Molecular genetics of resistance to *Spongospora subterranea* f. sp. *subterranea* infection in potato (*Solanum tuberosum* L.)

By

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Declaration

I, Moleboheng Lekota, declare that this dissertation, which I hereby submit for the degree of PhD in 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.

SIGNATURE: ……

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Abstract

Infection of potato by *Spongospora subterranea* (Wallr.) Lagerh. f. sp. *subterranea* Tomlinson (Sss) causes tuber lesions, which directly affect the quality and marketability of the tubers, as well as root infections, which are associated with tuber yield and weight reductions. The pathogen is also a vector of potato mop-top virus (PMTV), which is considered economically important in some potato growing countries. Management of Sss is difficult because of the ineffectiveness of the current control options and it therefore requires integration of different control measures, of which host resistance represents the most economical, long-term approach. However, the information on the mechanisms of potato resistance to Sss infection is limited. In this study, the molecular and biochemical mechanisms of different potato cultivars that vary in their susceptibility to Sss infection were assessed.

Potato cultivars commonly grown in South Africa were assessed for their differential responses to Sss root and tuber infection. A greenhouse pot trial was conducted to evaluate the susceptibility of 10 cultivars to Sss infection. Visual observation was performed to determine the severity of root galling and powdery scab infection, while root microscopic examination was carried out to assess root zoosporangia infection. Quantitative polymerase chain reaction (qPCR) was used to confirm and measure the quantity of Sss DNA in the inoculated and not-inoculated potato roots and tubers. No potato cultivar was found to be resistant to Sss root or tuber infection in this study, as all cultivars had zoosporangia and root galls on or in the roots, and powdery scab lesions on tubers, with the exception of cultivar Innovator, which did not show any powdery scab symptoms on progeny tubers. However, a range of susceptibility of potato cultivars to root infection, root gall formation and powdery scab, from tolerant to susceptible was observed in the study. There was no association between development of root diseases and powdery scab. A negative correlation was observed between powdery scab and number of root zoosporangia, with a weak positive correlation observed between powdery scab and number of root galls. Nevertheless, three cultivars; Fianna, Lanorma and Mondial were found to be moderately tolerant to all three diseases. These results were confirmed with qPCR, in which the amounts of Sss DNA confirmed the presence of Sss infection in roots and tubers of all 10 cultivars evaluated.

The molecular mechanisms underlying quantitative potato resistance to powdery scab; discovery of defense-related genes was undertaken by ribonucleic acid sequencing (RNA-seq) in a susceptible and tolerant potato cultivar in response to powdery scab infection. Validation of the RNA-seq results and gene expression profile analysis of the two cultivars were carried out using quantitative real-time reverse transcriptase PCR (RT-qPCR) on a subset of genes induced at different tuber developmental stages. Differentially expressed genes related to plant defense responses were identified in both cultivars. Expression of nine genes involved in defense was up-regulated more in the tolerant cultivar than in the moderately susceptible cultivar upon infection by Sss. The involvement of defense-related metabolites in potato root tolerance to Sss root infection was investigated.

Metabolic profiling of the chemicals in root exudates and root extracts of potato cultivars differing in susceptibility to Sss root infection, using a non-targeted metabolomics approach, was carried out. Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) analysis was used to compare metabolic profiles of tolerant cultivars and susceptible cultivars inoculated or not-inoculated with Sss, to identify and quantify the resistance-related (RR) metabolites involved. Significant differences between the moderately tolerant (Fianna, Innovator, Lanorma, Mondial and Valor) and the moderately susceptible/susceptible (Avalanche, BP1, Ronaldo Sifra and Up-to-date) cultivar groups were identified in the levels of several metabolites, including amino acids, organic acids, alkaloids, phenolics and sugars. These secondary metabolite classes play important roles in plant defense.

Monitoring Sss development in potato roots and tubers can enable efficient assessment of Sss infection in potatoes, resulting in proper, timely and improved control strategies for the diseases. Moreover, more information on the chemical composition of roots and root exudates as well as identification of defense-related genes is important for marker-assisted selection of potato germplasm in breeding programs for developing novel potato cultivars with broad and durable resistance to Sss.

Keywords: Potato, powdery scab, metabolite profiling, susceptibility, defense genes, RT-qPCR, RNA-sequencing, *Spongospora subterranea* f. sp. *subterranea*.

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Thesis Outputs

Statement

During the course of this research project the author has published and presented the following:

Scientific Publication

Lekota M, Muzhinji N, van der Waals JE (2019). Identification of differentially expressed genes in tolerant and susceptible potato cultivars in response to *Spongospora subterranea* f. sp. *subterranea* tuber infection. *Plant Pathology* **68**: 1196 - 1206.

Oral Presentations

National

Lekota M, Modisane JK, Apostolides Z, van der Waals JE (2019). Untargeted metabolomics reveals induced metabolites in potato cultivars differing in susceptibility to *Spongospora subterranea* f. sp. *subterranea* root infection. 51st Congress of the South African Society of Plant Pathology, Club Mykonos, Langebaan 20-24 January, Western Cape South Africa.

Lekota M and van der Waals JE (2017). Susceptibility of potato cultivars to *Spongospora subterranea* f. sp. *subterranea.* Annual Potatoes South Africa research symposium. Club Mykonos, Langebaan, 25-27 July Western Cape, South Africa.

Poster presentations

International

Lekota M and van der Waals JE (2016). Relationship between *Spongospora subterranea* f. sp. *subterranea* diseases: powdery scab, root galling and root infection in potatoes. European Association for Potato Research (EAPR), Pathology and Pests section meeting 7th – 11th August, Dundee Scotland UK.

National

Lekota M and van der Waals JE (2018). Analysis of differentially expressed genes in susceptible and tolerant potato cultivars upon *Spongospora subterranea* f. sp. *subterranea* infection. Annual Potatoes South Africa research symposium. Parys, 24-25 July, Free State, South Africa.

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List of abbreviations

BABA	β-aminobutyric acid
°C	Degrees Celsius
Ct	Cycle threshold
Cq	Quantification cycle
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immuno sorbent assay
ET	Ethylene
ESI	Electrospray ionization
ETI	Effector-triggered immunity
g	Gram
GABA	γ-aminobutyric acid
GC	Gas chromatogram
GO	Gene ontology
h	Hour
HCA	Hierarchical cluster analysis
ISR	Induced systemic resistance
JA	Jasmonic acid
kb	Kilobase
l	Litre
min	Minutes
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrometry
NIS	Nitrogen induced susceptibility
NMR	Nuclear magnetic resonance

OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PCR	Polymerase chain reaction
PTA	Plate-Trapped-Antigen
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcriptase PCR
Rt	Retention time
RNA	Riboxyribonucleic acid
s	Seconds
SA	Salicylic acid
SAR	Systemic acquired resistance
Sss	*Spongospora subterranea* f. sp. *subterranea*
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography

Chapter 1

1.1. General introduction

Global potato production has increased over the past two decades, especially in developing countries (Birch *et al.*, 2012). Approximately 2.5 million tonnes of potatoes are produced annually in South Africa (Potatoes South Africa, 2017); with an increase from 1.2 million tonnes in 1990 to 2.5 million tonnes per hectare in 2015 (www.nda.agric.za). The potato crop is vulnerable to attack by many insects and pathogens. *Spongospora subterranea* f. sp. *subterranea* (Sss), a biotrophic protozoan is one of the most important pathogens of potato. Sss causes the three potato diseases: root infection, root gall formation and powdery scab on tubers (Falloon *et al.*, 2016). Root infection by Sss is generally accompanied by reduced nutrient uptake and water absorption (Lister *et al.*, 2004; Falloon *et al.*, 2016), leading to reduced tuber yield and tuber weight (Shah *et al.*, 2005; 2012). The disease has been reported to be of economic importance in potato production as the infection of potato tubers causes lesions that affect tuber quality and marketability of the tubers (Merz, 1997). Moreover, Sss is also the vector of potato mop-top virus (PMTV), which causes internal damage in potato tubers rendering them unmarketable (Maldonado *et al.*, 2013). PMTV infects Sss spores and is transmitted during fungal attack by the zoospores demonstrating a close association between Sss and the virus (Carnegie *et al.*, 2010).

Spongospora subterranea f. sp. *subterranea* is both soil and tuber-borne and can be found in all potato producing areas of the world; although it has been reported to be prevalent in cool and moist climates (Merz *et al.*, 2008). Infected tubers are the mechanism of dispersal of the pathogen to other fields (Merz and Falloon, 2008). Additionally, the formation of root galls can be induced by resting spores on asymptomatic tubers in the absence of soil-borne inoculum (Tegg *et al.*, 2013). Resting spores formed by this plasmodiophorid pathogen are highly resistant to environmental stresses and remain infectious for many years in contaminated soils, making the disease difficult to control (Falloon, 2008). Therefore, development of new resistant potato cultivars may offer the best solution to the problem (Houser and Davidson, 2010; Merz *et al.*, 2012). Resistant cultivars have low levels of Sss zoosporangial root infection and few root galls as compared to susceptible cultivars, indicating that host resistance is expressed on the roots and stolon cells of potatoes (Falloon, 2003).

Control methods for diseases caused by Sss are limited and there is no method that is completely effective for Sss control, hence, integration of different disease management strategies is needed (Harrison *et al.,* 1997; Falloon, 2008; Merz and Falloon, 2009). These include very long crop rotations or the use of Sss-free land, as the pathogen can survive in the soil for more than ten years (Falloon, 2008; Sparrow *et al.,* 2015). The use of resistant cultivars (Falloon *et al.,* 2003) and certified clean seed tubers (Tegg *et al.,* 2015) is a further important control method. A limited number of chemicals, including fluazinam and flusulfamide, are registered in some countries for use against plasmodiophorids, including Sss (Falloon, 2008; Merz and Falloon, 2009). The usefulness of available chemicals is limited, moreover, extensive use of fungicides, especially systemic fungicides, not only increased the cost of the crop production and harmful effects on the environment but also enhanced fungicide resistance on crops, therefore non-chemical disease management options are desirable.

Differences in potato cultivars' response to Sss root and tuber infection were noted in several reports (Falloon *et al.,* 2003; Iftikhar *et al.,* 2007; Maldonado *et al.,* 2013; Bittara *et al.,* 2016) and resistance among different cultivars ranges from 'highly resistant' to 'highly susceptible', suggesting existence of a quantitative type of resistance to powdery scab (Falloon *et al.,* 2003 Harrison *et al.,* 1997; Merz *et al.,* 2004; Wastie 1991), though, none of the cultivars possesses true resistance to the pathogen (Nitzan *et al.,* 2008; Houser *et al.,* 2010; Brierley *et al.,* 2013). This suggests that several genes are involved in controlling inheritance of the disease resistance from one generation to the next. Unlike qualitative resistance, quantitative resistance is durable, even though the molecular and biochemical mechanisms underlying it are not known, and are utilized inefficiently in potato breeding programs (Yogendra *et al.,* 2014a). However, pathogenic variation in Sss has been reported, suggesting existence of different Sss strains (Bulman *et al.,*1998; Falloon *et al.,* 2003), therefore, cultivars that are identified as relatively resistant to a certain strain might become susceptible to a different strain.

In order to combat pathogens attack, plants have evolved complex strategies to recognize pathogen infection and develop effective defense responses (Jones and Dangl, 2006). The recognition of pathogen-associated molecular patterns (PAMPs) is the first line of defense that follows infection, triggering basal levels of plant defense responses referred to as PAMP-triggered immunity (PTI). Haustoria inside the plant cell are produced by the pathogens to

release effector proteins in order to combat PTI. This leads to development of a second line of defense in plants producing effector-specific resistance (R) proteins encoded by R genes (Kushalappa and Gunnaiah, 2013). PAMP activates phytohormones, such as Jasmonic acid, Salicylic acid, Ethylene, Abscisic acid, and gibberellins, to provide broad biochemical resistance and reduce pathogen growth (Robert-Seilaniantz *et al.*, 2011). Pathways involved in the production of secondary metabolites that impart quantitative resistance are modified by these phytohormones (Chisholm *et al.*, 2006), leading to the induction of target genes (Czernic *et al.*, 1999). A variety of transcription factors that regulate plant defense responses are included in the induced target genes, involving resistance related (RR) genes that bind to specific DNA (Yogendra *et al.*, 2014b).

1.2. Objectives of the study

1.2.1. General objective

The main aim of this study is to understand the molecular and biochemical mechanisms controlling susceptibility of potato tubers and roots to Sss infection.

1.2.2. Specific objectives

1. To assess the susceptibility of different potato cultivars commonly grown in South Africa to Sss disease development on roots and tubers under greenhouse conditions (**chapter 3**).

2. To identify and quantify resistance related (RR) metabolites in potato roots and root exudates in tolerant potato cultivars in comparison to susceptible cultivars **(chapter 4).**

3. To undertake gene expression profiling of the two cultivars differing in susceptibility to Sss tuber infection (**chapter 5**).

1.3. Thesis outline

Chapter 1 – An introduction and background related to the topic of the study.

Chapter 2 – The review of literature related to the topic of the study.

Chapter 3 – Relative **s**usceptibility of potato cultivars to *Spongospora subterranea* f. sp. *subterranea* infection.

Chapter 4 - Metabolic profiling of phytochemicals in potato roots and root exudates.

Chapter 5 - Transcriptome analysis of powdery scab resistance in potatoes.

Chapter 6 – General discussion.

1.4. Conclusion

In general, the studies compiled in this thesis provide significant information on the biochemical and molecular responses of different potato cultivars to Sss root and tuber infection. The data provided will contribute to an increased understanding of the potato-Sss host resistance interactions, which will in turn assist in the provision of biochemical and molecular biomarkers for marker assisted potato breeding.

1.5. References

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Chapter 2

Literature review

Epidemiology, control and genetic resistance of potato to *Spongospora subterranea* f. sp. *subterranea*

2.1. Introduction

Potato (*Solanum tuberosum* L.) is the world's most widely grown tuber crop; and the fourth most important crop after rice, wheat and maize (FAOSTAT, 2016). Potatoes are a staple food in countries of Africa, Asia, South America, and North Europe and to some extent the United States of America (Lutaladio and Castaidi, 2009). However, potato production is threatened by many biotic and abiotic factors (Black, 2008). One pathogen of importance is the protozoan *Spongospora subterranea* (Wallr.) Lagerh. f. sp. *subterranea* Tomlinson (Sss), which is one of the most devastating pathogens infecting potatoes, causing a significant reduction in quality and marketability of potato tubers (Falloon, 2008). *Spongospora subterranea* f. sp. *subterranea* also infects potato roots causing root galling and root zoosporangia infection that impairs plant growth and productivity due to reduced root function (Bittara *et al.*, 2016; Falloon *et al.*, 2016).

Spongospora subterranea f. sp. *subterranea* is a soil- and tuber-borne pathogen that can remain viable in the soil for over 10 years (Merz and Falloon, 2008). Control of Sss in the soil is difficult due to its wide host range and the longevity of sporosori in the soil (Falloon, 2008). Integrated management strategies to limit the risk of Sss disease development in potato production include certification schemes, irrigation management, cultural practices, chemical seed and soil treatments, crop rotation and use of biocontrol agents (Merz, 2008). However, these strategies yield inconsistent results in the management of Sss (Merz *et al.*, 2012; Balendres *et al.*, 2017). This lack of efficient and effective Sss control strategies has increased the importance of Sss diseases in potato production (Falloon, 2008). Planting pathogen-free seed tubers in uncontaminated fields and growing of resistant potato cultivars remain the most effective and sustainable long-term approaches for management of Sss (Merz and Falloon, 2008). *Spongospora subterranea* f. sp. *subterranea* resistance in potato

roots and tubers is under different genetic control mechanisms and is inherited independently (van de Graaf *et al.*, 2007; Merz *et al.*, 2012). Currently, none of the cultivars grown in South Africa are completely resistant to Sss root and tuber infection (Potatoes South Africa, 2017).

2.2. The potato industry in South Africa

Potato (*Solanum tuberosum* L.) belongs to the Solanaceae family, which includes tomato (*Solanum lycopersicum* L.), tobacco (*Nicotiana tabacum* L.), pepper (*Capsicum frutescens* L.) and eggplant (*Solanum melongena* L.). Potato originated from the highland regions of the Andes in South America (Hawkes and Francisco-Ortega, 1993). Potatoes were introduced for planting purposes into South Africa from the Netherlands for provision of food for mariners visiting the Cape in the 1600s (DAFF, 2013) and were first grown in South Africa in 1830 (Black, 2008). In South Africa, potatoes are produced in 16 potato-growing regions with different climatic and soil conditions (Figure 2.1) resulting in a constant supply of fresh potatoes throughout the year (Potatoes South Africa, 2017). The main potato -growing regions are the Eastern and Western Free State, Limpopo and the Sandveld (Steyn *et al.*, 2016; Potatoes South Africa, 2017). In South Africa, potatoes are produced by about 600 commercial potato growers including approximately 200 seed growers (Potatoes South Africa, 2017), with the Western Free State region having the highest number of producers.

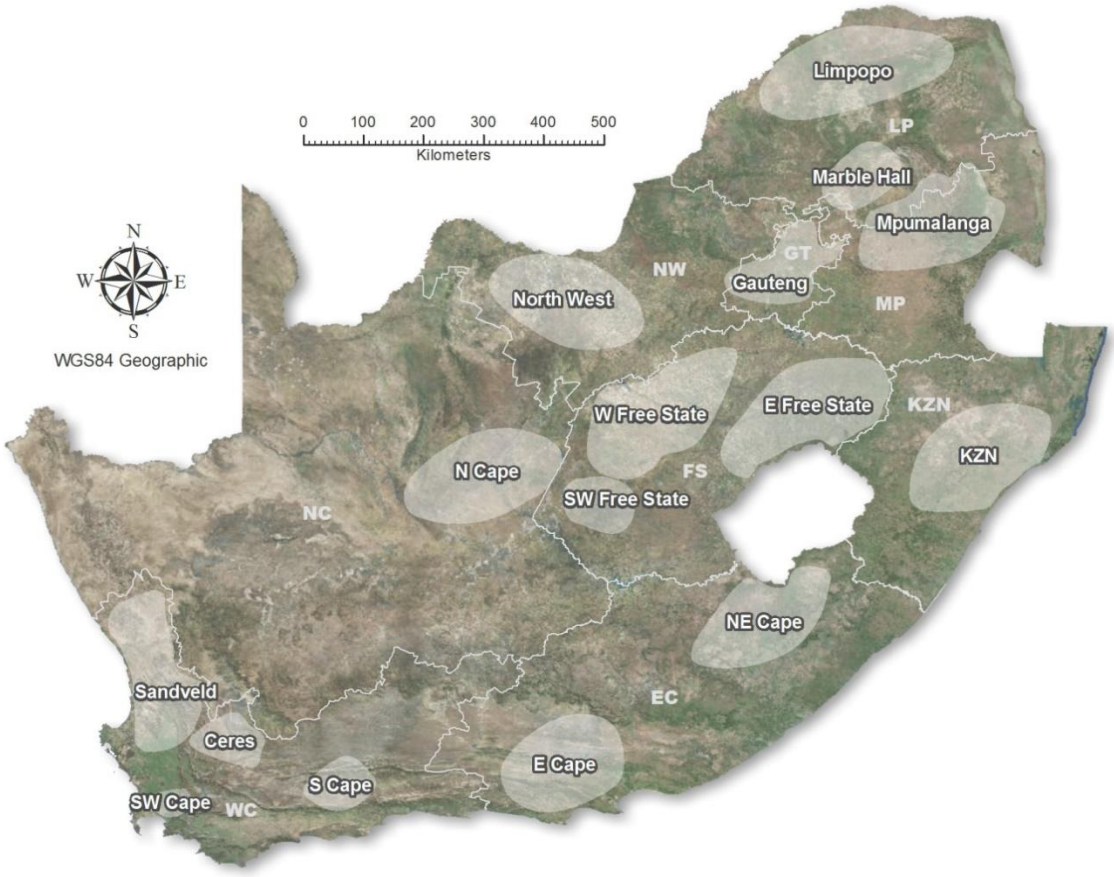

Figure 2.1. Map of the 16 potato growing regions (shaded in grey) of South Africa [Courtesy of L. van Zyl of TerraGIS]

2.2.1. Potato diseases

The majority of commercially grown potato cultivars are susceptible to diseases caused by abiotic and biotic factors; biotic factors include viruses, fungi, bacteria, protozoa, insects and nematodes, causing more than 40 diseases on potatoes (Stevenson *et al.*, 2001). Thirty fungal, 10 viral and three bacterial pathogens are reported to be of economic importance to potato production in South Africa (Nortje, 2015).

2.2.1.1. Fungi

Fungi are one of the most important groups of plant pathogens causing severe damage to potatoes (Friers *et al.*, 2012). Fungal diseases of economic importance in South Africa include stem canker and black scurf caused by *Rhizoctonia solani.* L (Muzhinji *et al.*, 2015),

powdery scab caused by *Spongospora subterranea* f. sp. *subterranea* (Wright *et al.*, 2012), black dot caused by *Colletotrichum coccodes* (Wallr.) Hughes (Nortjé, 2015), early blight caused by *Alternaria solani* Sorauer (van der Waals *et al.*, 2004), brown spot and black pit caused by *Alternaria alternata* (Fries.) Keissler (van der Waals *et al.*, 2011), and *Fusarium* dry rot and wilt caused by *Fusarium* spp. particularly *F. caeruleum* (Lib.) Sacc. and *F. sulphureum* L. (Theron, 1999). Late blight caused by the oomycete *Phytophthora infestans* is a potential threat to potato production in South Africa (McLeod *et al.*, 2001).

2.2.1.2. Bacteria

Various bacterial pathogens attack potato plants and tubers. Major bacterial diseases that cause considerable loss to potato production in the field and in storage are soft rot, blackleg, common scab, bacterial wilt and brown rot (Arora and Khurana, 2004). In South Africa, bacterial diseases of economic importance include bacterial wilt caused by *Ralstonia solanacearum* L. (Nortjé, 2015) and common scab caused predominantly by *Streptomyces scabiei* L. (Jordan and van der Waals, 2016). The most devastating bacterial diseases are soft rot and blackleg caused by *Pectobacterium* and *Dickeya* spp. (Ngadze *et al*., 2010).

2.2.1.3. Viruses

Several viruses and viroids affect potato by damaging leaves, stems and tubers thereby reducing plant vigor and productivity (Hooker, 1982). Potatoes are susceptible to about 40 viruses and two viroids (Jeffries *et al.*, 2005). The most devastating viruses in South Africa are potato virus Y (PVY) and potato leaf roll virus (PLRV) (Visser and Bellstedt, 2009; Thompson *et al.*, 2012). The PVY^{N}-Wilga and PYV^{NTN} strains are the most common potato viruses in South Africa (Potatoes South Africa, 2017). Most of the potato viruses cause mainly foliar symptoms that include vein necrosis, mosaic and leaf rolling. However, some viruses, like tobacco rattle virus (TRV), PVY, PLRV and tobacco necrotic virus (TNV), infect tubers causing tuber blemishes and an increase in undersized tubers with consequent reduction in marketable quality and yield (Agindotan *et al.*, 2007). Other than aphids, transmission of potato viruses is through infected seed tubers or groundkeeper tubers as the majority of them are tuber-borne (David *et al.*, 2010).

2.3. The pathogen

2.3.1. Classification of *Spongospora subterranea* f. sp. *subterranea*

Spongospora subterranea f. sp. *subterranea* is a biotrophic protozoan belonging to the plasmodiophorid family (Braselton, 2001). This family includes 10 genera and 36 species (Braselton, 2001). The genus *Spongospora* consists of four described species, two of which are defined as *formae speciales* and are morphologically indistinguishable, but differentiated based on their host range: *Spongospora subterranea* f. sp. *nasturtii* (*Ssn*), the cause of crook root of watercress, and *Spongospora subterranea* f. sp. *subterranea* (Sss), an economically important pathogen in potato production (Merz and Falloon, 2008).

Spongospora subterranea f. sp. *subterranea* is an obligate parasite requiring a living host to reproduce and complete its life cycle, and cannot be cultured or grown on artificial media; therefore, Koch's postulates cannot be fulfilled for the pathogen (Merz and Falloon, 2008). Nevertheless, Harrison *et al.*, (1997) attempted to culture Sss on agar plates and observed amoebae, but later concluded that the growth may have been due to contamination of Sss sporosori, as they could not fulfil Koch's postulates. Furthermore, Qu *et al.*, (2000) demonstrated clearly that the colonies isolated from surface-sterilized Sss-infected potato tubers and sporosori were amoebal contaminants and not Sss. This was further confirmed with the use of microscopy, bioassay and Sss species-specific primers (Qu and Christ, 2007).

Spongospora subterranea f. sp. *subterranea* has two important characteristic features: a sporosorus (Figure 2.2 A) containing resting spores (Figure 2.2 B) as its survival structure and the biflagellate zoospore with flagella shown in Figure 2.3 (Merz and Falloon, 2008). The significance of Sss zoospores and their role in spreading the disease was emphasized by Merz (1997), who described them as tools for infections in the field, as they are attracted to the hosts' root exudates and are carriers of the Potato mop-top virus (PMTV).

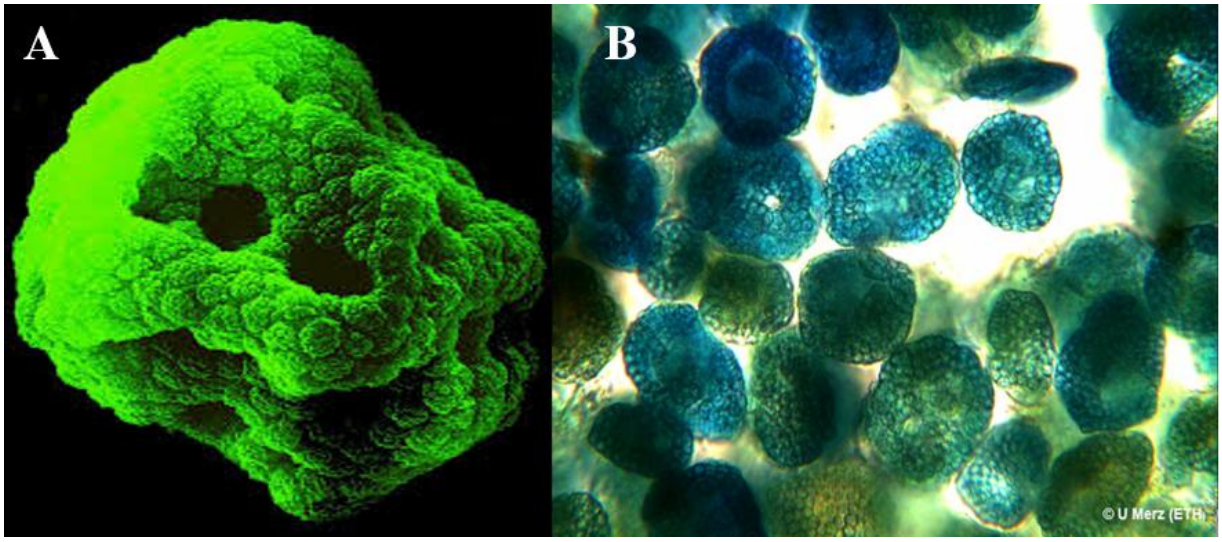

Figure 2.2. A: Sporosorus containing sporosori of *Spongospora subterranea* f. sp. *subterranea* (Merz, 2013) **B**: Microscopic observations of sporosori of *Spongospora subterranea* f. sp. *subterranea* stained with cotton blue (Merz, 2008).

Figure 2.3. Biflagellate zoospore with flagella at about 180˚ to each other (Merz, 1993).

2.3.2. Biology and genetic variation of *Spongospora subterranea* f. sp. *subterranea*

Knowledge of the presence of physiological races, genetic variation of Sss populations and its mode of reproduction is limited. Such information is needed for screening of new potato cultivars for resistance to powdery scab and effective deployment of Sss disease management strategies (Gau *et al.*, 2013; Qu and Christ, 2004). Moreover, knowledge of virulence of the pathogen populations is essential for effective and sustainable development of durable plant resistance (Qu and Christ, 2004). The size of sporosori has been used as an indicator for

variation of Sss strains (Gau *et al.*, 2013). Recently, DNA based techniques such as the Polymerase Chain Reaction (PCR) using specific primers and sequencing have become effective means for identifying and elucidating the genetic variation of plant pathogens. Bulman and Marshall (1998) and Qu and Christ (2004) identified two genetically different groups of Sss by sequencing the internal transcribed spacer (ITS) region. Both studies grouped Sss isolates into ITS Type I and ITS Type II. Sss field populations were found to be clonal and genetic variation within geographic locations was not evident, although it was observed among different Sss strains in North America (Qu and Christ, 2006). Gau *et al.*, (2013) reported a low global genetic diversity in Sss populations from different potato producing countries.

2.3.3. Life cycle of *Spongospora subterranea* f. sp. *subterranea*

Spongospora subterranea f. sp. *subterranea* survives in the soil for more than 10 years in the form of sporosori (Bittara *et al.*, 2013). These resting spores are highly resistant to environmental stresses due to their thick walls (Merz, 2008). The life cycle of Sss is divided into two phases, the primary phase that occurs in the epidermal cells and hairs of the host root, and the secondary phase that occurs in the cortical cells of the roots and tubers (Harrison *et al.*, 1997) as shown in Figure 2.4. At the beginning of the primary phase, an individual resting spore germinates and releases biflagellate primary zoospores that are able to swim short distances in moist soils to reach the host (Merz and Falloon, 2008). These primary zoospores swim to the roots, penetrate and infect the epidermal cells or host root hairs and produce multinucleate sporangial plasmodia.

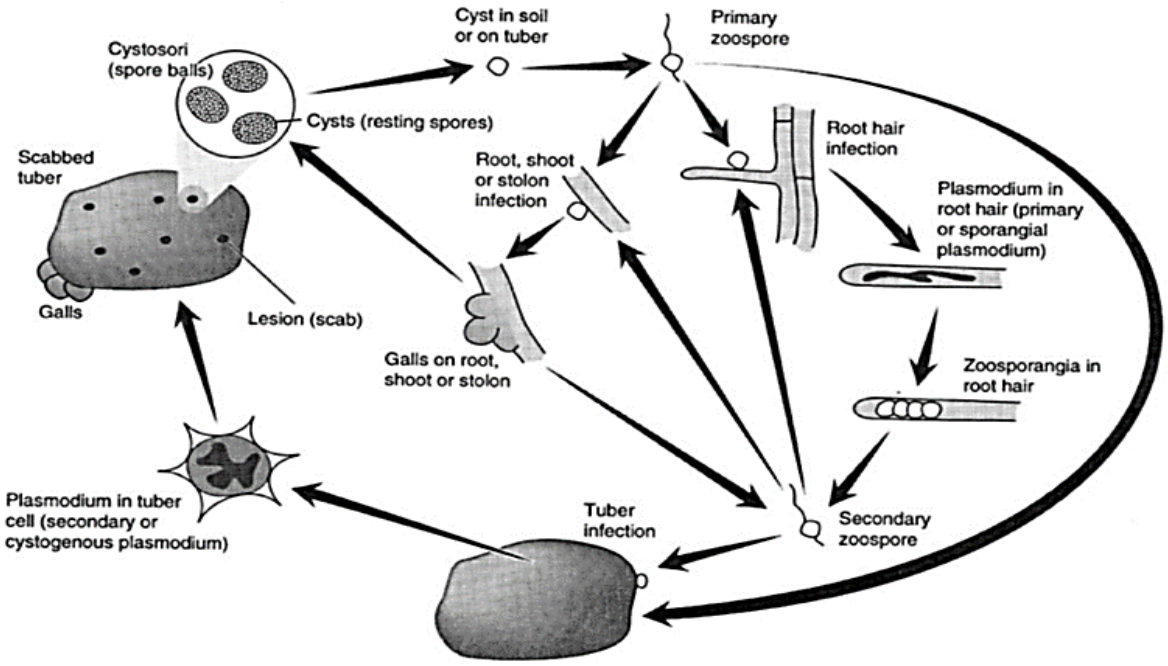

Figure 2.4. Life cycle of *Spongospora subterranea* f. sp. *subterranea* (Harrison *et al.*, 1997).

The zoosporangia release secondary zoospores, which are of equivalent size to the primary zoospores and also possess two anterior flagella, enabling them to swim and infect the cortical cells on roots, stolons and tubers of the host plant, resulting in development of root galls and tuber lesions (Nitzan *et al.*, 2008). Temperature influences the length of time a zoospore can swim before it encysts prior to infection to produce a tubular structure (Rohr) in which a projectile-like body (Stachel) is formed (Harrison *et al.*, 1997). Zoospore infection occurs through the injection of the cyst content, enabled by the formation of an adhesorium (Braselton, 1995; 2001).

Secondary zoospores can also re-infect the epidermal cells and root hairs, thus affording the pathogen an opportunity for continued vegetative multiplication by repetition of the primary phase of its life cycle (Kole and Gielink, 1963). Secondary zoospores are also released from the root galls and the tuber lesions, thus increasing the number of zoosporangia and zoospores (amount of inoculum) in the soil (Falloon, 2008; Nitzan *et al.*, 2008). The secondary cycle repeats during host growth and development for as long as the conditions are favourable, resulting in heavily infected potato plants (Tegg *et al.*, 2013).

Free water is needed for primary and secondary zoospore germination, and once germinated, they are able to swim for about two hours, and need to find host tissue to survive (Merz,

2008). Morphological structures and swimming patterns are similar in both primary (freed from sporosori) and secondary zoospores (released from zoosporangia in potato roots) (Merz, 1997). Secondary infection of tubers and adjacent plants provides the basis for multiple re-infections in a single growing season, hence the potential for very rapid Sss inoculum build-up. Tubers are most susceptible to Sss infection during the first two to three weeks of tuber initiation (van de Graaf *et al.*, 2005). Later infection of the lenticels is also possible as a result of lenticel propagation in mature tubers or a delay in lenticel suberization caused by low oxygen levels in wet soils (Wale *et al.*, 2008).

2.3.4. Host range of *Spongospora subterranea* f. sp. *subterranea*

Potato is the main host of Sss, but members of other plant families can be alternative hosts of the pathogen (Harrison, 1997; Anderson *et al.*, 2002; Iftikhar and Ahmad, 2005; Aristizabal *et al.*, 2013). The pathogen is able to complete its life cycle and produce sporosori in some hosts while in other hosts it is only able to produce zoosporangia (Qu and Christ, 2006; Merz and Falloon, 2008; Nitzan *et al.*, 2008; Aristizabal *et al.*, 2013).

Alternative hosts are able to maintain or increase the levels of inoculum in fields not planted with potatoes for many years (Burnett, 1991). Aristizabal *et al.*, (2013) characterized Sss alternative hosts into three categories: trapping plants (plants with only zoosporangia), Type I host (plants with only sporosori) and Type II host (plants with both zoosporangia and sporosori. Tomato (*Lycopersicon esculentum* L.), yellow mustard (*Brassica campestris* L.) and oat (*Avena sativa* L.) allow the proliferation of zoospores from zoosporangia and the development of sporosori, thereby assisting the survival of the pathogen in the soil (Qu and Christ, 2006). Other species like wheat and barley were reported as alternative type II hosts (Tsror, 2016b), as well as black nightshade *(Solanum nigrum* L.) (Shah *et al*., 2010). Non-Solanaceous plants like *Polygonum segetum* Kunth, which are zoosporangial hosts, could be used as trapping plants that prevent the completion of the pathogen's life cycle (Aristizabal *et al.*, 2013). This can be important in reducing the pathogen inoculum in the soil and contributing to the integrated management strategy of powdery scab (Aristizabal *et al.*, 2013). Moreover, a recent study by Clark *et al.*, (2018) confirmed opium poppy (*Papaver somniferum* L.) and pyrethrum (*Chrysanthemum cinerariifolium* L.) as new alternative hosts (trapping plants with zoosporangia only) for Sss root infection. Several other weeds and crops such as rye (*Secale cereal* L.), onion (*Allium cepa* L.), cabbage (*Brassica oleracea* var.

capitate L.), oilseed radish (*Raphanus sativus* L.), tomato (*Solanum lycopersicum* L.), wheat (*Triticum sativum* L.), maize (*Zea mays* L.), carrot (*Daucus carota* subsp. s*ativus* L.) and soybean (*Glycine max* L.), were also reported as Sss Type II hosts, with Indian mustard (*Brassica juncea* L.) and green beans (*Phaseolus vulgaris* L.) as Type I hosts in South Africa (van der Waals *et al.*, 2018).

