PCR products were cleaned using a sodium acetate precipitation step and sequenced on an ABI Prism DNA Automated Sequencer (Perkin-Elmer).

4.2.3 Sequence Analysis

Eight SSU rDNA sequences of *Sss* with the accession numbers: AY604171 (*Sss* Group Type I), AY604172 (*Sss* Group Type II), AF102819 (*Sss* Group Type II), EF593112 (*Sss* Group

Type II), AF305697 (*Sss* Group Type I), Y12829 (*Sss* Group Type I), AF102820 (*Sss* Group Type I) and AF104308 (*Sss* Group Type II) were obtained from the GeneBank database. Five other sequences that represent the Plasmodiophorids were also obtained from GeneBank with the following accession numbers: *Lignier* sp. (Y12828), *Plasmodiophora brassicae* (Y12831), *Polymyxa graminis* (Y12826), *Polymyxa beta* (Y12827) and *Spongospora subterranea* f.sp. *nasturtii* (AF257744).

Basecalling correction and sequence editing was done using the Chromas Lite version 2.01 program. Corrected sequences were edited and aligned using BioEdit Sequence Alignment Editor version 7.0.0 (Hall, 1999). Searches were performed on each consensus sequence generated in BioEdit using the BLAST program (Altschul *et al.*, 1990) from the GenBank database. All the selected sequences were aligned using Clustal W version 1.6 (Thompson *et al.*, 1994). Ambiguously aligned sequence regions were not included in the data for analysis. Phylogenetic trees for the data sets were based on distance and maximum parsimony (MP) in MEGA version 4 (Tamura *et al*., 2007) with the Archaea, *Giardia Lamblia*, as the outgroup in this study (as done by Ward & Adams, 1998). A neighbour-joining tree was obtained using Kimura's 2-parameter distances. The stability of the phylogenetic trees was assessed by performing bootstrap analysis from 1000 bootstrap replicates.

4.3 Results

4.3.1 Phylogenetic Analysis of *Spongospora subterranea* f.sp. *subterranea* SSU rRNA Sequences

The complete *Sss* sequences of the South African samples were aligned with the eight *Sss* sequences obtained from GeneBank. There were 2360 characters in the data set. Of the included characters, 178 were constant, 492 were variable uninformative, and 121 were parsimony informative.

The neighbour-joining analysis using Kimuora's two-parameter distance tree (Figure 4.1) gave similar topologies to those of the maximum parsimony tree. Both trees show the following phylogenetic patterns: all the samples from South Africa grouped together along with the GeneBank samples representing *Sss* Group Type II, while GeneBank samples representing *Sss* Group Type I formed a separate group. This is indicated in Figure 1 with bootstrap values of 77% and 99% for the MP tree, respectively (tree not shown).

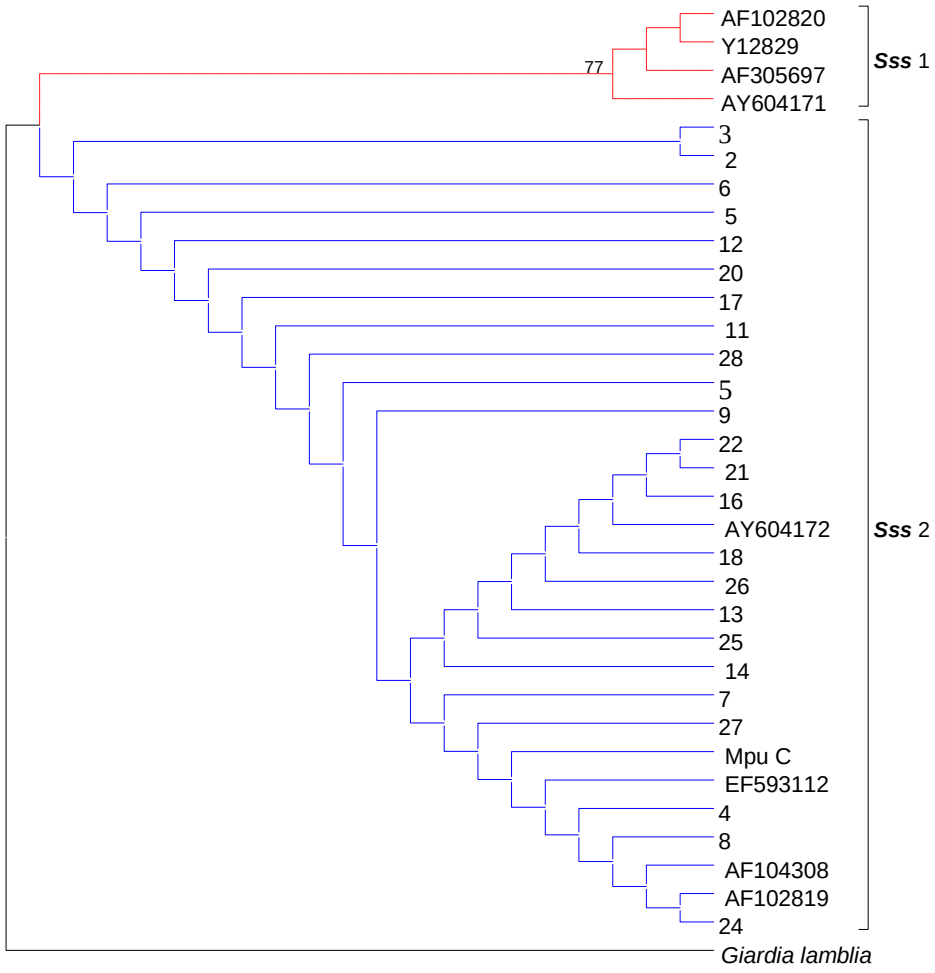

Figure 4.1: Phylogenetic tree based on ITS fragment sequences showing the phylogenetic relationships among South African, American and European samples of *Sss*. In the distance method, a neighbour-joining tree was obtained using Kimura's 2-parameter distances. The stability of the phylogenetic trees was assessed by performing bootstrap analysis from 1000 bootstrap replicates. (Only significant bootstrap values (>50%) are shown). *Sss* 1 indicates *Sss* Group Type I whereas *Sss* 2 indicates *Sss* Group Type II.

4.3.2 Phylogenetic Analysis of *Spongospora subterranea* f.sp. *subterranea* SSU rRNA Sequences with other Plasmodiophorids

The SSU rRNA sequences from *Sss* South African samples were aligned with the eight *Sss* sequences obtained from GeneBank as well as with five sequences representing the Plasmodiophorids. There were 2400 characters in the data set. Of the included characters, 232 were constant, 1259 were variable uninformative, and 415 were parsimony informative.

The neighbour-joining analysis using Kimura's two-parameter distance tree (Figure 4.2) and the maximum parsimony tree gave similar topologies. The trees have the following

phylogenetic patterns: the *Sss* samples from South Africa grouped with the GeneBank *Sss* samples and formed a monophyletic group with species from the *Plasmodiophorida*. Figure 2 indicates that *Sss* and *Ss* f.sp. *nasturtii* are distinct from one another.

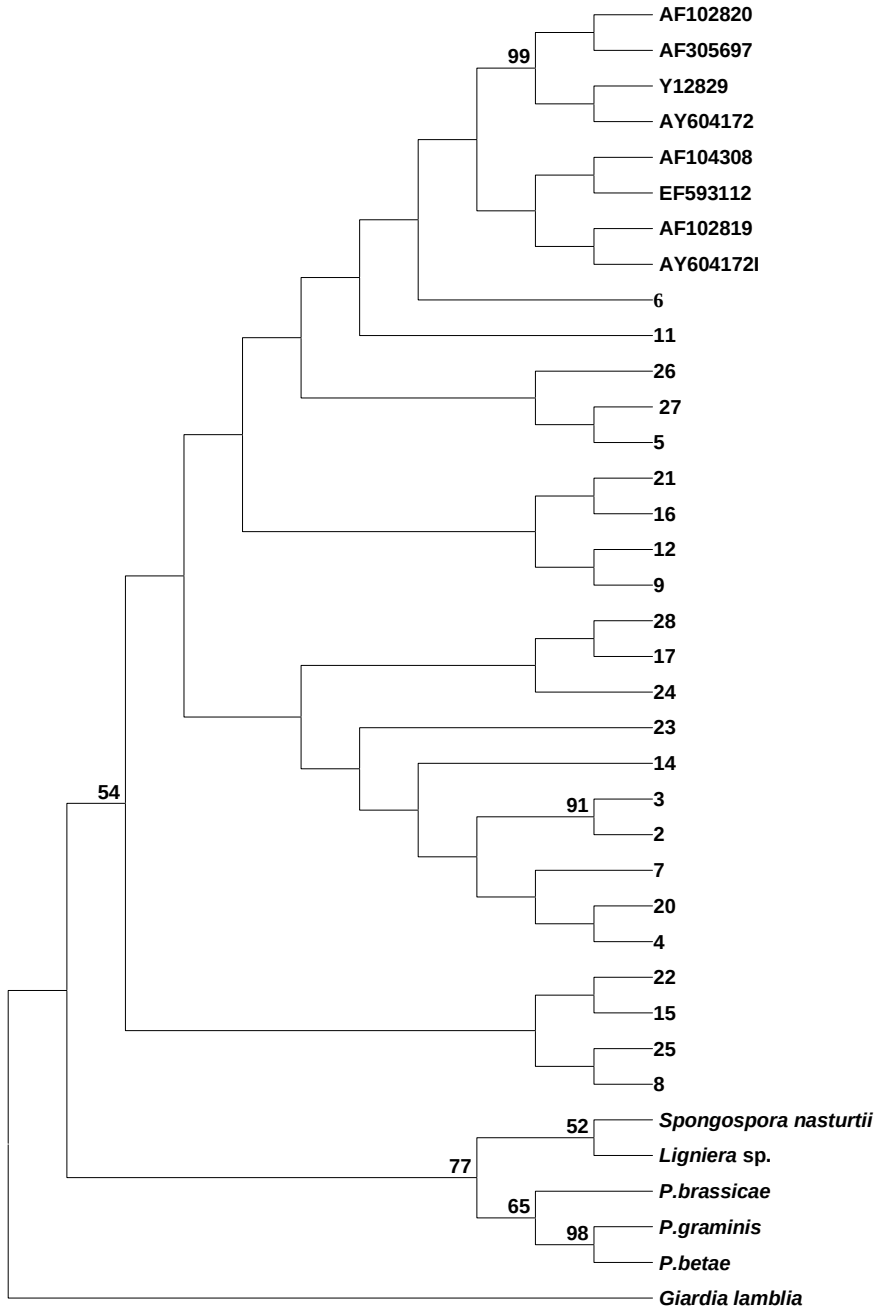

Figure 4.2: Phylogenetic tree based on *Sss* ITS fragments sequences and other Plasmodiophorid sequences downloaded from GeneBank. The distance method, neighbour-joining tree was obtained using Kimura's 2-parameter distances. The stability of the phylogenetic trees was assessed by performing bootstrap analysis from 1000 bootstrap replicates. (Only significant bootstrap values (>50%) are shown).

4.4 Discussion and Conclusion

There are two described groups of *Sss*, Group Type I and Group Type II (Bulman & Marshall, 1998; Qu & Christ, 2004). Current research indicates that *Sss* Group Type I originated from South America, yet it is unclear where and when exactly Group Type II originated (Qu & Christ, 2004). The populations of *Sss* in Europe consist of both Group Type I and II, whereas North America and Australasia only have Group Type II. Bulman & Marshall (1998) state that it is possible the European populations of *Sss* Group Type II may have been the source of the Australasian populations. Potatoes are native to South America and it is believed the crop and the powdery scab pathogen were introduced to Europe during 1570's (Niederwieser, 1999), spread to North America and afterwards to the rest of the world, including South Africa.