2.3.5. Symptoms caused by *Spongospora subterranea* f. sp. s*ubterranea* on potato tubers and roots

Primary Sss root infection causes zoosporangia on the root epidermal cells (Figure 2.5A), while scondary infections lead to development of creamy-white root galls (hyperplasia), which turn brown at maturity and are filled with sporosori (Harrison *et al.*, 1997; Merz, 2008) releasing Sss resting spores (Maldonado *et al.*, 2013) as shown in Figure 2.5B. Zoospore release causes disruption of root membrane selectivity in the epidermal cells of the host during infection (Falloon *et al.*, 2004). This reduces root function and leads to interrupted water and mineral uptake by roots as well as affecting photosynthetic sugar translocation to tubers, which is the main cause of reduced tuber weight (Falloon *et al.*, 2004; Lister *et al.*, 2004; Merz, 2008).

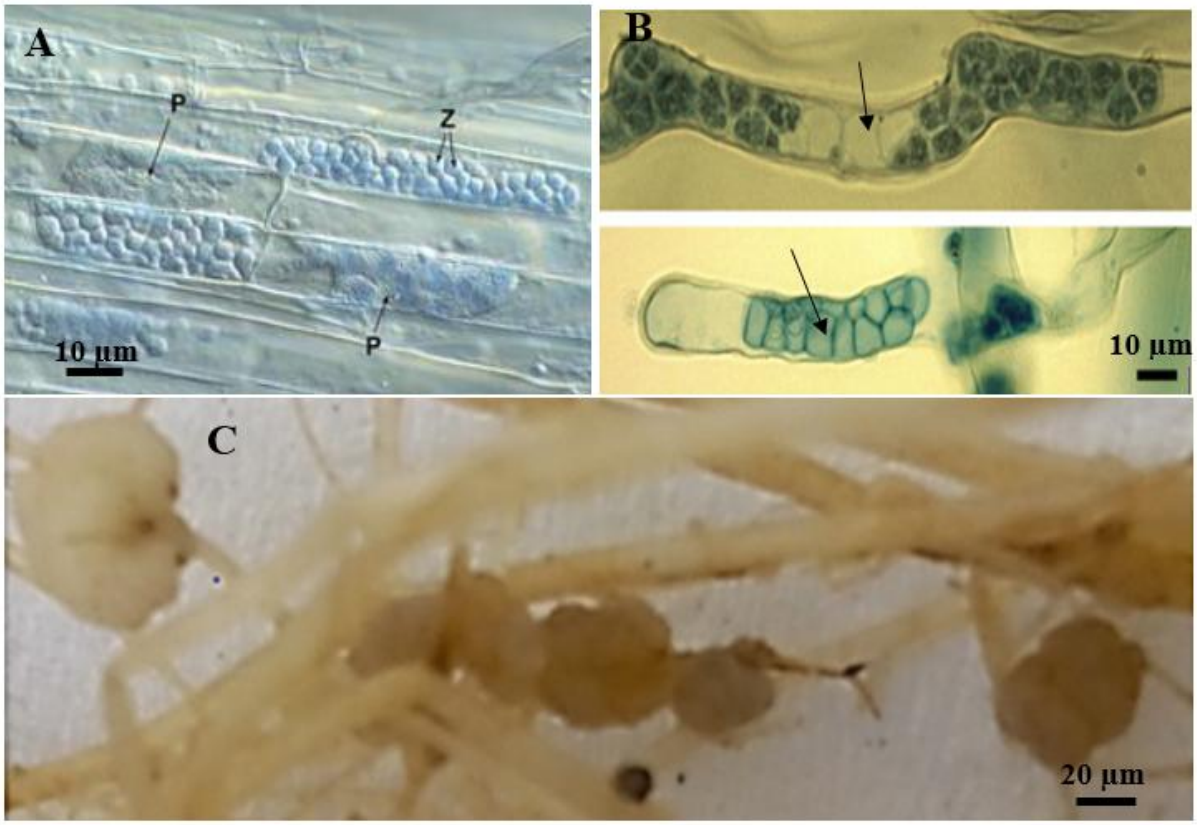

Figure 2.5. *Spongospora subterranea* f. sp. *subterranea* root infection **A:** Multinucleate plasmodia (P) and zoosporangia (Z) of *Spongospora subterranea* in root epidermal cells of *Solanum lycopersicum* (Falloon *et al*., 2015); **B:** Root hairs containing zoosporangia from which zoospores have been released**;** (Maldonado *et al.*, 2013); **C**: Root galls on BP1 cultivar (Photo: Moleboheng Lekota).

The pathogen also causes blemishes on the surface of potato tubers (Figure 2.6 A); reducing their market value (Nitzan *et al.*, 2008). Tuber infection is initially seen as purplish-brown pustules that are 0.5 to 2 mm in diameter, extending under the periderm and forming a raised pimple-like lesion; these lesions are the most noticeable damage caused by the disease (Burnett 1991; Harrison *et al.*, 1997; Merz, 2008). Lesions are weak skin areas on the surface of tubers with enhanced gas exchange, resulting in increased shrinkage and weight loss of stored potato tubers (Nitzan *et al.*, 2008). Moreover, the lesions serve as ports of entry for secondary infections by other pathogens (Falloon, 2008; Nitzan *et al.*, 2008). At maturity, lesions become hollow and are filled with a brown powder consisting of cystostori (sporosori). At this stage they are referred to as scabs, which feel sponge-like with smooth and raised margins (Harrison *et al.*, 1997). Scabs affect only the outer tissue of the tuber, but in rare cases can penetrate deep into the tuber, thus destroying a larger portion of the tuber

(Harrison *et al.*, 1997). Given prolonged favourable environmental conditions, namely cool and moist conditions, expansion in depth and width of the scabby lesions takes place, to form cankers and outgrowths (Lawrence and McKenzie, 1981). Large tumours commonly develop on mature tubers; with fresh tumours developing and continuing to grow, resulting in tuber deformities even during tuber storage (Harrison *et al.*, 1997). Cankers (Figure 2.6B) lead to formation of misshapen and deformed tubers and this form of the canker can be mistaken for wart disease of potatoes caused by *Synchytrium endobioticum* (Fornier, 1997).

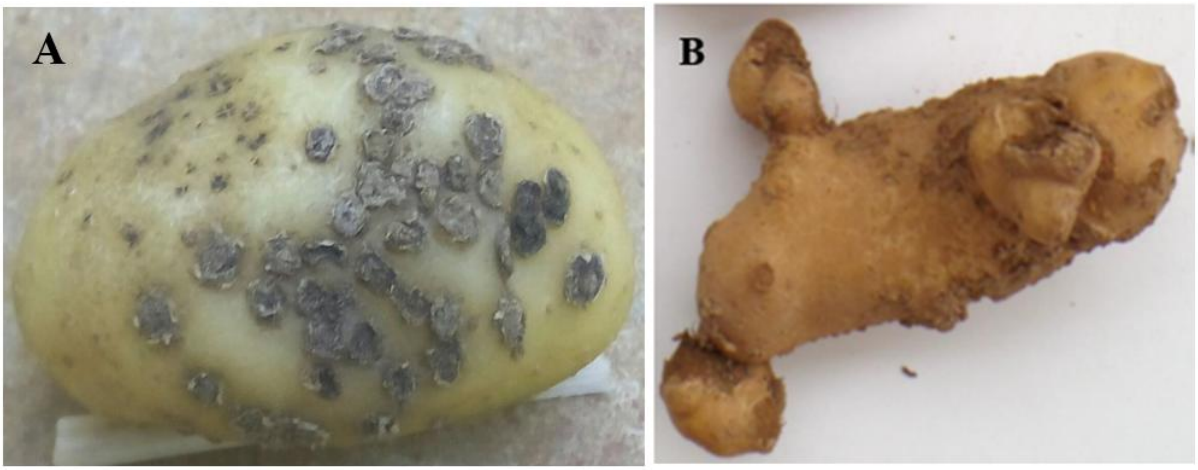

Figure 2.6. A. *Spongospora subterranea* f. sp. *subterranea* mature lesions on potato tubers (Photo: Moleboheng Lekota). **B**. Potato tuber cankers caused by *Spongospora subterranea* f. sp. *subterranea* (Tsror, 2017).

Powdery scab symptoms are still frequently confused with common scab symptoms caused by *Streptomyces scabies* (Harrison *et al.*, 1997; van der Waals, 2013). Symptoms of the two scab diseases on potato tubers look similar and are very difficult to distinguish with the naked eye (Harrison *et al.*, 1997; Qu *et al.*, 2011). However, common scab symptoms are irregularly shaped, raised or depressed cork-like blemishes on the tuber skin, while powdery scab symptoms on tubers are typically raised pustules with a central depression containing a powdery spore mass (Qu *et al.*, 2011). A precise identification of the symptoms and causes of the diseases on potatoes is necessary for proper control of the diseases and provision of information to potato growers and researchers who need assistance in disease diagnosis (Qu *et al.*, 2011).

2.3.6. Economic impact of *Spongospora subterranea* f. sp. *subterranea*

Powdery scab of potato tubers has long been regarded as the main disease of Sss infection worldwide, causing tuber lesions that lead to severe reductions in tuber quality. However, recent findings suggest that root infection by Sss can affect plant growth and functionality by disrupting water and nutrient uptake, leading to reduced tuber yield (Falloon *et al.*, 2004; Lister *et al.*, 2004; Nielsen and Larsen, 2004; Shah *et al.*, 2012; Falloon *et al.*, 2016). Root infection also has detrimental effects on plant height and shoot dry weight (Gilchrist *et al.*, 2011). Root galling caused by Sss may cause yield losses of up to 5 to 12 metric tons per hectare in potato (Brown *et al.*, 2007), while root infection reduced tuber weight by 42% (Falloon *et al.*, 2016).

Blemished tubers as a result of Sss infection are usually discarded before they reach the market, which is a great economic loss to the potato industry. Severely infected potato tubers are prone to weight loss (Gilchrist *et al.*, 2011). Even though the number of tubers per plant is not affected, the reduced tuber weight can become a problem in potato production for the fresh market or processing where tuber size is crucial (Gilchrist *et al.*, 2011). No quantification of yield losses to powdery scab has been made in South Africa; however, an estimation of A$13.4M loss per annum has been reported in the Australian potato processing industry (Ramsay, 2014), £7-9 million loss per annum in Great Britain (Prentice *et al.*, 2007) as well as an extra £10/tonne grading costs to Scotland seed growers (Wale, 2000). Moreover, there are no studies that have been carried out to confirm reduction in potato tuber weight in South Africa. However, it is plausable to speculate, given the fact that Sss also infects roots causing root galls that impair plant growth and productivity, that this may have an indirect effect on the number and size of the tubers produced (Falloon *et al.*, 2004; Lister *et al.*, 2004; Nielsen and Larsen, 2004; Brown *et al.*, 2007; Shah *et al.*, 2012; Falloon *et al.*, 2016).

Powdery scab has an important effect on the seed potato industry, as infected seed tubers are undesirable for establishment of a new crop and act as the mechanism of dispersal from infected fields to non-infected fields (Harrison *et al.*, 1997; Falloon, 2008). Tolerance level of an average tuber surface infection of about 1% powdery scab is used in some countries with seed certification systems, like Switzerland (Merz and Falloon, 2008). Similarly, South Africa has a certification scheme in place; for inspection and testing of potato seed to prevent

high levels of powdery scab infection on seed tubers. A certification standard of Generation 0 (G0) seed potatoes and Generation 1 (G1) seed potatoes (disease free *in-vitro* propagation material or microtubers from an approved laboratory) to Generation 8 (G8) seed potatoes (any earlier generation than the generation of the propagating material under cultivation) is used (DAFF, 1998).

2.3.7. Factors influencing *Spongospora subterranea* f. sp. *subterranea* infection

It is widely accepted that powdery scab development occurs in cool, moist and heavy soils (Merz, 2008), but several factors such as temperature, soil moisture, soil type, soil inoculum level and root exudates composition influence the incidence and severity of powdery scab (van de Graaf *et al.,* 2007).

2.3.7.1. Temperature

Different stages in the life cycle of Sss are favoured by different temperatures. For example, Sss zoospores are released at temperatures ranging from 5 to 25°C, while zoospore activity and potato tuber infection are highest during cool temperatures of 12 to 13°C, with root galls developing at slightly warmer temperatures of 17°C (van de Graaf *et al.,* 2007). van de Graaf *et al.,* (2005) and Lees *et al.,* (2008) recognised high tuber infection levels at 9°C, 12°C and 17°C in pot trials with severe symptoms occurring at 12°C. However, the average amount of DNA per mg tuber skin was significantly higher in plants grown at 9^{o}C than in those grown at 17^{o}C and 12°C (Lees *et al.,* 2008). Additionally, Sss DNA was detected in tubers that did not show any powdery scab symptoms at the three different temperatures of 9^{o}C, 12^{o}C, and 17^{o}C (Lees *et al.,* 2008). Warm temperatures ranging from 25 to 30°C reduce primary zoospore infectivity, but stimulate rapid release of secondary zoospores from infected roots (Fornier, 1997).

2.3.7.2. Soil moisture

Soil moisture is one of the most important factors affecting development of powdery scab on potato tubers as the presence of moisture in the soil is important for pathogen infection and subsequent stages of the disease. Therefore, determination of suitable moisture levels is crucial for the control of powdery scab (Tuncer, 2002). Timing and amount of water present

in the soil influence powdery scab occurrence and severity (Harrison *et al.*, 1997; Merz and Falloon, 2008).

Soil moisture affects resting spore germination (Fornier, 1997; Harrison *et al*., 1997; Balendres *et al*., 2016) and zoospores movement to the host tissues to initiate infection of roots and tubers (Fornier, 1997; Harrison *et al.*, 1997). High soil moisture levels deplete the mineral and oxygen availability in the soil, affecting root and tuber resistance to Sss infection and reducing the activity of potential antagonistic microbes in the soil (Fornier, 1997). Smith *et al.*, (1988) described powdery scab as a disease of wet seasons and waterlogged soils, while Tuncer (2002) considered alternating wet and dry soil conditions as conducive for large powdery scab lesions. Conversely, van de Graaf *et al.*, (2005) showed that constant soil moisture resulted in a higher rate of disease development than a fluctuating moisture regime.

Water availability at levels ranging between -0.01 and -0.03 bars is reported to stimulate high levels of root infection (de Boer *et al.*, 1985). Higher powdery scab incidence and severity were observed when plants were kept under constant moderate soil moisture (-1 bar), than those grown under fluctuating water regimes (van de Graaf *et al.*, 2005). However, low powdery scab incidences were found at the lowest moisture levels with limited yield reduction (Tuncer, 2002). On the other hand, powdery scab was a problem in areas where there was a high irrigation frequency of every one to two days (de Boer, 2000). Irrigation at 2 weeks' intervals or when applied during the second half of the season did not affect powdery scab disease incidence (Adams *et al*., 1987).

2.3.7.3. Soil type

Physical characteristics of the soil are responsible for water, gaseous movement and exchange required for potato roots and tubers development, while a condusive living environment for microorganisms is determined by soil structure (Harrison *et al*., 1997; Fiers *et al*., 2012). Soil with poor drainage influences lenticel opening that encourages decrease of gaseous exchange and lowers the levels of oxygen and carbon dioxide; thereby promoting tuber infection (Harrison *et al.*, 1997).

Powdery scab and root gall formation were reported in different soil textures; however, the diseases are reported to be prevalent in sandy and organic soils (van de Haar, 2000), though,

the amount of powdery scab did not differ significantly among soil types (van de Graaf *et al*., 2005). However, increased powdery scab severity was recorded in black sandy soils with high humus content compared to soils with low humus content (Nielsen and Nicolaisen, 2000). Shakoor *et al.*, (2015) and Brierley *et al*., (2009) further reported that powdery scab is more problematic in sandy soils than in loamy soils. The highest levels of root infection were recorded in clay loam soils than in sandy soil (de Boer, 2000). On the other hand, fewer root galls were found on potato plants grown on clay soil compared to plants grown on sandy and loam soils (van de Graaf *et al*., 2007).

2.3.7.4. Soil and seed inoculum level

Seed-borne inoculum is responsible for both short and long-distance spread of Sss (Brierley *et al*., 2013; Merz, 2000; Merz and Falloon, 2008). However, the inoculum source of Sss can be both soil-borne and/or seed-borne. The severity of the disease is directly related to several environmental factors, sporosori's ability to produce viable zoospores and the zoospore's rate of germination (Burnett, 1991). Initial soil inoculum level in the field cannot be used for determination of powdery scab severity, unless environmental factors are taken into account (Burnnet, 1991; Merz, 2008). Moreover, low numbers of sporosori can rapidly give rise to high numbers of zoospores under favourable conditions (van de Graaf *et al.*, 2005; Baldwin *et al.*, 2008; Merz and Falloon, 2008). However, initial amounts of soil inoculum are used as a risk predictor in other countries like Australia; where PreDicta® Pt (Pt = potato), a DNA-based soil testing service that helps potato growers recognize which soil-borne pathogens pose a significant risk to their crops, is used so that steps can be taken before planting to minimise the risk of yield loss (https://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_pt). Van de Graaf *et al.*, (2005) hypothesised that due to the simultaneous production of zoosporangia and secondary infections under favourable conditions, disease development may be high irrespective of initial amount of inoculum. Mature tuber lesions carry an average of 8500 sporosori (Bouchek-Méchiche *et al.*, 2005; Falloon *et al.*, 2011). The susceptible potato cultivar Bintje carried Sss tuber-borne sporosori that was ten times higher than the less susceptible cultivar Nicola (Bouchek-Méchiche *et al.*, 2005).

Powdery scab can develop from disease-free and symptomless tubers in soils having Sss inoculum, and from infected tubers planted in un-contaminated soil (Harrison *et al.*, 1997).

Moreover, the impact of both initial amount of inoculum and secondary infection on disease development is dependent on environmental factors, as Sss DNA was detected from symptomless root at all inoculum levels (van de Graaf *et al.*, 2007). Tegg *et al.*, (2015) also discovered development of root diseases from symptomless tubers bearing Sss sporosori and planted in pathogen-free soil. There is no association between the amount of seed tuber inoculum and powdery scab disease in the progeny tubers (Tegg *et al.*, 2015). Hence, varying quantities of seed tuber inoculum can result in different levels of powdery scab on the progeny tubers. Parker (1984) and Christ (1989) also discovered that disease incidence and severity resulting from naturally occurring soil inoculum were similar to that obtained in pots with additional inoculum added. On the contrary, several reports highlighted the effects of differing inoculum levels on powdery scab development (Makarainen *et al.*, 1994; van de Graaf *et al.*, 2005; Brierley *et al.*, 2013). A higher powdery scab incidence was reported in plants that were inoculated with Sss zoospore suspension than those grown in soil that naturally had Sss inoculum (Makarainen *et al*., 1994).

2.3.7.5. Root exudates

Root exudates are important in influencing the interactions between the plant and other soil microorganisms (Neumann and Romheld, 2007). Their function is to attract, inhibit and stimulate microbial growth (Bais *et al*., 2006) and influence root-infecting pathogens colonization and activation (Nelson, 2006). Response of soil microbes varies depending on the root exudate's composition (Balendres *et al.*, 2016).

Primary zoospores are the only motile phase of Sss (Merz *et al.*, 2008) that is responsible for Sss attachment and penetration of host cells (Merz, 1997). After release from the germinating sporosori, primary zoospores migrate to host roots where they encyst and establish infections, which develop into plasmodia and may mature as zoosporangia (Merz *et al.*, 2008). Phytochemicals in potato root exudates play an important role in stimulating Sss sporosori germination of Sss and chemotactic response of Sss zoospores to the host roots (Balendres *et al.*, 2016). Root exudates differing in low molecular weight organic (LMWO) influence spore germination of soil-borne pathogens differently (Carvalhais *et al.*, 2011; Li *et al.*, 2013; Zhang *et al.*, 2015). Twenty-four low molecular weight organic compounds in potato root exudates together with specific amino acids, sugars, organic acids, and other compounds that

were stimulatory to Sss resting spore germination were identified in tissue-cultured potato cultivars varying in resistance to Sss infection (Balendres *et al*., 2016).

2.3.8. Diagnosis and detection of *Spongospora subterranea* f. sp. *subterranea*

There are no effective control methods for powdery scab on potatoes (Merz and Falloon, 2008). The only practical control measure available to potato growers is planting of pathogen-free certified seed tubers in un-contaminated soil (Merz and Falloon, 2008). Therefore, detection and quantification of Sss levels in the soil and tubers is essential for epidemiological studies and risk assessment potential of the field soils and seed tubers (van de Graaf *et al*., 2003; Merz and Falloon, 2008).

2.3.8.1. Visual assessment

Visual inspection of seed tubers for powdery scab lesions and roots for root galls presence has been used for determination of Sss infection. However, symptomless infections of potato tubers have been reported (de Boer *et al*., 1982; van de Graaf *et al*., 2005; Tegg *et al*., 2015) resulting from contamination of symptomless tubers by contact with blemished tubers (de Boer *et al*., 1982). It is not easy to distinguish powdery scab from common scab lesions and this also makes visual assessment an unreliable method for Sss detection (Harrison *et al*., 1997; Merz, 2011).

2.3.8.2. Microscopy

One of the conventional methods used for identification of Sss is by observation of the sporosori and zoosporangia under a microscope. de Haan and van den Bovenkamp (2005) reported an overestimation of Sss concentration by microscopy as compared to ELISA and real-time polymerase chain reaction (PCR). It is likely that some sponge-like structures in potato roots may be confused with sporosori and therefore complicate the microscopic examination (de Haan and van den Bovenkamp, 2005). Furthermore, counting plasmodia under the microscope is time-consuming and laborious and different developmental stages of plasmodia can be difficult to identify (Bouchek-Mechiche *et al.*, 2011). *Spongospora subterranea* f. sp. *subterranea* sporosori from powdery scab tuber lesions are easily visualised under a light microscope, even though experience is required, as sporosori vary in

size (19-85 µm in diameter) and in shape (spherical, ovoid, polyhedral or irregular), hence making their identification difficult (Bouchek-Mechiche *et al.*, 2011). Use of a microscope and haemocytometer for quantification of Sss primary zoospores and sporosori is more time-consuming, less sensitive and less specific when compared to serological methods such as ELISA and molecular methods like real-time PCR (Harrison *et al.*, 1997; Qu and Christ, 2006).

2.3.8.3. Polymerase chain reaction (PCR)

Molecular methods are used to rapidly and accurately detect and quantify all forms of the pathogen (zoosporangia, zoospores, plasmodia, sporosori) in tubers, soils and roots (Bouchek-Mechiche *et al.*, 2000). Specific Sss primers SsF and SsR were designed from the internal transcribed spacer (ITS) regions of Sss for identification of Sss with a conventional PCR assay (Qu and Christ, 2006). Bulman and Marshall (1998) and Bell *et al.*, (1999) also designed species-specific primer pairs (Spo8/9 and Spsl/2) from ITS sequence data for the identification of Sss.

Real-time PCR is a sensitive, specific, quick and reliable tool for detection and quantification of Sss sporosori in water, plant tissue and field soil (van de Graaf *et al.*, 2003; Lees *et al.*, 2008). *Spongospora subterranea* f. sp. *subterranea* DNA can be quantified by measuring the fluorescence intensity over time during the amplification exponential phase (van de Graaf *et al.*, 2003). Ward *et al.*, (2004) highlighted that the TaqMan-based method is 100 times more sensitive than conventional PCR and it avoids post-PCR manipulation such as gel electrophoresis. Real-time PCR is increasingly replacing conventional PCR and ELISA for detection and quantification of Sss in plant tissue and soil (Thangavel *et al.*, 2016). For accurate quantitative risk assessment for a particular field, Bouchek-Mechiche *et al.*, (2002) suggested an automated and quantitative PCR system that quantifies the pathogen in potato tubers.

2.3.8.4. Lateral flow immunoassay

While all of the previously discussed diagnostic tools are useful in Sss identification, they require technical knowledge, are time-consuming and need expensive as well as laboratory equipment, which makes them inappropriate for routine detection of the pathogen during the

certification process (Bouchek-Mechiche *et al.*, 2011). Generally, seed potato ware inspection and field scoring require a quick and easy test to check confusing tuber symptoms (Bouchek-Mechiche *et al.*, 2011). The Sss AgriStrip was manufactured by BIOREBA AG, Reinach, Switzerland as a one-step assay for rapid on-site detection of Sss sporosori (Bouchek-Mechiche *et al.*, 2011). The assay is based on lateral flow immunochromatography and uses monoclonal antibodies, which are specific to sporosori of Sss (Merz *et al.*, 2005). According to Bouchek-Mechiche *et al.*, (2011) the Sss AgriStrip is an appropriate tool for routine identification of powdery scab symptoms on tubers in order to avoid the risk of misidentification between powdery and common scab lesions. However, the method recognises only one life-cycle stage of the Sss, the sporosori (Bouchek-Mechiche *et al.*, 2004).

2.3.9. Control of *Spongospora subterranea* f. sp. *subterranea*

There is no single effective method of controlling the pathogen due to the persistence of sporosori in the soil and the ability of its zoospores to infect and multiply within the roots of a susceptible host (Fornier, 1997; Harrison *et al.*, 1997; Falloon, 2008; Merz and Falloon, 2008). Reduction of the disease can be achieved by integrating different disease management strategies (Burgess and Wale, 1994; Harrison *et al.*, 1997; Falloon, 2008; Merz and Falloon, 2008). Strategies that have been promoted for control of Sss diseases have concentrated on targeting the sporosori stage of the pathogen cycle and on preventing zoospore infection of host root and tuber tissues, breaking the pathogen cycle at either or both of these stages (Falloon, 2008). Fornier (1997) reported the use of three main strategies related to the life cycle of the pathogen to tackle the disease. These include lowering the initial level of inoculum, reduction of pathogen multiplication within the host roots and reduction of tuber infection and development of powdery scab lesions, discussed below.

2.3.9.1. Cultural practices

Soil fumigants and seed treatments have shown to be successful in reducing many soil-borne diseases, however, their effects are generally short-lived; therefore, their use has not always been practical and effective. Options for effective approaches that could be both economically and environmentally sustainable would be highly desirable. Control of powdery

scab can be successful by avoiding conditions that favour the rapid infection of potato roots by the pathogen (Fornier, 1997). Hence, cultural methods for Sss diseases management lead to reduction of the amount of pathogen and disease development rate. These include sanitation, crop rotation, host eradication and agronomical practices such as avoiding waterlogged soil conditions and excessive nitrogen levels.

Avoidance of seed-borne inoculum by use of certified clean seed-tubers in un-contaminated soil is the best method of disease prevention (Merz, 2000). Once Sss is established in the soil, management of the disease becomes difficult due to increasing inoculum produced on roots and tubers of the potato, mainly when susceptible cultivars are planted (Maldonado *et al.*, 2013; Sparrow *et al.*, 2015). Powdery scab was reported to be more prevalent in areas where potato monoculture is practised; therefore, for reduction of Sss soil inoculum it is recommended that rotations between potato crops are practised (Harrison *et al.*, 1997). Rotational crops affect Sss by influencing changes in the soil microbial community (O'Brien and Milroy, 2017).

Spongospora subterranea f. sp. *subterranea* is capable of surviving for many years in the soil without the potato crop as sporosori and through infection cycles on alternative hosts, making it difficult to determine the length of the period between potato crops (Harrison *et al.*, 1997; Merz *et al.*, 2005; Falloon, 2008; Merz, 2008) in rotation schedules. Long rotations of five years are recommended (Falloon, 2008), although Wale (1987) considered the same duration too short for control of the disease. On the other hand, seven years of crop rotation excluding potatoes, was suggested in order to reduce the risk of developing the Sss disease (Sparrow *et al.*, 2015).

2.3.9.2. Biological control

Biological control is one of several measures for integrated management of potato diseases, by use of microorganisms that are antagonistic to the pathogens (Lodhi, 2004). Biological control of soil-borne plant pathogens has gained much popularity as an alternative to synthetic fungicides (Glare *et al.*, 2012) due to its safety for the environment and human health (Brimner and Boland, 2003). Mycoparasitism, production of antibiotics and induction

of host resistance or competition for resources with the pathogen are the mechanisms used by the biological control agents (BCAs) for effective disease suppression (Lal *et al.,* 2016).

Biological control agents (BCAs) are effectively used for management of seed-borne and soil-borne plant pathogens (Nakayama, 2017). Different BCAs, including fungal and bacterial, were reported by different researchers for managing of potato diseases (Lodhi, 2004; Restrepo *et al.,* 2009; Nakayama, 2017). *Bacillus* species and *Pseudomonas* species have been used in biological management of potato diseases such as common scab (Lin *et al.,* 2018) and potato late blight (Ajay and Sunaina, 2005; Hunziker *et al.,* 2015). Plant growth promoting rhizobacteria (PGPR) and vesicular arbuscular mycorrhizae (VAM) are also known to minimize plant diseases and increase potato yields (Yao *et al.,* 2002).

Use of BCAs for managing Sss was reported for the first time by Nielsen and Larsen (2004), where two *Trichoderma harzianum*-based BCAs (TRI 002 and Binab TF) significantly reduced the level of tomato root infection by Sss. Hoyos *et al.,* (2008) also suggested the possible use of these *Trichoderma* species for powdery scab control . Similarly, *Trichoderma viride* and *Trichoderma harzianum* Rifai were found to be effective in suppressing Sss infection in potatoes by Lodhi (2004) and Restrepo *et al.,* (2009), respectively; while *Trichoderma asperellum* was found to be ineffective in controlling Sss in potatoes (Gilchrist *et al.,* 2009). *Trichoderma harzianum* biocontrol is through competition, parasitism, and production of inhibitor compounds and inactivation of the pathogen's enzymatic systems (Leelavathi *et al*., 2014). A different antagonistic fungus, *Aspergillus versicolor,* was found to suppress potato root infection by Sss when applied directly on potato seed tubers and it suppressed powdery scab development in potato tubers by 54-70% (Nakayama and Sayama, 2013). Reduction in tuber infection was also realized with the application of FZB24, a product containing *Bacillus subtilis* (Nakayama and Sayama, 2013). Integration of BCAs with other control strategies like use of fungicides has a potential to better control Sss diseases on potato than when applied alone (Nakayama, 2017).

2.3.9.3. Chemical control

The use of fungicides plays a crucial role in the prevention and management of potato diseases. Different agro-chemicals are used together with other control measures like the use of resistant potato cultivars and suitable cultural practices in a combined disease management

strategy for powdery scab (Falloon *et al*., 1995; Falloon, 2008). The chemicals can be applied to seed tubers or to the soil at or before planting (Falloon, 2008). However, the use of chemicals aimed at reducing inoculum level and pathogen infectivity has shown inconsistent results; and the effectiveness of the treatments varies with the inoculum level (Burgess and Wale, 1994; Falloon *et al.*, 1996; Wale, 2000; Thangavel *et al.*, 2015).

There are no effective chemicals for control of Sss on potatoes in South Africa currently (Simango and van der Waals, 2017). Nevertheless, Simango and van der Waals (2017) reported reduction of Sss in the rhizospheric soil and potato roots with the use of metam sodium, fluazinam and calcium cyanamide soil treatments. Seed tuber or soil fungicide treatments at planting have successfully reduced powdery scab incidence and increased the yield of potato tubers in New Zealand and Europe (Braithwaite *et al.*, 1994; Falloon *et al.*, 1995). Fluazinam and mancozeb were effective for control of powdery scab and root gall formation (Falloon *et al.*, 1995; Wale *et al.*, 2003; Tsror, 2017), however, phytotoxic side effects were evident with the use of these chemicals (Wale *et al.*, 2003). Other than fluazinam, flusulfamide was also shown to be effective in reducing powdery scab in infested soil when used for seed disinfection (Falloon, 2008).

Fluazinam (Omega) is registered for the management of Sss diseases in roots and on tubers in United States (Bittara *et al.*, 2018). Fluazinam, fludioxonil, and mancozeb applied as soil treatments have been shown to be effective in reducing powdery scab severity (Tsror, 2017). Potato producers use fungicides such as fluazinam, mancozeb and dichlorophen-Na as dressings for seed tubers, applied as in-furrow applications at planting or during storage (O'Brien and Milroy, 2017). Incorporation of fluazinam, flusulfamide, mancozeb, cypronidil, dichlorophen-Na or sulfur as soil treatments before planting reduced powdery scab by 20% (Genet *et al.*, 1996).

The chemicals formaldehyde or copper sulphate solution can be used for sterilization and cleaning of any equipment used for handling of seed potato tubers (Harrison *et al.*, 1997). Reduction of tuber infections was observed when infested soils were treated with soil fumigants such as methyl bromide (James, 2004), metam sodium and chloropicrin (James 2004; Tsror, 2017). Moreover, promising results with the application of chloropicrin for the management of powdery scab was reported (Tsror *et al.*, 2009; Tsror 2014; Tsror *et al.*, 2016a) and powdery scab disease was reduced with tuber yield increases. Use of 61% 1,3-

dichloropropene, 35% chloropicrin (Telopic) and metam sodium in field experiments conducted in Israel over two consecutive years reduced powdery scab incidence by 70 - 90% (Tsror *et al.*, 2009). In another study, an effective reduction in the amount of Sss in the soil was achieved with chloropicrin fumigation; however, the chemical was not effective in the control of tuber powdery scab disease and root infection (Bittara *et al.*, 2016).

Seed-piece treatments with zinc compounds, formalin and sodium hypochlorite reduced the number of tuber lesions at harvest (Burnett *et al.*, 1993). Cyazofamid, a fungicide that is commonly used to control late blight (*Phytophthora infestans*) (Mitani *et al.*, 2002), was effective in the management of Sss in potato roots and tubers (Thomson *et al.*, 2006). Furthermore, pre-plant seed treatment with boric acid and stable bleaching powder (calcium hypochlorite) was reported to reduce powdery scab incidence and severity in field trials (Hamidullah *et al.*, 2002). Boric acid solution and boron (as sodium tetraborate) reduced Sss root infection in tomato plants and root galling in potato plants, when applied as a nutrient solution and as seed tuber treatment, respectively (Falloon *et al.*, 2001).

Although the chemicals registered against powdery scab can be effective, the cost of soil applications particularly, is extremely high and these do not offer complete control of the disease (Falloon *et al.*, 1996; Larkin and Griffin, 2006; Falloon, 2008). Furthermore, use of synthetic pesticides can result in poisoning of farmers through inappropriate spraying methods, poisoning of consumers through high residual levels of these chemicals, elimination of non-target organisms, as well as selection of phytopathogens, pests and weeds insensitive to certain active ingredients (Damalas and Eleftherohorinos, 2011). This has led to increased global awareness on the risks involved in use of pesticides, which necessitates the consideration of alternative disease management methods (Fan *et al.*, 2008). In most countries, methyl bromide and other synthetic pesticides like chloropicrin perceived to be harmful to humans and the environment have been banned under the Montreal Protocol on Substances that Deplete the Ozone Layer, due to their negative effects on the environment (Ibekwe, 2004).

2.3.9.4. Biofumigation

Biofumigation involves incorporation of different *Brassica* and other related species into the soil, leading to isothiocyanate compounds (ITCs) release, through hydrolysis of glucosinolate

(GSL) compounds in the plant tissues (Kirkegaard *et al.*, 1993). Use of *Brassica* plants as cover and green manure crops have been associated with reductions in soil-borne potato diseases (Larkin and Lynch, 2018). *Brassica* crops like broccoli (*Brassica oleracea* var *italica*), cabbage (*Brassica oleracea* var. capitata) , cauliflower (*Brassica oleracea* var. *botrytis*), kale (*Brassica oleracea* var. *sabellica*), turnip (*Brassica rapa* subsp. *rapa*), radish (*Raphanus raphanistrum* subsp. *sativus*), rapeseed (*Brassica napus* L.), and mustards, produce GSL, a sulfur compound that breaks down to produce ITCs which are toxic to several microorganisms in the soil (Smolinska *et al.*, 2003; Matthiessen and Shackleton, 2005). Soil Sss inoculum levels were significantly reduced in field rotations with Indian mustard (*Brassica juncea* L.) while rotations with rapeseed (*Brassica napus* L.) and yellow mustard (*Sinapis alba* L.) were less effective (Larkin and Griffin, 2006). On the other hand, *Brassica juncea* was found to be effective when applied to the soil as freeze-dried powder before tuber planting (Wale *et al.*, 2003).

2.4. Host resistance

An important objective of global potato breeding programmes is disease resistance and the use of resistant cultivars is an important strategy for disease management (Meiyalaghan *et al.*, 2009). The use of host resistance against Sss promises to be a long term, cost-effective approach for disease management (Johnson and Cummings, 2015). Genetic resistance is suitable on pathogen populations with low genetic diversity like Sss (Gau *et al.*, 2013). There is no potato cultivar that has been identified to be immune to infection by Sss (Merz *et al.*, 2006; Prentice *et al.*, 2007; Houser and Davidson, 2010; Falloon *et al*, 2013). Minor pathogenic variation has been reported with Sss, suggesting that different strains of this pathogen exist, and that cultivars identified as relatively resistant to a certain strain might become susceptible to a different strain (Bulman and Marshall, 1998; Falloon *et al.*, 2003). Consequently, development of potato varieties with genetic resistance to Sss tuber and root infection can be beneficial in the control of Sss diseases especially when combined with other control strategies in an integrated management of the disease.

2.4.1. Mechanisms of resistance to *Spongospora subterranea* f. sp. *subterranea* in potato

The mechanisms of potato resistance to Sss infection are not fully understood. Resistance differs among cultivars; cultivars that are resistant to tuber infection may be highly susceptible to root infection and galling, or vice versa; suggesting that the mechanisms of resistance differ for roots and tubers in different potato cultivars, indicating that host resistance is expressed at the sites of zoospore penetration of root and tuber cells (Falloon *et al.,* 2003). Resistance to powdery scab was found to be inherited from one generation to the next (Wastie, 1991; Harrison *et al.,* 1997; Merz *et al.,* 2004).

2.4.2. Genetics of resistance to *Spongospora subterranea* f. sp. *subterranea* in potato

Generally, plants resistance against microbial attack is either qualitative or quantitative, and the mechanisms of resistance are controlled by different genes (Kushalapa *et al.,* 2016). Plants use a combination of active and passive defense mechanisms to defend themselves against pathogens (Jibril *et al.,* 2016). Inhibition of pathogens entrance and spread in the plant in passive defence is activated by structural characteristics that act as physical barriers (Jibril *et al.,* 2016). Toxic substances are produced and they create conditions that inhibit growth of the pathogen by the biochemical responses that take place in the plant cells and tissues during active defence (Gouveia *et al.,* 2017).

Two-branched innate immune system is used by plants to respond to pathogen attack (Jones and Dangl, 2006). In the first branch of the immune system, the plant cells recognize and respond to molecules common to many classes of microbes, including non-pathogenic organisms (Dangl and Jones, 2001; Jones and Dangl, 2006). Pathogen-associated molecular patterns (PAMPs) recognition by plant cell surface pattern recognition receptors (PRR) induces resistance, initiating PAMP triggered immunity that prevents pathogens infection before invasion. PAMPs activate defence responses that are collectively termed PAMP triggered immunity (PTI) or basal resistance (Chisholm *et al.,* 2006; Jones and Dangl, 2006). PAMP triggered immunity (PTI) induces pathogen-responsive genes, production of reactive oxygen species, mitogen-activated protein kinase signalling and deposition of callose to reinforce the cell wall at sites of infection, thereby preventing pathogen growth at an early stage of infection (Jones and Dangl, 2006). In the second branch of disease resistance (R) proteins recognize pathogen-delivered effectors inside the plant cell. Cognate pathogen

avirulence (*Avr*) gene products are recognized by protein products of plant resistance (R) genes and trigger strong resistance response (Gouveia *et al*., 2017; Jones and Dangl, 2006). Resistance proteins (R protein) mediated defenses are called effector triggered immunity (ETI) or gene-for-gene resistance (Jones and Dangl, 2006; Robatzek and Saijo, 2008).

The hypersensitive response (HR) is induced by ETI with restricted cell death and expression of defense gene that suppresses pathogens growth and spread (Giraldo and Valent, 2013). PTI and ETI are two mechanisms seen as stepwise evolution schematized in the zigzag model (Figure 2.7) described by Jones and Dangl (2006). Several genes for resistance to potato diseases have been identified in potato germplasm and are available to potato breeders. Antimicrobial peptides (AMPs) such as Snakin-1 (SN1) and Snakin-2 (SN2) have been identified and isolated from potato tubers (Segura *et al.,* 1999; Berrocal-Lobo *et al.,* 2002; Almasia *et al*., 2008; Lekota *et al.,* 2019). Overexpression of the *StSN1* and *StSN2* genes in potatoes is known to provide broad-spectrum activity against a wide range of bacterial and fungal pathogens (Berrocal-Lobo *et al.,* 2002). Perla *et al.,* (2014) also discovered resistance to powdery scab in repeated glasshouse experiments on 20 potato cultivars, where the level of lipoxygenase (*StLOX*) gene expression was positively correlated with disease incidence in the russet skinned cultivars and negatively correlated with tuber disease severity.

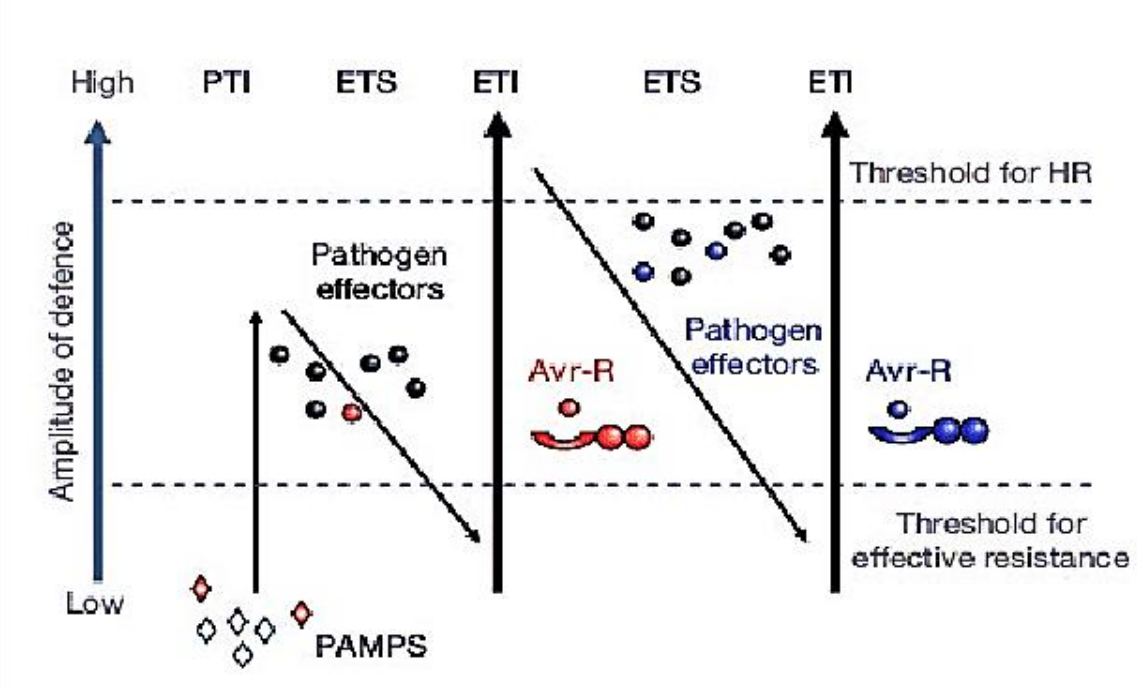

Figure 2.7. A zigzag model illustrating the quantitative output of the plant immune system. In the scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS1 ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by nucleotide-binding site leucine-rich repeat (NB-LRR) protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue); these can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI (Jones and Dangl, 2006).

Few studies have reported genes involved in the interaction of Sss and its host, potato (Rodríguez-Fuerte *et al.*, 2014; Lekota *et al.*, 2019). Methalothionein, phosphate 2C, and pectin methylesterase inhibitor genes were overexpressed in Sss infected susceptible *Solunum phureja*, and two different genes associated with α-Galactosidase were transcribed several times more than in not-inoculated cultivar (Rodríguez-Fuerte *et al.*, 2014). Several defense-related genes were identified in the tolerant (Innovator) and moderately susceptible (Vanderplank) cultivars of the tetraploid *Solunum tuberosum* group in Lekota *et al*., (2019).

Nevertheless, contrasting expression patterns between the tolerant and susceptible cultivars were recorded in the study. Genes identified included the marker genes involved in the salicylic acid hormonal response pathway MRNA, 1346 bp sequence (*StMRNA)*, UDP-glucoseglucosyl transferase (*StUDP)* and WRKY transcription factor 6 (*StWRKY6*). Induction of six defense-related genes *StWRKY6*, Tospovirus resistance protein B (*StTOSB*), *StSN2*, *StLOX*, *StUDP* and *StSN1* persisted until harvest of the tubers, 15 weeks after emergence (WAE), while three other genes NBS-LRR protein (*StNBS)*, *StMRNA* and PRF (*StPRF*) were highly up-regulated in the tolerant cultivar at 7 WAE, which was during the initial stages of disease development (Lekota *et al*., 2019).

2.4.3. Resistance-related metabolites

Little information is available about the biochemical and cellular interactions of Sss with its host cells (Maldonado *et al.,* 2015). Following pathogen attack, plants, including potatoes, produce several pathogenesis and defense-related compounds such as pathogenesis-related (PR) proteins, signal molecules and phytoalexins to deter the pathogen (Kombrink and Schmelzer, 2001). Resistance-related metabolites (RRMs) are produced in growing plants and are stored in trichomes, oil glands and epidermal cell layers as nontoxic glycosides, with toxic forms bieng released after hydrolysis (Kushalappa *et al.,* 2016). After pathogen attack, de novo biosynthesis of resistance metabolites may take place; known as phytoalexins (Ahuja *et al.,* 2012; Piasecka *et al.,* 2015). The amount of the metabolite synthesized by the plant, and the antimicrobial property of a given metabolite determines the resistance of the plant (Piasecka *et al.,* 2015). Primary cell walls are imposed by the deposition of secondary metabolites which are induced (RRI) following pathogen invasion (Bashline *et al.,* 2014; Eudes *et al.,* 2014; Nakano *et al.,* 2015).

Generally, plants produce antimicrobial carotenoids, phenols, flavonoids, terpenes, fatty acids and alkaloids (Tsao, 2009). However, only carotenoids, alkaloids and phenolics are found in significant amounts in potatoes (Tsao, 2009). Quantitative data of compounds produced in potatoes is not readily available in the literature as not all compounds in potatoes have been detected or reported. However, some compounds or groups of compounds are common in all potato cultivars, such as chlorogenic acid, which is the predominant phenolic acid found in all potatoes. Some compounds are unique to certain cultivars; for instance, anthocyanin is only

found in red and purple-fleshed potatoes and carotenoids are only found in yellow and orange-fleshed potatoes (Brown, 2005).

It had been shown in several molecular studies that enhanced resistance is induced when plants are treated with defense-inducing agents such as β-aminobutyric acid (BABA), thiadiazole-7-carbothioc acid S-methyl ester (BTH), and thiamine (vitamin B1) before pathogen attack (Ahn *et al.*, 2005; Lobato *et al.*, 2010). Moreover, application of BABA has been reported to induce resistance in potatoes against Sss root infection (Maldonado *et al.*, 2015) and against *Phytophthora infestans* (Eschen-Lippold *et al.*, 2010; Liljeroth *et al.*, 2010). Other studies on potato disease defense have demonstrated induction of resistance with BABA treatments to *Phytophthora infestans* (Altamiranda *et al.*, 2008; Olivieri *et al.*, 2009; Eschen-Lippold *et al.*, 2010; Liljeroth *et al.*, 2010; Bengtsson *et al.*, 2014), and the necrotrophic potato pathogens *Fusarium solani* and *Fusarium sulphureum* (Olivieri *et al.*, 2009; Yin *et al.*, 2010).

2.5. Conclusion

The review has highlighted the knowledge and gaps in the Sss potato pathosystem that require further elucidation and research. Powdery scab disease is a serious economic threat to the potato crop all over the world as it reduces tuber quality and tuber marketability value. In this review, the factors contributing to Sss diseases development, the methods used for Sss detection and management strategies for the Sss diseases were discussed based on published work. According to this review, no method has yet been found effective in controlling Sss diseases; hence control can only be achieved by the integration of different disease management strategies. Several aspects of the Sss potato pathosystem, such as the mechanisms of resistance to Sss in potato, are poorly understood and further research is needed to understand the genetic and phytochemical mechanisms that are involved. Incorporation of host resistance into other disease management strategies such as crop rotation and fungicide application could be effectively implemented if the genes and metabolites responsible for potato resistance to Sss infection are well documented. This could assist in development of genetically modified potato cultivars with more of the phytochemicals enhancing resistance of potato to the Sss disease. Compounds released in potato roots and root exudates in response to pathogen attack can also be identified and used as resistance biomarkers for screening of cultivars in potato-breeding programs. Additionally,

identified genes for resistance can also be used as genetic markers for marker-assisted selection in the breeding programs. Studies on sources of resistance to Sss and evolution of resistance genes are needed for developing novel potato cultivars with broad and durable resistance to Sss root and tuber infection.

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Chapter 3

Relative susceptibility of ten potato cultivars to diseases caused by *Spongospora subterranea* f. sp. *subterranea* infection

Abstract

The biotrophic protozoan *Spongospora subterranea* f. sp. *subterranea* (Sss) causes powdery scab, root infection and root galling diseases on potato. The susceptibility of 10 potato cultivars commonly grown in South Africa to Sss diseases was assessed in pot trials in a greenhouse. Disease severity of root gall formation and root infection was determined 60 days after planting, while powdery scab severity was evaluated at harvest, 120 days after planting. DNA extracted from potato roots and tubers was quantified using quantitative polymerase chain reaction (qPCR) to determine the concentration of pathogen DNA in root and tuber tissues. None of the cultivars tested were resistant to root or tuber infection by Sss, however, significant differences ($P < 0.05$) were observed among potato cultivars in susceptibility to the diseases. All the cultivars tested developed powdery scab lesions, except for Innovator, which showed no powdery scab symptoms, but Sss DNA was detected in the progeny tubers, indicating possible latent infection of the pathogen. Pearson's correlation analyses of the disease indices showed no association ($P > 0.05$) between root gall severity, root infection severity and powdery scab severity in the same cultivar. These results provide potato growers with information on susceptibility of cultivars to Sss, allowing them to make informed strategic decisions in management programmes.

Keywords: Plasmodiophorid, powdery scab, quantitative PCR, root galling, *Solanum tuberosum*

3.1. Introduction

Spongospora subterranea (Wallr.) Lagerh. f. sp. *subterranea* Tomlinson (Sss) is a plasmodiophorid obligate pathogen that causes powdery scab of potato tubers worldwide (Falloon *et al.*, 2015). Powdery scab is a disease of economic importance in potato production because it reduces the quality of potato tubers destined for seed and ware markets (Harrison *et al*., 1997; Tegg *et al*., 2014). In addition to powdery scab on potato tubers, Sss infects potato root hairs, causing root galls (hyperplasia), subsequently impairing plant growth and productivity through reduced water use and nutrient uptake (Falloon *et al*., 2015; Bittara *et al*., 2016). Sss is not only important as a potato pathogen, but is also a vector of the potato mop-top virus (PMTV), which causes internal damage to potato tubers, reducing their market value (Andersen *et al*., 2002; Kirk, 2008).

The inoculum source of Sss can be sporosori present on seed tubers and/or in root galls and the soil, although the role of each source of inoculum in Sss disease epidemics is not fully understood (Merz and Falloon, 2009). Scabs on potato tubers contain masses of sporosori, which are aggregates of resting spores (sporeballs), and release primary zoospores under conducive environmental conditions, initiating disease epidemics on potato (Merz, 2008). Root galls also contain powdery masses of sporosori that are released into the soil. The sporeballs are thick cell-walled, highly recalcitrant to environmental stresses and can persist being dormant in the soil for more than 10 years (Wale, 2000; Falloon, 2008; Bittara *et al.*, 2013). Sporeballs have a high reproductive potential and when triggered by root exudates they gradually release zoospores into the soil at staggered periods making control and eradication of Sss very difficult (Merz, 2008; Balendres *et al*., 2014; 2016).

The only reliable method for controlling Sss on potato is to plant disease-free seed tubers in uncontaminated fields (Merz, 2008). Many of the management strategies used by potato growers to minimise risk of Sss diseases are based on crop rotation; cultural practices and the use of biocontrol agents (Merz, 2008). Various reports, however, have shown that these strategies, more often than not, yield inconsistent results in the management of Sss (Merz *et al*., 2012; Balendres *et al*., 2017). For example, sporosori in fields are difficult to eradicate by crop rotation due to longevity of the resting spores in the soil (Falloon *et al*., 2011). Furthermore, Sss has a relatively wide host range that could allow the pathogen to survive and multiply for long periods of time (Falloon, 2008). With the lack of efficient and effective

Sss control strategies, powdery scab has increased in importance in all potato production areas globally (Falloon, 2008). The increase in disease incidence and severity has been aggravated by increased cultivation of potato cultivars susceptible to Sss.

Planting of potato cultivars that are resistant to Sss infection is one of the most effective and sustainable long-term strategies for management of Sss on potato (Merz and Falloon, 2009). However, efforts to breed for Sss resistance in potato have been hampered by lack of gene sources that are resistant to both tuber and root infections. Many studies have indicated that Sss resistance in potato roots and tubers are under different genetic control mechanisms and are inherited independently (van de Graaf *et al*., 2007; Merz *et al*., 2012). Some of the cultivars are more susceptible to powdery scab than to root infection and root gall formation or vice-versa (Falloon *et al*., 2003; Genet *et al*., 2005; Nitzan *et al*., 2008; Houser and Davidson, 2010). Different studies have reported that in some cultivars susceptibility to powdery scab is not related to the development of root galls, highlighting the importance of root infection in the epidemiology of Sss (Merz *et al*., 2012; Maldonado *et al*., 2013).

Until recently, assessment of potato cultivar susceptibility to Sss was primarily through monitoring the development of powdery scab symptoms on potato tubers, disregarding root infections (Falloon *et al.*, 2003). However de Boer (2000) and Tegg *et al*., (2013) noted that if control of root gall development is ignored, the level of Sss inoculum in the soil could still increase during the cultivation of a potato crop, even in the absence of powdery scab lesions on the tubers. Studies to evaluate susceptibility of commercial potato cultivars to Sss infection of roots and tubers are gaining attention and have been done in different countries including USA (Houser and Davidson, 2010), Switzerland (Merz *et al*., 2004), New Zealand (Falloon *et al*., 2003; Genet *et al*., 2007) and Australia (Thangavel *et al*., 2015). In South Africa, there is little information available on the relationship between the susceptibility of roots and tubers of commonly grown potato cultivars to Sss infection. Powdery scab susceptibility screening in South Africa has however been done under field conditions in naturally infested soils (van der Waals, 2015). Screening for susceptibility of cultivars to Sss in the field often results in inconsistency in disease development and symptom expression, due to the influence of environmental conditions (Hiltunen *et al*., 2011). Screening of potato cultivar susceptibility to Sss under greenhouse conditions provides consistent optimal conditions necessary for effective cultivar evaluation.

Assessment of powdery scab and root diseases has conventionally been done using visual observations and by microscopy. Although these methods have been proven to be useful, they are subjective and require an experienced eye. Molecular methods such as quantitative PCR (qPCR) are quick, reliable and specific for the detection and quantification of Sss DNA in host tissues (van de Graaf *et al*., 2003). Furthermore, qPCR can detect all life stages of Sss (zoosporangia, zoospores, plasmodia and sporeballs) (Bell *et al*., 1999; Bouchek-Mechiche *et al*., 2004). In different studies, qPCR has proven to be a useful method for assessment of susceptibility levels of different potato cultivars to Sss infection of potato roots and tubers (Maldonado *et al*., 2013; Thangavel *et al*., 2015).

The aim of the current study was to assess the susceptibility of 10 potato cultivars commonly grown in South Africa to root and tuber diseases caused by Sss under greenhouse conditions. The relationship between susceptibility to root diseases and powdery scab development of each of the 10 cultivars screened was also determined. The findings from this study may have important implications for future potato breeding programs and cultivar choices for potato growers in South Africa.

3.2. Materials and methods

3.2.1. Plant material and soil inoculation

Spongospora subterranea f. sp. *subterranea* inoculum was produced by scraping powdery scab lesions from heavily infected field-grown tubers of cultivar BP1 using a sterile scalpel. The lesions were air-dried and crushed into powder using a sterile pestle and mortar. The powder was sieved through a sterile 75 µm mesh sieve. The concentration of sporosori per gram of inoculum was determined using a haemocytometer (Houser and Davidson, 2010). Lateral flow immunoassay using Sss AgriStrip (BIOREBA AG, Reinach, Switzerland) was used to confirm Sss pathogen in the inoculum (Bouchek-Mechiche *et al.*, 2011).

Sprouted certified mini-tubers of 10 potato cultivars (Table 3.1) commonly grown in South Africa were used in this study. A total of twenty-four sprouted mini-tubers per cultivar were individually planted in twenty-four plastic pots (13.5 cm height x 15 cm diameter), each filled with 2 kg of pasteurised sandy-loam soil and grown in a greenhouse maintained at 22 ± 2 °C with a 16-hr photoperiod. The experiment was laid out in a Randomized Complete Block

Design (RCBD) with two treatments (inoculated and not-inoculated control plants) per cultivar and six replicates per treatment (total of 120 pots). For the inoculated treatment, 2 kg of pasteurised sandy loam soil in greenhouse pots were thoroughly mixed with four grams of Sss inoculum, equivalent to 1.09 x 10^7 sporosori per gram of soil, suspended in 50 ml of distilled water (Houser and Davidson, 2010) 2 h before planting. Pots with pasteurised sandy loam soil mixed with 50 ml of sterile water, served as a negative control (un-inoculated treatment). Each pot was planted with a single mini-tuber of each cultivar. Plants were irrigated every second day with 200 ml of sterile distilled water per pot for maintenance of moist soil conditions favorable for root and tuber infection by Sss. Plants were fertilized fortnightly with 100 ml solution per pot of Dr Fisher's MultiFeed Classic (NPK 19:8:16) (Nulandis Ltd) containing trace elements. The greenhouse experiment was repeated twice; 28 September 2015 to January 2016, trial 1; 15 February to June 2016, trial 2; and 19 July to November 2018, trial 3. All ten cultivars emerged after approximately seven days.

Table 3.1. Ten potato cultivars that were evaluated under greenhouse conditions for susceptibility to root and tuber diseases caused by *Spongospora subterranea* f. sp. *subterranea*

Potato cultivar	**Skin colour**	**Maturation period**[a]	**Use**	**Reference**
Avalanche	White	Medium to late	Mashed potatoes and French fries	Potato Seed Production, 2013
BP1	White	Medium to late	Mashed potatoes	Potato Seed Production, 2013
Fianna	Light Yellow	Medium to late	Chips and frozen chips	Potato Seed Production, 2013
Innovator	Brown/Russet	Early to medium	French fries	ADHB Potato Variety Database, 2019
Lanorma	White	Early	French fries	ADHB Potato Variety Database, 2019
Mondial	White	Late	Mashed potatoes and French fries	HZPC Potatoes, 2019
Sifra	Light Yellow	Late	French fries	HZPC Potatoes, 2019
Up-to-date	Cream White	Medium to late	French fries	Potato Seed Production, 2013
Valor	Cream White	Medium to late	Baking	Potato Seed Production, 2013
Vanderplank	White	Early to medium	French fries and frozen chips	Potato Seed Production, 2013

[a]Maturation period: early = less than 90, medium = 90-110 and late = 110-150 days.

3.2.2. Root infection, root galls and powdery scab assessment

Approximately 60 days after planting (53 days after emergence), two plants per treatment were gently uprooted, washed with sterile distilled water, blotted dry with paper towels and assessed for root infection and root gall formation. A sample (100 g) of roots from each plant was air dried at 25ºC and stored at -20°C for DNA extraction and Sss DNA quantification. Another sample of roots (100 mg) was excised approximately 30 mm below the crown of each plant for light microscopy. A zoosporangium staining procedure was carried out as described by Merz (1989), where roots were destained in ethanol/chloral hydrate/water (1:1:1 w/w/w) for 10 min and then stained for 5 min in a staining solution of 3% formaldehyde, 6% lactic acid, 3.5% phenol, 87.2% ethanol/water (1:1 v/v) and 0.3% water blue (all w/w). The solution was heated to 80°C before use. Roots were then fixed in lactic acid for five minutes, before examination under a compound microscope for root infection. A modification of the scale proposed by Merz (1989) was used for rating root infection from five roots per plant: 0 = no sporangia, 1 = only a few sporangia (1 to 50), 2 = several roots with little infection, 3 = several roots with moderate infection, 4 = sporangia regularly present, moderate infection, 5 = sporangia regularly present, heavy infection. Root gall severity scores were recorded for each plant according to a 0 to 4 scale where: 0 = no galls; 1 = 1 to 2 galls; 2 = 3 to 10 galls; 3 = >10 galls, mostly in clusters; 4 = many galls, regularly distributed over the roots (van de Graaf *et al*., 2007).

At harvest, approximately 120 days after planting, the remaining two plants per block were assessed for powdery scab symptoms on progeny tubers. Tubers were scored for powdery scab severity according to a standard disease assessment scale of zero to 10 (Falloon *et al*., 1995), where 0 = no visible disease symptoms and 10 = tuber surface completely covered with powdery scab lesions (Figure 3.1). After disease assessment, four tubers from each replication were randomly selected, air dried at 25ºC and stored at -20°C for DNA extraction and Sss DNA quantification.

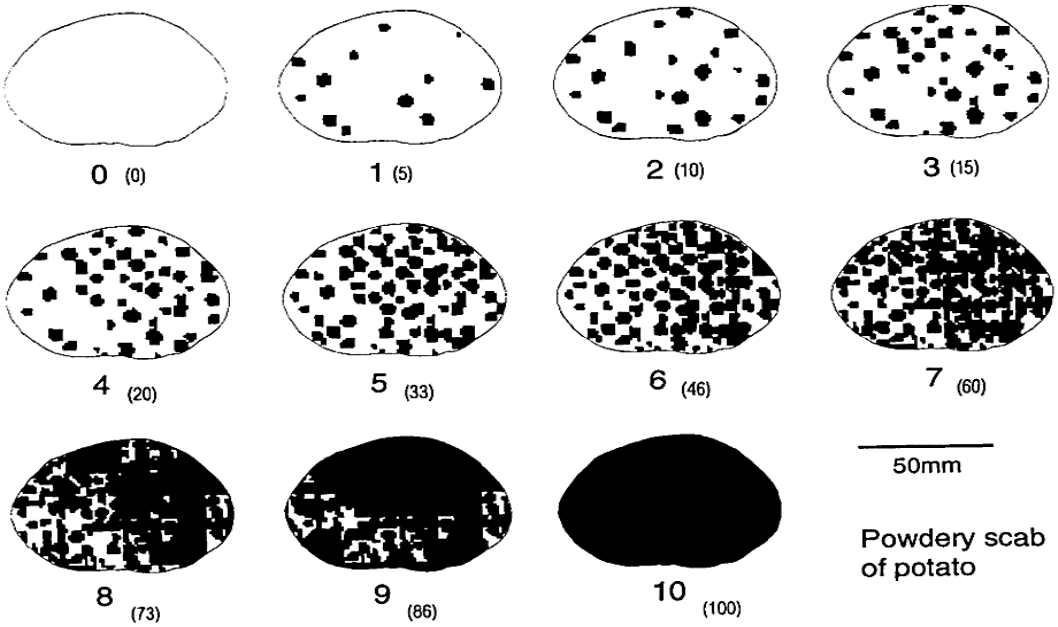

Figure 3.1. Powdery scab assessment scale used to determine disease severity on potato tubers (Falloon *et al*., 1995).

3.2.3. DNA extraction and quantification

Samples of 100 g each of tuber peels and root samples were ground using a mortar and pestle. DNA was extracted from 50 mg ground tuber peels and 50 mg root samples of each plant using the ZR plant/seed DNA kit™ (Zymo Research Corp, USA) according to the manufacturer's recommendations. DNA purity and quantity were measured using the NanoDrop 2000c Spectrophotometer (Thermo Scientific). Quantitative PCR (qPCR) was carried out to confirm the presence of the pathogen and to determine the concentration of Sss DNA in the tubers and root samples. Using the standard curve with an efficiency of 100%, the amount of Sss DNA in each unknown sample was determined on the basis of the Ct values (van de Graaf *et al*., 2003). The qPCR standard curve of the critical threshold (Ct) values against the logarithm of the number of sporosori was obtained using DNA extracted from Sss suspensions with known sporosori concentrations, diluted with Tris-EDTA buffer (pH 8.0) to obtain a dilution series of DNA equivalent to 10 000, 1 000, 100, 10 and 1 sporosori μl^{-1}, respectively (van de Graaf *et al.*, 2003). A non-template control with 1 µl double distilled water instead of DNA was included in every assay as a negative control. All the samples were tested in duplicate and the results were averaged. A final volume of 25 µl containing 1 µl (10 ng μl^{-1}) template DNA, added to 24 µl

reaction mix consisting of 12.5 µl TaqMan® Universal PCR Master Mix, 9.5 µl sterile HPLC water, 0.75 µl of each of the Sss specific primers (0.3 µM), SsTQF1 (5'-CCGGCAGACCCAAAACC-3') and SsTQR1 (5'-CGGGCGTCACCCTTCA-3'), and 0.5 µl TaqMan® probe (0.1 µM) SsTQP1 (5'-CAGACAATCGCACCCAGGTTCTCATG-3') (van de Graaf *et al*., 2003) was used for qPCR. Quantitative PCR reactions were performed in a Piko Real Real-Time PCR system (Thermo Scientific) with the following conditions: 50°C for 2 min, followed by 95°C for 10 minutes, then 45 cycles at 95°C for 15 sec and 60°C for 1 min. Data was expressed as the ng of DNA per gram of tuber or root tissue.

3.2.4. Data analysis

Data on powdery scab, root infection, root galling severity and DNA amounts (ng/µl) for the roots and tubers were analysed by one-way ANOVA using GenStat® (VSN International, 2017; 19th Edition). Homogeneity of variances was assessed during data analysis using Bartlett's test ($\alpha = 0.05$). Normal distribution of the data was evaluated using the Shapiro-Wilk test ($\alpha = 0.05$). Data were back transformed and analysed by ANOVA as randomized complete block design with potato cultivar as a sub-sample. A general linear model (GLM) was used for the analysis, with a binomial error structure to allow for smaller variance of the scores of tolerant and susceptible cultivars (Freund *et al*., 2010). Treatment means were compared using Fisher's protected least significant difference (LSD) test at the 5% level of significance. Powdery scab severity, root infection severity and root gall formation severity of the cultivars were averaged across the three trials and the relationships between root infection disease severity and powdery scab severity aa well as between root gall formation severity and powdery scab severity were determined for each cultivar using the Pearson's correlation analysis. Based on the root infection, root galling and powdery scab disease severity values recorded in this study, potato cultivars were classified according to arbitrary cut-off points as tolerant (0), moderately tolerant (0.1 to 1.4), moderately susceptible (1.5 to 2.4) and susceptible (2.5 to 3.4).

3.3. Results

3.3.1. Susceptibility of cultivars to root infection and root gall formation

None of the 10 potato cultivars screened in this study were found to be resistant to root infection or gall formation, as Sss zoosporangia and galls were observed in or on roots of all cultivars evaluated (Table 3.2; Figure 3.2 A and B). No root gall formation or root zoosporangia infection was observed on the not-inoculated plants. Differences among cultivars in the degree of susceptibility to root symptoms were observed ($P < 0.001$) in all the three trials with no statistical differences ($P > 0.05$) between the three trials for each cultivar.

Cultivar BP1 was consistently the most susceptible to root infection in all three trials, with an average severity score of 3, which was the highest root infection severity value. This was not significantly different ($P > 0.05$) from Avalanche, Sifra and Up-to-date with average severity scores of 2.6, 2.4 and 2.1 respectively. The lowest mean root infection severity across the three trials with the average score of 0.8 was observed on Vanderplank (Table 3.2), which was thus found to be the least susceptible cultivar. Root infection severity was not significantly different ($P > 0.05$) between cultivars Fianna, Innovator, Lanorma, Mondial and Valor.

Root gall severity varied substantially between the 10 cultivars, ranging from 0.3 to 2.5 (Table 3.2). The highest average root gall severity of 2.5 was observed in Valor followed by Up-to-date with a severity of 1.9 (Table 3.2) and there was no significant difference between the two cultivars. Root gall severity was not statistically different ($P > 0.05$) in the eight remaining cultivars Avalanche, BP1, Fianna, Innovator, Lanorma, Mondial, Sifra, Up-to-date and Vanderplank (Table 3.2); with Fianna having the lowest root gall severity of 0.3.

Table 3.2. Root infection and root gall disease severity caused by *Spongospora subterranea* f. sp. *subterranea* on potato cultivars assessed under greenhouse conditions on scales of 0-5 and 0-4, respectively

	Root infection (0 – 5)								Root galling (0 – 4)							
Cultivar	**Trial 1**		**Trial 2**		**Trial 3**		**Mean**		**Trial 1**		**Trial 2**		**Trial 3**		**Mean**	
Avalanche	3.3[x]	cd*	2.3	bcd	1.5	bcd	2.4	cd	0.6	abc	1.2	abcd	1.0	b	0.9	b
BP1	3.8	d	2.8	d	2.4	d	3.0	d	1.2	abcd	0.7	ab	1.0	b	1.0	b
Fianna	1.8	abc	1.2	ab	0.7	ab	1.2	ab	0.2	a	0.3	a	0.7	ab	0.4	a
Innovator	2.3	abcd	1.5	abc	1.0	abc	1.3	abc	0.3	ab	0.8	abc	1.2	bc	0.8	ab
Lanorma	1.5	ab	1.5	abc	1.3	bcd	1.4	abc	1.3	bcd	1.3	abcd	1.3	bcd	1.3	ab
Mondial	1.5	ab	1.3	abc	0.8	abc	1.2	ab	0.2	a	0.5	a	0.2	a	0.3	ab
Sifra	2.8	bcd	2.7	bcd	1.3	bc	2.3	cd	1.2	abcd	0.5	a	0.8	b	0.8	ab
Up-to-date	3.6	d	2.5	cd	1.6	cd	2.6	d	1.5	cd	2.3	cd	2.0	cd	1.9	c
Valor	2.0	abc	1.2	ab	1.1	bc	1.4	abc	2.3	d	2.8	d	2.5	d	2.5	c
Vanderplank	1.3	a	0.7	a	0.5	a	0.8	a	0.3	ab	0.5	a	0.8	b	0.5	ab
LSD	1.5		1.2		0.9		0.9		0.9		1.1		0.7		0.6	

*Means followed by the same letter(s) in a column are not significantly different at $P \leq 0.05$ (LSD test).