This study indicated that *Sss* samples from the different potato growing regions in South Africa are all the same Type, namely *Sss* Group Type II. This result is similar to that of Qu & Christ (2004), who noted that there was no variation among samples from different locations in Canada and USA. Qu & Christ (2006), however, noted that samples from the same geographical location clustered together within the phylogenetic tree, showing two distinct evolutionary lineages. This was not the case with the South African samples, as there was no distinct grouping within the trees.

This study shows that *Sss* is distinct from other Plasmodiophorids, as a monophyletic *Sss* group was observed in this study. There are two forma speciales in *S. subterranea*, *Ss* f. sp. *subterranea* and *Ss* f. sp. *nasturtii* and according to this study they are distinct from each other. Bulman *et al.* (2001) and Down *et al.* (2002) report that there is a considerable phylogenetic distance separating *Sss* and *Ss* f. sp. *nasturtii* and suggest they be considered as two distinct species.

The primary aim of this study was to determine *Sss* Group Types found in South Africa. The results are, however, based on a relatively small sample size. Future studies involving long term sampling from South African fields contaminated with *Sss* may indicated the presence of *Sss* subgroups which will be of importance for future resistance breeding programs.

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CHAPTER 5

Infection of Tomato Seedling Roots by *Spongospora subterranea*

Abstract

Spongospora subterranea f.sp. *subterranea* (*Sss*) is an economically important potato pathogen causing scabby lesions on tubers, thus reducing tuber marketability. Bioassays with bait plants such as tomato are used to assess plant tissues and soils for contamination with *Sss*. The aim of this experiment was to determine the time required by *Sss* to release zoospores and infect bait plant roots. Tomato seeds (cv. Rodade) were planted in seedling trays containing sterilised vermiculite grown at 22˚C and irrigated with Merz's nutrient solution without zinc. Two-leaf stage seedlings were then transferred to 60 ml plastic pots (one seedling per pot) filled with nutrient solution. At day seven, seedlings were inoculated with 0, 100 or 10 000 sporeballs per 60 ml nutrient solution. Samples of tomato seedling roots were taken every second day for microscopic evaluation, PCR testing and root infection rating. Results indicated that it takes four days from inoculation for zoospores to be released; while root infection occurred six days after inoculation. Plasmodia and zoosporangia were observed in the root hairs on day 12 and 14, respectively. The average disease severity resulting from the different inoculum concentrations did not differ significantly. These results and those from similar studies provide a better understanding of the biology of *Sss*, which can be used in the development of new management strategies for powdery scab.

5.1 Introduction

Powdery scab of potato is caused by *Spongospora subterranea* (Wallroth) Lagerheim f.sp. *subterranea* Tomlinson (*Sss*), a biotrophic pathogen. *Spongospora subterranea* f.sp. *subterranea* survives for many years as extremely resistant thick-walled resting spores which are found clumped together within sporeballs (cystosori). Under favourable conditions, and only in the presence of water, the resting spores germinate and release single uninucleate, flagellated zoospores (primary zoospores) (van de Graaf *et al.*, 2007).

The length of time that primary zoospores swim before they infect a susceptible host is highly dependent on free water and temperature (Wallace *et al.*, 1995; Qu & Christ, 2006; Merz, 2008). Zoospores need to find and infect a susceptible host in order to survive and this

presumably occurs by means of chemotaxis (Merz, 2008). Once the zoospore enters into the host cell it becomes an unwalled protoplast, also known as a multinucleate plasmodium, which is separated from the host by a single unit membrane (Merz, 2008). The plasmodia develop and enlarge to form zoosporangia in the root hairs (Wallace *et al.,* 1995; Qu & Christ, 2006), which when mature give rise to between four and eight secondary zoospores per zoosporangium (van de Graaf *et al.,* 2003; de Haan & van den Bovenkamp, 2005; Merz, 2008). Primary and secondary zoospores are indistinguishable from one another and both have the ability to re-infect roots and subsequently release more zoospores, thus increasing the inoculum levels in the plant and soil (Qu & Christ, 2006).

A baiting technique with tomato plants, also known as a bioassay is used to assess plant tissues and soils for contamination with *Sss* (Flett, 1983; Merz, 1989; Wallace *et al*., 1995; Jeger *et al.,* 1996; Montero-Astua *et al.,* 2005). Root hair infections of the tomato seedlings are visually estimated by assessing the root hairs under a microscope after growing the seedlings in an inoculated test solution (Flett, 1983; Merz, 1989). Bioassays are time consuming as they can take several weeks to complete (Walsh *et al.,* 1996; Harrison *et al*., 1997; Qu *et al.,* 2006). Although the visual assessment of the baited roots requires experience (Montero-Astua *et al.,* 2005; Qu *et al.,* 2006), it can however, be a sensitive technique for detecting and quantifying *Sss* inoculum (Merz, 1989; Harrison *et al.,* 1997; Merz *et al.,* 2004).

Only resting spores that release zoospores are detected (Merz *et al.*, 2004). This means that caution must be taken if the results are going to be used for estimating risk because factors controlling dormancy and germination are currently unknown (Merz, 1989; Harrison *et al.,* 1997; Merz & Falloon, 2009). Merz *et al.* (2004) recently developed a bioassay using potato roots which has the potential to screen and select for resistant material at an early breeding stage.

Previous work by van de Graaf *et al.* (2000) investigated the relationship between inoculum level and infection as well as the course of zoospore release from *Sss* sporeballs over time and concluded that zoospore release and root infection occurs within one week after inoculation. The experiment by van de Graaf *et al.* (2000) was adapted in this study to investigate the dormancy period of *Sss* sporeballs, by determining more accurately the time

required for the pathogen to release zoospores and infect bait plant roots after inoculation. This information will contribute to the knowledge of *Sss* biology and epidemiology.

5.2 Materials and Methods

5.2.1 Inoculum Preparation

Powdery scab lesions were cut from infected tubers (cv. Mondial) using a sterile scalpel and were ground using a pestle and mortar. The powdery mass was passed through a series of mesh sieves (200, 100, 75 and 53 µm). The sieved sporeballs were then suspended in 10 ml of sterile distilled water (SDW) and centrifuged at 4000 *g* for one minute. The supernatant was discarded and the pellet was resuspended in 10 ml SDW and centrifuged at 4000 *g* for 1 minute. This process was repeated five times. The final pellet was suspended in 5 ml SDW and transferred to a Petri dish. Using a dissection microscope and a pipette, single sporeballs could be transferred to microcentrifuge tubes to make up a sporeball concentration of 100 sporeballs per ml. Where a sporeball concentration of more than 100 sporeballs per ml was required for inoculation, a haemocytometer was used to estimate the number of sporeballs.

5.2.2 Growth and Inoculation of Tomato Seedlings

Tomato seeds (cv. Rodade) were planted in seedling trays containing sterilised vermiculite and grown at 22˚C. Seeds were irrigated with Merz's nutrient solution (Merz, 1989) without zinc. Once the seedlings reached the two-leaf stage, 252 seedlings were transferred to individual 60 ml plastic pots, which were wrapped in aluminium foil and filled with 60 ml Merz's nutrient solution (126 seedlings for root infection and zoospore detection and 126 seedlings for root infection rating). The seedlings were incubated at 15˚C for one week with a 15 hour light regime. After 7 days the plants were inoculated with sporeball solutions containing 0, 100 and 10 000 sporeballs per 60 ml nutrient solution and maintained at 15˚C. One bait plant was used per replicate, with three replicates per treatment. Samples of tomato seedling roots were taken every second day for 14 days for evaluation and further testing.

5.2.3 Root Infection and Zoospore Detection

To determine root infection, tomato plants were removed from the nutrient solution. The roots were washed with SDW and allowed to air dry overnight. The roots were then frozen at -20˚C for one day (to kill the zoospores) followed by DNA extraction and PCR.

The remaining nutrient solution in each pot was filtered using a sterile syringe with a 5 µm syringe filter to separate the zoospores (2-4 µm) from the sporeballs (40-80 µm). The filtered solutions were frozen overnight at -20˚C to kill (burst) the zoospores followed by DNA extraction and PCR.

5.2.4 DNA Extraction and PCR Conditions

DNA was extracted from each individual root and nutrient solution sample using the ZR Soil Microbe DNA kit™ (Zymo Research Corp, USA). PCR was carried out in a final volume of 25 µl containing 0,5 µl of dNTPs (250 µM of each dATP, dTTP, dGTP and dCTP) (Bioline, London, UK), 2.5 µl PCR reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL at pH 8.8) (Bioline), 0.25 µl *Taq* polymerase (1 U) (Bioline), 1.25 µl $MgCl_2$ (2.5 mM) (Bioline), and 0.25 µl (0.5 µM) *Spongospora subterranea*-specific primers, *Sps*1 (5'-CCT GGG TGC GAT TGT CTG TT-3') and *Sps*2 (5'-CAC GCC AAT GGT TAG AGA CG-3') (Bulman & Marshall, 1998). Five µl of DNA template was added to each reaction.

200 µl thin walled PCR tubes and a thermocycler were used in the PCR process. The thermal profiles were: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of melting (95°C for 20 seconds), annealing (64°C for 25 seconds) and extension (72°C for 50 seconds) with a final cycle of 72°C for 10 min. Amplified DNA, a positive and a negative control stained with ethidium bromide and were subjected to electrophoresis on 1% agarose gel. Standard size markers (HyperLadder II, Bioline, London, UK) were incleded on the agarose gel. The gels were viewed under ultraviolet illumination.

5.2.5 Root Infection Rating

Seedlings were removed from the nutrient solution. The roots were washed with SDW and stained with 1% (w/v) cotton blue in lactophenol for 5 minutes and viewed under a light microscope. The roots were rated according to Merz (1989): 0 = no sporangia, 1 = few sporangia, 2 = several roots with sporangia, 3 = sporangia regularly present (moderate infection) and 4 = sporangia regularly present (heavy infection).

5.3 Results

Tomato plants growing in nutrient solution were removed and tested every second day for the presence of zoospores in the nutrient solution and infection in the seedling roots. PCR results indicated that *Sss* zoospores were present in the nutrient solution four days after inoculation

whereas detectable root infection occurred six days after inoculation (Table 5.1). All tomato seedlings inoculated with 0 sporeballs were negative for infection. Although two different sporeball inoculum concentrations (100 and 10 000 sporeballs per 60 ml) were used in this experiment, both concentrations yielded similar results (Figure 5.1).

Table 5.1: Presence of zoospores, root infection, plasmodia and zoosporangia in nutrient solution and tomato bait plants 2 – 14 days after inoculation with *Sss*

Day	**Zoospores**	**Root infection**	**Plasmodia**	**Zoosporangia**
2	No	No	No	No
4	Yes	No	No	No
6	Yes	Yes	No	No
8	Yes	Yes	Yes	No
10	Yes	Yes	Yes	No
12	Yes	Yes	Yes	Yes
14	Yes	Yes	Yes	Yes

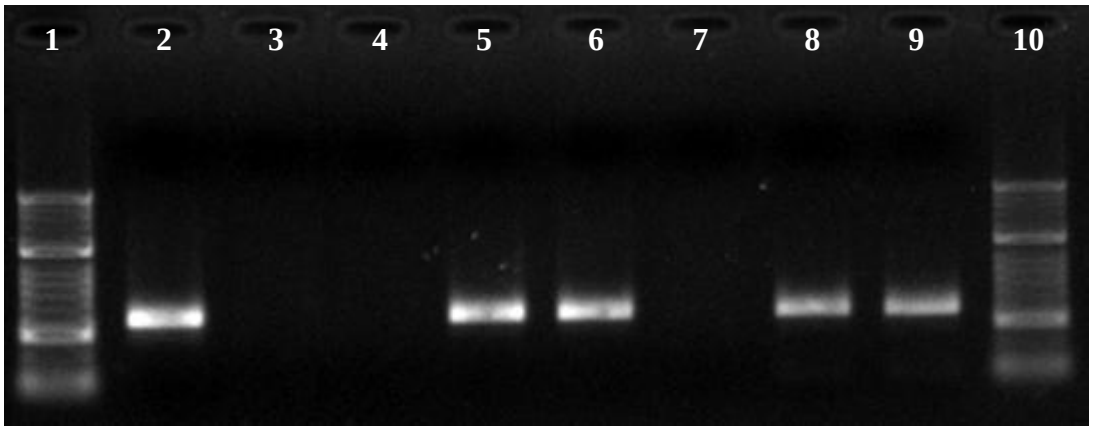

Figure 5.1: EtBr-stained agarose gel of PCR products (using *Spongospora subterranea*-specific primers) obtained from DNA extracted from nutrient solution and tomato roots six days after inoculation (Lane 1: Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4: Root 0 Sporeballs, Lane 5: Root 100 Sporeballs, Lane 6: Root 10 000 Sporeballs, Lane 7: Solution 0 Sporeballs, Lane 8: Solution 100 Sporeballs, Lane 9: Solution 10 000 Sporeballs and Lane 10: Ladder).

Root infection was observed microscopically after staining the tomato roots with 1% (w/v) cotton blue in lactophenol . Plasmodia were present in the root hairs of tomato seedlings eight

days after inoculation (Figure 5.2A), but not in the seedlings that were inoculated with 0 sporeballs. On day 12 zoosporangia were visualized in the root hairs (Figure 5.2B).

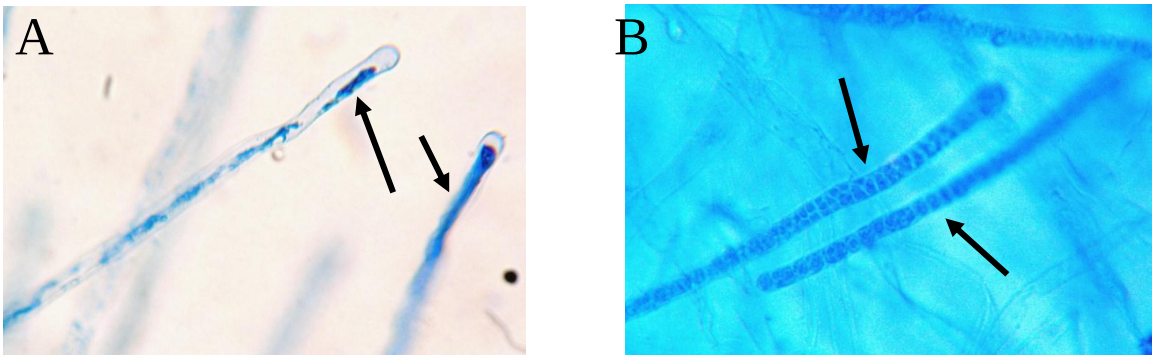

Figure 5.2: A) Plasmodia present in tomato root hairs eight days after inoculating with *Spongospora subterranea* f.sp. *subterranea* sporeballs and B) *Spongospora subterranea* - zoosporangia in root hairs of tomato 12 days after inoculation.

An average root infection severity of 2 ± 0.41 and 2.7 ± 0.75 was observed on day 12 and 14, respectively, at an inoculum concentration of 100 sporeballs per 60 ml of nutrient solution. Similar results of 2.3 ± 0.58 and 2.7 ± 0.57 were observed at an inoculum concentration of 10 000 sporeballs per 60 ml nutrient solution.

5.4 Discussion and Conclusion

A baiting technique using tomato seedlings was used to determine the time between inoculation of *Spongospora subterranea* f.sp. *subterranea* and zoospore release and root infection, respectively. Two detection techniques; bioassay with microscopy and PCR were used.

Bioassays have been used in various studies on the biology of *Sss.* Merz (1989) used a bioassay to study the infectivity of sporeballs, whereas van de Graaf *et al.* (2000) studied zoospore release using a bioassay. Although bioassays are sensitive enough in that zoospores and root infections in the form of as plasmodia and/or zoosporangia, can be observed using a microscope, visual assessment of the presence of *Sss* requires experience as *Sss* zoosporangia may be similar to those of other soil organisms (van de Graaf *et al.*, 2000; Montero-Astua *et al.*, 2005; Qu *et al.*, 2006). PCR, using *Spongospora subterranea*-specific primers, was also used in this experiment as DNA from all *Sss* life stages including sporeballs, zoospores, plasmodia and zoosporangia can be detected (van de Graaf *et al.*, 2003; Lees *et al.*, 2008).

Thus PCR was used to confirm the bioassay results as well as to detect root infections before plasmodia could be observed microscopically.

The nutrient solution in each pot was filtered using a sterile syringe with a 5 µm syringe filter to separate the zoospores from the sporeballs. No zoospore, plasmodia or zoosporangia where observed or detected from the seedlings that were inoculated with 0 sporeballs.

The results of this experiment indicated that *Sss* zoospores were present in the nutrient solution four days following inoculation. Merz (1989) also observed resting spore germination and zoospore release three days after inoculation. The temperature of the experiment was maintained between 12 and 20˚C, which, according to Merz & Falloon (2009) promotes zoospore release. PCR results confirmed the findings of van de Graaf *et al.* (2000), which showed that infection of tomato roots occurred six days after inoculation.

The presence of plasmodia in the root hairs was confirmed microscopically eight days after inoculation, followed by zoosporangia observations on day 12. These findings are similar to those of Merz (1989), which stated that zoosporangia can develop five days after root infection has occurred.

A single sporeball contains approximately 500-1000 resting spores, which under favourable conditions can germinate and release biflagellate primary zoospores (Harrison *et al.,* 1997; Iftikhar *et al.*, 2002). This could be the reason that the average severity values of root infection did not differ for inoculum of 100 and 10 000 sporeballs per 60 ml nutrient solution.

The information obtained in this experiment and previous experiments provides a better understanding of the biology of *Sss*, which can be used to assist in the development of new management strategies for powdery scab (Wale, 2004; Falloon, 2008; Merz & Falloon, 2009). For example, the incorporation of sulphur or zinc in soils affects zoospore viability, and could thus reduce the severity of powdery scab (Wale, 2004; Falloon, 2008). Therefore, the combined results of bioassays and PCR can be a valuable tool for future studies on the life cycle of *Spongospora subterranea* f.sp. *subterranea.*

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CHAPTER 6

Assessment of Host Resistance to Powdery Scab in Potato Cultivars Grown in South Africa

Abstract

Spongospora subterranea f. sp. *subterranea* (*Sss*) causes root galls, cankers and powdery scab lesions on potato tubers. To date no potato cultivars have shown complete immunity to powdery scab. The availability of resistant potato cultivars with commercially acceptable quality is essential for long term control of powdery scab. The aim of this experiment was to determine the susceptibility of six potato cultivars, commonly grown in South Africa, to powdery scab. Individual pots were filled with steam pasteurised soil, inoculated with either, 5, 25 or 50 *Sss* sporeballs per gram of soil and planted with seed of six South African cultivars. The plants were grown in a greenhouse under conditions that favour powdery scab development. The progeny tubers and roots were harvested after four months and assessed for the incidence and severity of root galls and the tubers were rated for severity of powdery scab. To confirm that the root galls and tuber lesions were caused by *Sss*, DNA was extracted and subjected to PCR using *Sss* specific-primers. As all the cultivars had root galls and tuber lesions it can be concluded that none of the tested South African cultivars are immune to infection by the pathogen. In addition there were no significant differences in disease incidence and severity of powdery scab (and root galling) between the cultivars. Inoculum concentration had no effect on the final amount of disease. The results of this experiment are a valuable tool for future potato breeding.

6.1 Introduction

Powdery scab caused by *Spongospora subterranea* (Wallroth) Lagerheim f. sp. *subterranea* Tomlinson (*Sss*) may severely affect potatoes (*Solanum tuberosum* L.), causing root galls, cankers and scab-like lesions on tubers (Wastie *et al.,* 1988). This disease reduces the quality and marketability of both seed and ware tubers throughout the world (Karling, 1968; van de Graaf *et al.,* 2005). The cultivation of susceptible cultivars, increased use of irrigation, earlier planting, mechanization and the requirement for washed table potatoes have all contributed to the importance of this disease in recent years (Kirkham, 1986; Merz *et al.,* 2004; Baldwin *et al.,* 2008).

The plasmodiophorid, *Sss*, produces sporeballs that are filled with masses of resting spores (Braselton, 1995; de Haan & van den Bovenkamp, 2005). When environmental conditions are favourable, the resting spores germinate and give rise to zoospores which infect the tubers and roots of the potato. *Spongospora subterranea* f. sp. *subterranea* requires temperatures of between 12-17°C and free water for infection to occur (de Haan & van den Bovenkamp, 2005; Baldwin *et al.*, 2008). Low levels of inoculum may result in significant powdery scab symptom development under these conditions (van de Graaf *et al.*, 2007; Baldwin *et al.*, 2008; Houser & Davidson, 2010).

Controlling powdery scab of potato is difficult due to the lack of effective chemical treatments (Harrison *et al.*, 1997; Merz *et al.*, 2004; Falloon 2008). Thus, integrated crop management with resistant cultivars, long crop rotations and pathogen-free seed may be the only feasible tools for the management of powdery scab (Elad *et al.*, 1980; Thurston 1990; Honeycutt *et al.*, 1996; Wale, 2004; Genet *et al.*, 2005; Iftikhar *et al.*, 2007; Falloon, 2008). Yet, potato cultivars are usually chosen by the grower for their marketability rather than on their ability to resist powdery scab (Harrison *et al.*, 1997; Merz & Falloon, 2009). Currently there are no potato cultivars with complete immunity to powdery scab, but different levels of resistance have been recorded (Iftikhar *et al.*, 2007; Falloon, 2008). Even cultivars that do not develop tuber symptoms can still support the multiplication and survival of *Sss* through root infections (Merz *et al.*, 2004; Merz & Falloon, 2009; Houser & Davidson, 2010).

The aim of this experiment was to determine the susceptibility to infection by *Sss* of six potato cultivars that are commonly grown in South Africa. Visual assessment using a disease severity key and conventional PCR to confirm *Sss* infection were used to rate cultivar susceptibility.

6.2 Materials and Methods

6.2.1 Potato Cultivars

Six potato cultivars that are planted in South Africa were used in this experiment, namely Argos, BP1, Buffelspoort, Caren, Up-to-Date (UTD) and Valor. Seed tubers were inspected visually for the presence of powdery scab to ensure that pathogen-free seed tubers were used.

6.2.2 Inoculum Preparation

Scabby tubers (cv. Mondial) were collected from the potato growing area of the Sandveld,

Western Cape and subjected to an ELISA test. Tubers that tested positive for powdery scab were used for inoculum preparation. Powdery scab lesions were cut from infected tubers using a sterile scalpel and were ground using a pestle and mortar. The powdery mass was passed through a series of mesh sieves (200, 100, 75 and 53 µm), then suspended in 10 ml of sterile distilled water and centrifuged at 4000 *g* for one minute. The supernatant was discarded and the pellet was resuspended in 10 ml sterile distilled water and centrifuged at 4000 *g* for 1 minute. This process was repeated five times. The final pellet was suspended in 5 ml sterile distilled water. Inoculum concentrations of 5, 25 and 50 sporeballs per gram of sterile sand were estimated using a haemocytometer. A negative control of 0 sporeballs per gram of soil was included in the experiment.

6.2.3 Pot Trial

72 pots (six potato cultivars with three replicates per treatment) were each filled with 4 kg of steam pasteurised sandy loam soil and the experiment was repeated twice. The different inoculum concentrations resulted in 0, 5, 25 and 50 sporeballs per gram of soil in the pot. Once the inoculum was added, the seed tuber was placed in the pot and covered with soil. The plants were grown in a greenhouse where the temperature was maintained at 16°C and each plant received 200 ml of sterile water every second day to maintain cool and damp soil conditions favourable for powdery scab disease development. The potato plants were harvested four months after emergence. Potato roots and tubers were carefully removed from each pot. Roots and tubers were washed with distilled water and allowed to air dry. Once dry the roots were assessed for the presence of root galls and the galls were rated according to van de Graaf *et al.* (2007), where 0 = no root galls, 1 = one or two root galls, 2 = several galls, mostly small (< 2 mm in diameter), 3 = many galls, some > 2 mm in diameter, and 4 = most major roots with galls, some or all > 4 mm in diameter. Tuber disease severity was rated according to Falloon *et al.* (1995) (Figure 6.1). To confirm that the symptoms observed were caused by *Sss* infection, DNA was extracted from the all root on which root galls were observed as well as all tubers resembling powdery scab lesions and subjected to PCR using *Sss* specific-primers.