[x]Values are back transformed means of the six blocks for each cultivar per trial (n = 6).

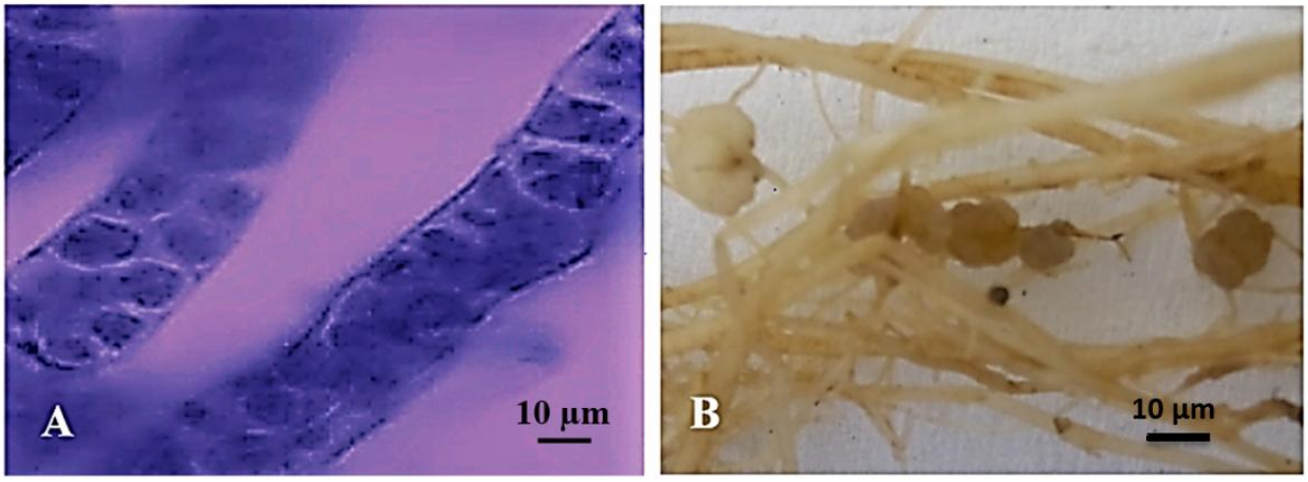

Figure 3.2. A. Micrograph at 100x magnification of *Spongospora subterranea* f. sp. *subterranea* zoosporangia in stained potato roots **B**. Root galls on potato roots.

3.3.2. Susceptibility of cultivars to powdery scab

No powdery scab lesions were observed on progeny tubers of the not-inoculated plants. Statistically significant differences ($P < 0.01$) were observed for powdery scab severity between inoculated potato cultivars. Cultivar Vanderplank had the highest powdery scab severity across all three trials compared to the other cultivars; with a mean severity of 1.6 (Table 3.3; Figure 3.3 A) and was arbitrarily classified in this study as moderately susceptible to the disease (Table 3.4). This was followed by Up-to-date with a mean disease severity value of 1.0, then Avalanche and Lanorma (0.8). These were not significantly different from the mean disease severity of the other five cultivars BP1, Fianna, Mondial, Sifra and Valor and were categorized as moderately tolerant. There were no visible powdery scab lesions on progeny tubers of cultivar Innovator in any of the three trials (Table 3.3) and the cultivar was categorized as tolerant to powdery scab (Table 3.4).

Table 3.3. Powdery scab disease severity caused by *Spongospora subterranea* f. sp. *subterranea* on potato cultivars assessed on a scale of 0-10 under greenhouse conditions

	Powdery scab severity (0 – 10)							
Cultivar	**Trial 1**		**Trial 2**		**Trial 3**		**Mean**	
Avalanche	0.3 [x]	cdef*	0.2	de	1.8	f	0.8	cd
BP1	0.4	cdef	0.8	de	0.6	f	0.6	de
Fianna	0.1	bc	0.1	b	1.4	def	0.5	bc
Innovator	0.0	a	0.0	a	0.0	a	0.0	a
Lanorma	0.1	bcde	0.6	de	1.8	cde	0.8	cd
Mondial	0.1	b	0.9	bc	0.1	b	0.4	b
Sifra	0.2	bcd	0.2	bc	0.6	cd	0.3	bcd
Up-to-date	0.5	bcdef	0.7	de	1.7	ef	1.0	ef
Valor	0.2	b	0.7	de	0.2	bc	0.4	bcd
Vanderplank	0.9	f	1.3	e	2.6	f	1.6	f
LSD	0.4		0.6		0.9		0.4	

*Means followed by the same letter(s) in a column are not significantly different at $P \leq 0.05$ (LSD test).

[x] Values are back transformed means of the six blocks for each cultivar per trial (n = 6).

Figure 3.3. A. Potato tuber of a moderately susceptible cultivar Vanderplank covered with powdery scab lesions caused by *Spongospora subterranea* f. sp. *subterranea* **B**. Potato tuber of a moderately tolerant cultivar BP1 with few powdery scab lesions.

Table 3.4. Classification of ten potato cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea* under greenhouse condition according to their susceptibility ranks to powdery scab, root infection and root gall formation

	*Susceptible	Moderately susceptible	Moderately tolerant	Tolerant
Powdery Scab		Vanderplank	Avalanche BP1 Fianna Lanorma Mondial Sifra Up-to-date Valor	Innovator
Root galling	Valor	Up-to-date	Avalanche BP1 Fianna Innovator Lanorma Mondial Sifra Vanderplank	
Root infection	BP1 Up-to-date	Avalanche Sifra	Fianna Innovator Lanorma Mondial Valor Vanderplank	

*Potato cultivars were arbitrarily categorised into tolerant (0), moderately tolerant (0.1 to 1.4), moderately susceptible (1.5 to 2.4) and susceptible (2.5 to 3.4) according to their average powdery scab, root gall and root infection severity scores.

3.3.3. Detection and quantification of *Spongospora subterranea* f. sp. *subterranea* DNA in potato roots and tubers

Quantitative PCR analysis of root and tuber samples confirmed the presence of Sss DNA in all the inoculated plants of the 10 cultivars including Innovator, which had no visible powdery scab symptoms on the tubers (Table 3.5; Figure 3.4). The correlation coefficient of the standard curves used for calculating the amount of DNA in the unknown samples was consistently greater than 0.9 (Fiure 3.5). Lanorma yielded low amount of Sss DNA from both the roots and tubers of

1.2 ng/µl DNA per 50 mg root weight, and 0.9 ng/µl DNA per 50 mg tuber weight. On the other hand, Avalanche the highest amounts of DNA in the roots (2.8 ng/µl DNA per 50 mg root weight) followed by Up-to-date (2.7 5 ng/µl DNA) and BP1 (2.5 ng/µl) DNA per 50 mg root weight, with no significant differences between the three cultivars. Vanderplank yielded the highest tuber DNA (4.3 ng/µl per 50 mg tuber) weight followed by BP1 (4.1 ng/µl per 50 mg tuber weight), and there was no significant difference between the two cultivars.

Table 3.5. Amounts of *Spongospora subterranea* f. sp. *subterranea* (Sss) DNA detected and measured in roots and tubers of 10 potato cultivars inoculated with Sss

	Sss **DNA in roots (ng/µl)**								Sss **DNA in tubers (ng/µl)**							
Cultivar	**Trial 1**		**Trial 2**		**Trial 3**		**Mean**		**Trial 1**		**Trial 2**		**Trial 3**		**Mean**	
Avalanche	2.4[x]	b*	4.4	a	1.6	c	2.8	a	1.8	ef	1.9	e	0.6	de	1.4	e
BP1	1.3	c	4.0	a	2.1	b	2.5	a	5.3	a	4.9	b	2.0	ab	4.1	a
Fianna	1.4	c	2.5	bcd	2.5	a	2.1	b	1.1	fg	1.2	f	1.2	c	3.1	bcd
Innovator	1.0	d	2.8	bc	2.4	a	2.1	b	4.2	abcd	4.1	c	0.0	e	2.8	d
Lanorma	0.7	e	2.3	cd	0.8	d	1.2	e	0.5	g	1.3	f	1.0	cd	0.9	f
Mondial	1.1	d	2.1	cd	2.0	b	1.8	c	4.5	abc	5.4	a	1.1	cd	3.7	b
Sifra	0.5	f	1.8	d	2.4	a	1.6	cd	3.0	de	3.0	d	0.8	cd	3.0	cd
Up-to-date	2.6	a	3.1	b	2.4	a	2.7	a	3.9	bcd	4.0	c	2.6	a	3.5	bc
Valor	0.5	f	2.3	cd	2.1	b	1.6	cd	3.5	cd	3.6	c	1.4	bc	2.8	d
Vanderplank	1.3	c	1.0	e	1.9	b	1.4	de	4.9	ab	5.7	a	2.3	a	4.3	a
LSD	0.2		0.7		0.2		0.3		1.2		0.6		0.7		0.6	

*Means followed by the same letter (s) in a column are not significantly different at $P \leq 0.05$ (LSD test).

[x]Values are back transformed means of the three replications for each cultivar per trial (n = 3).

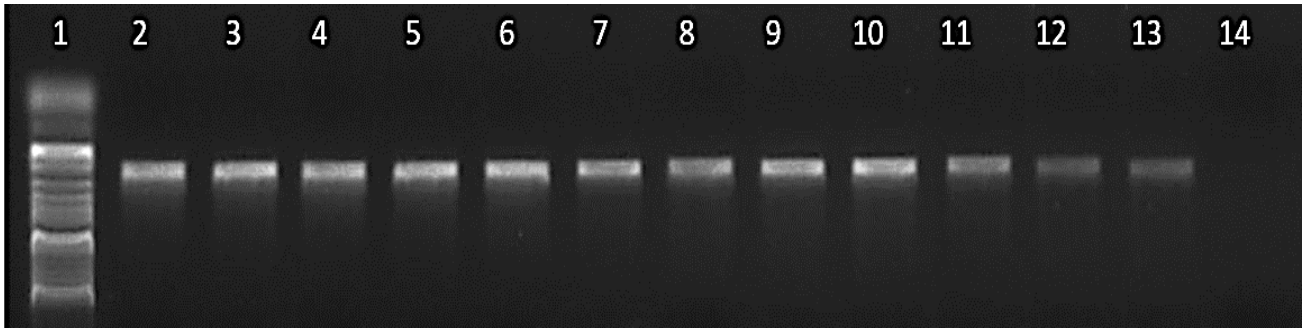

Figure 3.4. Agarose gel of PCR products with *Spongospora subterranea* f. sp. *subterranea* specific primers obtained from DNA extracted from tuber samples of twelve cultivars. (Lane 1: Ladder, Lane 2: Positive control, Lanes 3 – 13: Samples, and Lane 14: Negative control).

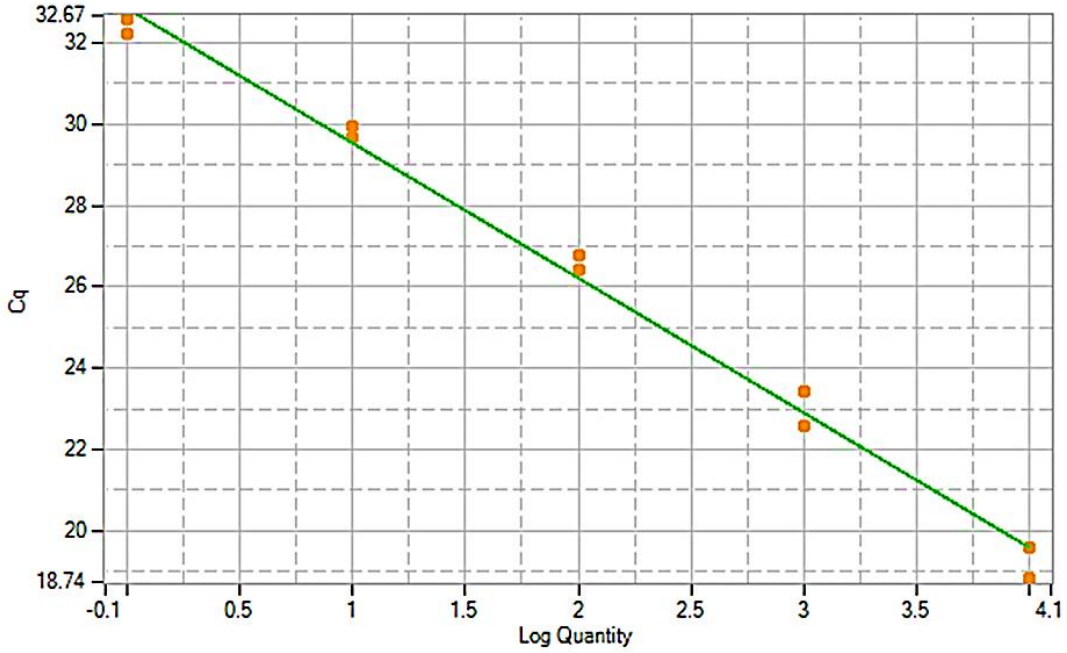

Figure 3.5. Standard curve used in the real-time PCR assay for the quantification of *Spongospora subterranea* f. sp. *subterranea* DNA samples extracted from potato roots and tubers. Cq is the quantification cycle.

No relationship was observed ($P > 0.05$) between root gall severity and powdery scab severity, or between the number of zoosporangia in roots and powdery scab. There was a weak positive correlation between powdery scab severity and root gall severity for all 10 cultivars evaluated, as

shown by a Pearson's correlation coefficient (r) value of 0.17. A negative correlation coefficient (r) of -0.06 was observed between powdery scab severity and root infection severity.

3.4. Discussion

This study evaluated the susceptibility of 10 potato cultivars commonly grown in South Africa to Sss root and tuber infection, using visual assessments, microscopic examination and qPCR. Previous studies have shown that the response to Sss differs among potato cultivars; some cultivars are tolerant to tuber infection, but highly susceptible to root infection and/or root gall formation, or vice versa; suggesting that different mechanisms of resistance exist for roots and tubers of the same plant (Falloon *et al.*, 2003).

All 10 potato cultivars evaluated in this study were found to be either moderately tolerant, moderately susceptible or susceptible to Sss root and tuber infection as all cultivars had zoosporangia and root galls on or in the roots, and powdery scab lesions on tubers, with the exception of cultivar Innovator, which did not show any powdery scab symptoms on progeny tubers. Sss DNA was however, detected in symptomless Innovator tuber tissue samples, possibly due to latent infection of the pathogen. Sss can remain latent in infected potato tubers as has been reported in field trials conducted by de Haan and van den Bovenkamp (2005) and a greenhouse study by van de Graaf *et al*., (2007). van de Graaf *et al*., (2005) and Bell *et al*., (1999) also reported amplification of DNA from symptomless potato tuber peels in a conventional PCR assay with Sss species-specific primers. The other reason for amplification of Sss DNA in symptomless Innovator tuber peels could be the presence of sporosori trapped on the tuber surface or in tuber lenticels (de Boer *et al*., 1982). Presence of Sss DNA in symptomless tubers is an indication that visual disease assessment alone cannot be used as a reliable predictive indicator of potato cultivar susceptibility to Sss infection. Sss DNA was not detected in roots or tubers of any of the not-inoculated controls. Even though low powdery scab and root disease pressure was observed in the study, high amounts of DNA were detected in both roots and tubers of inoculated plants. Vanderplank had the highest amount of Sss DNA in tubers, with the lowest amount of Sss DNA in roots. The study showed a positive relationship between tuber/root infection visual scores and tubers/roots DNA, as Vanderplank also had the highest powdery scab

severity and the second lowest in terms of root zoosporangia severity. Maldonado *et al*., (2012), also recognized increasing amounts of root DNA with increasing density of root zoosporangium infection 2–3 weeks after inoculation.

The findings of this study highlighted a range of susceptibility of potato cultivars to root infection, root gall formation and powdery scab, from tolerant to susceptible. Differing susceptibility levels of potato cultivars to powdery scab (Kirkham, 1986; Wastie *et al*., 1988; de Boer, 1991; Merz *et al*., 2004; Iftikhar *et al*., 2007; Houser and Davidson, 2010; Merz *et al*., 2012; Falloon *et al*., 2013), root gall formation (Nitzan *et al*., 2008; Houser and Davidson, 2010; Nitzan *et al*., 2010) and root zoosporangia infection (Merz *et al*., 2004; Merz *et al*., 2012; Maldonado *et al*., 2013) have been reported by other researchers.

Results from field trials conducted in South Africa demonstrated similar responses of potato cultivars Fianna, Lanorma, Mondial, Sifra and Valor, to powdery scab infection (van der Waals, 2015). These cultivars were found to be moderately tolerant in the greenhouse trial while they were tolerant in the field trial. However, Avalanche and BP1 were found to be susceptible in the field studies (van der Waals, 2015), while they exhibited a moderately tolerant response in the current greenhouse study. In the same field study only Valor (susceptible) had similar responses to root gall formation to the findings of the current study. Avalanche, BP1, Fianna and Mondial were susceptible in the field studies while they exhibited a moderately tolerant response in the greenhouse trials. The disparity between field and greenhouse studies was also reported by van de Graaf *et al*., (2005) who reported a differential reaction of cultivar Swift to powdery scab lesions; which was resistant in field trials but highly susceptible in greenhouse experiments. Powdery scab severities were also found to be higher in the greenhouse than in the field (de Boer, 1991). Inconsistency in susceptibility levels of potato cultivars in the field and greenhouse pot trials have been ascribed to different factors such as genotype x environment interactions (Gans and Vaughan, 2000) and patchy distribution of the Sss inoculum in the field soil (Lees, 2000).

The susceptibility responses of some of the potato cultivars evaluated in this study are in agreement with studies that assessed the same cultivars in New Zealand (Falloon *et al*., 2003;

Genet *et al*., 2007). In New Zealand, Fianna was found to be highly resistant, while Innovator and Valor showed a moderately resistant response to powdery scab (Falloon *et al*., 2003; Genet *et al*., 2007). Even though not fully resistant; Innovator was found to be tolerant to powdery scab in the current study, while Fianna and Valor were moderately tolerant. In this study Mondial was also found to be moderately tolerant which is in contrast with the results reported by Falloon *et al*., (2003) who reported Mondial to be moderately susceptible to powdery scab in a field trial. Similarly, Lees (2000) and Bus (2000) found Bintje and Desiree cultivars to be moderately susceptible, while they had been ranked as highly resistant in screening trials in other countries like USA (Houser and Davidson, 2010), Swizerland (Merz *et al*., 2004) and New Zealand (Falloon *et al*., 2003; Genet *et al*., 2007) and Australia (Thangavel *et al*., 2015). This may be an indication of the variation in virulence of Sss strains to potato in different geographic regions (Muzhinji and van der Waals, 2019; Gau *et al*., 2013; 2015). Genetic variation of Sss strains was evident among but not within locations of the United States (Qu and Christ, 2006) as well as South Africa (Muzhinji and van der Waals, 2019).

While a range of susceptibility levels of the 10 potato cultivars to root infection, root gall formation and powdery scab was observed in this study, very low tuber and root disease pressure was observed in all three trials. This could be the reason for the discrepancies experienced between the field trials conducted in South Africa and the current greenhouse study. This is attributed to the fact that the temperatures of 22 ºC ± 2 in the greenhouse were not favourable for tuber infection and root gall formation, while suitable for root zoosporangium development. Tuber infection is normally high at low temperatures of 12 ºC to 13 ºC, while root galling is optimal at 17 ºC (Van de Graaf *et al*., 2007; Lees *et al*., 2008). Moreover, roots and tubers have different periods of susceptibility to Sss infection; potato tubers have a narrow susceptibility window, whereas roots are susceptible to Sss infection throughout the whole plant development stages; with root infections beginning 15–20 days after inoculation (Thangavel *et al*., 2015).

The results from this study showed that there is no association between tuber and root symptoms in most of the commercially grown potato cultivars in South Africa. A negative correlation was observed between powdery scab and number of root zoosporangia, with a weak positive correlation observed between powdery scab and number of root galls, even in plants that

developed all the three diseases. However, this could have been due to the low tuber and root diseases preasure in all three trials. Vanderplank was found to be moderately susceptible to powdery scab, while it showed a moderately tolerant behavior to both root galling and root infection. On the other hand, Innovator was tolerant to powdery scab, while it was moderately tolerant to root infection and root galling. Only three cultivars, Fianna, Lanorma and Mondial were found to be moderately tolerant to all the three diseases. This corroborates with the findings of Tegg *et al*., (2013), van de Graaf *et al*., (2007) and Merz *et al*., (2012), where no association was observed between powdery scab development and root gall formation in potato. Houser and Davidson (2010) and Zink *et al*., (2004) also concluded that cultivar susceptibility to tuber lesion development does not necessarily indicate cultivar susceptibility to root gall formation; hence a cultivar may be resistant to powdery scab lesion development, but be susceptible to root gall formation or root infection. No cultivar showed a similar response to all the three diseases in the present study. This was in contrast with the findings by Merz *et al*., (2004), where cultivar Desiree ranked similar in root galling, root infection severity and tuber infection severity.

A combination of field and greenhouse trials is important in screening cultivars for susceptibility to Sss infection. Screening potato cultivars in the greenhouse was proposed as preliminary to the evaluation of cultivars in the field (de Boer, 1991). Effective and efficient screening of cultivar susceptibility to Sss infection is important in breeding programs. Findings from this study suggest that none of the cultivars tested are resistant to root gall formation, root infection or tuber infection by Sss, however cultivars that were found to be tolerant to root infection, root gall formation and powdery scab infection may be useful in breeding programmes for cultivars resistant to Sss. Moreover, the results from this study will allow potato growers in South Africa to make informed decisions regarding choice of cultivars and whether to apply other Sss control strategies. Future research is recommended in order to elucidate the differences in susceptibilities of these cultivars under field conditions and at varying inoculum levels naturally found in fields.

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Chapter 4

Metabolic profiling of phytochemicals in potato roots and root exudates

Abstract

Plants defend themselves from pathogens by producing bioactive defense chemicals. The biochemical mechanisms relating to quantitative resistance of potato to root infection by *Spongospora subterranea* f. sp. *subterranea* (Sss) are however, not understood, and are not efficiently utilized in potato breeding programs. An untargeted metabolomics approach using UPLC-Q-TOF/MS was used to elucidate the biochemical mechanisms of susceptibility to Sss root infection. Potato root metabolic profiles of five tolerant cultivars were compared with those of five susceptible cultivars, following Sss inoculation; to identify tolerance-related metabolites. Comparison of relative metabolite abundance of tolerant versus susceptible cultivars revealed contrasting responses to Sss infection. Metabolites belonging to amino acids, organic acids, fatty acids, phenolics and sugars were putatively identified and most of them were especially abundant in the tolerant cultivars relative to the susceptible cultivars. Among these metabolites, phenylalanine, proline, solanidine and tryptophan significantly increased in the tolerant cultivars compared to susceptible cultivars following Sss inoculation. These metabolites are known to activate plant secondary defense metabolism, particularly the phenylpropanoid pathway that produces several antimicrobial compounds including flavonoids and phytoalexins. Cultivars that were tolerant to Sss infection had high levels of flavonoids and alkaloids, well-known cell wall thickening compounds. Root-exuded compounds belonging to the chemical class of phenolics were also found in abundance in the tolerant cultivars compared to susceptible cultivars. This study illustrated that Sss infection of potato roots leads to differential expression of metabolites in tolerant and susceptible potato cultivars. The metabolic profiles in this study have the potential to be used in screening of potato breeding lines for tolerance against Sss root infection.

Keywords: Potato, chromatogram, antimicrobial, metabolic profiles, phytoalexins, UPLC-MS

4.1. Introduction

The potato plant is susceptible to attack by many insects and pathogens. *Spongospora subterranea* f. sp. *subterranea* (Sss), the causal agent of powdery scab in potatoes, is one of the most destructive pathogens of potato (Falloon, 2008). Powdery scab of tubers has long been considered the main disease caused by Sss infection of potato, resulting in a reduction in quality of harvested tubers. However, it has recently been shown that the pathogen affects plant growth and tuber yields through infection of roots (Falloon *et al.,* 2004; Maldonado *et al.,* 2012; Shah *et al.,* 2012; Falloon *et al.,* 2016). Root infection by Sss has not been well studied compared with tuber infection. Reports from field and pot experiments indicated that root function, in terms of water and nutrient uptake, was compromised and plant growth reduced following Sss infection (Lister *et al.,* 2004; Falloon *et al.,* 2004; 2016). Maldonado *et al.,* (2012) also demonstrated in pot experiments that inoculation of potato plants with Sss reduced root mass in susceptible cultivars compared to resistant cultivars. The root infection stages of the pathogen (zoosporangia and root galling) are important, both due to effects on plant productivity and because of their contribution to increased soil inoculum for development of powdery scab (tuber disease) epidemics. Moreover, potato roots are susceptible to infection at all stages of development (Thangavel *et al.,* 2015).

Potato root exudates play an important role in initiating interactions between the plant and soil microbes (Balendres *et al.,* 2016). The functions of root exudates include among others, chemotaxis-induction, inhibition and stimulation of microbial growth (Bais *et al.,* 2006) as well as influencing the colonization and activation of root-infecting pathogens (Nelson, 1990; Singh *et al.,* 2004). Therefore, root exudates function in belowground plant defense (Baetz and Martinoia, 2014).

The mechanisms of potato resistance to Sss root infection are not completely understood; and there is little information about the biochemical and cellular interactions of Sss with its host cells (Maldonado *et al.,* 2015). Following pathogen attack, plants, including potatoes, produce several pathogenesis and defense related compounds such as pathogenesis related (PR) proteins, signal molecules and phytoalexins, to deter the pathogen (Kombrink and Schmelzer, 2001). Metabolites

produced in potato plants in response to pathogen attack can be identified and used as resistance biomarkers for screening of cultivars in potato breeding programs. Molecular studies have shown that enhanced resistance is induced when plants are treated with defense inducing agents such as β-aminobutyric acid (BABA), thiadiazole-7-carbothioc acid S-methyl ester (BTH), and thiamine (vitamin B1) before pathogen attack (Ahn *et al.*, 2005; Lobato *et al.*, 2010). Moreover, application of BABA has been reported to induce resistance in potatoes against Sss root infection (Maldonado *et al.*, 2015) and against *Phytophthora infestans* (Eschen-Lippold *et al.*, 2010; Liljeroth *et al.*, 2010). Metabolic profiling allows detection of unknown compounds and provides functional information on metabolic phenotypes of plants (Kopka, 2006; Torras-Claveria *et al.*, 2010).

A comprehensive study of metabolite changes in response to Sss infection of potato roots will increase the knowledge of plant defense responses, interactions between metabolic networks and basic plant metabolism. The general objective of the current study was to undertake root and root exudate metabolic profiling using roots and root exudates of tolerant and susceptible potato cultivars not-inoculated or inoculated with Sss. Differentially expressed metabolites were identified and quantified using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) chromatograms with unsupervised and supervised data mining methods. The study provides an unbiased, quantitative view of a wide range of Sss infection-induced changes of secondary metabolites in potato roots and root exudates. Biochemical understanding of the interactions between Potato and Sss is important for the development of new Sss management strategies such as natural plant defense mechanisms enhancement and development of resistant cultivars in plant breeding programs.

4.2. Materials and methods

4.2.1. Plant growth and *Spongospora subterranea* f. sp. *subterranea* inoculation of plants

Sprouted certified mini-tubers of 10 potato cultivars commonly grown in South Africa (Table 4.1) were planted in plastic pots (13.5 cm height X 15 cm top diameter and 12 cm bottom diameter) filled with 800g of pasteurized sandy loam soil on 15 May 2017. Ten tubers were planted for each

cultivar. Plants were grown in the greenhouse at a temperature of 22 ± 2°C with a 16 h photoperiod and were irrigated every second day with 200 ml of sterile distilled water for maintenance of moist soil conditions, favourable for root and tuber infection by Sss. Plants were fertilized fortnightly with 100 ml solution per pot of Dr Fisher's MultiFeed Classic (NPK 19:8:16) (Nulandis Ltd), containing trace elements. The experiment was laid out in a Randomized Complete Block Design (RCBD) with 10 cultivars, two treatments (inoculated and not-inoculated control plants) and five replicates per treatment (100 plants).

Spongospora subterranea f. sp. *subterranea* inoculum was produced by removing powdery scab lesions from heavily infected field-grown tubers of BP1 cultivar using a sterile scalpel. The peels were air-dried and ground into powder using a sterile pestle and mortar. The resulting powder was sieved through a sterile 75 µm mesh sieve. Five plants of each cultivar were inoculated with four grams of Sss inoculum suspended in 50 ml of distilled water, equivalent to 5 x 10^4 cystosori per gram of soil, determined using a haemocytometer at planting. The other five plants of each cultivar were treated with 50 ml sterile distilled water per pot to serve as the not-inoculated control treatment.

Table 4.1. List of ten potato cultivars differing in susceptibility levels to *Spongospora subterranea* f. sp. *subterranea* root infection

Cultivar	Root infection response*
Fianna	Moderately tolerant
Innovator	Moderately tolerant
Lanorma	Moderately tolerant
Mondial	Moderately tolerant
Valor	Moderately tolerant
Avalanche	Moderately susceptible
Sifra	Moderately susceptible
BP1	Susceptible
Ronaldo	Susceptible
Up-to-date	Susceptible

* Potato cultivars were arbitrarily categorised into moderately tolerant, moderately susceptible and susceptible according to their root infection severity scores (Chapter 3).

4.2.2. Potato root exudates collection and root sample preparation

All 10 plants from each cultivar (five inoculated and five not-inoculated) were gently uprooted from the soil seven weeks after emergence for root sampling and root exudates collection. Roots were washed with Sterile Distilled Water (SDW), and then blotted on sterile tissue paper. Each plant was transferred into a 750 ml polypropylene bottle containing 500 ml of fresh sterile distilled water (SDW) and kept under the same temperature conditions as described for plant growth. Seven days after transferring to SDW root exudates were aseptically collected from the SDW solutions, filtered through a 2.5 µm filter paper (Whatman™ GE Healthcare UK Limited, Amersham Place UK) and stored at -20°C in the dark until metabolites extraction, following the method of Balendres *et al.*, (2016).

Immediately after collecting the root exudates, the roots were frozen in liquid nitrogen and stored at -80°C until metabolites extraction. Roots were put in 50 ml conical Falcon centrifuge tubes and freeze-dried for two days in a Modulyo® Freeze Dryer (Thermo Electron Corporation, USA). Root samples were prepared according to a method by Tawaraya *et al.*, (2014). Briefly, a 100 mg sample of freeze-dried root tissue from each plant was ground into a fine powder with a mortar and pestle. Root and root exudates samples were prepared from each of the 100 potato plants in the pot trial. Another sample of roots (100 mg) was excised approximately 30 mm below the crown of each plant for evaluation of root infection by Sss zoosporangia using light microscopy. A zoosporangium staining procedure and examination under a compound microscope for rating of zoosporangia root infection were carried out as described by Merz (1989) as outlined in chapter 3.

4.2.3. Metabolite extraction

One hundred mg of freeze-dried powdered roots and 1 ml of root exudates samples were placed in sterile 2 ml Eppendorf tubes with 1 ml of water: methanol (30:70 v/v) extraction buffer for roots, and in triple distilled water for root exudates. The mixture was transferred to sterile 2 ml Eppendorf tubes, immersed in an ultrasonic bath (UMCS Ultrasonic Pty Ltd Kenware, Krugersdorp SA), sonicated for 20 minutes at 100 W ultrasonic power and were then centrifuged at 14,000 g for six minutes with a minispin® microcentrifuge (Eppendorf AG, Germany). The

supernatant was transferred to a sterile centrifuge tube and evaporated on a heat block (Accublock ™ Digital dry bath, Labnet international, Inc. Woodbridge, USA) at 60°C overnight to remove excess solvent.

After evaporation, the samples were cooled at room temperature for five minutes. The dry residues were re-dissolved in 50:50 v/v acetonitrile: water solution to make up a volume of 1 ml and the mixture was vortexed for 30 seconds. Potato root extracts were centrifuged at 14,000 g for 5 minutes in a micro-centrifuge. The extracts were filtered through 0.22 µm filters (Whatman™ GE Healthcare UK Limited, Amersham Place UK) into pre-labelled UPLC vials fitted with slit caps (Waters, Milford, MA, USA). Metabolites were extracted for all 200 samples prepared (100 root samples and 100 root exudates samples) and all the samples were then sent to the LC-MS (SYNAPT) section of the Chemistry Department at the University of Pretoria for UPLC/MS analysis.

4.2.4. Ultra-performance liquid chromatography-time of flight coupled with mass spectrometry (UPLC–TOF-MS) analysis

To investigate the effect of Sss infection on phytochemical responses of potato roots, UPLC-MS (ACQUITY UPLC; Waters, Milford, MA, USA) analysis was employed to undertake metabolite profiling of ten potato cultivars, not-inoculated or inoculated with Sss. Chromatographic separation of root exudates and root extracts was performed on an ACQUITY UPLC column (HSS T3 100 mm × 2.1 mm, 1.8 µm; Waters) using mobile phase A (0.1% formic acid in deionized water) and mobile phase B (0.1% formic acid in 100% acetonitrile). Mobile phase B was isocratic at 1% until 0.2 minutes and then increased linearly to 100% at 16 minutes and then held at 100% from 16.1 until 20 minutes. Finally, solvent B was decreased to 1% at 20.1 minutes and held at 1% until 30 minutes. The injection volume of the samples was 5 µl.

For mass data acquisition the SYNAPT G2 was used in V-optics and was operated in both positive electrospray ionization-positive (ESI+) and negative (ESI-) modes. Sodium formate was used to calibrate the instrument and leucine encephalin was used as a reference calibrant to obtain typical mass accuracies. The mass spectrometer accuracy was < 0.005 Da, operated in

both ESI positive and negative mode with a capillary voltage of 2.5 kV and the sampling cone voltage of 25 volts. The source and desolvation temperature were set at 120 °C and 400°C respectively. Data were acquired in MS^e mode, consisting of a scan using low collision energy of 5 electron volts and a scan using a collision energy ramp from 8 to 20 volts. Helium gas was used as the nebulization gas at a flow rate of 90 ml/minute.

4.2.5. Multivariate statistical analysis

The UPLC–ESI-MS multivariate data analysis of the 200 samples was done using MassLynx version 4.1 software (Waters Corporation, Milford, MA, USA) with an added statistical programme, in which the ESI positive and negative raw data were extracted and analysed. Primary data was analysed by Markerlynx XS™ software (Waters Corporation, Milford, USA) for alignment, peak finding, peak integration and retention time (Rt) correction with parameters as follows: Rt range of 1–27 min, mass range of 100–1000 Da, mass tolerance of 0.02 Da, Rt window of 0.2 min. Data was normalized to total intensity (area) using Markerlynx. For qualitative visualisation, isotopic peaks were excluded from the analysis but included for quantitative and identification purposes. The dataset obtained from MarkerLynx™ processing was exported to the SIMCA-P software version 12.0 (Umetrics, Umea, Sweden) programme in order to perform principal component analysis (PCA) and orthogonal to latent structures discriminant analysis (OPLS-DA) models, and Pareto scaling was used for both models. Multivariate statistical approaches, such as PCA, Hierarchical Cluster Analysis (HCA) and OPLS-DA, were employed to explore the relationships between metabolites in order to detect differences between the tolerant and susceptible cultivars. Using the elemental composition and molecular formula of each compound, monoisotopic mass was automatically calculated and compared to pre-defined databases or manually searched against freely available online databases such as ChEBI, Chemspider, BioCyc, PlantCYC, KEGG, NIST, LifeChemicals and PubChem. Data was then exported and subjected to the Student's t-test ($P \leq 0.05$) statistical analysis in the Statistical Analysis Software computer program (SAS 9.4; SAS Institute, 2006, Cary, NC).