6.2.4 DNA Extraction and PCR Conditions

DNA was extracted from each macerated root gall and tuber lesion sample using the ZR Soil Microbe DNA kit™ (Zymo Research Corp, USA).

PCR was carried out in a final volume of 25 µl which included 0,5 µl of dNTPs (250 µM of each dATP, dTTP, dGTP and dCTP) (Bioline, London, UK), 2.5 µl PCR reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL at pH 8.8) (Bioline), 0.25 µl *Taq* polymerase (1 U) (Bioline), 1.25 µl $MgCl_2$ (2.5 mM) (Bioline), and 0.25 µl (0.5 µM) *Spongospora subterranea*-specific primers, *Sps*1 (5'-CCT GGG TGC GAT TGT CTG TT-3') and *Sps*2 (5'-CAC GCC AAT GGT TAG AGA CG-3') (Bulman & Marshall, 1998). Five µl of DNA template was added to each reaction.

A thermocycler and 200 µl thin walled PCR tubes were used in the PCR process. PCR conditions included: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of melting (95°C for 20 seconds), annealing (64°C for 25 seconds) and extension (72°C for 50 seconds) with a final cycle of 72°C for 10 min. Amplified DNA, as well as a positive and negative control stained with ethidium bromide and were subjected to electrophoresis on 1% agarose gel alongside standard size markers (HyperLadder II, Bioline, London, UK). The gels were viewed under ultraviolet illumination.

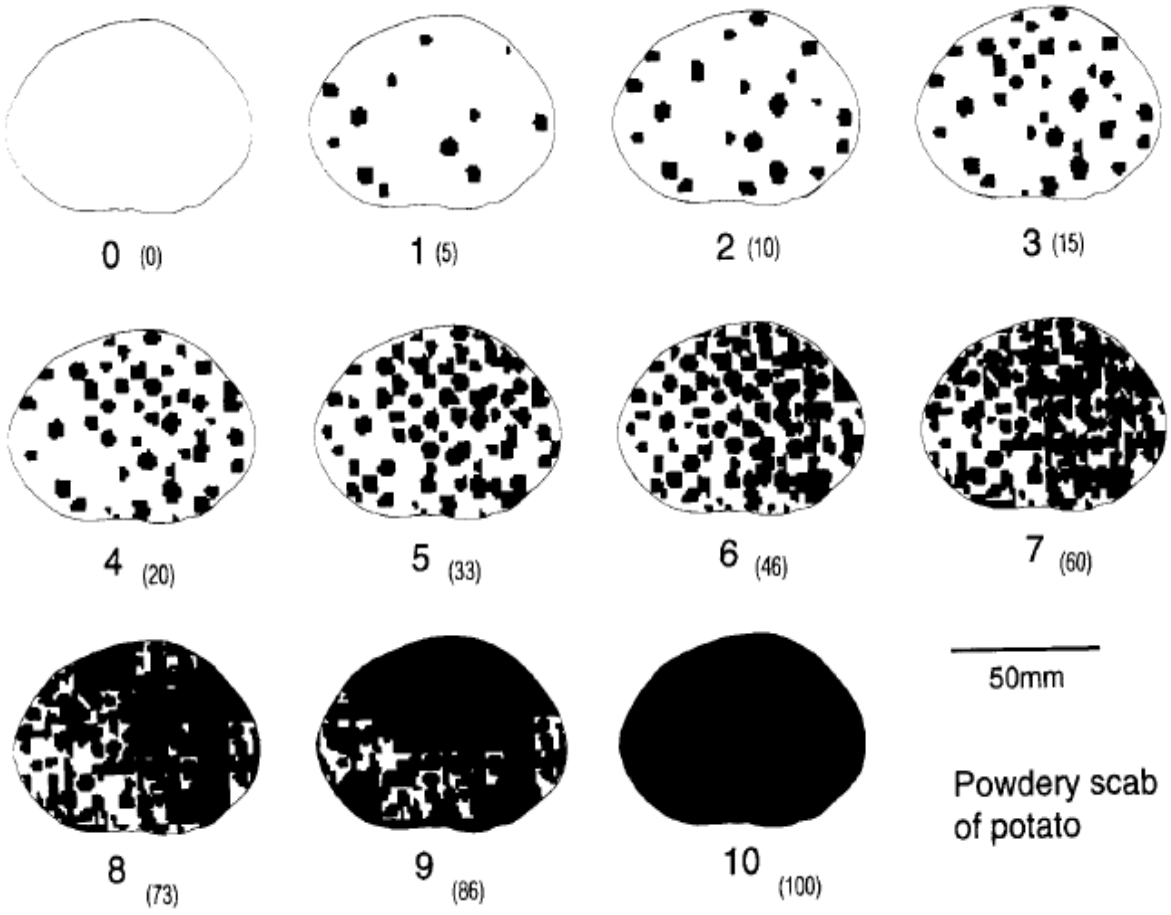

Figure 6.1: Disease severity assessment key for powdery scab of potato (Falloon *et al.*, 1995).

6.2.5 Statistical Analysis

Statistical analysis of the data from the root galls and tuber lesions was performed using the Kruskal-Wallis non-parametric ranking test and multiple comparisons were done using the Mann-Whitney tests ($P \leq 0.05$). The ranked root galls and tuber lesions data were compared using Spearman's ranking correlation coefficient (r_s).

6.3 Results

At harvest root galls and tuber lesions were observed on all the cultivars. There seemed to be no differences between the different inoculum concentrations, except for the control (0 *Sss* sporeballs per gram of soil), where no symptoms were observed. PCR results indicated that roots and tubers with powdery scab symptoms were caused by *Sss* (Figure 6.2). No cultivar was immune to the pathogen.

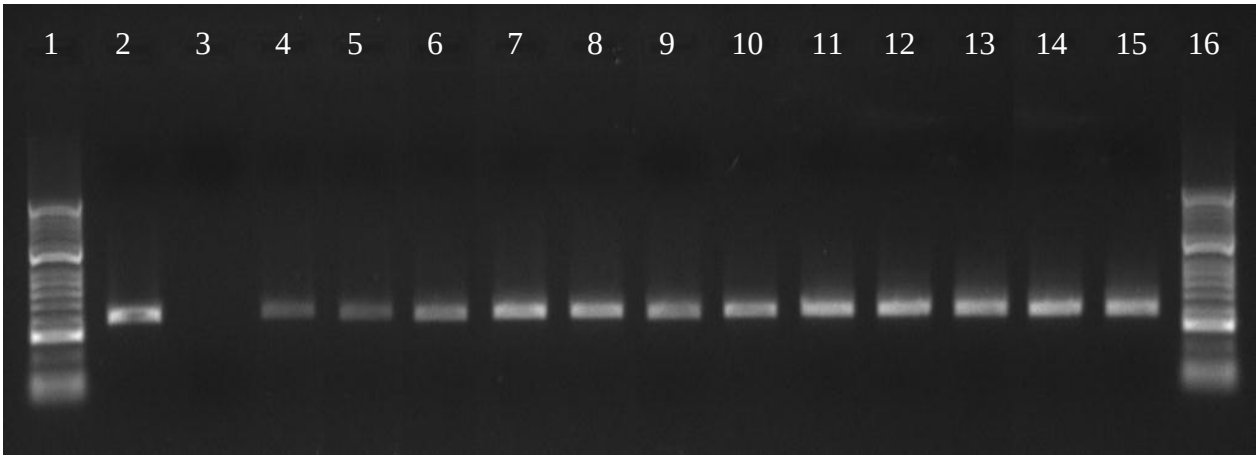

Figure 6.2: EtBr-stained agarose gel of PCR products (using *Spongospora subterranea*-specific primers) obtained from DNA extracted from five root galls and five tuber lesions which were selected randomly (Lane 1: Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4: Root gall (Argos), Lane 5: Root gall (Caren), Lane 6: Root gall (BP1), Lane 7: Root gall (Buffelspoort), Lane 8: Root gall (UTD), Lane 9: Root gall (Valor), Lane 10: Tuber lesion (Argos), Lane 11: Tuber lesion (Caren), Lane 12: Tuber lesion (BP1), Lane 13: Tuber lesion (Buffelspoort), Lane 14: Tuber lesion (UTD), Lane 15: Tuber lesion (Valor) and Lane 16: Ladder).

The washed roots were assessed for the incidence and severity of root galls. The statistical analysis of the root gall data showed no significant difference in average root infection between the different cultivars (Figure 6.3A). The washed tubers were assessed for severity

of powdery scab lesions. Statistical analysis of this showed no significant differences between the six cultivars (Figure 6.3B).

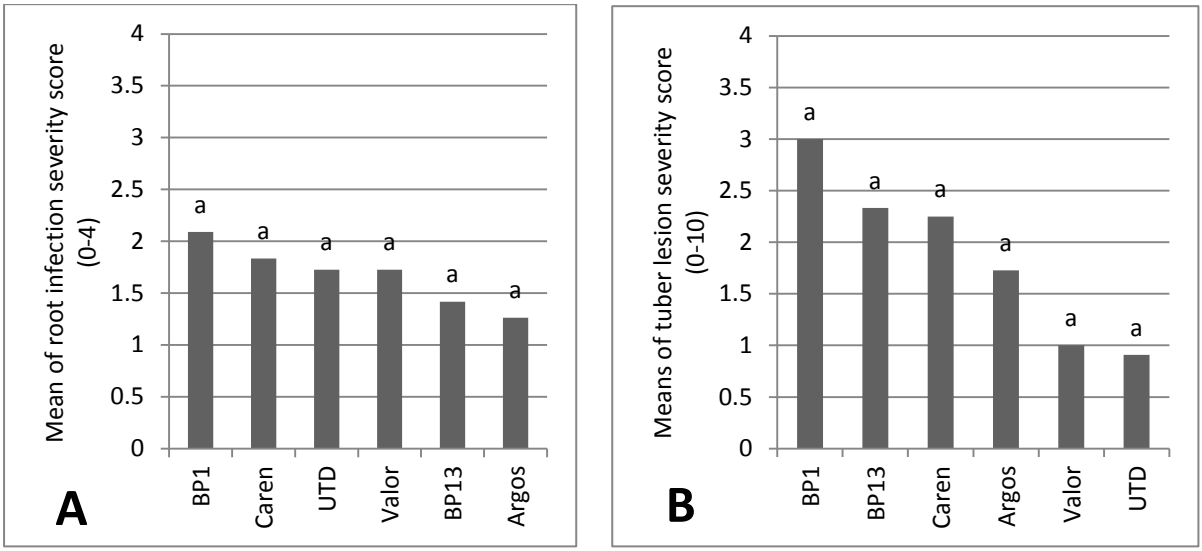

Figure 6.3: The means from both pot trials were statistically analysed. A) Mean root gall severity and B) Mean tuber lesion severity ratings of six cultivars infected by *Sss*. Means accompanied by the same letters are not significantly different at $P \leq 0.05$ (Mann-Whitney test).

The Spearman's rank correlation coefficient (r_s) is a measure of statistical dependence between two variables. The Spearman's rank correlation coefficient between root gall severity and lesion severity ratings for all cultivars was significant (Table 6.1), as the r_s values are between 0.5 and 1. The closer the r_s value is to 1, the stronger the positive correlation will be.

Table 6.1: Spearman's rank correlation coefficient between root gall severity and tuber lesion severity ratings of the six South African potato cultivars

Cultivar	**r_s value**	**P value**	**Degrees of freedom**
Buffelspoort	0.659	< 0.001	22
Up-to-date	0.689	< 0.001	20
Argos	0.790	< 0.001	22
Valor	0.843	< 0.001	21
BP1	0.848	< 0.001	21
Caren	0.859	< 0.001	22

6.4 Discussion and Conclusion

This experiment aimed to compare the levels of susceptibility of six cultivars that are grown in South Africa, Argos, BP1, Buffelspoort, Caren, UTD and Valor. According to de Boer (1991) and Houser & Davidson (2010) resistant cultivars offer one of the most reliable means of powdery scab control.

To limit the effect of variation in soil temperature during the experiment, all the plants were grown in the same soil mix with constant moisture in a greenhouse where the temperature was maintained at 16˚C throughout the experiment. According to Baldwin *et al.* (2008) these environmental conditions are the most conducive to disease development and will thus give more accurate results than traditional field trials.

All root galls and tuber lesions tested positive for the presence of *Sss*, as confirmed by conventional PCR. As all the cultivars had root galls and tuber lesions it can be concluded that none of the tested cultivars are resistant to infection by *Sss*.

According to Kirkham (1986), Genet *et al.* (2005), Baldwin *et al.* (2008) and Houser & Davidson (2010) different cultivars have a wide range of reactions to powdery scab. In this experiment when the root gall ratings and tuber lesion ratings were subjected to statistical analysis, no significant differences between the reactions of the cultivars to powdery scab were observed. These results concur with those of Iftikhar *et al.* (2007) who noted that although there may appear to be visual differences in disease severity between varieties, statistical analysis of results often shows no significant differences between varieties.

Varying initial inoculum concentrations used in this experiment did not result in significant differences in the final severity of disease. This was also observed by Baldwin *et al.* (2008) and Houser & Davidson (2010) who stated that an increase in the amount of initial inoculum has only minimal impact on powdery scab severity. According to the Spearman's rank correlation coefficient there was a positive correlation between the severity of root galls and the severity of tuber lesions. The correlation was highly significant for Argos, Valor, BP1 and Caren and the correlation was significant for Buffelspoort and UTD. This indicates that there is likely a relationship between root and tuber infections, as it is probable that the young tubers and root hair were infected simultaneously by primary zoospores which resulted in root galls and tuber lesions. Experiments done by Merz *et al.* (2004) showed that this

relationship was more evident when tuber lesion severity was compared with zoosporangial root infection instead of root gall infections.

This experiment shows that greenhouse trials can be used to screen cultivars for resistance to powdery scab. Further experiments for South African cultivars are needed to determine a set of reference cultivars relative to their tuber susceptibility to *Sss* to assist in standardizing this test, making this an even more valuable tool for future potato breeding in South Africa.

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CHAPTER 7

Alternate Hosts for *Spongospora subterranea* f.sp. *subterranea* in Crop Rotation Systems

Abstract

Spongospora subterranea f.sp. *subterranea* Tomlinson (*Sss*), the cause powdery of scab of potato only multiplies within living tissues of a susceptible host plant. An ideal trap plant for use in crop rotation systems is one in which the pathogen is unable to complete its life cycle, thus reducing the soil-borne inoculum. The aim of this study was to determine the host range of *Sss* in rotation crops in South African potato growing regions and identify possible trap crops for *Sss*. Five crops: cabbage (cv. Copenhagen market), mustard (cvs. Florida Broadleaf and Southern Giant Curled), soybean (cv. PAN737R), wheat (cv. SST882) and tomato (cv. Rodade) were selected. 48 seeds per crop were germinated in vermiculite. Seedlings were transferred to containers filled with Merz's nutrient solution and inoculated with 0 or 100 *Sss* sporeballs per ml. After 14 days seedling roots were microscopically examined for *Sss* plasmodia or zoosporangia. Seedlings were transferred to 2L pots containing soil in a greenhouse. Plants were harvested after four months and the roots were examined for galls and sporeballs. The dry weight of each plant was recorded. Results showed that *Sss* zoospores infected cabbage, both mustard cultivars, wheat and tomato 14 days after inoculation. Soybean was not a host for *Sss*. The galls containing sporeballs in the roots of tomato and both mustard cultivars indicate that the pathogen completes its life cycle in these hosts. Wheat and cabbage may be used as trap/decoy crops as the pathogen is unable to complete its life cycle within these hosts thus reducing *Sss* inoculum in the soil. *Spongospora subterranea* f. sp. *subterranea* infection did not significantly influence the dry weight of mustard (cv. Southern Giant Curled), wheat or cabbage. The results of this experiment are useful in the selection of rotation crops for the management of powdery scab of potato.

7.1 Introduction

Spongospora subterranea (Wallroth) Lagerheim f.sp. *subterranea* Tomlinson (*Sss*) causes powdery scab of potatoes (*Solanum tuberosum* L.). This economically significant disease causes unsightly blemishes on tuber surfaces and reduces the quality and marketability of seed and ware tubers (Karling, 1968). *Spongospora subterranea* f. sp. *subterranea* is an

obligate parasite that can only multiply within living tissues of a susceptible host plant (Andersen *et al.,* 2002; Qu & Christ, 2006; Kirk, 2008). It survives for many years on tubers and in soil as extremely resistant thick-walled resting structures known as sporeballs or cystosori (Harrison *et al.,* 1997). Resting spores germinate in moist, cool soil (12 to 18˚C) giving rise to primary zoospores, which infect susceptible hosts (Andersen *et al.,* 2002; van de Graaf *et al.,* 2005; Qu & Christ, 2006; Nakayama *et al.,* 2007). The development of plasmodia and subsequently zoosporangia, gives rise to secondary zoospores that infect tubers, roots and stolons of potato plants and roots of other susceptible host species (van de Graaf *et al.,* 2005; Qu & Christ, 2006).

Control of powdery scab of potato is difficult due to the lack of resistant varieties or chemical treatments (Harrison *et al.,* 1997; Merz *et al.,* 2004; Falloon 2008). Currently the only reliable way to manage this disease is to plant pathogen-free seed tubers in uninfested soils. In the absence of effective control measures, integrated crop management with long crop rotations may be a feasible control method for powdery scab (Elad *et al.,* 1980; Thurston 1990; Honeycutt *et al.,* 1996; Katan, 2000; Qu & Christ, 2006). However, sporeballs of *Sss* may remain viable in soil for many years and also probably survive through infection cycles on alternate hosts (Qu & Christ, 2006; Falloon, 2008). Therefore, the normal crop rotation of three or four years may not be enough to reduce the viable *Sss* soil inoculum (Wale, 2004; Nakayama *et al.,* 2007). It is important to note that even low soil inoculum levels result in significant powdery scab infections when environmental conditions are favourable (Harrison *et al.,* 1997; van de Graaf *et al.,* 2007). Thus, trap or decoy crops in crop rotations could be used in the management of powdery scab.

The ideal trap plant will be one in which the pathogen is unable to complete its life cycle, thus reducing the soil-borne inoculum (Harrison *et al.,* 1997; Qu & Christ, 2006). Harrison *et al.* (1997) and Andersen *et al.* (2002) stated that *Sss* can infect many plant species, including weeds and crop plants. Nitzan *et al.* (2009) and Shah *et al.* (2010) noted that nightshade weeds (*Solanum* ssp.) are hosts of *Sss*. Qu & Christ (2006) identified yellow mustard, oat and tomato as hosts of *Sss* in which the pathogen was able to complete its life cycle and produce sporeballs; whereas rye and oilseed radish are hosts of *Sss* in which the pathogen does not complete its life cycle.

Various detection techniques, such as bioassays, ELISA, conventional polymerase chain reaction (PCR) and quantitative PCR (real-time PCR) are used to detect *Sss* from soil and various plant tissues, including root tissues of potatoes and other host species (Merz, 1989; Bell *et al.,* 1999; Merz e*t al.,* 2004; van de Graaf *et al.,* 2003). The aim of this study was to determine the host range of *Sss* in crop plants commonly rotated in South African potato growing areas using bioassays and conventional PCR techniques.

7.2 Materials and Methods

7.2.1 Inoculum Preparation

Powdery scab lesions were cut from infected tubers using a sterile scalpel and ground with a pestle and mortar. The fine mass was passed through a series of mesh sieves (200, 100, 75 and 53 µm). The sieved sporeballs were then suspended in 10 ml of half strength Merz's nutrient solution (Merz, 1989) without zinc, and centrifuged at 4000 *g* for one minute. The supernatant was discarded, the pellet resuspended in 10 ml half strength Merz's nutrient solution and centrifuged at 4000 *g* for 1 minute. This process was repeated five times. The final pellet was suspended in 5 ml half strength Merz's nutrient solution. The sporeball concentration was estimated using a haemocytometer to be 1000 sporeballs per ml.

7.2.2 Plant Materials and Inoculation

Five rotation crops commonly used in South African potato production were selected, viz. cabbage (cv. Copenhagen market), mustard (cvs. Florida Broadleaf [A] and Southern Giant Curled [B]), soybean (cv. PAN737R) and wheat (cv. SST882). Tomato (cv. Rodade) was used as a control as it is known from previous studies (Merz, 1989; Qu & Christ, 2006) to become infected by *Sss*, and to develop galls, plasmodia and zoosporangia. Due to the destructive nature of the sampling procedure, a minimum of 48 seeds per crop were germinated in vermiculite at 22˚C until the two-leaf stage, after which the seedlings were transferred to 60 ml plastic containers filled with 50 ml Merz's nutrient solution. The seedlings were placed in a growth chamber at 18˚C with 15 hours light and 9 hours dark for one week (Figure 7.1). After one week, half of the seedlings were inoculated with 6 ml of the sporeball solution (1 000 sporeballs per ml) and 6 ml Merz's nutrient solution was added to the remaining plants to serve as negative control (0 sporeballs per ml).

After 14 days seedling roots (three per treatment) were examined microscopically for *Sss* plasmodia or zoosporangia by staining the roots with 1% (w/v) cotton blue in lactophenol for

five minutes. DNA was extracted from six additional roots (three per treatment) and tested using a PCR assay for the presence of *Sss*. Remaining seedlings were transferred to 2 L pots containing steam sterilized soil and placed in a greenhouse for four months. The temperature in the greenhouse was maintained between 14-20˚C and plants were watered with 200 ml of sterile distilled water every second day.

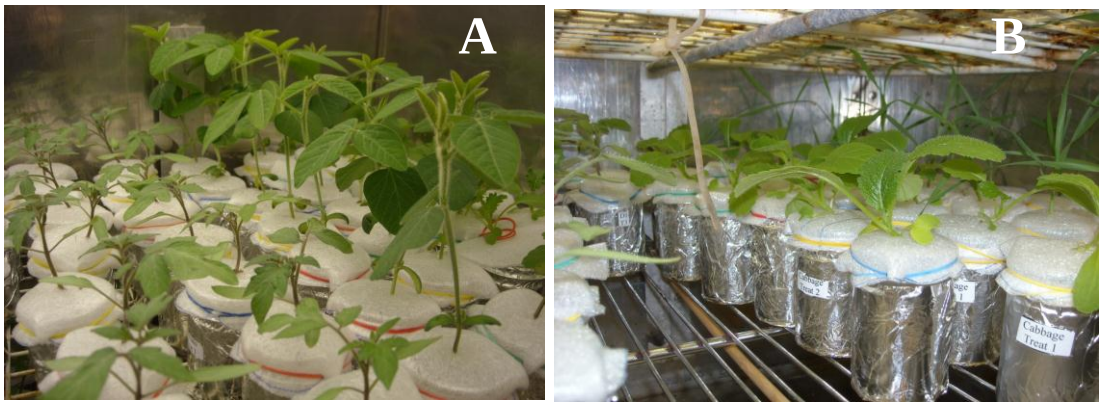

Figure 7.1: A) Tomato and soybean seedlings and B) cabbage and wheat seedlings in the growth chamber.