4.3. Results

4.3.1. Zoosporangia root infection

Plants inoculated with Sss inoculum resulted in root zoosporangia development in all 10 cultivars assessed (Figure 4.1), with no infection observed on the not-inoculated controls of all the cultivars. The results confirmed the observation that no cultivar was found to be resistant to root zoosporangia infection in chapter 3.

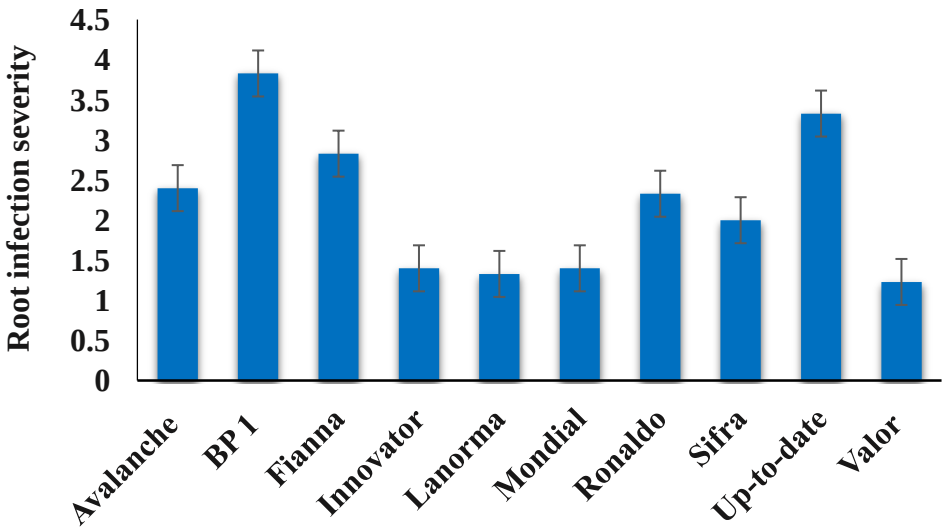

Figure 4.1. Mean of zoosporangia root infection severity of ten potato cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea* sporeballs. Values are log 10 transformed means of the five biological replicates for each cultivar. Bars represent the standard error.

4.3.2. Determination of metabolite levels by chemometric models

The representative base peak intensity (BPI) of UPLC/MS chromatograms of root exudates and roots of the two potato cultivars, tolerant not-inoculated/or tolerant inoculated and susceptible not-inoculated/or susceptible inoculated with Sss are shown in Figures 4.2 and 4.3, respectively. The BPI chromatograms display the complexity of the root extracts and root exudates obtained from different cultivars and show clear qualitative (presence and absence of peaks) and quantitative variation in peak intensities for each sample.

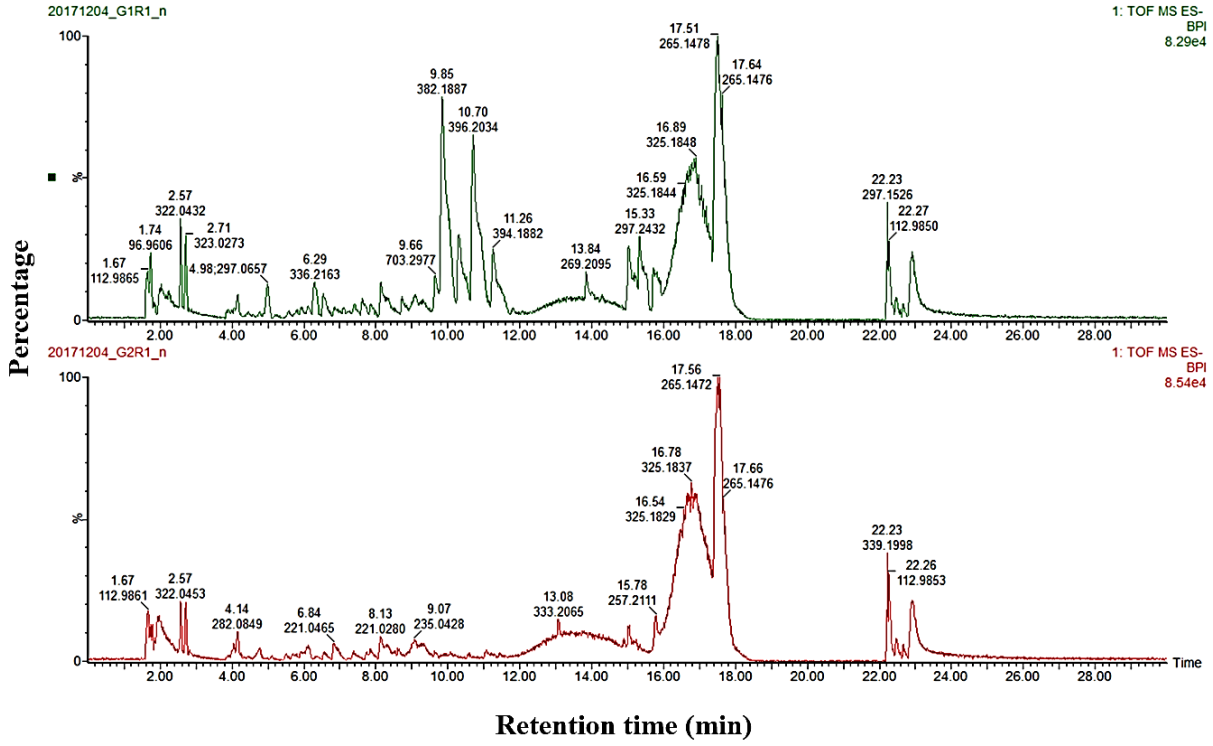

Figure 4.2. Base peak chromatogram spectra of potato root extracts from a moderately tolerant potato cultivar Innovator. Represented: G1R1 (inoculated) and G2R1 (not-inoculated) with *Spongospora subterranea* f. sp. s*ubterranea*.

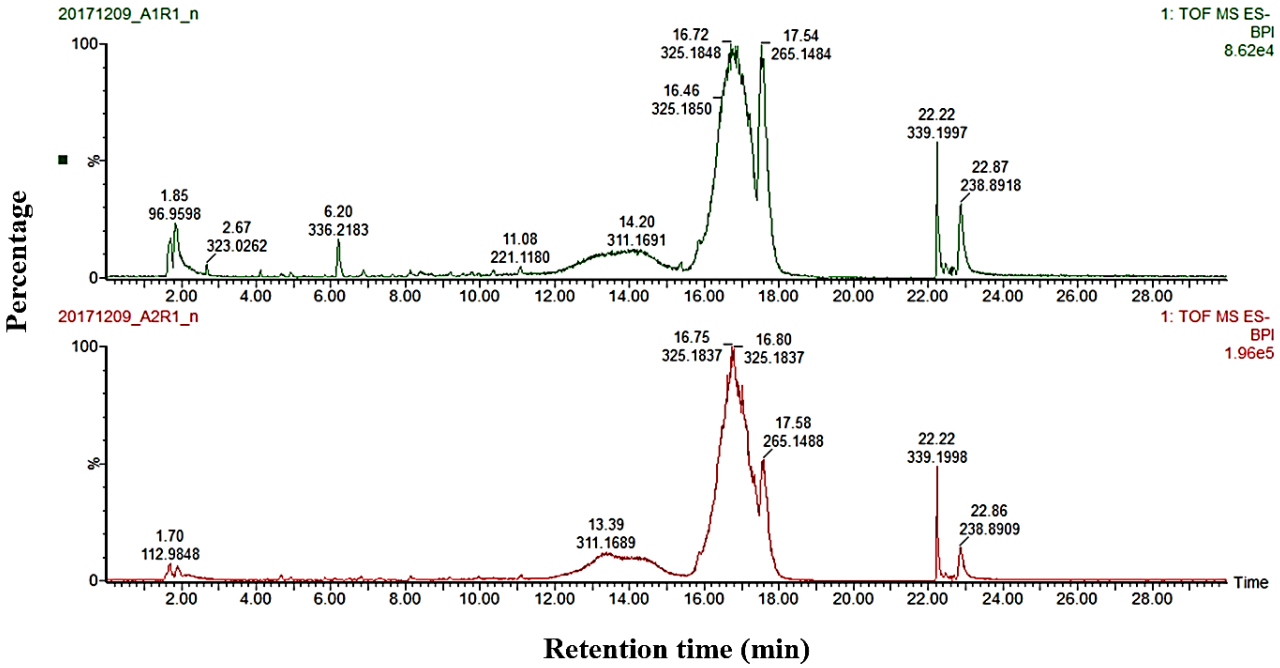

Figure 4.3. Base peak chromatogram spectra of potato root exudates collected from a susceptible potato cultivar, Up-to-date. Represented: A1R1 (inoculated) and A2R1 (not-inoculated) *with Spongospora subterranea* f. sp. s*ubterranea*.

To provide comparative interpretations and visualization of metabolic changes under different treatments, principal component analysis (PCA) was first applied to the UPLC-MS spectral datasets. A good discrimination between the tolerant inoculated and the susceptible inoculated as well as between the tolerant not-inoculated and the susceptible not-inoculated treatments was observed from the PCA score plots, which demonstrated that significant differences in the metabolite profiles are a result of the susceptibility or tolerance of the cultivars to Sss root infection. The PCA scores plots in Figure 4.4 illustrate the distinct clustering of the two cultivar groups (susceptible and tolerant cultivars), and the similarities or dissimilarities between and within the sample clusters.

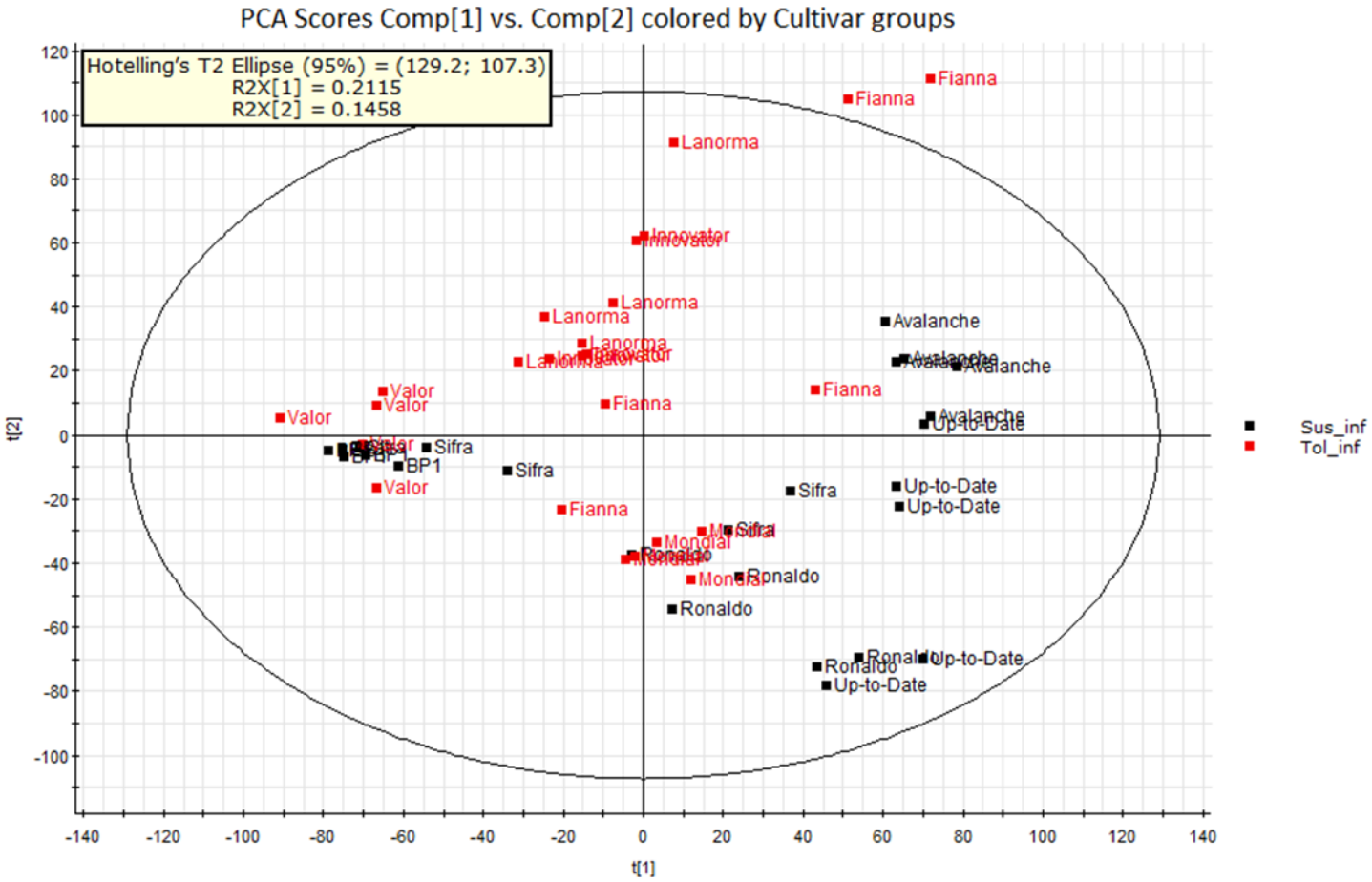

Figure 4.4. PCA plot of the UPLC-MS data illustrating the clustering of the two cultivar groups, moderately tolerant inoculated cultivars (red) vs moderately susceptible/susceptible inoculated cultivars (black).

Orthogonal partial least squares discriminant analysis (OPLS-DA) was subsequently performed to refine the separation of the cultivar groups obtained by PCA and showed clear differences in metabolites produced between susceptible and tolerant cultivar groups. In Figure 4.5, the OPLS-DA scores plots show distinct sample clustering and clear cultivar separation.

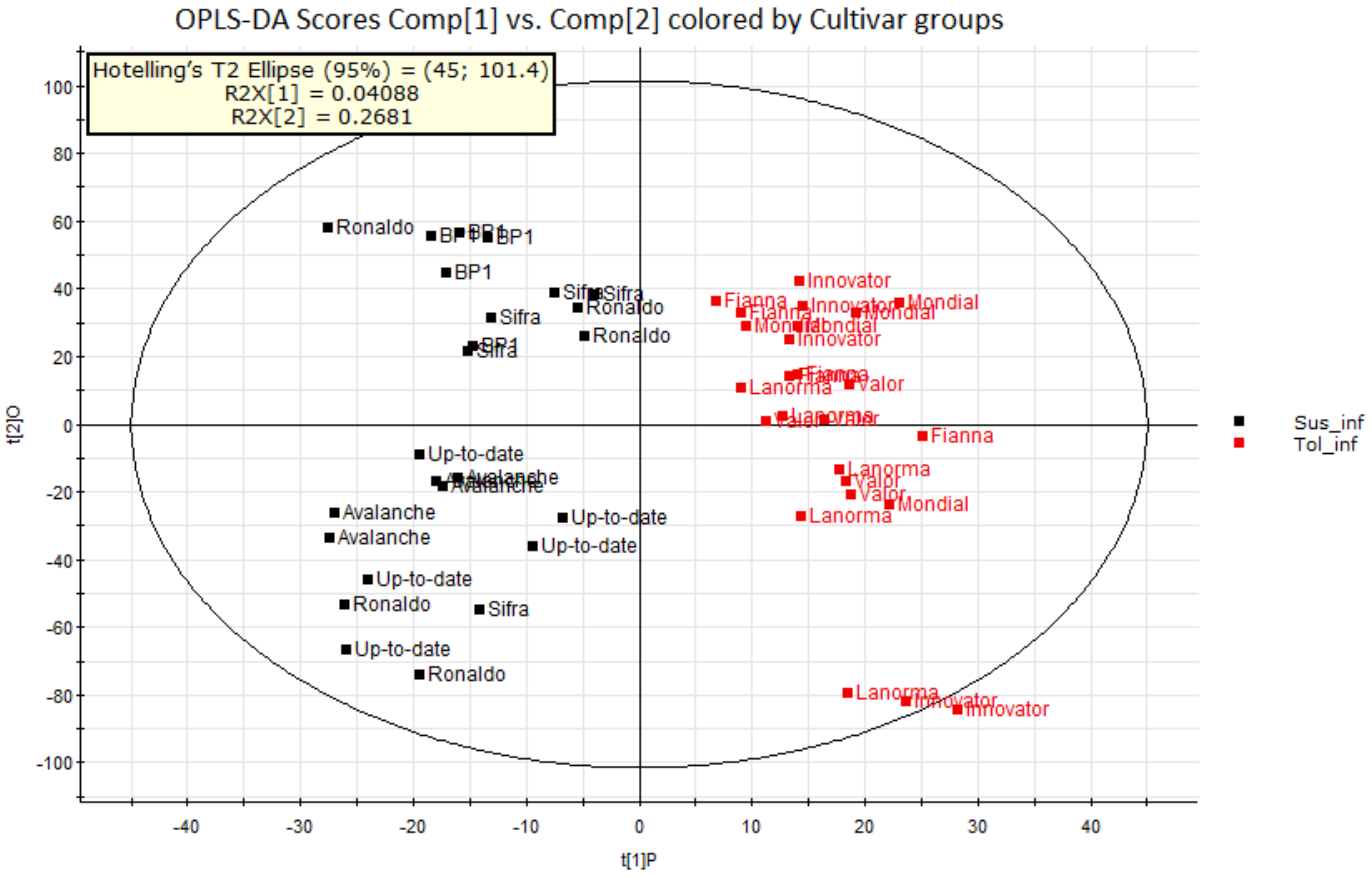

Figure 4.5. OPLS-DA score plots showing a distinct separation between moderately tolerant inoculated cultivars (red) vs moderately susceptible/susceptible inoculated cultivars (black).

The corresponding loadings S-plot (Figure 4.6A and 4.6B) were used to select discriminating ions between the cultivars. The S-plots allow a visual interpretation of the OPLS-DA models, to facilitate the targeting of statistically significant biomarkers. The biomarkers at the bottom left occur predominantly in the moderately susceptible/susceptible cultivars and those at the top right of the curve in the moderately tolerant cultivars, with p corr < −0.5 and > 0.5. The mass numbers marked with red squares (top and bottom markers) were uploaded to the online metabolite databases from MarkerLynx for putative identification of the biomarkers.

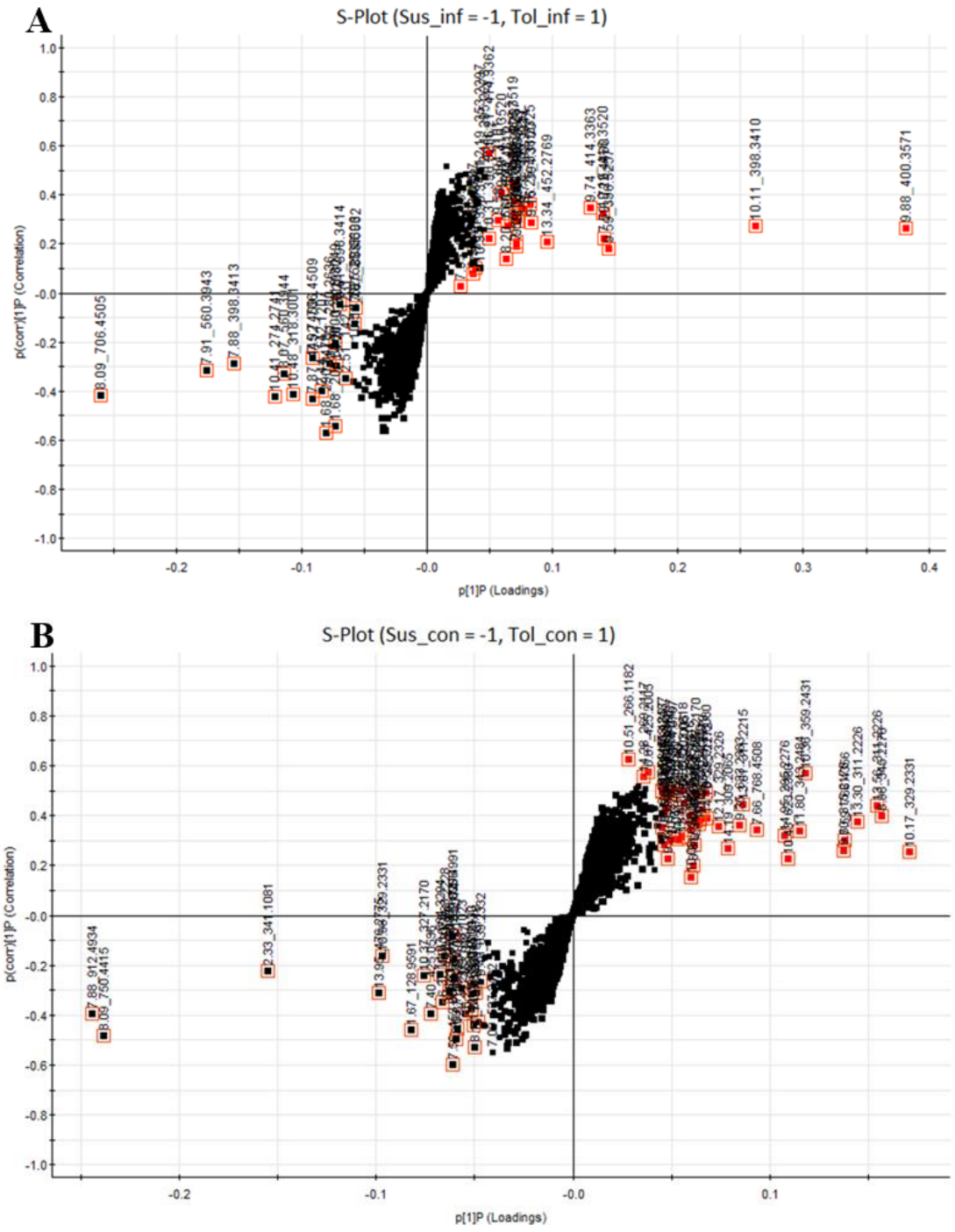

Figure 4.6. OPLS-DA loadings S-plot. The markers selected in the bottom left and top right of the S-curve indicate statistically significant biomarkers identified from the OPLS-DA analysis and occurring predominantly in: **A**- moderately tolerant inoculated (Tol-inf), top right vs moderately susceptible/susceptible inoculated (Sus-inf), bottom left and **B**- moderately tolerant not-inoculated (Tol-con), top right vs moderately susceptible/susceptible not-inoculated (Sus-

con), bottom left. The black unselected squares indicate biomarkers that are common to both the susceptible and the moderately tolerant cultivar groups.

The distribution of metabolites was further analysed using hierarchical cluster analysis *(*HCA), which highlights the similarity between different cultivars based on the metabolites they contain. The computed HCA plots in Figure 4.7 indicated that the samples were separated into two major groups; with the tolerant cultivars clustering together and separately from the susceptible cultivars, except for one tolerant cultivar, Valor, that clustered with the susceptible cultivars.

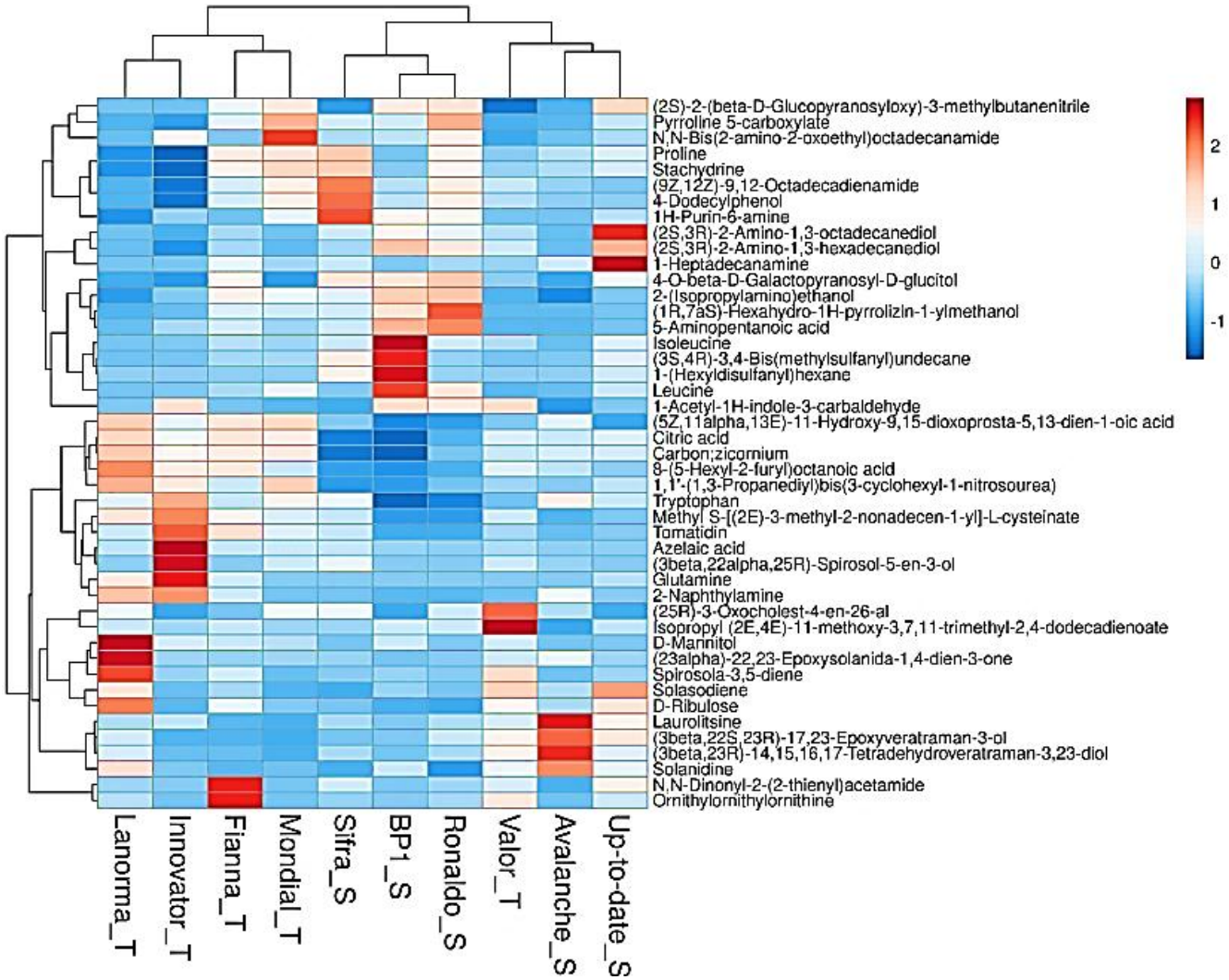

Figure 4.7. Two-way hierarchical clustering analysis visualized using a dendrogram combined with a heat map. The heat map represents the intensities of the metabolites in the roots of 10 cultivars (T-moderately tolerant cultivar and S- moderately susceptible/susceptible cultivar).

4.3.3. Metabolic profiling of potato cultivars

Changes in metabolites associated with Sss root infection of different potato cultivars were evaluated in this study. In order to detect changes in expression of metabolites due to Sss infection and susceptibility of cultivars to the pathogen, three models were built for comparing: moderately tolerant inoculated cultivars vs moderately susceptible/susceptible inoculated cultivars; moderately tolerant not-inoculated cultivars vs moderately susceptible/susceptible not-inoculated cultivars and inoculated vs not-inoculated cultivar groups in positive and negative electrospray ionization (ESI) modes.

After merging the peak intensity variables from the same metabolite, several compounds were selected as statistically significant $P<0.05$ (Table 4.2; Table S4.1). One hundred and two and 79 major metabolites were putatively identified and selected as potential biomarkers for root exudates and potato roots, respectively. Of the 181 metabolites identified for both the roots and root exudates, only 13 metabolites were common to both the susceptible and the tolerant cultivar groups (six for roots and seven for root exudates). The identified metabolites were further classified into chemical groups, such as fatty acids, sugars, sugar alcohols, amino acids, alkaloids, organic acids and phenolics (Figure 4.8). Other than the putatively identified 181 compounds, 164 other compounds (52 from the roots and 112 from the root exudates) were detected as unknown compounds.

T**able 4.2.** Metabolites putatively identified in roots and root exudates from potato cultivars differing in susceptibility to *Spongospora subterranea* f. sp. *subterranea* detected using UPLC-MS

Putative identity	ESI*	m/z	Rt (min)	Roots	Root exudates	Tolerant inoculated	Tolerant not-inoculated	Susceptible inoculated	Susceptible Not-inoculated
Sugars									
D-mannitol	+	181.0716	1.94	+			+		
D-ribulose	-	149.0454	2.34	+		+			
Heterodendrin	+	262.1291	2.57	+		+	+	+	+
Linamarin	+	248.1132	2.01	+			+		
Nystose	-	341.1081	2.33	+			+	+	
Sugar alcohol									
Lactitol dehydrate	-	343.1237	2.15	+				+	
Amino acids									
Arginine	-	175.1197	1.85		+		+	+	+
4-Aminobutanoic acid	-	102.0556	2.17	+		+			
Glutamine	-	145.0617	1.85	+				+	
Kinetin	+	136.0623	2.58	+				+	
Leucine	-	130.0870	2.66	+	+	+	+		+
Methionine	-	150.0603	2.76		+	+			
Methionine sulfoxide	-	166.0543	4.40		+	+			
5-Oxoproline	-	130.0506	3.95		+			+	
Phenylalanine	-	166.0846	4.77		+	+			
Proline	+	116.0711	2.15	+	+	+		+	
Pyrroline 5-carboxylate	+	130.0504	1.84	+			+		
Stachydrine	+	144.1025	2.63	+	+		+		
Tryptophan	-	203.0820	5.55	+	+	+	+		
Valine	-	118.0869	2.82		+	+	+		+
Fatty acids									
(6Z)-6-Decosenamide	+	338.3443	6.22		+	+	+		
Linoleic acid amide	+	280.2636	11.55	+			+		+
Laestisaric acid	-	295.2276	14.95	+					+
Octanoic acid	-	145.1259	2.73		+	+			
16-Oxohexadecanoic acid	-	269.2117	14.28	+					+
C16 sphinganine	+	274.2741	10.41	+	+	+			
(11E)-9,10,13-Trihydroxy-11-Octadecenoic acid	+	329.2329	10.98	+				+	
Alkaloids									
Cyclopamine	+	706.4505	8.09	+		+	+		

Dehydrosolasodine	+	398.3254	9.93	+		+				
Laurolitsine	+	314.1389	8.91	+			+			
Melicopicine	-	328.1187	7.45	+						+
Solanine	+	722.4457	7.59	+			+			
Solanidine	+	398.3412	10.10	+	+	+	+	+		
Solanidane	+	383.6895	9.10	+		+				
Solasodiene	+	396.3255	10.31	+	+	+	+	+		
Solasodine	+	414.3361	8.53	+	+	+	+	+		
Swainsonine	+	174.1113	2.74		+		+			
Trachelanthamidine	+	142.1207	2.44	+	+			+		+
Veratramine	+	410.3049	9.00	+		+				
Tomatidine	+	416.3519	11.28	+			+			
Organic acids										
Azelaic acid	-	187.0971	8.26	+		+		+		+
Citric acid	-	191.0194	2.58	+		+				
Erythronic acid	+	136.1032	2.72	+						+
Phenolics										
p-coumaric acid	+	165.0557	2.91		+		+			
6-Hydroxymellein	+	193.0501	7.63	+						+
Quinic Acid	-	191.0536	2.22	+			+			

+ The compound was abundant in the sample.
Blank means that the compound was not present in the sample.
*Electrospray ionization.
m/z: mass-to-charge ratio.
Rt (min): Retention time in minutes.

The most frequent and abundant metabolite groups were amino acids (13) and alkaloids (13), which were present in the highest proportions, followed by fatty acids, sugars, phenolics and organic acids, with sugar alcohols being the least represented group (Figure 4.8). Of the 14 amino acids identified, five of them, namely aminobutanoic acid, methionine sulfoxide, proline, tryptophan and phenylalanine were more abundant in the moderately tolerant cultivars than in the moderately susceptible/susceptible cultivars. The other six amino acids (arginine, glutamine, kinetin, methionine, stachytrine and 5-oxoproline) were more abundant in the moderately susceptible/susceptible cultivars with the remaining two (leucine and valine) being equally abundant between both cultivar groups shown in Table 4.2.

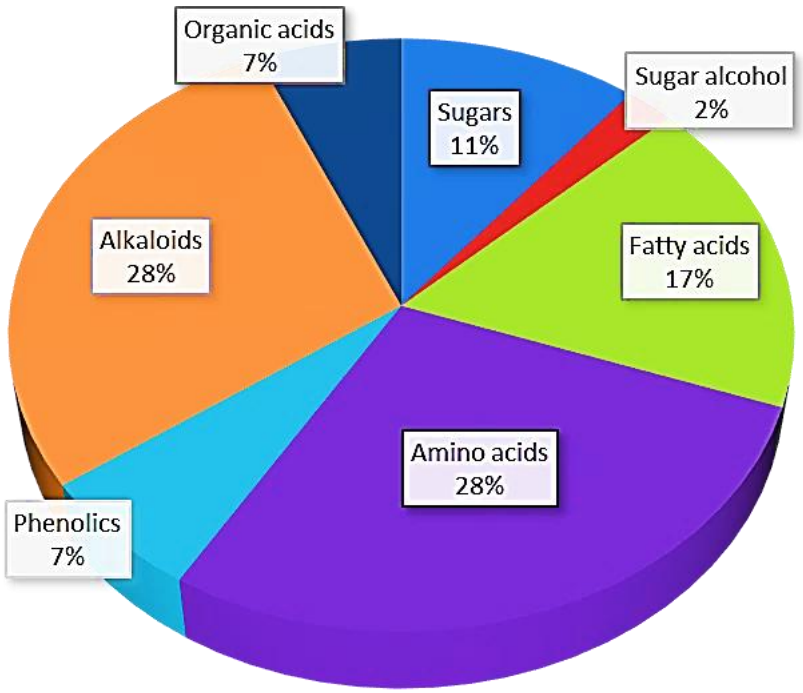

Figure 4.8. Pie chart representing proportions of major metabolites according to their chemical groups identified in roots and root exudates of the 10 potato cultivars not-inoculated or inoculated with *Spongospora subterranea* f. sp. *subterranea.*

Among the Sss inoculated plants, alkaloids were up-regulated in greater abundance (77%) in the moderately tolerant cultivars than in susceptible cultivars. Cyclopamine, laurolitsine, dehydrosolasodine, solanine, solanidane swainsonine, tomatidine and veratramine were identified only in the moderately tolerant cultivars, while only two alkaloids (melicopicine and trachelanthamidine) were identified only in the moderately susceptible/susceptible cultivars, and three alkaloids, namely solanidine, solasodiene and solasodine were common to

both the moderately susceptible/susceptible and the moderately tolerant inoculated cultivars. However, solanidine and solasodiene were more abundant in the tolerant cultivars than in the susceptible cultivars, P = 0.003 and P = 0.0092, respectively (Table 4.3).

The results from this study highlighted that all the five Sugars identified, D-mannitol, D-ribulose, heterodendrin, linamarin and nystose were higher in the tolerant cultivars than the susceptible cultivars. With only three sugars, nystose and heterodendrin being equally abundant in both the tolerant and the susceptible cultivars, though heterodendrin was significantly increased (P = 0.0100) in the tolerant cultivars compared to the moderately susceptible/susceptible cultivars. Of the seven fatty acids identified, three of them, (11E)-9, 10, 13-trihydroxy-11-octadecenoic acid, laestisaric acid and 6-oxohexadecanoic acid were found in abundance in the susceptible cultivars compared to the tolerant cultivars. Linoleic acid amide was the only fatty acid that was common to both the cultivar groups; though the moderately tolerant cultivars had a higher concentration of linoleic acid amide (P = 0.00315) than the tolerant cultivars (Table 4.3).

Even though phenolics, organic acids and sugar alcohols signal strength was lower compared to the other compound groups, it is worth noting that two phenolics identified (6-hydroxymellein and quinic acid) were more abundant in the moderately susceptible/ susceptible cultivars than the moderately tolerant cultivars. Of the three organic acids identified, the concentrations of azelaic acid and citric acid were high in the tolerant cultivars than in the moderately susceptible/susceptible cultivars at P = 0.0136 and P = 0.0017 respectively, while there was no significant difference in abundance of quinic acid among the cultivar groups. Only one sugar alcohol, lactitol dehydrate was putatively identified in greater abundance in the susceptible cultivars than in the moderately tolerant cultivars.

The Venn diagrams (Figure 4.8A and 4.8B) show a minimal metabolite overlap in potato roots and root exudates between the four sample groups (moderately tolerant inoculated, moderately susceptible/susceptible inoculated, moderately tolerant not-inoculated and moderately susceptible/ susceptible not-inoculated). Very few of the metabolites shown in Table 4.2 were shared among different cultivar groups, suggesting a high metabolite variation between the tolerant and the susceptible cultivars. Figure 4.9A and 4.9B highlight that only one metabolite (1,2-dihydroaceanthrylene) was shared between the moderately tolerant not-inoculated and moderately susceptible/susceptible not-inoculated groups and another one

(isorubijervine) between the moderately tolerant inoculated and the moderately susceptible/susceptible inoculated groups in potato roots. Heterodendrin was found in all the four treatment groups (moderately tolerant not-inoculated and moderately susceptible/susceptible not-inoculated, as well as the moderately tolerant inoculated and the moderately susceptible/susceptible inoculated groups). Four of the amino acids (arginine, solasodiene, solasodine and valine) were present in the root exudates of both the moderately tolerant not-inoculated and the moderately susceptible/susceptible not-inoculated groups, while one compound, solanidine, was present in both the tolerant inoculated and the susceptible inoculated groups.

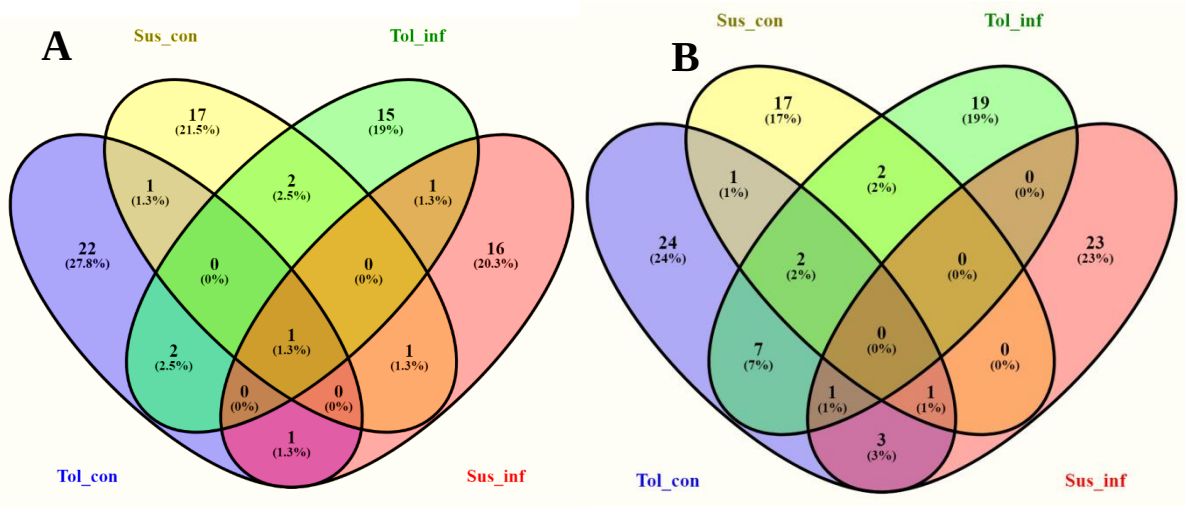

Figure 4.9. Venn diagrams displaying partial overlap of statistically significant biomarkers selected from the four OPLS-DA models comparing extracts from **A**: roots and **B:** root exudates of four sample groups: Tol-con (moderately tolerant not-inoculated), Sus-con (moderately susceptible/susceptible not-inoculated), Tol-inf (moderately tolerant inoculated) and Sus-inf (moderately susceptible/susceptible inoculated). The numerical values in the diagrams depict the number of metabolites that are unique to certain cultivar groups or shared between the cultivar groups.

Table 4.3. Statistically significant differences in regulation of metabolites identified in roots and root exudates from potato cultivars differing in susceptibility to *Spongospora subterranea* f. sp. *subterranea* root infection detected using UPLC-MS

Putative identity	m/z	P-value[a]	Fold-change[b]
Sugars			
D-mannitol	181.0716	0.0426*	2.43
D-ribulose	149.0454	0.0272*	1.68
Heterodendrin	262.1291	0.0100**	2.17
Linamarin	248.1132	0.0049**	3.17
Nystose	341.1081	0.0229*	0.62
Sugar alcohol			
Lactitol dehydrate	343.1237	0.2417^{ns}	0.54
Amino acids			
Arginine	175.1197	0.1687^{ns}	0.57
4-Aminobutanoic acid	102.0556	0.0086**	3.58
Glutamine	145.0617	0.0141*	0.74
Kinetin	136.0623	0.0198*	0.63
Leucine	130.0870	0.6421^{ns}	0.87
Methionine	150.0603	0.9756^{ns}	1.01
Methionine sulfoxide	166.0543	0.6894^{ns}	1.49
5-Oxoproline	130.0506	0.4268^{ns}	1.23
Phenylalanine	166.0846	0.0331*	1.58
Proline	116.0711	0.0393*	1.67
Stachydrine	144.1025	0.6924^{ns}	0.74
Tryptophan	203.0820	0.0019**	2.56
Valine	118.0869	0.0024*	1.50
Fatty acids			
(6Z)-6-Decosenamide	338.3443	0.4288^{ns}	1.19
Linoleic acid amide	280.2636	0.0315*	1.75
Laestisaric acid	295.2276	0.0275*	1.58
Octanoic acid	145.1259	0.7256^{ns}	0.45
16-Oxohexadecanoic acid	269.2117	0.5041^{ns}	0.28
C16 Sphinganine	274.2741	0.0102**	0.55
(11E)-9,10,13-Trihydroxy-11-Octadecenoic acid	329.2329	0.5878^{ns}	0.65
Alkaloids			
Cyclopamine	706.4505	0.2417^{ns}	1.58
Dehydrosolasodine	398.3254	0.2844^{ns}	0.82
Laurolitsine	314.1389	0.3227^{ns}	1.53
Melicopicine	328.1187	0.9641^{ns}	1.57
Solanine	722.4457	0.2390^{ns}	1.69
Solanidine	398.3412	0.0003***	3.11
Solanidane	368.6895	0.0145*	2.04
Solasodiene	396.3255	0.0092**	2.87
Solasodine	414.3361	0.1975^{ns}	1.34

Swainsonine	174.1113	0.2843[ns]	1.75
Trachelanthamidine	142.1207	0.0473*	0.41
Veratramine	410.3049	0.0091**	4.15
Tomatidine	416.3519	0.0025**	4.91
Organic acids			
Azelaic acid	187.0971	0.0136*	1.72
Citric acid	191.0194	0.0017**	2.04
Erythronic acid	136.1032	0.1572[ns]	0.25
Phenolics			
p-coumaric acid	165.0557	0.5164[ns]	1.69
6-Hydroxymellein	193.0501	0.8348[ns]	1.27
Quinic Acid	191.0536	0.0225*	0.52

[a]P-values calculated using student t-test showing significance differences between mean peak areas of the tolerant and susceptible cultivar groups.

The asterisks indicate significant differences between the two cultivar groups (* means $P \leq 0.05$; ** means $P \leq 0.01$; *** means $P \leq 0.001$, [ns] means $P > 0.05$, not statistically different).

[b]Fold-change = mean peak areas of tolerant cultivars divided by mean peak areas of susceptible cultivars. Fold change > 1.5 means the compound is up-regulated in tolerant cultivars. Fold change <1.5 means the compound is down-regulated in tolerant cultivar.

4.4. Discussion

Plants have developed efficient mechanisms to combat pathogen attack. Timely recognition of an attacking pathogen together with the rapid and effective activation of host defense is the main difference between resistant and susceptible plant cultivars (Del Pozo *et al.*, 2004; Pedley and Martin, 2004). The present study reports the potential application of metabolite profiling as a tool for phenotyping potato cultivars varying in susceptibility to Sss root infection.

The diversity of phytochemicals in potato roots and root exudates of five Sss tolerant and five susceptible potato cultivars was elucidated by UPLC-MS. Based on untargeted metabolomics, polar extracts (mainly amino acids, organic acids, sugars, and sugar alcohols) and nonpolar extracts (fatty acids) were putatively identified. A wide range of metabolites, including p-coumaric acid, one of the phenolics that plays an important role in plant disease defense, were abundant in the root exudates of the moderately tolerant cultivars; offering considerable scope for selecting germplasm for breeding programmes. Most of the

metabolites identified in the tolerant cultivars, such as D-fructose, phenylalanine, proline and Tryptophan belonged to four metabolic pathways: The phenylpropanoid pathway, amino acid metabolism, sphingolipid metabolism, and phospholipid metabolism. These metabolites are well-known for their antimicrobial properties including cell wall thickening that is initiated at the pathogen penetration sites in response to cell wall damage (Yogendra *et al.*, 2014a). Plant phenylpropanoids are involved in signal transduction, synthesis of several defense-related metabolites and development of physical barriers in plants (Dixon *et al.*, 2002). Moreover, Phenylalanine is a precursor of several secondary metabolites such as phenolics, coumarines, flavones, isoflavones, isoflavanones, lignins, tannins, and salicylic acid (Abu-Nada *et al*, 2007). These metabolites are important in defense response against pathogens and these signal molecules can activate several defense pathways leading to more complex defense mechanisms in plants (Dixon *et al.*, 2002; Barabasi and Oltvai, 2004).

Interestingly, among the amino acids identified in potato roots, the non-protein 4-aminobutanoic acid (γ-aminobutyric acid - GABA) was expressed in abundance in the roots of inoculated tolerant cultivars compared to inoculated susceptible cultivars and thus, could be a potential biomarker for tolerance to Sss in potato roots. GABA is an isomer of β-a'minobutyric acid (BABA), which was shown by Maldonado *et al.*, (2015) to induce resistance to Sss root infection. Some other studies on potato disease defense have demonstrated induction of resistance with BABA treatments to *Phytophthora infestans* (Altamiranda *et al.*, 2008; Olivieri *et al.*, 2009; Eschen-Lippold *et al.*, 2010; Liljeroth *et al.*, 2010; Bengtsson *et al.*, 2014), and the necrotrophic potato pathogens *Fusarium solani* and *Fusarium sulphureum* (Olivieri *et al.*, 2009; Yin *et al.*, 2010). The current study is the first to report GABA's importance in potato disease defense.

Alkaloids are found in potatoes in the form of glycosides of alkaloids (Lachman *et al.*, 2001). The importance of glycoalkaloids to potato-pathogen interactions was suggested by Aliferis and Jabaji (2012). The number and abundance of alkaloids was higher in the Sss inoculated moderately tolerant cultivars compared to the Sss inoculated moderately susceptible/susceptible cultivars in the current study. This suggests their involvement in the defense response of the moderately tolerant cultivars to Sss. In a similar study, the concentrations of the glycoalkaloid metabolite, solanidine as well as of the amino acids alanine and valine were increased in potato leaves following inoculation with *Phytophthora infestans* (Abu-Nada *et al.*, 2007). Moreover, increases in solanidine and solasodine were

observed in potato sprouts infected with *Rhizoctonia solani* (Aliferis and Jabaji, 2012). Solanidine has been reported in inhibiting pathogen infection (Lachman *et al.*, 2001) and is produced through the mevalonate pathway from acetyl-CoA, which is in the cytosol, and can also be produced by the enzyme ATP-citrate lyase from the organic acid citrate (Fatland *et al.*, 2005). Remarkably, solanidine was the most abundant metabolite identified in the roots of moderately tolerant inoculated potato cultivars.

An organic acid, citric acid, was identified in higher amounts in the moderately tolerant cultivars compared to the inoculated susceptible cultivars, suggesting its potential as a biomarker for Sss tolerance in potato roots. Citric acid is an intermediate of the Krebs cycle that is directly involved in the production of different amino acids belonging to glutamic and aspartic acid families (Berg *et al.*, 2002). Citric acid was identified in the moderately tolerant inoculated cultivars but not in the moderately susceptible/susceptible inoculated cultivars proving its role in potato roots Sss defense. This was true in Martinez-Pacheco *et al.*, (2011) who discovered citric acid's role of improving plant vigour against pathogen attack in *Zea mays*.

In this study, the abundance of amino acids of the glutamic acid family, such as proline and glutamine, increased following Sss inoculation of both the moderately tolerant and moderately susceptible/susceptible cultivars. Similarly, proline and glutamine were identified in potato root exudates of both the resistant and susceptible cultivars (Balendres *et al*., 2016). Yogendra *et al.*, (2014b) found increased concentrations of proline, isoleucine, leucine and valine in potato leaves of both resistant and susceptible cultivars upon *Phytophthora infestans* infection, while alanine was increased only in the resistant cultivar. L-proline is the main precursor in the production of cell wall proteins such as proline-rich proteins (PRPs) and hydroxyproline-rich glycoproteins (HRGPs) (Showalter, 1993). Extensin, a subgroup of the HRGP family, is known for its ability to cross-link and is covalently linked to different cell wall components such as pectin, thus increasing the mechanical strength and rigidity of the plant cell walls (Jackson *et al.*, 2001).

Glutamine is a shuttle for carrying nitrogen in many essential intermediate reactions in plant cells, and is a primary precursor for the production of the porphyrin ring of chlorophyll (Coruzzi and Last, 2000; Taiz and Zeiger, 2002). The results of this study suggest that glutamine is a biomarker for potato root susceptibility to Sss as its concentration was higher

in the moderately susceptible/susceptible cultivars than in the moderately tolerant cultivars. This is consistent with the findings by Huang *et al.,* (2017), who observed the role of glutamine in nitrogen-induced susceptibility (NIS) to the rice blast fungus in rice plants. Their study concluded that nitrogen fertilization increased susceptibility of rice plants to blast due to an increase in glutamine concentration (Huang *et al.,* 2017). This was due to the accumulation of the glutamine synthetase OsGS1-2 enzyme, which is responsible for conversion of glutamate into glutamine in infected plants (Huang *et al.,* 2017). Moreover, glutamine was found to play an important role of stimulating Sss resting spore germination for root and tuber infection (Balendres *et al*., 2016); hence, higher glutamine concentrations were observed in the moderately susceptible/susceptible cultivars than in the moderately tolerant cultivars.

Fatty acids and lipids are important sources of reserve energy, which is particularly important for the energy-intensive processes that underlie the plant defense response (Lim *et al.*, 2017). Lipids are also precursor molecules for the synthesis of various phytohormones, such as the fatty acid-derived Jasmonic acid (JA), which has known defense gene regulating capabilities (Bosch *et al.,* 2014), although JA was identified in abundance in the moderately susceptible/susceptible cultivars in this study. This could be attributed to the fact that the JA hormonal defense pathway is related to plants' responses to necrotrophic pathogens (Bari *et al.,* 2009; Derksen *et al.,* 2013), while Sss is an obligate biotroph (O'Brien and Milroy, 2017).

Sugars are precursors of many metabolic pathways and are the building blocks of cell wall middle lamellae, and participate in the modification of proteins and fatty acids (Abu-Nada *et al.,* 2007). Moreover, they are important in the production of structural defense materials such as callose and papillae in response to pathogen attack (Abu-Nada *et al.,* 2007). Hence, more of the sugars identified in the current study were found in abundance in the tolerant cultivar with only three identified in the moderately susceptible/susceptible cultivars.

Root exudates are comprised of low molecular-weight compounds such as amino acids, organic acids and phenolics (Balendres *et al.,* 2016; Gargallo-Garriga *et al.,* 2018) as well as high-molecular-weight compounds like proteins (Walker *et al.,* 2003). The current study showed that more amino acids were detected in the root exudates than in the potato roots. Similarly, Balendres *et al.,* (2016) detected higher amounts of amino acids, including

glutamine, proline, arginine, DL-methionine and tryptophan compared to other compound groups like the sugar alcohols, sugars and organic acids. Root-exuded phenolics have strong antibacterial and antifungal qualities (Lanoue *et al.*, 2010; Wurst *et al.*, 2010; Vukovic´ *et al.*, 2013) and act in stimulating chemotaxis of soil-borne beneficial microorganisms towards plant roots as well as influencing the native soil microbial community beneficially (Badri *et al.*, 2013). On the other hand, soil microorganisms influence the production of metabolites produced by the host in response to pathogen infection and interacting microorganisms in the rhizosphere (Bais *et al*., 2006; Neumann and Romheld, 2007; Balendres *et al.*, 2016). Even though there is no scientific evidence for this on potato, Neumann *et al*., (2014) confirmed that soil type also affects root exudates composition of lettuce (*Lactuca sativa* L. cv. Tizian). Powdery scab has been observed in different soils in South Africa, indicating that soil type plays no role in disease development; but rather, in the severity of Sss diseases (Nielsen and Nicolaisen, 2000; van de Haar, 2000; Brierley *et al*., 2009; Shakoor *et al.*, 2015) and perhaps in release of metabolites.

The study demonstrated cultivar groups' clustering, which was evident in the HCA; where all the moderately tolerant cultivars clustered together and separate from the moderately susceptible/susceptible cultivars. However, an exception was noted with one tolerant cultivar, Valor which grouped together with the moderately susceptible/susceptible cultivars. This suggests that some cultivars phenotypically classified as tolerant to Sss, might share certain metabolic features associated with the susceptible cultivars. Moreover, other amino acids like leucine, valine, proline and arginine including one alkaloid (solanidine) and one organic acid (azelaic acid) were common to both the moderately tolerant and the moderately susceptible/susceptible cultivars.

Other than the putatively identified compounds, other compounds from both the roots and the root exudates were detected as unknown compounds; hence, further investigation using different analytical chemistry techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) as well as use of different online databases is necessary to identify more compounds.

4.5. Conclusion

Untargeted metabolomics analysis successfully identified several metabolites related to infection of potato roots by Sss. Significant differences between the moderately tolerant and the moderately susceptible/susceptible cultivar groups were identified in the levels of several metabolites including amino acids, organic acids, alkaloids, phenolics and sugars. These secondary metabolite classes play important roles in plant defense. The dissimilarities in the metabolic profiles and metabolites identified in the current study indicate that untargeted metabolomics can be used to distinguish between cultivars with differential levels of susceptibility to Sss. The data of the present work will be useful in the development of a comprehensive biomarker list for use in breeding programs for resistant potato cultivars. The results also indicate that the presence or absence of specific metabolites is not the only determining factor in tolerance to Sss, but the relative concentrations or ratios of metabolites between cultivars also play an important role in conferring tolerance phenotype in potato cultivars.

4.6. References

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Chapter 5

Transcriptome analysis of powdery scab resistance in potatoes

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Abstract

Powdery scab caused by *Spongospora subterranea* f. sp. *subterranea* (Sss) has recently become one of the most devastating potato diseases of economic importance in South Africa. The use of resistant cultivars has long been considered the most effective and sustainable strategy to manage the pathogen. However, little is known about the molecular mechanisms underlying resistance of potato tubers to Sss. Using RNA-sequencing (RNA-seq), 2058 differentially expressed genes (DEGs) were identified from two potato cultivars (tolerant and susceptible) in response to Sss infection. Analysis of the expression patterns of ten selected defense-response genes was carried out at two different stages of tuber growth using RT-qPCR to validate the RNA-seq data. Several defense related genes showed contrasting expression patterns between the tolerant and susceptible cultivars, including marker genes involved in the salicylic acid hormonal response pathway (*StMRNA*, *StUDP* and *StWRKY6*). Induction of six defense related genes (*StWRKY6*, *StTOSB*, *StSN2*, *StLOX*, *StUDP* and *StSN1*) persisted until harvest of the tubers, while three other genes (*StNBS*, *StMRNA* and *StPRF*) were highly up-regulated during the initial stages of disease development. The results of this study suggested that the tolerant potato cultivar employs quantitative resistance and salicylic acid pathway hormonal responses against tuber infection by Sss. The identified genes have the potential to be used in the development of molecular markers for selection of powdery scab resistant potato lines in marker assisted breeding programs.

Keywords: Differential gene expression, potato, powdery scab, RT-qPCR, RNA-sequencing, *Spongospora subterranea* f. sp. *subterranea*

5. 1. Introduction

Potato (*Solanum tuberosum* L.) production is constrained by a number of diseases including powdery scab caused by *Spongospora subterranea* f. sp. s*ubterranea* (Sss). The disease has been associated with devastating losses resulting from reduced yields, low quality and poor marketability of potato tubers (Nitzan *et al.*, 2008). Currently, there are no effective methods for the control of powdery scab disease of potatoes in South Africa (van der Waals, 2018). A limited number of chemicals, including fluazinam and flusulfamide, are registered in some countries for control of Sss (Falloon, 2008). However, the use of chemicals is not effective in controlling the pathogen, therefore integrated pest management strategies such as the use of certified disease-free seed tubers (Tegg *et al.*, 2015) and planting in uninfested soil (Falloon *et al.*, 2003) are recommended to minimize yield losses. Until now, most studies on the potato-Sss pathosystem have focussed on the symptoms (Merz and Fallon, 2008), occurrence (Kim *et al.*, 2003), epidemiology (Bittara *et al.*, 2016), lifecycle (Merz, 1997), chemical control (Simango and van der Waals, 2017) and biological control of the disease (Nakayama, 2017).

The use of potato cultivars resistant to Sss is considered as an effective, durable and environmentally friendly approach to manage the disease (Merz and Falloon, 2008). Potato cultivars exhibit differential response to Sss tuber infection from highly resistant to highly susceptible, suggesting that resistance to powdery scab is quantitatively inherited (Merz *et al.*, 2004). Nevertheless, the genetic and molecular mechanisms of powdery scab resistance in potato tubers have not yet been fully elucidated. Plant defense to pathogens requires a complex interaction between the host and the pathogen that involves the up- or down-regulation of genes (Berger *et al.*, 2007).

Identification of the differentially expressed genes (DEGs) involved in potato tuber defense to Sss is important in understanding molecular mechanisms related to resistance. This will serve as a valuable potential resource for potato breeders in understanding the dynamic interactions between potato and the pathogen. Transcriptomic analysis during the interaction between plants and pathogens is commonly used to provide new insights into the underlying

molecular mechanisms of plant resistance. Transcriptome comparisons between resistant and susceptible varieties have been used to elucidate gene expression profiling of potato in response to infection by *Phytophthora infestans* (Gao *et al.*, 2013), *Streptomyces turgidiscabies* (Dees *et al.*, 2016), PVY (Goyer *et al.*, 2015) and *Pectobacterium carotovorum* subsp. *brasiliense* (Kwenda *et al.*, 2016). Many genes were found associated with resistance signal transduction and defense mechanisms in potatoes. For example, WRKY transcription factors were significantly up-regulated in potatoes after *Phytophthora infestans* infection (Gao *et al.*, 2013).

In order to gain insight into the transcriptional reprogramming that occurs during the compatible/incompatible interaction of potatoes with Sss, discovery of genes by RNA-seq and gene expression profiling by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were carried out on two potato cultivars with variable susceptibility to powdery scab at two different time points of potato plant growth. Results from this study will improve the understanding of potato tuber resistance to Sss and aid in defining future potato breeding programs.

5.2. Materials and methods

5.2.1. Plant material and inoculation

Two potato cultivars, namely Innovator (tolerant) and Vanderplank (moderately susceptible) as determined in chapter 3 of the current study were selected for this greenhouse experiment. Briefly, 10 potato cultivars commonly grown in South Africa were evaluated in the greenhouse for their susceptibility to Sss root and tuber infection in three trials. Visual assessments, microscopic examination and qPCR were used to evaluate susceptibility of cultivars to Sss potato diseases namely; powdery scab, root infection and root galls. Cultivars were ranked according to average powdery scab, root infection and root gall disease indices for three greenhouse trials. Innovator, a processing cultivar used for the French fry market, has russet skin with yellow fresh, and was the most tolerant cultivar to powdery scab, as confirmed by the ADHB Potato Varieties Database http://varieties.ahdb.org.uk/varieties/view/Innovator). Vanderplank is an all-purpose cultivar with white skin and flesh (http://potatoseed.co.za/index.php?c=cultivar_characteristics/cultivar_characteristics), and

was the most susceptible to powdery scab. Both cultivars are open pollinated with short to medium growing periods.

Two pot trials for gene expression were planted in 2017 in a temperature-regulated greenhouse at the University of Pretoria. A total of twenty-four sprouted mini-tubers per cultivar were individually planted in plastic pots (13.5 cm height x 15 cm diameter) filled with pasteurised sandy-loam soil and grown in a greenhouse maintained at 22 ± 2 °C with a 16-hr photoperiod. Plants were watered every second day with 200 ml of sterile distilled water for maintenance of moist soil conditions, favourable for tuber infection by Sss. Seven days after planting, 12 plants of each cultivar were inoculated with 4 g of Sss inoculum suspended in 50 ml of distilled water, equivalent to 5 x 10^4 sporeballs per pot, determined using a haemocytometer. The remaining 12 plants per cultivar were inoculated with sterile distilled water and served as the not-inoculated control treatment. The experiment was set up with three biological replications of the two treatments (inoculated and not-inoculated) for each cultivar. To determine suitable time points for RNA isolations, extra pots were planted to monitor tuber development on the selected cultivars and tuber initiation on both cultivars started at 5 weeks after emergence. Diriwachter and Parbery, (1991) indicated that tubers are susceptible to Sss only during the first 14-20 days of tuber initiation; hence 7 weeks after emergence (7 WAE) was selected as the early stage of tuber development.

5.2.2. Tuber sampling

Twelve plants per treatment (inoculated and not-inoculated) for both cultivars, Innovator and Vanderplank were destructively sampled at 7 WAE and at 15 weeks after emergence (15 WAE) for powdery scab disease assessment and RNA extractions. Seven tubers from two plants of each replication were randomly selected, washed and scored for powdery scab severity according to a standard disease assessment key derived by Falloon *et al.*, (1995), where 0 was no visible disease symptoms and 10 was when tuber surface was completely covered with tuber lesions. Powdery scab disease indices for the two cultivars were calculated as disease index = diseases incidence x disease severity (Merz *et al.*, 2012). For gene expression analysis, whole potato tubers were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction (diseased and symptomless tubers from two plants were pooled for each of the three biological replicates).

5.2.3. RNA sample preparation and quality check

For total RNA extraction, 100 mg of homogenized tuber samples from two plants of each treatment were ground into a fine powder. All RNA extractions were performed in three biological replications. RNA was extracted using a Direct Zol™ RNA extraction kit (Zymo Research, USA) together with Tri-Reagent® (Sigma-Aldrich, Germany) following the manufacturer's instructions. Contaminating DNA was removed from the samples using Invitrogen™ Ambion™ TURBO DNA-free kit, according to the manufacturer's instructions. The yield and purity of RNA was evaluated by measurement of absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Thermo Scientific Technologies). RNA integrity of the samples (Figure 5.1) was assessed using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

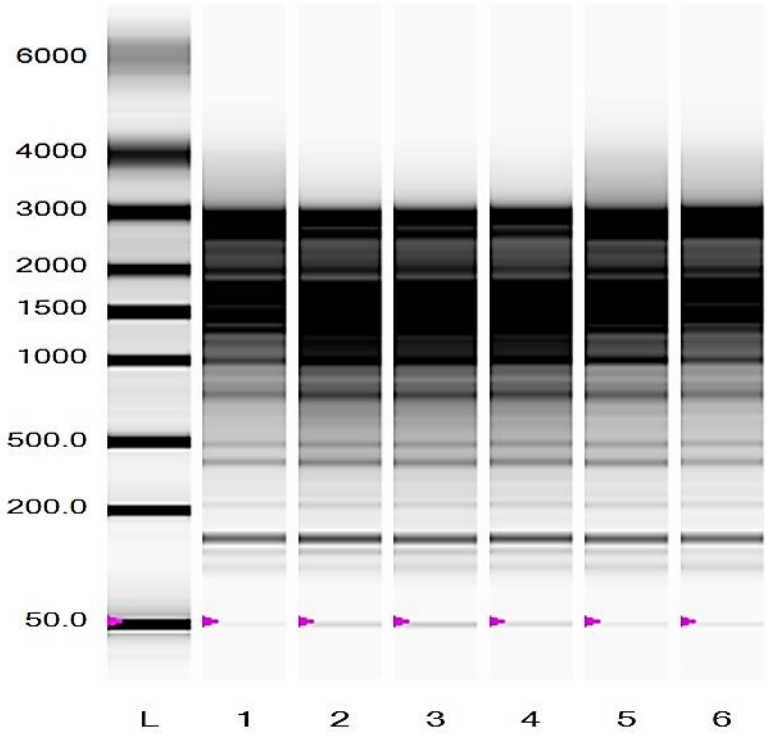


Figure 5.1. RNA integrity of the six RNA samples from three potato cultivars assessed using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

5.2.4. Preparation of cDNA libraries and illumina sequencing

Construction of cDNA libraries and sequencing was carried out at the Beijing Genomics Institute (BGI-Shenzhen, China; http://www.genomics.cn/en/index). Aliquots of high quality

total RNA (5ug, 100 ng/µl) from the three biological replicates for each treatment combination were sent to BGI and were used for poly(A) + mRNA isolation and preparation of cDNA libraries using the TruSeq RNA sample Prep Kit v2 (Illumina, San Diego, CA, USA) following manufacturer's instructions. The quality of the libraries was re-checked and quantified using an Agilent BioAnalyzer 2100 system and qPCR. Finally, the cDNA libraries were sequenced with an Illumina Hi Seq 2000 sequencer generating 90 bp paired-end reads.

5.2.5. Quality validation, read mapping and functional enrichment analysis

Quality of individual sequences was evaluated using TrimGalore (Cutadapt and FastQC) tool (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) analysis. High quality sequence reads (Figure 5.2) were aligned and mapped to the potato reference genome sequence (*Solanum tuberosum* Group Phureja DM v4.04) (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml), using Hisat2 v2.1 spliced aligner. Transcript assembly and quantification of mapped reads (counting reads mapped against exons), were performed using StringTie v1.3.3 and featureCounts (http://subread.sourceforge.net/), respectively. Generated read counts for each sample were used for post mapping quality check prior to performing differential expression analysis. The percentage of uniquely mapped reads ranged from 73 to 85%. A False Discovery Rate (FDR) threshold of 10% and an absolute log 2-fold change > 1 were used to determine differentially expressed genes. G-fold (v1.1.4) analysis tool was used to perform differential expression (DE) analysis. Functional enrichment analysis of differentially expressed genes obtained from each comparison (direct pairwise comparison between cultivars Innovator and Vanderplank or cultivar specific comparisons of inoculated samples and not-inoculated controls) was performed using g: Profiler web Server (Reimand *et al.*, 2016) Venny 2.1. (http://bioinfogp.cnb.csic.es/tools/venny/) was used to make an illustration of the differentially expressed transcripts displayed in Venn diagrams (Oliveros, 2007-2015).

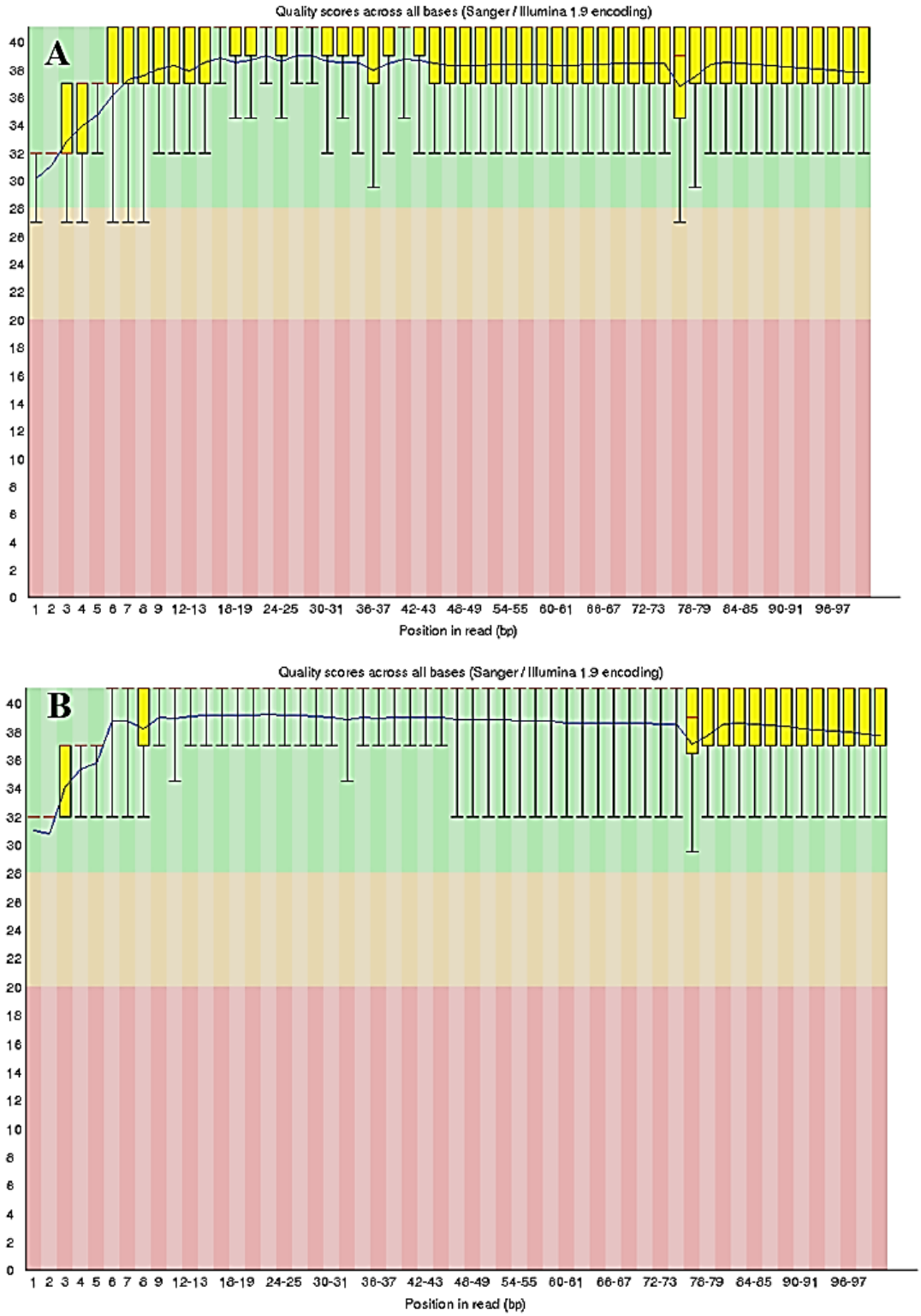


Figure 5.2. High quality sequence reads of **A**: Innovator and **B**: Vanderplank cultivars used for alignment and mapping of reads to the potato reference genome sequence (*Solanum tuberosum* Group Phureja DM v4.04) using Hisat2 v2.1 spliced aligner.