After four months the plants were harvested (plants were cut at soil level to separate roots and foliage), roots were washed carefully and all plant roots were examined for galls. Microscopic examination was done to determine whether plasmodia/sporeballs had developed. DNA was extracted from any roots with observed plasmodia/sporeballs as well as from root galls for confirmation of the presence of *Sss* using PCR. The plant material was then placed overnight in an oven to dry. The dry weight of each plant's roots and foliage was recorded.

7.2.3 DNA Extraction and PCR Conditions

DNA was extracted from each macerated root sample using the ZR Soil Microbe DNA kit™ (Zymo Research Corp, USA) according to the manufacturer's instructions.

PCR was performed in a final volume of 25 µl containing 0.5 µl of dNTPs (250 µM each of dATP, dTTP, dGTP and dCTP) (Bioline, London, UK), 2.5 µl PCR reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL at pH 8.8) (Bioline), 0.25 µl *Taq* polymerase (1 U) (Bioline), 1.25 µl $MgCl_2$ (2.5 mM) (Bioline), and 0.25 µl (0.5 µM) *Spongospora subterranea*-specific primers, *Sps*1 (5'-CCT GGG TGC GAT TGT CTG TT-3') and *Sps*2 (5'-CAC GCC AAT

GGT TAG AGA CG-3') (Bulman & Marshall, 1998). Five µl of DNA template was added to each reaction.

A thermocycler and 200 µl thin walled PCR tubes were used in the PCR process. The thermal profiles were: Initial denaturation at 95°C for 2 minutes, followed by 35 cycles of melting (95°C for 20 seconds), annealing (64°C for 25 seconds) and extension (72°C for 50 seconds) with a final cycle of 72°C for 10 min. Amplified DNA, as well as positive and negative controls were stained with ethidium bromide and subjected to electrophoresis on 1% agarose gel alongside standard size markers (HyperLadder II, Bioline, London, UK). The gels were viewed under ultraviolet illumination.

7.2.4 Statistical Analysis

Statistical analysis of the dry weight data for the roots and foliage of the different crop plants was performed using multiple t-test statistics (LSD test; $P \leq 0.05$) using SAS 9.2 (SAS Institute, Inc., Cary, North Carolina).

7.3 Results

When seedling roots were removed from the nutrient solution and examined microscopically for plasmodia and/or zoosporangia 14 days after inoculation, plasmodia were observed in the roots of cabbage, both mustard cultivars, wheat and tomato (Table 7.1). Zoosporangia were only observed in tomato roots (Figure 7.2). No plasmodia or zoosporangia had developed in the roots of soybean. PCR results confirmed that the observed plasmodia and zoosporangia were those of *Sss*. Figure 7.3 shows the results of one of three PCRs performed.

Observations at harvest showed that galls had developed on roots of tomato and both mustard cultivars (Figure 7.4). Microscopic examination revealed that zoosporangia were also present in the roots of tomato and both mustard cultivars, whereas the roots of cabbage and wheat contained only *Sss* plasmodia. Dissection of the galls showed the presence of sporeballs in tomato and both mustard cultivars. PCR specific-primers confirmed that the observed plasmodia, zoosporangia and sporeballs were *Sss*. This indicates that *Sss* is able to complete its life cycle in tomato and mustard hosts, but not in cabbage or wheat.

Table 7.1: Number of seedling roots where Plasmodia or Zoosporangia were observed 14 days after being inoculated with *Sss*

Crop	**Plasmodia**	**Zoosporangia**
Cabbage (cv. Copenhagen market)	5/6	0/6
Mustard (cv. Florida Broadleaf)	5/6	0/6
Mustard (cv. Southern Giant Curled)	4/6	0/6
Soybean (cv. PAN737R)	0/6	0/6
Wheat (cv. SST882)	3/6	0/6
Tomato (cv. Rodade)	6/6	3/6

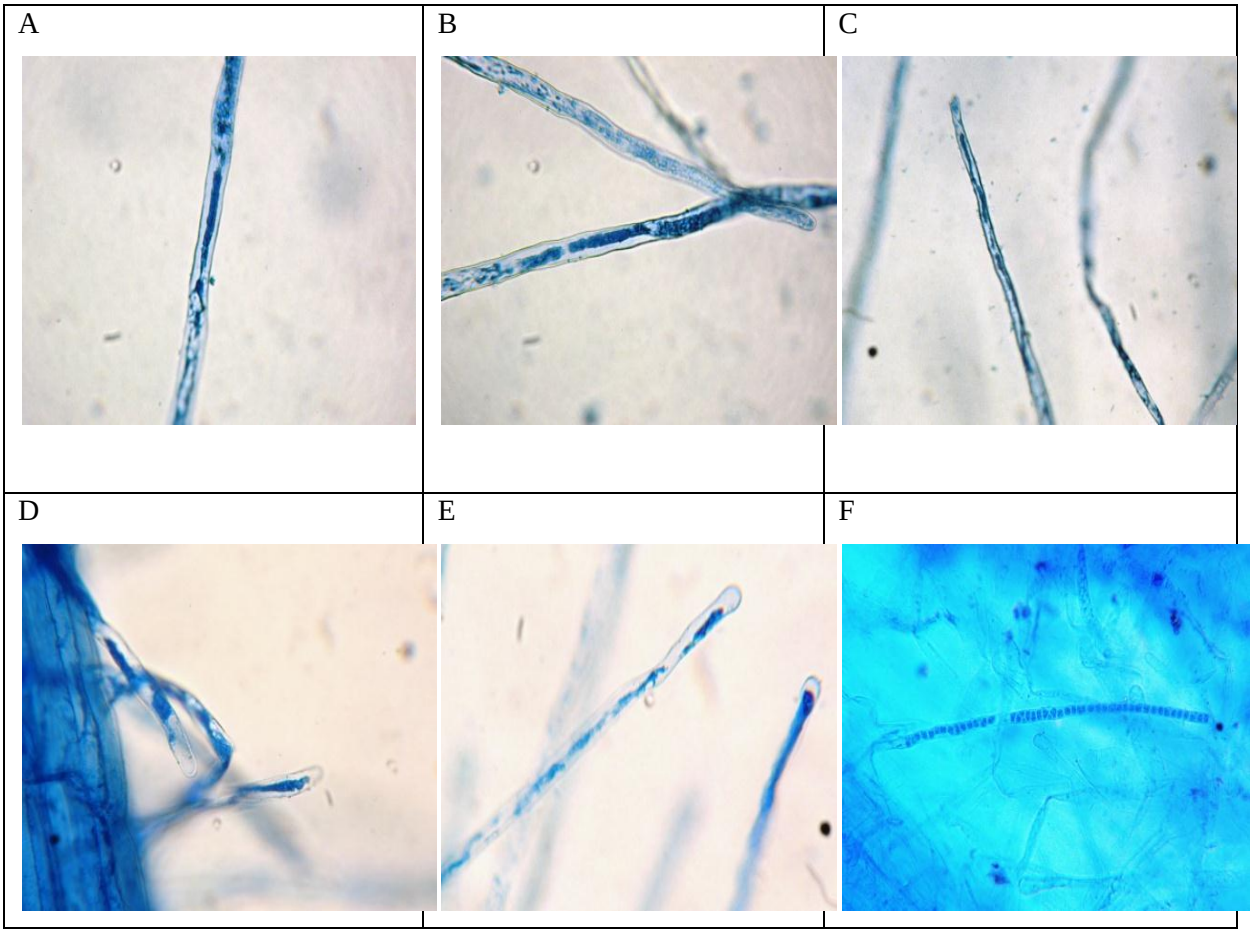

Figure 7.2: Plasmodia in cabbage (A), mustard A (B), mustard B (C), wheat (D) and tomato (E) and zoosporangia in tomato (F) seedling roots.

The effect of *Sss* infection on the growth of each plant was evaluated by comparing the dry weight of the infected and uninfected plants. There were significant differences in the dry

weight of tomato, soybean, mustard A, mustard B and wheat roots between the two treatments (Figure 7.5). Significant differences in dry weight of foliage were, however, only observed in tomato, soybean and mustard A plants (Figure 7.6). This indicates that *Sss* infection is not likely to influence the yield of mustard B, wheat or cabbage.

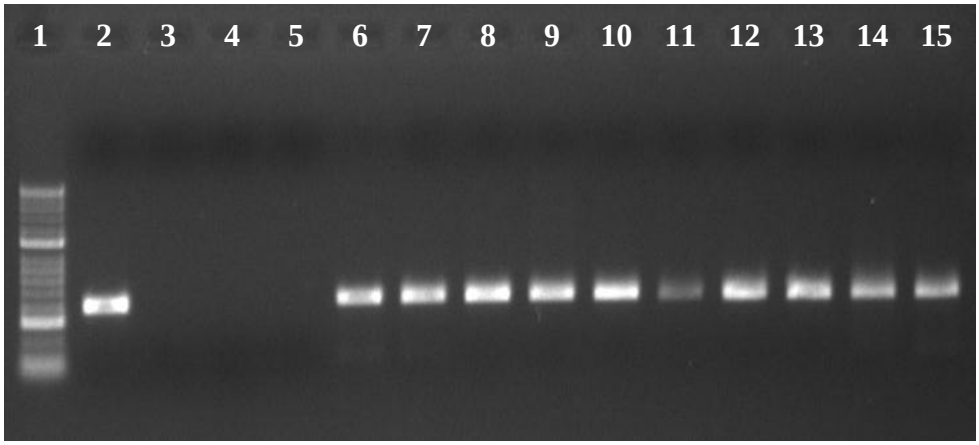

Figure 7.3: EtBr-stained agarose gel of PCR products (amplified using *Spongospora subterranea*-specific primers) obtained from DNA extracted from the roots of six crop roots 14 days after inoculation with 100 *Sss* sporeballs per ml (Lane 1: Ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4: Soybean 1, Lane 5: Soybean 2, Lane 6: Wheat 1, Lane 7: Wheat 2, Lane 8: Mustard A 1, Lane 9: Mustard A 2, Lane 10: Mustard B 1, Lane 11: Mustard B 2, Lane 12: Cabbage 1, Lane 13: Cabbage 2, Lane 14: Tomato 1, Lane 15: Tomato 2).

Figure 7.4: *Spongospora subterranea* root galls formed on the roots of a tomato seedling.

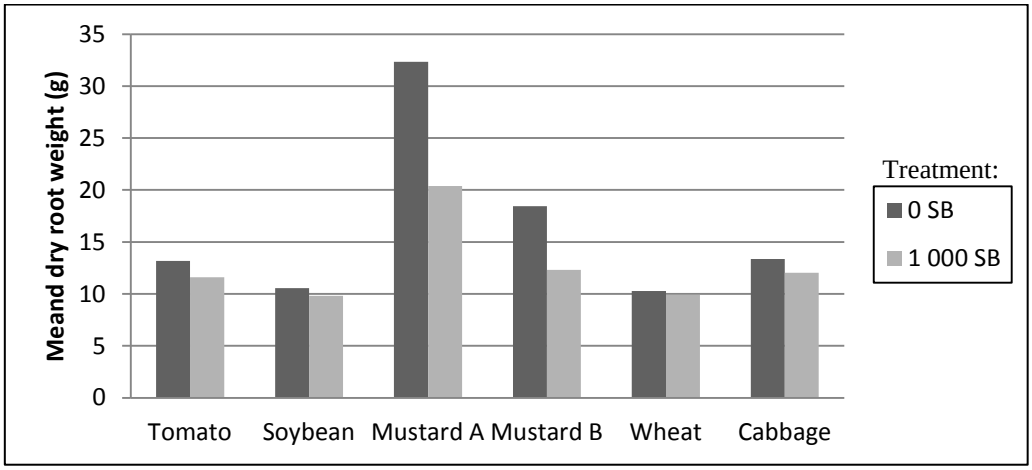

Figure 7.5: Mean dry root weights (g) of Tomato, Soybean Mustard A and B, Wheat and Cabbage. Means accompanied by the same letters are not significantly different at $P \leq 0.05$ (t-test).