5.2.6. RT-qPCR gene expression analysis

Ten randomly selected DEGs were analysed by RT-qPCR for gene expression profiling. The expression levels were monitored in potato tubers of tolerant (Innovator) and susceptible (Vanderplank) cultivars, with the two treatments (inoculated and not-inoculated) at the two different timed harvest points, 7 WAE and 15 WAE. All RNA extractions were performed in three biological replicates and each cDNA sample was analyzed three times (three technical replications) per treatment (24 samples). First-strand cDNA was synthesized from 1 µg of total RNA using Superscript™ III First-Strand cDNA Synthesis ImProm-II™ Reverse Transcriptase kit according to the manufacturer's protocol (Promega, USA). Gene expression analysis was performed by RT-qPCR, using a SYBR green dye system, according to Minimum Information for Publication of Quantitative qPCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). Specific primer sets were designed using an online primer design software Primer3Plus (http:// primer3plus.com/cgi-bin/dev/primer3plus.cgi) from the specific sequence of *Solanum tuberosum* Group Phureja DM v4.04 (http://*solanaceae*.plantbiology.msu.edu/pgsc_download.shtml). The primer pairs were chosen and validated *in-silico* using primer BLAST specific analysis (http://www.ncbi.nlm.nih.gov/Blast.cgi) and according to RT-qPCR efficiency calculation. Primer sequences as well as expected amplicon product sizes are listed in Table 5.1.

Table 5.1. Primer information for reverse transcriptase quantitative polymerase chain reaction validation of RNA sequencing data and gene expression analysis of potato tubers

Gene name	Gene description	Accession number	Primer sequence (5'-3')	Expected amplicon size (bp)	PCR efficiency (%)[a]
StSN2	Snakin-2 protein	NW_006239862.1	F-TGCATGCCAAAACTCATCAT/ R-GATCGACGGCTTCAATCACT	162	85
StSN1	Snakin-1 protein	NW_006240278.1	F-TGCAAGCAAGTGATTGAAGC/ R-AAGCCAGGAAAACGAGAGGT	190	91
StTOSB	Tospovirus resistance protein B	NW_006239163.1	F-CACCTTTGCGACATCTCTGA/ R-CTCAGTTTCCCACGGTTTGT	203	96
StNBS	NBS-LRR protein	NW_006239040.1	F-CAACTTCAGCAGATCGTCCA/ R-GGATTGCCTCTTGTGGTTGT	235	97
StDEF	Defensin J1-2	NW_006239640.1	TCCAATATGGCTGGCTTTTC/ R-AGTGTCTGGTGCAGAAGCAA	226	110
StLOX	Lipoxygenase	NW_006238985.1	F-CAAATCGCCCCACAGTAAGT/ R-CAAATTTGTCGAAAGCAGCA	243	89
StMRNA	MRNA, 1346 bp sequence	NW_006239280.1	F-AGTCGAACAAATGGCGTCTT/ R-CCGCATGGTGTTTCTCTACA	242	118
StUDP	UDP-glucoseglucosyl transferase	NW_006239431.1	F-TGGTGCAATCAGCTCAAGAC/ R-TAGGCCTCACACCAATCTCC	184	112
StWRKY6	WRKY transcription factor 6	NW_006238949.1	F-GGGTTAATTCGTGGTCGAGA/ R-TAATCTTCCGGCGACTTGAC	203	114
StPRF	PRF	NW_006239027.1	F-TCATTTGCTGAGTGCTGGAC/ R-AGCCACTTTGGAAGCTGAAA	218	107
ef1α[b]	Elongation factor 1-α (ef1α)	NW_006238970.1	F-ATTGGAAACGGATATGCTCCA/ R-TCCTTACCTGAACGCCTGTCA	101	86
β-tubulin[b]	β-tubulin	NW_006238934.1	F-ATGTTCAGGCGCAAGGCTT/ R-TCTGCAACCGGGTCATTCAT	101	101

[a]PCR amplification efficiencies and regression coefficients for the standard curves are reported for each primer pair.
[b]Reference genes.

The RT-qPCR reactions were performed in a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). For RT-qPCR, 2 µl of cDNA was added to 10 µl iTaq Universal SYBR® Green supermix (Bio-Rad, USA) and primers at a concentration of 0.5 µm under the following conditions: an initial activation cycle (10 min at 95 °C), followed by 40 cycles of denaturation, annealing and polymerization (15 sec at 95 °C, 60 sec at 60 °C). PCR amplification was conducted in a total volume of 20 µl, with 1 µl of diluted (1:20) cDNA, 50 nmol l^{-1} primers and 10 µl of SYBR® Green Supermix. To determine the specificity of the amplicons, melting curve analysis was performed over the temperature range of 60 to 95°C. The RT-qPCR efficiency (E) of each primer pair was determined using standard curves generated according to the equation E = 10^{-1}/slope of five triplicate cDNA pool dilutions (undiluted, 0.25, 0.0625, 0.015, and 0.003). Standard curves were used to validate primer pairs. All standard curves had a PCR efficiency of between 90 - 110% with a R^2 value higher than 0.9. The comparative $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) was used to evaluate the relative expression level of each gene. The samples were normalized to β-tubulin and elongation factor1-α (*EF1-α*) as the reference genes and the not-inoculated samples were used as calibrators (Nicot *et al.*, 2005). Each sample was run in three technical replications. In the amplification process, the detection of fluorescence was carried out during the combined annealing step. Melting curve analysis was performed to verify amplification of the specific target DNA (Figure 5.3). The melting curves step started with slow heating at 60°C with a rate of 1°C up to 90°C and each step followed as per the manufacturer's instructions.

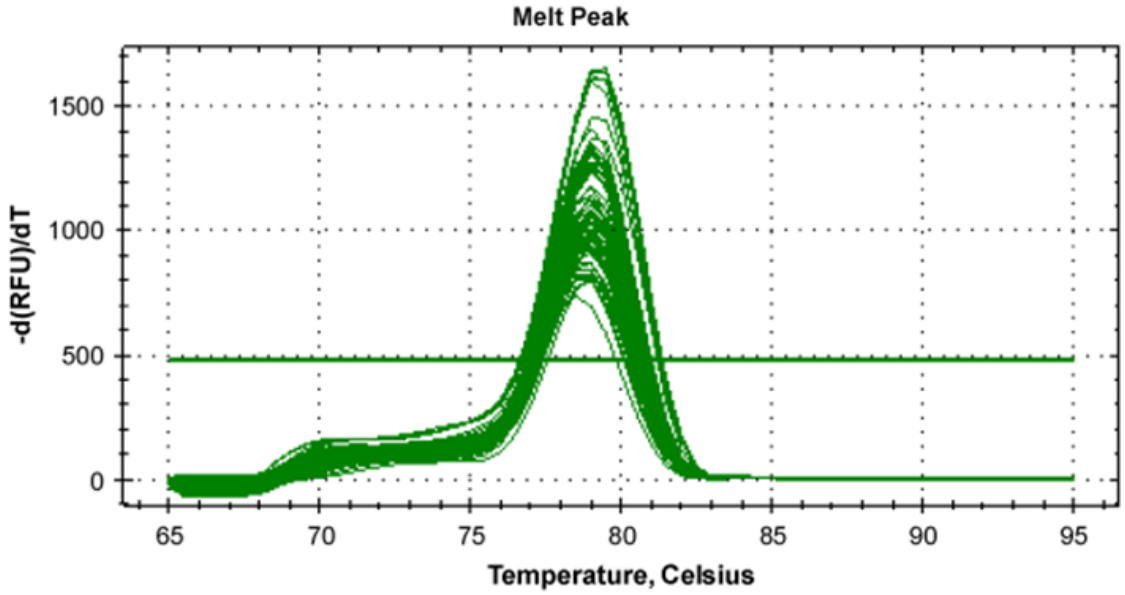


Figure 5.3. Melting curve analysis of twelve genes' primers to verify amplification of the specific target DNA for the two cultivars.

5.2.7. Statistical analysis

Data were analyzed with the General Linear Model (GLM) procedure in the Statistical Analysis System computer program (SAS 9.4; SAS Institute, 2006, Cary, NC). Mean values were separated using the Least Significant Difference (LSD) test at 5% to check whether RT-qPCR results were statistically different when comparing inoculated to not-inoculated samples and the difference between the two cultivars.

5.3. Results

5.3.1. Powdery scab development on potato tubers

Plants inoculated with Sss resulted in powdery scab lesion development in the susceptible cultivar Vanderplank with disease incidence of 26.2% and 41.7% at 7 WAE and 15 WAT, respectively. The disease severity of 0.2 at 7 DAE and 0.7 was observed at 15 DAE (Figure 5.4). There were no visible powdery scab lesions on the tolerant cultivar Innovator at either time points (Figure 5.4). This confirmed that Innovator maintained its tolerance to Sss tuber infection. Moreover, no symptoms were observed on the not-inoculated controls of both cultivars at the two time points.

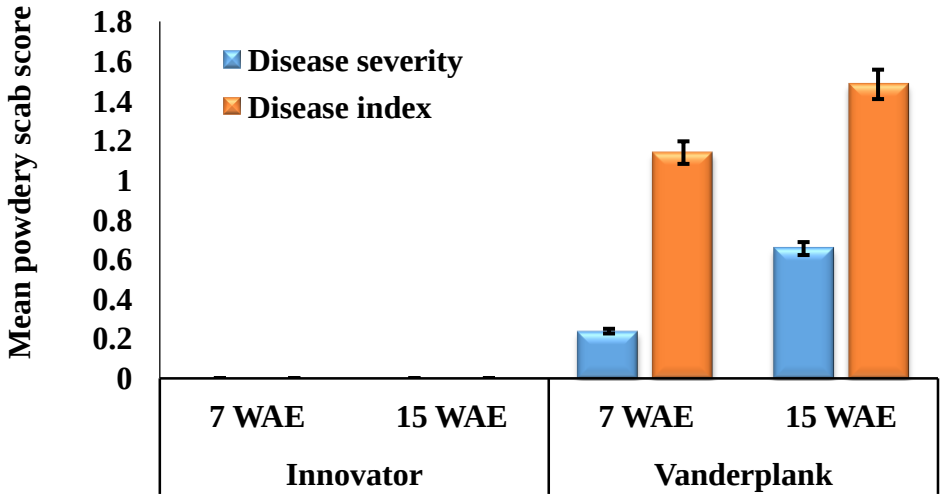


Figure 5.4. Mean powdery scab disease, severity and index of two potato cultivars evaluated for *Spongospora subterranea* f. sp. *subterranea* tuber infection in two greenhouse pot trials. Values are log 10 transformed means of the three biological replicates for each cultivar. Bars represent the standard error.

5.3.2. Identification and functional classification of differentially expressed genes

A total of 2058 DEGs were identified in both the tolerant and susceptible cultivars; of these, 1076 and 954 DEGs related to growth and / or development of the plants were found in Innovator and Vanderplank cultivars, respectively. In addition, 28 DEGs were present in both cultivars at 7WAE) The heat map created to highlight the relationship and clustering between expressed genes in the inoculated and not-inoculated samples showed a clear difference between the samples (Figure 5.5). The Venn diagrams constructed showed the distribution of expressed genes in both cultivars (Figures 5.6A and 5.6B). A total of 61% of the genes were up-regulated (Supplementary Table 5.1), while only 39 % of the genes were down-regulated in both cultivars (Supplementary Table 5.2).

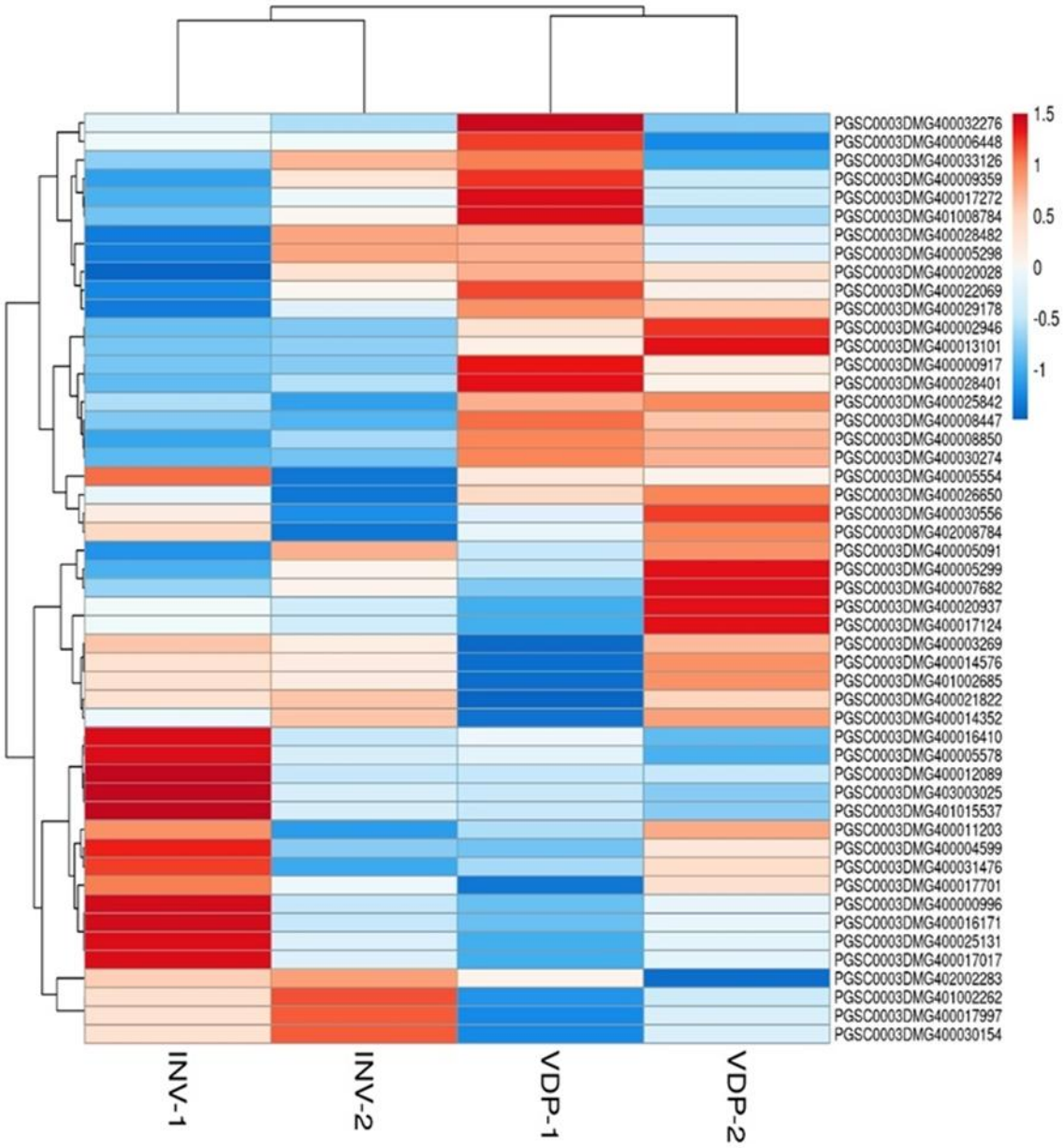


Figure 5.5. Heat map showing expression profiles of differentially expressed genes in response to *Spongospora subterranea* f. sp. *subterranea* infection between the two cultivars, INV-1 (inoculated Innovator), INV-2 (not-inoculated Innovator) and VDP-1 (inoculated Vanderplank). VDP-2 (not-inoculated Vanderplank). The up-regulated genes are shown in red while the down-regulated genes are shown in blue.

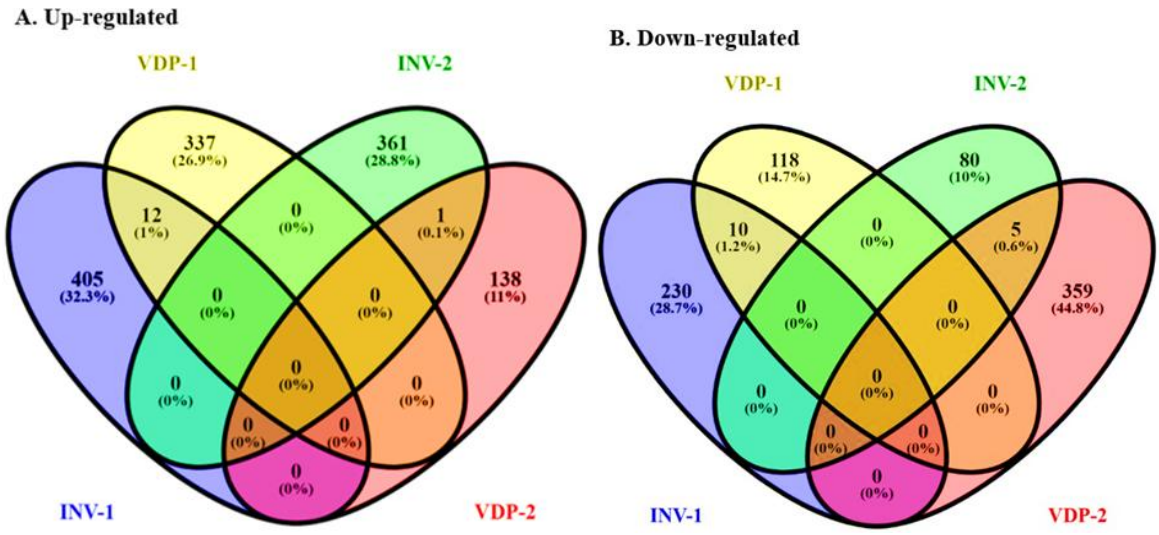


Figure 5.6. Venn diagrams illustrating the differentially expressed genes in the treatments **A:** up-regulated genes and **B:** down-regulated genes in Innovator (tolerant) and Vanderplank (susceptible) cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea* in comparison to not-inoculated control plants. INV = Innovator; VDP = Vanderplank; 1 = Inoculated with *Spongospora subterranea* f. sp. *subterranea;* 2 = not-inoculated control.

To identify the potential relationships between the gene expression patterns and their biological functions, the DEGs were classified into functional categories (Figure 5.7) according to their putative function obtained using Gene Ontology (GO) in g: Profiler webserver (http://biit.cs.ut.ee/gprofiler/). Candidate genes were assessed for their involvement in potato defense responses based on differential expression in the two cultivars. Genes were grouped into the following functional groups: transport, defense response, metabolic processes, response to stress, reduction processes and gene regulation. All six functional groups were present in both the Innovator and Vanderplank cultivars. Among the 1076 DEGs specific to the tolerant cultivar, GO enrichment analyses revealed that 129 DEGs were overrepresented in the gene regulation biological process category, followed by genes belonging to the metabolic process category. Enrichment analysis showed a comparable response of the two cultivars to Sss infection. A significant increase in up-regulation of defense related genes was observed in plants inoculated with Sss than in the not-inoculated control plants.

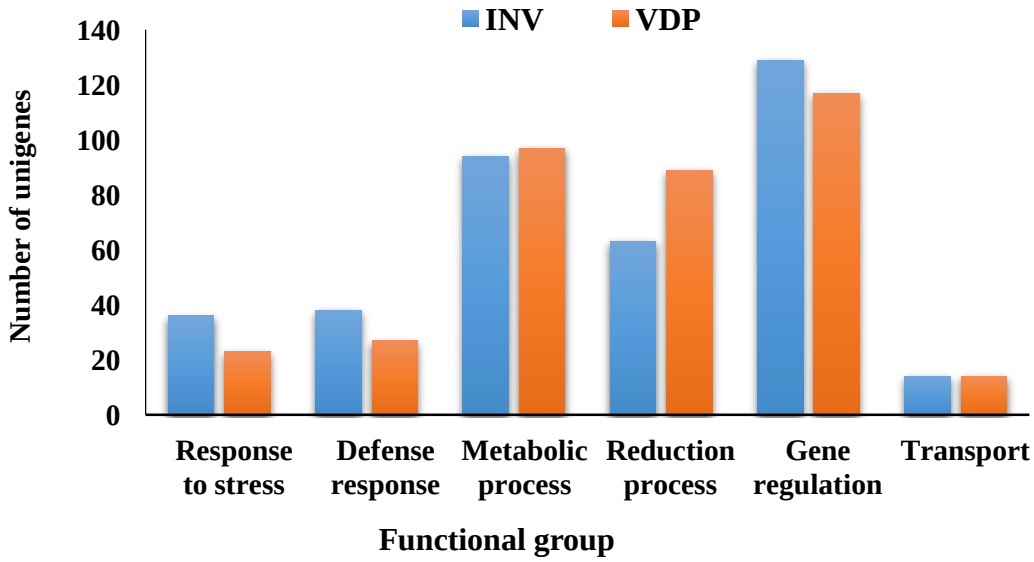


Figure 5.7. Gene Ontology categories of differentially expressed genes showing functional classification of expressed genes derived from each cultivar (INV = Innovator; VDP = Vanderplank).

5.3.3. Analysis of differentially expressed defense related genes using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

For confirmation of RNA-seq results and gene expression analysis, 10 genes (*StWRKY6*, *StSN2*, *StLOX*, *StTOSB*, *StNBS*, *StMRNA*, *StDEF*, *StUDP*, *StPRF* and *StSN1*) were analyzed in inoculated and not-inoculated plants of two potato cultivars differing in Sss susceptibility, using RT-qPCR. The RT-qPCR provided a detailed picture of how changes occurred in gene expression levels of the selected genes at 7 WAE and 15 WAE in the tolerant and susceptible cultivars. The RT-qPCR results were in agreement with the RNA-seq expression patterns in the tolerant and susceptible cultivars (Figure 5.8). Figure 5.9 demonstrates a comparison of fold changes between the two cultivars inoculated and not-inoculated with Sss in gene expression levels, as measured by RNA-Seq and RT-qPCR. This includes 10 selected genes identified by RNA-Seq that are associated with functions related to disease defense. There was a significant Spearman correlation between fold change expression by the two techniques ($R^2 = 0.79$, $P < 0.05$).

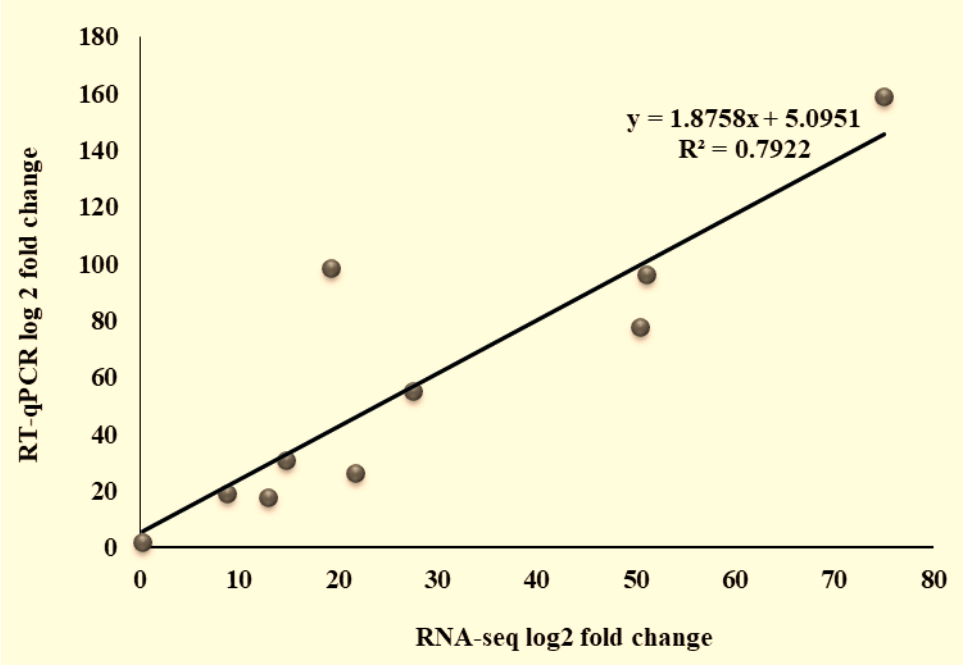


Figure 5.8. Comparisons of relative gene expression as determined by RNA-sequencing and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for the 10 selected defense related genes.

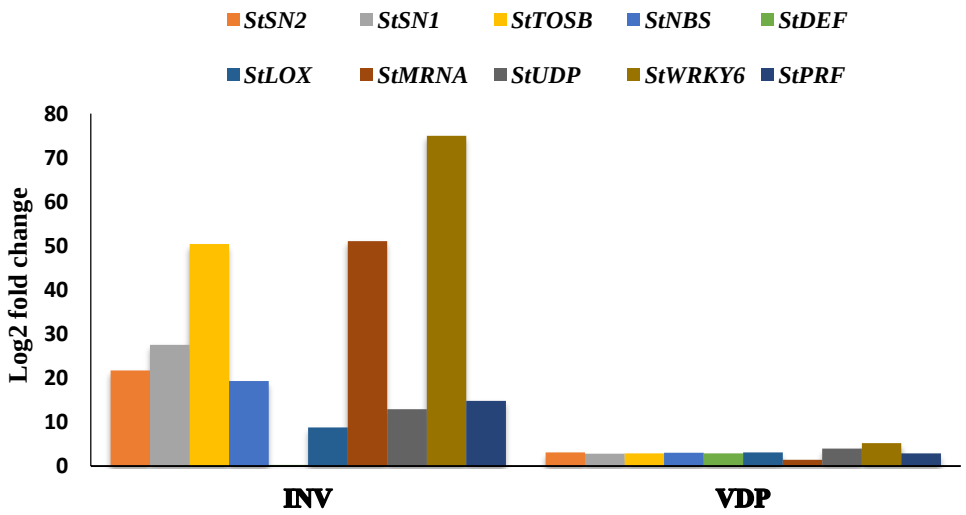
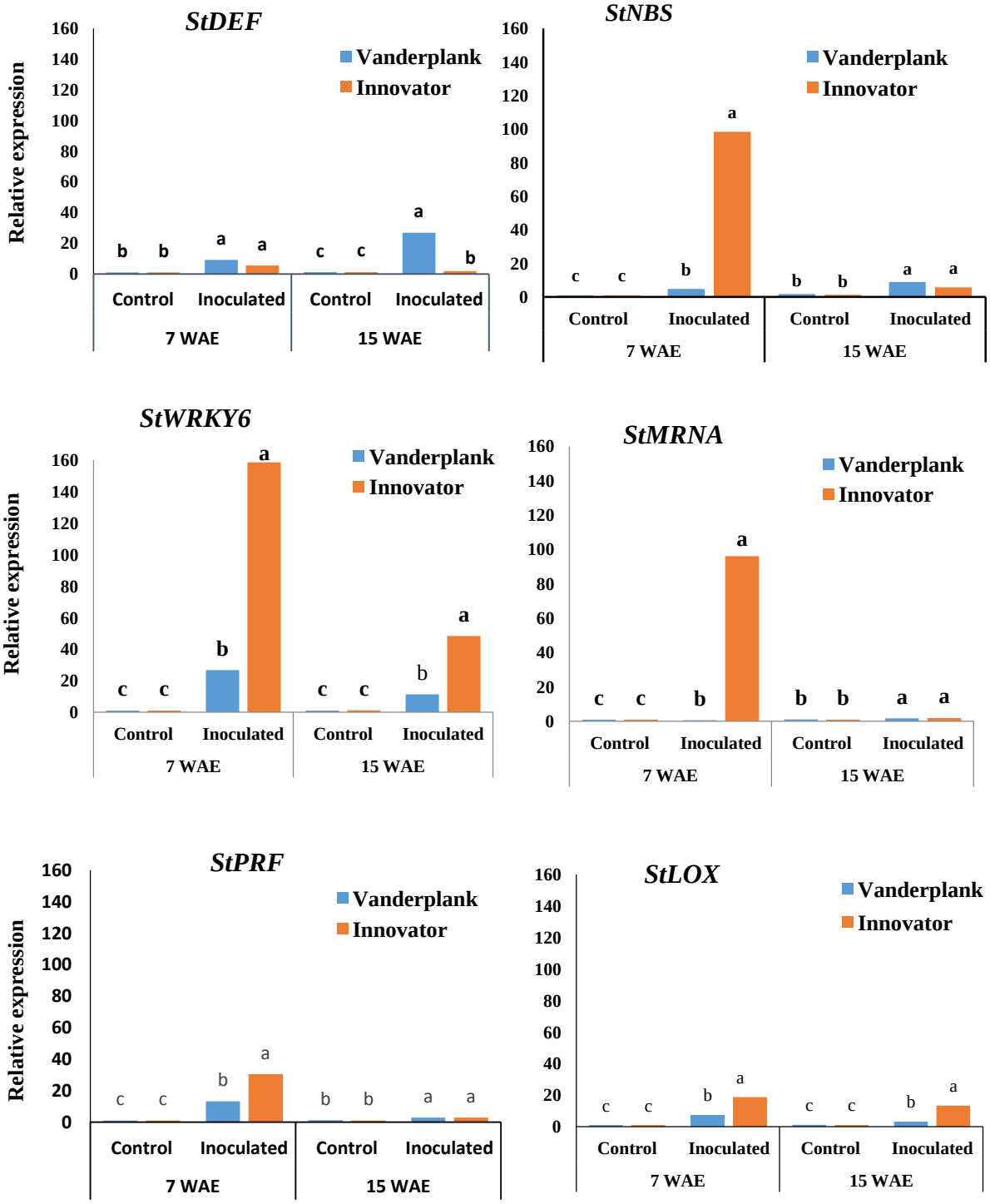


Figure 5.9. Relative expression levels of selected potato defense genes in tolerant and susceptible potato cultivars determined by RNA-sequencing. Expression levels of *StTOSB, StSN2, StUDP, StSN1 StPRF, StLOX, StWRKY6, StMRNA, StDEF* and *StNBS* were measured in Innovator (INV) and Vanderplank (VDP) cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea.*

The RT-qPCR analysis performed indicated that nine of the selected defense-related genes were significantly up-regulated in the inoculated plants compared to the not-inoculated control plants (Figure 5.10). Even though the expression of the genes increased at 7 WAE in both cultivars, all the genes assessed remained constant (1-fold) for all the control plants at both time points. Differences in the gene expression levels were observed between the inoculated tolerant and susceptible cultivars at 7 WAE and 15 WAE. The highest gene up-regulation with a change of 158.6-fold was observed with the *StWRKY6* gene in the tolerant cultivar compared to the susceptible cultivar, which had a lower up-regulation of 26.8-fold at 7 WAE. The expression level of the *StNBS* gene was up-regulated by 98.5-fold and 21.9-fold in the tolerant and susceptible cultivars, respectively at 7 WAE. This was followed by up-regulation of *StMRNA* (96.1-fold), *StTOSB* (77.3-fold), *StSN1* (54.9-fold), *StPRF* (30.4-fold), *StSN2* (26-fold), *StLOX* (18.7-fold) and *StUDP* (17.6-fold) in the tolerant cultivar, which was significantly higher ($P < 0.05$) than the up-regulation levels of the same genes in the susceptible cultivar at 7 WAE. Conversely, no increase in expression was observed for the *StDEF* gene in the tolerant cultivar at either time-point. However, *StDEF* was up-regulated by 9-fold at 7 WAE, with an increase of 26.8-fold at 15 WAE in the susceptible cultivar. Expression of *StNBS*, *StMRNA*, *StUDP*, *StTOSB* and *StPRF* genes was highly up-regulated at 7 WAE in the tolerant cultivar, but showed a sudden decrease of 3.7-fold, 2-fold, 2.2-fold, 7.5-fold and 2.8-fold, respectively at 15 WAE. On the other hand, expression of the four genes, *StWRKY6*, *StSN2*, *StLOX*, and *StSN1*, was highly up-regulated at 7 WAE, persisted and remained up-regulated with fold increases of 48.3-fold, 14-fold, 13.3-fold and 48.7-fold respectively, at 15 WAE in the tolerant cultivar.


StDEF
StNBS
StWRKY6
StMRNA
StPRF
StLOX
Relative expression
Vanderplank
Innovator
Control
Inoculated
7 WAE
15 WAE

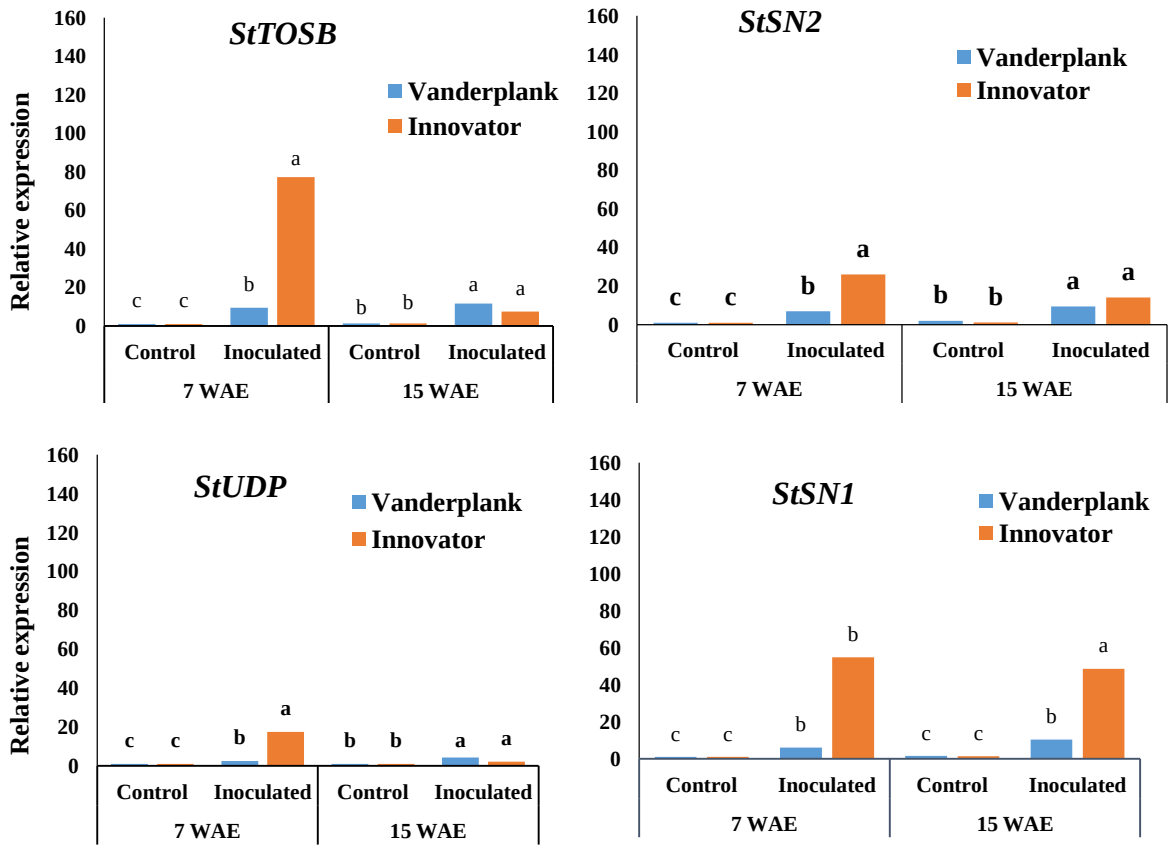


Figure 5.10. Relative expression levels of selected potato defense genes in tolerant and susceptible potato cultivars determined by RT-qPCR. Expression levels of *StTOSB, StSN2, StUDP, StSN1 StPRF, StLOX, StWRKY6, StMRNA, StDEF* and *StNBS* were measured in Innovator (tolerant) and Vanderplank (moderately susceptible) cultivars inoculated and not-inoculated with *Spongospora subterranea* f. sp. *subterranea*. Amplification of *StEFα*-1 and β-tubulin gene expression was used to normalize the expression value in each sample. The relative expression values were determined against the average values of the not-inoculated control samples. Data represent fold change of gene expression at 7 weeks after emergence and at 15 weeks after emergence. The average of three replicates is shown and different letters show significant differences (Student's t-test: $P < 0.05$) between the two cultivars.