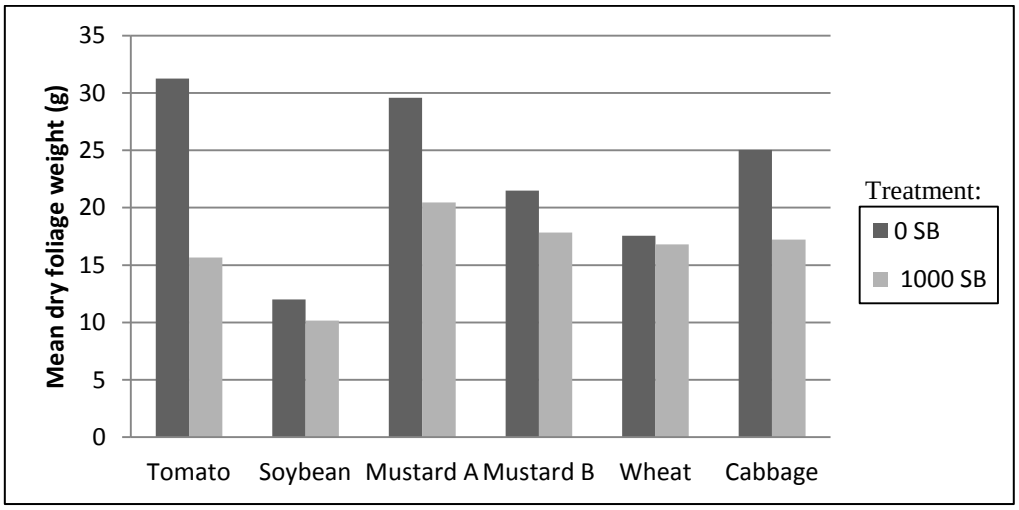

Figure 7.6: Mean dry foliage weights (g) of Tomato, Soybean Mustard A and B, Wheat and Cabbage. Means accompanied by the same letters are not significantly different at $P \leq 0.05$ (t-test).

7.4 Discussion and Conclusion

Crops commonly rotated with potatoes in South Africa were evaluated to determine their status as hosts of *Sss.* Results showed that primary zoospores of *Sss* were able to infect roots of cabbage, mustard A and B, wheat and tomato 14 days post inoculation. These results are similar to those of van de Graaf *et al.* (2000), who showed that infection of tomato roots occurs six days after inoculation, and Merz (1989), who stated that zoosporangia developed in tomato roots five days after infection had occurred.

Selecting a crop plant as a trap or decoy plant for the management of powdery scab, one should ensure that the pathogen is unable to complete is life cycle in that host (Harrison *et al.*, 1997; Qu & Christ, 2006). According to Shah *et al.* (2010) hosts in which *Sss* completes its life cycle will probably allow zoospore proliferation from zoosporangia in roots and thus development of sporeballs. Nitzan *et al.* (2009) and Shah *et al.* (2010) noted that nightshade weeds (*Solanum* ssp.) are hosts of *Sss* and that the pathogen is able to complete its life cycle in these weeds. Qu & Christ (2006) identified yellow mustard, oat and tomato as hosts of *Sss* in which the pathogen is able to complete its life cycle; whereas rye and oilseed radish are hosts of *Sss* where the pathogen does not complete its life cycle.

This study determined that soybean is not a host for *Sss*, whereas cabbage, mustard A and B, wheat and tomato are *Sss* hosts. The galls containing sporeballs present on the roots of tomato, mustard A and mustard B, indicate that the pathogen completes its life cycle within these hosts. Thus, these crops should be avoided when planting in fields contaminated with *Sss* as they may contribute to an increase in *Sss* inoculum. Honeycutt *et al.* (1996) states that crop rotation can be an effective mechanism for reducing disease incidence in a field. Wheat and cabbage may be used as trap/decoy crops as the pathogen is unable to complete its life cycle within these hosts, thus reducing the amount of *Sss* inoculum in the field.

When the dry weight of inoculated (1 000 sporeballs per ml) and control plants (0 sporeballs per ml) was statistically analysed, significant differences in the root dry weight of tomato, mustard A and B and wheat were observed. Foliage dry weight of tomato and mustard A plants also show statistical differences. Merz & Falloon (2009) state that when compared with uninfected plants, those with *Sss*-infected roots used less water, had disrupted nutrient uptake and produced less dry matter, smaller shoots and fewer leaves. This study showed that *Sss* infection does not significantly affect the foliage dry weight of mustard B, wheat or cabbage. These crops can thus be used as economic rotation crops in potato production regions. The results of this experiment are useful for the selection of rotation crops for the management of powdery scab of potato.

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CHAPTER 8

Detection and Eradication of *Spongospora subterranea* in Mini-Tuber Production Tunnels

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Wright J, Lees AK, van der Waals JE, 2012. Detection and eradication of *Spongospora subterranea* in mini-tuber production tunnels. *South African Journal of Science* **108(5/6),** 1-4.

Abstract

Powdery scab, a root and tuber disease caused by the pathogen *Spongospora subterranea* f.sp. *subterranea* (*Sss*), poses a major problem to potato producers worldwide because it affects potato quality. Inoculum can be seed-borne or originate from contaminated growing media or contaminated equipment. During 2006, a potato mini-tuber production facility in Ceres in the Western Cape Province of South Africa had an outbreak of powdery scab. The purpose of this study was to detect *Sss* in the production facility and identify the source or sources of contamination so that corrective measures could be taken to eradicate the pathogen. Swab samples were taken from numerous points in the facility in 2009 and *Sss*-specific primers (*Sps1* and *Sps2*) were used in a polymerase chain reaction to detect *Sss*. Of 11 surfaces tested, 6 were positive for *Sss*. A second set of swab samples was taken after efforts were made to eradicate the pathogen through improved facility hygiene measures to determine whether these corrective measures were efficient. Corrective measures resulted in a disease-free harvest from 2009 onwards. This novel study has value for the mini-tuber industry as production tunnels can be tested for the presence of *Sss* and other pathogens before planting to ensure that, where suitable control measures are available, disease-free mini-tubers are produced.

8.1 Introduction

Potato (*Solanum tuberosum* L.) is one of the most important food crops, both in developed and developing countries. Over the past two decades, potato production has more than doubled in developing countries (Schulz, *et al.*, 1998; Donnelly, *et al.*, 2003; Badoni & Chauhan, 2009) Thus, there is an increasing demand for seed tubers that are true-to-type,

disease-free and high yielding (Ebbels, 1983; Kotkas & Rosenberg, 1999; Rolot & Seutin, 1999). In potato seed-tuber production, each cycle of newly produced tubers are progeny of plants that developed from previously planted seed-tubers. These are commonly known as potato seed-tuber generations. As potato seed-tubers are commercially produced in the field, each generation accumulates, and further transmits viral, fungal or bacteriological disease-causing agents to the next generation. Hence, as the age of the generations increases, the plant's production potential tends to decline (Schulz, *et al.*, 1998). To reduce this problem growers use tissue culture multiplication, also known as mini-tuber technology (Struik, 2007; Badoni & Chauhan, 2009). In this system, potato seed tubers are first multiplied *in vitro* via nodal cuttings in tissue culture and then in the field giving rise to true-to-type and disease-free plant materials (Struik, 2007; Badoni & Chauhan, 2009).

A disease of major concern to potato producers globally, including in South Africa, is powdery scab. This disease is caused by the obligate, plasmodiophorid pathogen *Spongospora subterranea* (Wallroth) Lagerheim f.sp. *subterranea* Tomlinson (*Sss*) (Braselton, 1995). The most common visible symptoms of powdery scab on tubers include purple-brown pimple-like swellings or necrotic spots, usually first observed at the rose end of the tubers, and the development of root galls (Cook, 1932; Christ & Weidner, 1988; Harrison, *et al.*, 1997). As the swellings increase in size, the epidermis ruptures (Genet, *et al.*, 2005). Mature lesions become hollow and filled with a powdery mass of sporosori (aggregates of resting spores), which can survive in growing media and soil for many years (Christ & Weidner, 1988; Merz, *et al.*, 2005). When conditions are favourable, (van de Graaf, *et al.*, 2005) that is, when temperature is between 12 °C and 17 °C and free water is present, the resting spores (within the sporosori) release zoospores, which infect new host tissues (Harrison, *et al.*, 1997). Powdery scab diminishes potato quality and marketability, which results in significant economic losses to growers (Jeger, *et al.*, 1996; Bulman & Marshall, 1998; van de Graaf, *et al.*, 2005; Nakayama, *et al.*, 2007).

Infected seed tubers play an important role in the dissemination of *Sss* to areas where the disease was not previously present (Tsror *et al.*, 1999). Sporosori of the scab pathogen can, however, also be transmitted in infested growing media and/or on contaminated farm equipment (Merz, 1993; Muro, *et al.*, 1997; Iftikhar, *et al.*, 2000). Thus, the most reliable management strategy for controlling powdery scab is to ensure the use of only pathogen-free

seed tubers, the use of pathogen-free growing media and strict farm and production area hygiene (Harrison, *et al.*, 1997).

A number of techniques are currently used for the detection and quantification of *Sss*, including enzyme-linked immunosorbent assay (ELISA) systems, bioassays, conventional polymerase chain reaction (PCR) techniques and real-time PCR techniques (van de Graaf, *et al.*, 2003; Falloon, 2008). PCR can be used to detect *Sss* in infected potato roots and tubers, in infected symptomless host plant roots and in infested field soils as this technique is highly specific, relatively fast and reliable (Bulman & Marshall, 1998; McCartney, *et al.*, 2003; van de Graaf, *et al.*, 2003; Nakayama, *et al.*, 2007). PCR is of immense value in powdery scab disease management because it can be used to identify sources of powdery scab contamination.

Starting in 2006, a potato mini-tuber production facility in Ceres (Western Cape Province, South Africa) experienced a series of outbreaks of powdery scab. These outbreaks were the first report of powdery scab in this facility and no other diseases had previously been recorded from this facility. Several possible sources of inoculum were considered at the time, including contamination from the surrounding environment, the source of plant material used, water, workers and the growing media. Potatoes or other potential *Sss* hosts are not produced near the mini-tuber facility, nor have they been produced in the area in the past. The only agriculture that occurs within the surrounding area is fruit production. The fruit is predominantly citrus and fertilised with organic fertiliser and not compost. No animal farms are located in the immediate vicinity of the mini-tuber production facility.

Despite attempts at removing possible sources of pathogen contamination, by changing the growth medium, using chemically treated borehole water, placing copper sulphate foot baths at the entrance to the tunnels, installing new drippers and using new crates, powdery scab outbreaks continued in the following two years. Before planting in 2009, the management personnel of the tunnels requested help in determining the source or sources of powdery scab inoculum. The aim of this study was thus to trace the presence of *Spongospora subterranea* f.sp. *subterranea* in the mini-tuber production tunnel and identify potential sources of contamination, so that corrective measures could be taken to eradicate the pathogen.

8.2 Materials and Methods

8.2.1 Sampling

Two sets of swab samples were taken from the potato production facility in Ceres. The first set of sampling was done in August 2009 and the second set in October 2009, after various measures were taken to eradicate the pathogen, based on the findings of the first sampling.

A number of swab samples (Transwab©, Medical Wire and Equipment, Corsham, Wiltshire, England) were taken from various surfaces throughout the production facility in Ceres. These surfaces included the concrete floor of the tunnels, the wooden bridge over the run-off channel, the run-off channel, water troughs, drain pipes, shade net, wet wall, the tunnel frame, the entrance floor to the tunnel, the outside wash bath and cleaned crates. Swab samples were transported in a cooler box and processed the following day.

8.2.2 Sample Analysis

DNA was extracted from each of the swab samples using the ZR Soil Microbe DNA kit™ (Zymo Research Corporation, Irvine, CA, USA). PCR was carried out in a final volume of 25 µL containing 0.5 µL of dNTPs (250 µM of each dATP, dTTP, dGTP and dCTP) (Bioline, London, UK), 2.5 µL PCR reaction buffer (16 mM (NH4)2SO4, 67 mM Tris-HCL at pH 8.8) (Bioline), 0.25 µL *Taq* polymerase (1 U) (Bioline), 1.25 µL MgCl2 (2.5 mM) (Bioline) and 0.25 µL (0.5 µM) *Spongospora subterranea* specific primers, *Sps*1 (5'-CCT GGG TGC GAT TGT CTG TT-3') and *Sps*2 (5'-CAC GCC AAT GGT TAG AGA CG-3'), (Bulman & Marshall, 1998) which were designed to yield an amplification product of 391 base pairs. DNA template (5 µL) was added to each reaction.

A thermocycler and 200-µL thin-walled PCR tubes were used in the PCR process. The thermal profiles were initial denaturation at 95 °C for 2 min, followed by 35 cycles of melting (95 °C for 20 s), annealing (64 °C for 25 s) and extension (72 °C for 50 s) with a final cycle at 72 °C for 10 min. Amplified DNA, as well as positive and negative controls, were subjected to electrophoresis on 1% agarose gel, stained with ethidium bromide and run alongside standard size markers (HyperLadder II, Bioline). The results were viewed under ultraviolet illumination. PCR reactions were repeated twice.

8.3 Results

8.3.1 Sampling

A total of 40 swab samples were taken during August 2009. After various measures were taken to eradicate the pathogen, a second set of 40 swab samples were taken during October 2009. Both sets of samples were collected from the same sites (Table 8.1).

8.3.2 Sample Analysis

Of the 11 surfaces sampled in August, 2009, 6 tested positive for *Sss*. These six were the tunnel floor, the run-off channel, water troughs, drain pipes, the entrance floor and cleaned crates (Table 8.1).

Based on our findings from the first sampling, corrective measures were taken prior to the 2009 production period to eradicate the inoculum. These measures included the laying of a new concrete floor, new drippers, new pipes, use of new growing medium, new crates and sterilisation of all equipment and surfaces, including the outside water bath.

Table 8.1: Presence of *Spongospora subterranea* on swab samples taken in mini-tuber production tunnels before corrective measures were taken

Sample taken from	Number of samples taken	Samples positive for *S. subterranea*
Tunnel floor	10	4
Wooden bridge	1	0
Run-off channel	3	3
Water troughs	6	1
Drain pipes	6	1
Shade net	2	0
Wet wall	3	0
Tunnel frame	1	0
Entrance floor	2	1
Outside water bath	3	0
Cleaned crates	3	1

PCR analysis of the swab samples taken after the corrective measures were carried out indicated that *Sss* inoculum had been eradicated from the production tunnel.

8.4 Discussion

The occurrence of powdery scab in this South African mini-tuber production facility was of

great concern as the planting material used in this mini-tuber production facility was from a certified laboratory and good phytosanitary measures were followed, indicating that the *Sss* contamination was most likely introduced through the imported coconut peat. Coconut peat is organic in origin, which increases the possibility that it may harbour pathogens (Rolot & Seutin, 1999). Hanafi (2003) recently found that the sanitary quality of coconut peat is lower than previously presumed, because of its organic origin. Rolot and Seutin (1999) and Hanafi (2003) observed common scab (*Streptomyces scabies* (Thaxter) Lambert & Loria), powdery scab (*Spongospora subterranea*) and even soft rots (*Pectobacterium* and *Dickeya* spp.) in mini-tuber production facilities as a result of contaminated organic growing media. Supporting the hypothesis that *Sss* contamination was most likely introduced through the imported coconut peat is the fact that no previous outbreaks of powdery scab had ever been reported at this facility until the use of coconut peat. However, after the outbreak of powdery scab in 2006, the mini-tuber facility destroyed the coconut peat, so PCR tests could not be conducted on the peat to conclusively prove that it was infested with *Sss* sporosori.

Ideal conditions for powdery scab development include high moisture and low temperature (12 °C – 17 °C) (Ebbels, 1983; Harrison, *et al.,* 1997; van de Graaf, *et al.,* 2003). During the production of mini-tubers the temperature in the tunnels is maintained at 15 °C – 18 °C, (Donnelly, *et al.,* 2003) thus favouring the development of powdery scab. As a result of the polycyclic nature of powdery scab, (Merz, *et al.,* 2004; Merz, 2008; Nitzan, *et al.,* 2009) zoospores continue infecting roots and new zoosporangia develop in roots until the environmental conditions are no longer favourable (Harrison, *et al.,* 1997; Nitzan, *et al.,* 2009).

The results of this study confirm that the sporosori of *Sss* were able to survive in the tunnel in the absence of a host, from one season to the next (van de Graaf, *et al.,* 2005). The sporosori that remained in the run-off channels, water troughs and the drain pipes were thus the most likely sources of primary inoculum that led to the re-occurrence of disease in the 2007 and 2008 seasons following the use of coconut peat as a growing medium. Corrective measures taken to eliminate the pathogen from the mini-tuber production tunnel resulted in a disease-free harvest from 2009 onwards.

This is a novel study that has significant potential for the potato industry, particularly the mini-tuber industry, as surfaces and equipment in production tunnels can be tested for the presence of *Sss* before planting, allowing ample time for the application of corrective

measures if and where necessary. Producers should nonetheless ensure that growing material is pathogen free to prevent introduction of the powdery scab pathogen into tunnels. This study is the first in which swabs have been used for the detection of *Sss*; this method can be adapted and used to detect other potato pathogens in tunnels before planting to ensure that disease-free mini-tubers are produced and sold to specialist growers for later generation of high-quality seed tubers in the field.

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CHAPTER 9

General Discussion/Summary

Potato (*Solanum tuberosum* L.) is one of the most important food crops throughout the world and potato production has more than doubled over the past two decades in developing countries (Schulz *et al.*, 1998; Donnelly *et al.*, 2003; Badoni & Chauhan, 2009). Powdery scab of potato caused by *Spongospora subterranea* (Wallroth) Lagerheim f.sp. *subterranea* Tomlinson (*Sss*) is becoming a major problem for fresh and seed-tuber producers globally, due to the negative effects it poses on tuber quality and marketability. The incidence of powdery scab and its severity varies by season as well as field to field. There is currently a lack of complete information on the epidemiology and control of powdery scab. The research presented in this thesis will in part contribute to the current knowledge and understanding of powdery scab.

The main objectives of this research were to detect and quantify *Sss* from tuber lesions, infested water and soil (Chapter 3), identify the specific Group Type/s of *Sss* found in South Africa (Chapter 4), investigate the dormancy of *Sss* sporeballs (Chapter 5), evaluate South African potato cultivars for their susceptibility to powdery scab (Chapter 6) and determine the host range of *Sss* in crops that are commonly planted in rotation with potatoes in South Africa (Chapter 7). A case study was also conducted to trace the presence of *Sss* in a mini-tuber production facility and identify potential sources of contamination, so that corrective measures could be taken to eradicate the pathogen (Chapter 8).

In order for detailed studies into the epidemiology and management of powdery scab to be done, methods for detecting the pathogen were necessary. Methods for detecting and quantifying *Sss* included enzyme linked immunosorbent assay (ELISA) (Merz *et al.*, 2005), conventional PCR (Bell *et al.*, 1999) and real-time PCR (van der Graaf *et al.*, 2003). ELISA detected sporeballs from both tuber lesions and water sample and the accuracy of ELISA improved when concentrations of 100 sporeballs/ml or greater were tested. In this experiment ELISA did not however detect sporeballs from soil samples, which may have been due to the matrix effect of soil components interfering with the antigen-antibody reaction, thus masking the detection of the sporeballs. The reasonable cost of ELISA tests makes it common practice

for diagnostics in seed-tuber certification programmes. Both conventional PCR and qPCR detected *Sss* DNA from tuber, soil and water samples. Conventional PCR is more sensitive than ELISA as it detects sporeball DNA from both tuber and soil samples. The high sensitivity of qPCR ensured consistent detection of standard DNA quantities ranging from 1, 10, 100, 1 000 and 10 000 sporeballs per ml. The benefit of qPCR is that it can be used to reliably detect and quantify the different life stages of *Sss* in a range of sample types (van de Graaf *et al.*, 2003; de Haan & van den Bovenkamp, 2005).

To date, only two ITS Group Types of *Sss* have been internationally described; Group Type I and Group Type II (Bulman & Marshall, 1998; Qu & Christ, 2004). The South African *Sss* samples from Sandveld, Ceres, KwaZulu Natal and Mpumalanga were all found to be *Sss* Group Type II. The results were similar to that of Qu & Christ (2004), who noted that there was no variation among samples from different locations in Canada and USA. This study paves the way forward for long term sampling from South African fields contaminated with *Sss* to perhaps indicate *Sss* subgroups which can be of value for future resistance breeding programs.

Bioassays can be used to study the biology of *Sss*. For example Merz (1989) used a bioassay to study the infectivity of sporeballs and van de Graaf *et al.* (2000) studied zoospore release using a bioassay. The results from this experiment indicate that *Sss* zoospores are released four days after inoculation and infection of tomato roots occurs six days after inoculation. Plasmodia in the root hairs develop eight days after inoculation, followed by zoosporangia development by day 12. The result obtained from this experiment improves the understanding of the biology of *Sss*, which in turn may benefit management strategies as these strategies can be applied during susceptible developmental stages of *Sss*, hence disrupting the life cycle of the pathogen.

Controlling powdery scab of potato is difficult and thus, integrated crop management with resistant cultivars, long crop rotations with non-host crops and pathogen-free seed may be the only practical solution for managing powdery scab (Elad *et al.*, 1980; Thurston 1990; Honeycutt *et al.*, 1996; Wale, 2004; Genet *et al.*, 2005; Falloon, 2008). Currently no potato cultivar is completely immune to powdery scab, but different levels of resistance have been recorded by Iftikhar *et al.* (2007) and Falloon (2008). Evaluation of South African cultivars Argos, BP1, Buffelspoort, Caren, UTD and Valor showed that they are all susceptible to

infection by *Sss* and that there is a positive correlation between the severity of root galls and of tuber lesions. Thus it is of immense importance to develop a standard test that can not only be used to evaluate and rate the susceptibility of all South African cultivars, but also be used in future potato breeding programmes.

When selecting an alternate crop as a trap plant for the management of powdery scab, it is of importance that the pathogen is unable to complete is life cycle in that host (Qu & Christ, 2006), thus the soil-borne inoculum will be reduced. Of the tested crops, wheat and cabbage indicated that the pathogen was unable to complete its life cycle, whereas soybean is a non-host crop. Galls containing sporeballs observed in the roots of tomato and mustard cultivars signify that the pathogen completes its life cycle in these hosts. This finding was similar to that of Qu & Christ (2006) who identified yellow mustard, oat and tomato as hosts of *Sss* as the pathogen completes its life cycle within these crops. The results from this experiment can aid the selection of rotation crops for the management of powdery scab of potato.

Infected mini-tubers and/or seed tubers play an important role in the dissemination of *Sss*, especially to areas where the disease was not previously present (Merz *et al.*, 2005). Mini-tubers planted by the seed potato growers must be of a high standard. A case study at a potato mini-tuber production facility in Ceres, Western Cape Province, South Africa was done due to an outbreak of powdery scab during 2006. Swab samples indicated areas in the production facilities that were infested with *Sss* sporeballs. After eradication efforts to improve facility hygiene measures a second set of swab samples was taken in 2009 to determine the efficacy of the corrective measures. From 2009 onwards, disease-free mini-tubers have been harvested. This innovative study using the swab sampling methods can become a common practice in the mini-tuber industry, as it can be used to evaluate the presence of *Sss* sporeballs as well as other pathogens before, during and after planting. This allows ample time to implement control measures to make certain that disease-free mini-tubers are produced.

The results of the studies presented in this thesis will contribute to the knowledge of powdery scab not only in South Africa but internationally too. *Spongospora subterranea* f.sp. *subterranea* is a pathogen that needs to be studied further, especially regarding strategies to reduce the economic impact that it has on potato production around the world.

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