5.4. Discussion

Next-generation transcriptomic sequencing was used in this study to identify genes that are differentially expressed in tolerant and moderately susceptible potato cultivars in response to Sss infection at two different time points (7 WAE and 15 WAE). The differentially expressed genes involved in potato tuber Sss defense were validated using RT-qPCR. Several putative defense related genes were identified and were differentially expressed in the inoculated plants compared with the not-inoculated plants.

Differentially expressed genes related to plant defense responses, including the Defensin J1-2 gene, were identified in both cultivars. Furthermore, genes involved in pathogen perception were differentially expressed in both cultivars. These included signalling genes such as WRKY transcription factors (e.g. *StWRKY6*) and pathogen detection genes (e.g. NBS-LRR protein). However, the highest number of up-regulated defense response genes was observed in the inoculated tolerant cultivar. This observation is similar to the findings by Dees *et al.,* (2016) who reported a pronounced up-regulation of defense related genes including the disease resistance protein At4g27190 in a *Streptomyces turgidiscabies* infected potato cultivar, compared to the susceptible cultivar. Evers *et al.,* (2003) also found that most of the differentially expressed genes were up-regulated in the resistant compared to the susceptible potato cultivar upon infection by *Phytophthora infestans*.

The WRKY domain-containing protein is a well-established marker for defense responses of plants against pathogens (Rushton *et al.,* 2010). Hence, *StWRKY6* was significantly up-regulated in the tolerant potato cultivar compared to the susceptible cultivar. The WRKYs are one of the largest families of transcriptional factors in plants and are associated with regulation of transcriptional reprogramming related to plant immune responses (Yogendra *et al.,* 2015). However, several studies have focused on model plants like arabidopsis (Pandey and Somssich, 2009) and tobacco (Menke *et al.,* 2005). Few members of the WRKY superfamily in potatoes have been isolated or functionally characterized. For instance, a significantly higher up-regulation of the *StWRKY1* gene was reported in the resistant potato cultivar compared to the susceptible cultivar upon infection of potatoes by *Phytophthora infestans* (Yogendra *et al.,* 2015). Similarly, *AtWRKY33* was highly up-regulated in the tolerant cultivar compared to susceptible cultivar in response to *Pectobacterium carotovorum* subsp. *brasiliense* potato stem infection (Kwenda *et al.,* 2016).

Lipoxygenase (*StLOX*) gene is one of the potato tubers' main storage proteins and is known to play a key role in resistance of tubers to powdery scab, through deposition of suberin to the skin (Perla *et al.*, 2014). Comparably in this study, *StLOX* was highly up-regulated at the early stages of disease development and remained high until harvest of the tubers. More so, the *StLOX* gene is one of the key markers associated with late blight resistance in potatoes (Trognitz *et al.*, 2001); regulating a number of suberin- and/or non suberin- mediated pathways in potatoes (Perla *et al.*, 2014). Other than the *StLOX* gene, three more genes (*StWRKY6*, *StSN2* and *StSN1*) showed a persistent expression behavior, from the early stage of disease development to maturity of the tubers, in the tolerant cultivar as opposed to the susceptible cultivar. Similarly, Dees *et al.*, (2016) reported an early and sustained response of potatoes to *S. turgidiscabies* infection in a resistant cultivar but not in a susceptible cultivar. In-depth research is suggested for further exploration into the expression and role of these genes during potato tuber storage.

The up-regulation of *StSN1* and *StSN2* in the tolerant cultivar compared to the susceptible cultivar underlines the importance of these genes during plant disease defense. Snakin-1 (SN1) and snakin-2 (SN2) are two cell wall antimicrobial peptides that were isolated from potato tubers and were found to be active against important fungal and bacterial plant pathogens (Berrocal-Lobo *et al.*, 2002). They are components of both the constitutive and inducible defense barriers, specifically in storage and reproductive plant organs (Berrocal-Lobo *et al.*, 2002). Therefore, the *StSN1* and *StSN2* expression levels were highly up-regulated in the tolerant cultivar, while only a slight up-regulation was observed in the susceptible cultivar during infection of the tubers. This was in agreement with Berrocal-Lobo *et al.*, (2002) who reported *StSN2* up-regulation after infection of potato tubers with *Botrytis cinerea*. Moreover, overexpression of the *StSN1* gene was reported to result in transgenic potato resistance to *Rhizoctonia solani* and *Pectobacterium carotovorum* subsp. *carotovorum* (Almasia *et al.*, 2008).

The NBS-LRR protein (*StNBS*) up-regulation was induced at early stages of powdery scab disease development in the tolerant cultivar, as opposed to the susceptible cultivar. Defense response by NBS-LRR proteins induces effector-triggered immunity (ETI) and several of these NBS-LRR proteins recognize effectors secreted by pathogens that in turn activate downstream signaling pathways, leading to activation of plant defense response against

various classes of pathogens (Dubey and Singh, 2018). Phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play an important role in defense responses against pathogens in different plants (Shah, 2003). Therefore, the expression levels of potato SA marker genes were monitored in the current study. The findings of this study suggested that the tolerant potato cultivar Innovator employs the salicylic acid (SA) pathway hormonal responses against infection by Sss. This was evident in the up-regulation of three SA marker genes; *StWRKY6* (PGSC0003DMG400029207), *StUDP* (PGSC0003DMG400015601) and *StMRNA*, (GSC0003DMG402007388), while *StUDP* and *StMRNA* were down-regulated in the susceptible cultivar. The involvement of these genes in SA signaling in potatoes was previously reported by Wiesel *et al.,* (2015). These genes were specifically up-regulated when potato plants were treated with SA hormone compared to ET and JA treatments (Wiesel *et al.,* 2015). Generally, the SA hormonal pathway is related to plants' responses to biotrophic pathogens such as Sss; whereas JA and ET are related to defenses against necrotrophic pathogens (Derksen *et al.,* 2013).

It was noted that the *StDEF* gene was significantly up-regulated in the susceptible cultivar, while down-regulated in the tolerant cultivar, suggesting its involvement as a susceptibility-related gene to Sss in potatoes, although further investigations are required to validate this finding. Late blight susceptibility genes were identified in potatoes, where WRKY transcription factor 6 was found to be one of the susceptibility proteins (Sahu *et al.,* 2014), while it was highly up-regulated in the current study. Similarly, susceptibility-related genes against necrotrophic pathogens such as MYC2 basic helix-loop-helix-leucine zipper (bHLH) transcriptional factors (e.g. PGSC0003DMG400007010 and PGSC0003DMG400012237) were differentially expressed in a susceptible cultivar upon potato stem infection by *Pectobacterium carotovorum* subsp. *brasiliense* (Kwenda *et al.,* 2016).

The results of this study showed that increased mRNA accumulation for many plant defense genes is more rapid and pronounced during the early stages of Sss disease development. The products of major resistance (R) genes recognize and interact with elicitors produced by pathogens, and the proteins encoded by defense response genes initiate signal transduction, leading to defense responses of host plants. The defense-response genes are usually race non-specific (Wen *et al.,* 2003), and thus, may have potential for improving the disease resistance of crop plants.

Quantitative analysis of defense response genes identified by RNA-seq contributes to the understanding about the roles of genes involved in powdery scab resistance. The RT-qPCR analysis revealed up-regulation of nine defense response genes in the tolerant cultivar, compared to the susceptible cultivar, indicating their potential involvement in the defense responses to Sss infection. While the limited number of cultivars and timed harvest points used in this study somewhat limit the interpretation of results in this study, there is an indication that Innovator mounts an early and durable response to infection by Sss, while Vanderplank is not able to sustain the defense response until harvest of plants. Future studies screening a larger number of cultivars at numerous timed harvest points will be beneficial in supporting the outcomes of this study. Selection of disease response genes from potatoes, their expression and functional analysis may facilitate the development of biomarkers, which then could be utilised in the development of Sss resistant cultivars by marker-assisted breeding.

5.5. References

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Chapter 6

Summary and future research

6.1. Summary

Spongospora subterranea f. sp. *subterranea* is a soil- and tuber-borne pathogen that causes powdery scab on tubers, root zoosporangia infection and root gall formation in potato (*Solanum tuberosum* L.). Powdery scab affects the quality and marketability of tubers while root diseases are associated with tuber yield reduction and weight loss (Nitzan *et al.,* 2008; Falloon *et al.,* 2016). Control of Sss is difficult due to the nature of the pathogen that produces resting spores, which can survive in the soil for more than 10 years, making them resistant to environmental stresses (Brierley *et al.,* 2013; Merz and Falloon, 2008). *Spongospora subterranea* f. sp. *subterranea* undergoes two phases of its life-cycles that increase the amount of inoculum available to cause tuber and root infection. Larger amounts of Sss inoculum in soil increase the rate at which the pathogen infects and causes root and tuber diseases (Brierley *et al.,* 2013; Harrison *et al.,* 1997).

There is no single effective method of controlling Sss diseases (Harrison *et al.,* 1997). Therefore, management of the diseases can be achieved by integrating different disease control strategies (Burgess and Wale, 1994; Harrison *et al.,* 1997) as well as evaluation of control measures that can be easily adopted into cropping systems (Bittara *et al.,* 2015). However, no commercial potato cultivars used by growers are immune to Sss infection in South Africa (van der Waals, 2018). The main objectives of the current study were to evaluate susceptibility of different potato cultivars to Sss infection and to elucidate the genetic and biochemical interactions underlying potato tuber and roots tolerance to powdery scab and root infection.

The first chapter of the thesis involved a greenhouse experiment that focused on disease management through cultivar resistance. DNA quantification by qPCR was carried out to confirm the greenhouse results and to accurately measure root and tuber infection. Ten potato cultivars were evaluated for their susceptibility to Sss infection on potato tubers and roots under greenhouse conditions. Variability in the degree of susceptibility to powdery scab, root

infection and root gall formation was observed among potato cultivars and no cultivar was found to be immune to the three diseases. Response to Sss diseases followed a continuum, and cultivars were ranked from moderately susceptible to tolerant in powdery scab and from susceptible to moderately tolerant in both potato roots diseases. This was confirmed by the presence of Sss DNA in the roots and tuber samples of all the cultivars tested; even in tuber samples that did not show any powdery scab symptoms. Furthermore, no correlation was observed between root infection and root gall formation or between powdery scab and root infection. Hence, the mechanisms for resistance differ for root and tuber infection (Harrison *et al.,* 1997; Falloon *et al.,* 2003). However, this finding was based on a trial that had quite low powdery scab tuber infection in a greenhouse environment with relatively high temperatures that perhaps didn't favour tuber disease development. Hence, more greenhouse cultivar screening trials need to be done in greenhouses with temperatures favouring tuber and root disease development, so as to ensure higher disease pressure and greater differences between treatments.

To study the interactions between Sss with potato roots and tubers, genetic and phytochemical responses were investigated in the tolerant and susceptible potato cultivars. This was achieved through ultra performance chromatography and mass spectrometric analysis (UPLC-MS) in chapter four as well as RNA-seq and RT-qPCR gene expression analysis in chapter five. Comparison of differentially expressed genes (DEGs) between the tolerant cultivar, Innovator and the susceptible cultivar, Vanderplank, was undertaken in chapter four using RNA-seq. Comparison of differentially expressed genes (DEGs) between the tolerant cultivar, Innovator and the susceptible cultivar, Vanderplank, was undertaken in chapter four using RNA-seq. It revealed expression of several defense-related genes in Sss inoculated and not-inoculated control plants. Differentially expressed genes related to disease defense, growth and / or development of the plant were identified in Innovator and Vanderplank cultivars. Analysis of the expression patterns of ten selected defense-response genes was carried out at two different stages of tuber growth, 7 WAE and 15 WAE using qRT-PCR to validate the RNA-seq data. Several defense-related genes showed contrasting expression patterns between the tolerant and susceptible cultivars. Induction of some genes persisted until harvest of the tubers; other genes were highly up-regulated during the initial stages (7 WAE) of disease development with regulation lowering at the later stage (15 WAE). Salicylic acid (SA) pathway hormonal response was evident with the up-regulation of the marker genes; *StWRKY6*, *StUDP* and *StMRNA* in the tolerant cultivar as opposed to the

susceptible cultivar. Hence, potato cultivar Innovator employs quantitative resistance and salicylic acid pathway hormonal responses against tuber infection by Sss. The identified genes have the potential to be used in the development of molecular markers for selection of powdery scab resistant potato lines.

For detection of the resistant-related metabolites involved in potato roots tolerance and susceptibility to Sss infection, the infected potato roots and root exudates were collected and extracts were prepared. These extracts were analysed using UPLC-MS. Various compounds were detected and quantified in different potato cultivars (five moderately tolerant and five moderately susceptible/susceptible) and these were compared with the quality and quantity of compounds detected in the control/ not-inoculated plants. Metabolites belonging to amino acids, organic acids, fatty acids, phenolics and sugars were putatively identified and most of them were especially abundant in the moderately tolerant cultivars relative to the moderately susceptible/susceptible cultivars. Phenylalanine, proline, solanidine and tryptophan were significantly increased in the moderately tolerant cultivars compared to moderately susceptible/susceptible cultivars following Sss inoculation. These metabolites are known to activate plant secondary defense metabolism, particularly the phenylpropanoid pathway that produces several antimicrobial compounds including flavonoids and phytoalexins. Cultivars that were moderately tolerant to Sss infection had high levels of flavonoids and alkaloids, well-known cell wall thickening compounds. Root-exuded compounds belonging to the chemical class of phenolic acids were also found in abundance in the tolerant cultivars compared to moderately susceptible/susceptible cultivars.

Host selection is highly recommended for disease management. The current study provides an indication of susceptibility status of potato cultivars to powdery scab, root infection and root gall formation and this will assist potato growers with cultivar choice in relation to the disease. Background information for development of potato breeding initiatives to incorporate Sss host resistance into new cultivars will be provided. Monitoring of Sss development in potato roots and tubers can enable efficient assessment of the disease, hence proper, timely and improved Sss control strategies can be achieved. Moreover, more information of the phytochemical composition of root exudates is important as primary and secondary metabolites released play an important role in defense mechanisms of plants as well as sporeballs germination and zoospores attraction. Therefore, genetically modified potato cultivars with more of the chemicals enhancing resistance of potatoes to the disease

can be attained in future. Furthermore, identified resistance genes can be used as genetic markers for marker-assisted selection in the breeding programs. Studies on sources of resistance to Sss and evolution of resistance genes are needed for developing novel potato cultivars with broad and durable resistance to Sss. Further research is required in order to assist breeding programs in the selection and development of cultivars conferring resistance to powdery scab, root infection and root gall formation.

6.2. Future research

The results of the studies presented in this thesis will contribute to the knowledge of Sss as well as potato cultivar response to the diseases it causes in South Africa. Control of Sss 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 (Wale, 2004; Genet *et al*., 2005; Falloon, 2008). There are no potato cultivars that are completely immune to Sss diseases, but different levels of resistance have been recorded in chapter 3 of the current syudy. Host selection is highly recommended for disease management and more information on the mechanisms of resistance of potato to Sss infection is required for development of potato cultivars resistant to Sss diseases. Moreover, effective disease management strategies can be achieved if the genetic nature of the Sss pathogen is well documented. Identification and characterisation of host genes associated with and expressed during infection by Sss would facilitate the elucidation of the mechanisms of the compatible host-pathogen interaction, root gall and tuber lesion formation, and identification and characterisation of host defence processes and putative (R) genes. Gene sequences designed by Bulman *et al.*, (2011) and Gutiérrez *et al.*, (2014) should be further annotated for their structure and functions. Whilst it is expected that multiple (R) and susceptible genes may be involved, quantitative trait loci (QTL) mapping and association analysis will be essential to characterise the gene(s) controlling resistance to Sss. Genes associated with metabolic pathways during Sss infection also need to be identified in order to understand the genetic factors that trigger disease development in potato plants.

To document a comprehensive list of resistant related metabolites released upon Sss infection, different analytical chemistry techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) as well as use of different online databases are necessary to identify different other compounds. Many cultivars with

high levels of resistance must be evaluated and the cultivars must be tested at several time points after pathogen inoculation as the rate of gene expression and production RR-metabolites differs at different potato growth stages. Furthermore, more greenhouse cultivar screening trials must be conducted and followed by field screening on similar cultivars for accurate categorizing of cultivars' response to Sss root and tuber infection. In addition, greenhouse trials wth root disease assessment at different potato developmental stages is recommended as root zoosporangia infection and root gall formation may change throughout the growing period of the plants.

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Appendix

Supplementary Table 4.1. Organic compounds in roots and root exudates from potato cultivars differing in susceptibility to *Spongospora subterranea* f. sp. *subterranea* detected using UPLC-MS

Putative identification	ESI*	M/z	Rt (min)	Roots	Root exudates	Susceptible cultivars	Tolerant cultivars
Dipentyl sulphite	-	221.1177	11.08		+	+	
Cerulenin	-	224.1285	3.83		+	+	
8-Quinolinol	+	146.0611	5.54		+		+
1-Octanamine	+	130.1597	5.89		+	+	
Indoline	-	120.0815	4.77	+	+	+	+
Isocyanatocyclohexane		126.0925	2.74		+		+
Lauraldehyde	-	185.1867	7.16		+		+
2-Allyl-4(2-methyl-2butanyl)phenol	-	205.1595	11.78		+		+
N,N,N,N-Tetramethylphosphonous diamide	-	121.0848	4.79		+	+	+
6-Ethoxy-1,3-benzothiazole-2(3H)-thione	-	212.0207	10.37		+		+
Bis(2-ethylhexyl)1,2-cyclohexanedicarboxylate	-	397.3297	10.10		+		+
Phenylbis(diethylamino)phosphine	-	253.1807	10.80		+		+
3,20-Bis(dimethylhydrazone)pregn-4-ene	-	399.3452	10.04		+		+
Estralutin	+	429.2992	13.36		+		+
6-Propyl -3,4,5-petrahydro-3-pyridinol	+	142.1245	2.74		+		+
7-Cyano-7-deazaguanine	-	176.0564	2.27		+		+
Cyclopamine	-	412.3211	8.56		+		+
4-(Dodecylamino)-4-oxo-3-sulfobutanoic acid	-	366.1946	9.88		+		+
Methysergide	-	354.2179	7.27		+		+
Octylphenol	-	207.1747	8.98		+		+
6-Aminohexanoic acid	-	132.1023	2.74		+		+
3-Oxododecanoic acid	-	213.1491	13.61		+		+

1-(2-Methoxyphenyl)piperazine	-	193.1338	8.39		+			+
6-Azido-1-hexanamine	-	143.1271	2.74		+	+		+
2,3,4,4-Tetrahydroxybutanoic acid	-	153.0414	2.7		+			+
4-(3-Pyridinyl)butanoic acid	-	166.0678	5.01		+			+
3,5-Dithiaheptane	-	137.0465	2.74		+			+
4-vinylguaiacol	-	151.0762	8.65		+			+
1,1'-(1,6-Hexanedyl)bis(1-nitrosourea)	+	254.1522	5.14		+			+
N-Acetyleucine	+	173.1051	2.52		+			+
6-Ethoxy-1,3-benzothiazole-2(3H)-thione	+	211.2974	10.36		+			+
N-(3-(aminomethyl)benzyl)-acetamidine	+	177.2515	5.36		+			+
4-Hydroxy-4-(3-pyridinyl)butanoic acid	-	182.0822	2.91		+			+
Hypoglycine A	-	142.0872	1.98		+			+
4-(3-Pyridinyl)butanoic acid	-	166.0872	4.77		+			+
Dethiobiotin	-	215.1398	4.4		+			+
4-(Dodecylamino)-4-oxo-3-sulfobutanoic acid	-	366.1951	9.88		+			+
8-Amino-7-oxononanoic acid	-	188.1287	2.69		+			+
1-Acetyl-1H-indole-3-carbaldehyde	-	188.0712	5.48	+	+			+
Ally bis(1-aziridinyl)phosphinate	-	188.0745	5.48		+			+
2-(Phenylsulfanyl)cyclododecanone	-	291.1741	6.17		+			+
(9Z)-1,9,16-Heptadecatriene-4,6-dyn-3-one	-	241.1552	4.24		+			+
3,20-Bis(dimethylhydrazono)pregn-4-ene	-	399.3449	10.02		+			+
Diethylene glycol, amino, N-octyl	-	218.2117	7.77		+			+
Methysergide	-	354.2173	7.26		+			+
Obscuraminol A	+	278.2485	13.4		+	+		
Farnesyl acetone	+	263.2375	14.24		+	+		
Trimethylolmelamine	-	217.1053	4.74		+	+		
Queuosine	-	410.1652	11.64		+	+		
Diprogulic Acid	-	275.1108	5.25		+	+		
Monocyclohexyl phthalate	+	249.1125	11.37		+	+		

Lauric monoethanolamide	+	244.2276	8.28		+	+		
2-[2-(Octylamino) ethoxy]ethanol	+	218.2118	7.78		+	+		
Heptaethylene Glycol	+	327.2018	5.75		+	+		
Drofenine	+	318.2408	13.4		+	+		
6-Amino-2(1H)-pyrimidinone	+	112.0614	2.64		+	+		
Hexadecanodoale	+	265.2041	13.14		+	+		
Linoleohydroxamic acid	+	280.2617	13.88		+	+		+
P,P-Di-1-piperidinylphosphinic hydrazide	+	247.1672	4.24		+	+		
2-Amino-1,3,4,5-icosanetetrol	+	362.3263	10.33		+	+		
N-Benzyloctadecan-1-amine	+	360.3624	14.25		+	+		
2-Amino-1,3-octadecanediol	+	302.3053	11.37		+	+		
Dihexylphthalate	+	335.2215	13.14		+	+		
11-Aminoundecanoic acid	+	202.1809	2.72		+	+		+
1,3,5-Benzenetriamine	+	124.0875	4.25		+	+		
9,12,15-Octadecadrienoic Acid	+	279.2313	13.63		+	+		
6-Azido-1-hexanamine	+	143.1271	2.73		+	+		+
6-phenyl-4 hexyn-2-cn	+	158.244	2.72		+	+		
Betonicine	+	159.1851	1.98		+	+		
2-methoxy-4-vinylphenol	+	150.1772	8.67		+	+		
4-(Dodecylamino)-4-Oxo-3-sulfobutanoic acid	+		9.88		+	+		
N,N,N,N-Tetramethylphosphonous diamide	+	253.1804	4.77		+	+		+
Dopamantine	+	315.4133	4.64		+	+		
(9Z)-1,9,16-Heptadecatriene-4,6-diyn-3-one	+		4.24		+	+		
3-20-Bis(dimethylhydrazono)progn-4-one	+		10.02		+	+		
L-Leucyl-L-Leucyl-aspartic acid	-	360.2131	4.7		+	+		+
N-Cyclododecyl-2-(4-methoxyphenoxy) acetamide	-	348.2537	7.17		+	+		+
p-Heptyloxybenzylidene p-heptylaniline	-	394.3099	9.2	+			+	
2-Naphthylamine	-	144.0809	5.84	+			+	

Glutarylcarnitine	-	276.1443	2.59	+					+	
6-Ethoxybenzothiazolethiol	-	212.0198	10.47	+					+	
4,6-Nonanedione	-	155.1077	8.83	+					+	
Alafosfalin	-	209.0996	1.97	+					+	
Dimethylphosphoramidocyanidic acid	-	133.0145	2.57	+					+	
Inosine		267.0712	2.22	+					+	
2,3-Dinor-8-iso PGF1α		327.2165	9.41	+					+	
Threonic acid		136.1033	2.72		+		+			
N-Undecanoylglycine	-	242.1734	10.14	+					+	
8-(5-Hexyl-2-furyl)octanoic acid	-	295.2273	12.11	+			+		+	
15-Keto-PGE2	-	394.3100	9.15	+					+	
1-acetyl-3-formylindole	-	188,0707	5.44	+				+		
Methoprene	+	311.2583	13.05		+				+	
Docosapentaenoylcarnitine	+	474.3581	13.50		+				+	
1-18:2-lysophosphatidylethanolamine	+	478.2932	13.68		+				+	
Ornithylornithylornithine	-	359.2427	10.42		+				+	
Medemo	+	210.0760	6.57		+		+			
Ureidopropanoic acid	+	131.0463	1.78		+		+			
1,2-Dihydroaceanthrylene	+	203.0819	5.51		+		+		+	
N-[2-(Dimethylamino)propyl]hydrazinecarbothioamide	+	175.0980	7.24		+		+			
2,2'-(Cyclohexylphosphinediyl)dipyridine	-	281.2116	14.37		+		+			
D-Glucose diethyl dithioacetal	+	285.0804	8.32		+		+			
Pyrroline hydroxycarboxylic acid	-	128.0345	3.87		+		+			
(+)-Jasmonic acid	+	209.1176	13.97		+		+			
Glycerophosphoglycerol	+	245.0424	1.985		+		+			
2-(isopropylamino)ethanol	+	104.1076	1.81		+		+			
5-Aminopentanoic acid	+	118.0868	1.99		+		+			
1-Heptadecanamine	+	256.3001	12.42		+		+			

Hexyl disulphide	+	235.1554	5.88	+		+				
N,N-Dinonyl-2-(2-thienyl)acetamide	+	394.3111	10.48	+		+				
Vorinostat	+	398.2411	13.01	+		+				
2,3-Dinor-8-iso PGF1α	-	685.3636	13.33	+		+				
Carbon;zicornium	-	128.9594	1.68	+		+				
4-Dodecylphenol	+	263.2370	14.21	+		+				
N-Benzyl-Linoliamide	+	280.2636	14.21	+		+				
Phenylpyruvic Acid	+	164.1632	2.91		+	+				
4-Hydroxy-4-(3-pyridinyi)butanoic acid	+	181.1911	2.91		+	+				
Ethosuximide	+	141.1684	1.98		+	+				
1H-Purin-6-amine	-	136.0634	2.74		+		+			
Spiroxamine	+	298.2751	12.61		+	+	+			
Guanine	-	152.0576	4.17		+	+	+			
Leucylproline	+	229.1552	5.16		+	+	+	+	+	

*Electrospray ionization
+ The compound was present in the sample
Blank – the compound was not present in the sample
M/z-Atomic mass
Rt(min)-Retention time in minut

Supplementary Table 5.1. List of genes up-regulated in tolerant and moderately susceptible potato cultivars inoculated/not-inoculated with *Spongospora subterranea* f. sp. *subterranea.*

INV 1	VDP 1	INV 2	VDP 2	Common INV/VDP 1	Common INV/VDP 2
PGSC0003DMG400018630	PGSC0003DMG400031457	PGSC0003DMG400001049	PGSC0003DMG400022953	PGSC0003DMG400025000	PGSC0003DMG400006448
PGSC0003DMG400046798	PGSC0003DMG400004037	PGSC0003DMG401018223	PGSC0003DMG400030771	PGSC0003DMG400005974	
PGSC0003DMG400002582	PGSC0003DMG400003962	PGSC0003DMG400034091	PGSC0003DMG400030470	PGSC0003DMG400011016	
PGSC0003DMG400027146	PGSC0003DMG400003084	PGSC0003DMG400003667	PGSC0003DMG400012653	PGSC0003DMG400011953	
PGSC0003DMG401021841	PGSC0003DMG400016887	PGSC0003DMG400009559	PGSC0003DMG400018395	PGSC0003DMG400013412	
PGSC0003DMG400035539	PGSC0003DMG400006709	PGSC0003DMG400000917	PGSC0003DMG400022340	PGSC0003DMG400007244	
PGSC0003DMG400029822	PGSC0003DMG400016908	PGSC0003DMG400033126	PGSC0003DMG400003880	PGSC0003DMG400029879	
PGSC0003DMG400023922	PGSC0003DMG400000811	PGSC0003DMG400008433	PGSC0003DMG400012798	PGSC0003DMG400017334	
PGSC0003DMG400024980	PGSC0003DMG400029419	PGSC0003DMG400026719	PGSC0003DMG400004940	PGSC0003DMG400004036	
PGSC0003DMG400004599	PGSC0003DMG400028984	PGSC0003DMG400030144	PGSC0003DMG400027180	PGSC0003DMG400011170	
PGSC0003DMG400010320	PGSC0003DMG400005884	PGSC0003DMG400031093	PGSC0003DMG400021838	PGSC0003DMG400014740	
PGSC0003DMG400002414	PGSC0003DMG400006788	PGSC0003DMG400014986	PGSC0003DMG400044492	PGSC0003DMG401032203	
PGSC0003DMG400025842	PGSC0003DMG400006789	PGSC0003DMG401029877	PGSC0003DMG400002728		
PGSC0003DMG400013210	PGSC0003DMG400027914	PGSC0003DMG400006630	PGSC0003DMG400008276		
PGSC0003DMG400006389	PGSC0003DMG400022188	PGSC0003DMG400000200	PGSC0003DMG400041062		
PGSC0003DMG400046823	PGSC0003DMG400015746	PGSC0003DMG400026783	PGSC0003DMG400030212		
PGSC0003DMG400019106	PGSC0003DMG400004800	PGSC0003DMG400017103	PGSC0003DMG400022037		
PGSC0003DMG402002685	PGSC0003DMG400028165	PGSC0003DMG402004840	PGSC0003DMG400029736		
PGSC0003DMG400000981	PGSC0003DMG400005835	PGSC0003DMG400014756	PGSC0003DMG400016284		
PGSC0003DMG400005170	PGSC0003DMG400030623	PGSC0003DMG400019646	PGSC0003DMG400016247		
PGSC0003DMG400020028	PGSC0003DMG400007073	PGSC0003DMG400032746	PGSC0003DMG400010113		
PGSC0003DMG400006248	PGSC0003DMG400026762	PGSC0003DMG402031520	PGSC0003DMG400014267		
PGSC0003DMG400043752	PGSC0003DMG400027283	PGSC0003DMG400017017	PGSC0003DMG400020264		
PGSC0003DMG400023237	PGSC0003DMG400005515	PGSC0003DMG400005464	PGSC0003DMG400033693		
PGSC0003DMG400021822	PGSC0003DMG400008985	PGSC0003DMG400018381	PGSC0003DMG400018398		
PGSC0003DMG400024964	PGSC0003DMG400017301	PGSC0003DMG402004883	PGSC0003DMG400005719		
PGSC0003DMG400030587	PGSC0003DMG400024755	PGSC0003DMG400018375	PGSC0003DMG400007258		

PGSC0003DMG400035146	PGSC0003DMG400014778	PGSC0003DMG400018480	PGSC0003DMG400022386
PGSC0003DMG400003099	PGSC0003DMG400028687	PGSC0003DMG400016616	PGSC0003DMG400010112
PGSC0003DMG400001679	PGSC0003DMG400027937	PGSC0003DMG400024182	PGSC0003DMG400046334
PGSC0003DMG400016023	PGSC0003DMG400006461	PGSC0003DMG401005669	PGSC0003DMG400018400
PGSC0003DMG400022069	PGSC0003DMG400004087	PGSC0003DMG400010601	PGSC0003DMG400016121
PGSC0003DMG400030198	PGSC0003DMG400026346	PGSC0003DMG400005234	PGSC0003DMG400025333
PGSC0003DMG400047102	PGSC0003DMG400011633	PGSC0003DMG400018020	PGSC0003DMG400011751
PGSC0003DMG400004168	PGSC0003DMG400024771	PGSC0003DMG400005997	PGSC0003DMG400024754
PGSC0003DMG400028401	PGSC0003DMG400018131	PGSC0003DMG400031848	PGSC0003DMG400018396
PGSC0003DMG400007005	PGSC0003DMG400001923	PGSC0003DMG400000549	PGSC0003DMG400026446
PGSC0003DMG400029242	PGSC0003DMG400007613	PGSC0003DMG400000983	PGSC0003DMG400010388
PGSC0003DMG400017997	PGSC0003DMG400017976	PGSC0003DMG400017101	PGSC0003DMG400025171
PGSC0003DMG400013929	PGSC0003DMG400009955	PGSC0003DMG400016496	PGSC0003DMG400025747
PGSC0003DMG400001154	PGSC0003DMG400031365	PGSC0003DMG400010612	PGSC0003DMG400017179
PGSC0003DMG400012538	PGSC0003DMG400001508	PGSC0003DMG400010498	PGSC0003DMG400006899
PGSC0003DMG400015156	PGSC0003DMG400030683	PGSC0003DMG400000866	PGSC0003DMG400019245
PGSC0003DMG400030274	PGSC0003DMG400006995	PGSC0003DMG400005537	PGSC0003DMG400012120
PGSC0003DMG400028710	PGSC0003DMG400014779	PGSC0003DMG400032496	PGSC0003DMG403009323
PGSC0003DMG400000816	PGSC0003DMG400023028	PGSC0003DMG400025961	PGSC0003DMG400002804
PGSC0003DMG401030252	PGSC0003DMG400032555	PGSC0003DMG400020855	PGSC0003DMG400008978
PGSC0003DMG400004204	PGSC0003DMG400021421	PGSC0003DMG401002262	PGSC0003DMG400017578
PGSC0003DMG400040321	PGSC0003DMG400020252	PGSC0003DMG400027338	PGSC0003DMG400030636
PGSC0003DMG400011406	PGSC0003DMG400019872	PGSC0003DMG400017215	PGSC0003DMG400026260
PGSC0003DMG400014056	PGSC0003DMG400003518	PGSC0003DMG400013619	PGSC0003DMG400018401
PGSC0003DMG400007682	PGSC0003DMG400030165	PGSC0003DMG400005243	PGSC0003DMG400017261
PGSC0003DMG400029490	PGSC0003DMG400022893	PGSC0003DMG400031424	PGSC0003DMG400006903
PGSC0003DMG400022951	PGSC0003DMG400018651	PGSC0003DMG400030102	PGSC0003DMG400024600
PGSC0003DMG400011690	PGSC0003DMG400019693	PGSC0003DMG400006692	PGSC0003DMG400014597
PGSC0003DMG400001135	PGSC0003DMG400013108	PGSC0003DMG400018399	PGSC0003DMG402003286
PGSC0003DMG400028331	PGSC0003DMG400006044	PGSC0003DMG400006207	PGSC0003DMG400030927
PGSC0003DMG400031031	PGSC0003DMG400016928	PGSC0003DMG400025706	PGSC0003DMG400035783

PGSC0003DMG400006939	PGSC0003DMG400000783	PGSC0003DMG400015832	PGSC0003DMG400026261
PGSC0003DMG400017096	PGSC0003DMG400016477	PGSC0003DMG400039596	PGSC0003DMG400006309
PGSC0003DMG400025192	PGSC0003DMG400006887	PGSC0003DMG400018624	PGSC0003DMG400007328
PGSC0003DMG400032276	PGSC0003DMG400009965	PGSC0003DMG400018397	PGSC0003DMG402022401
PGSC0003DMG400003609	PGSC0003DMG400007122	PGSC0003DMG400029617	PGSC0003DMG400002068
PGSC0003DMG402008784	PGSC0003DMG400000211	PGSC0003DMG401017076	PGSC0003DMG400012620
PGSC0003DMG400023040	PGSC0003DMG400025163	PGSC0003DMG400031347	PGSC0003DMG400019517
PGSC0003DMG400042469	PGSC0003DMG400025480	PGSC0003DMG400017211	PGSC0003DMG400014547
PGSC0003DMG400021725	PGSC0003DMG400011752	PGSC0003DMG400024474	PGSC0003DMG400013367
PGSC0003DMG400026650	PGSC0003DMG400031832	PGSC0003DMG400010166	PGSC0003DMG400009207
PGSC0003DMG401015537	PGSC0003DMG400018650	PGSC0003DMG400005262	PGSC0003DMG400017358
PGSC0003DMG400018170	PGSC0003DMG400033317	PGSC0003DMG400017481	PGSC0003DMG400026360
PGSC0003DMG400007672	PGSC0003DMG400030309	PGSC0003DMG400006394	PGSC0003DMG400018445
PGSC0003DMG400009915	PGSC0003DMG400030246	PGSC0003DMG402014659	PGSC0003DMG400034296
PGSC0003DMG400033354	PGSC0003DMG400014312	PGSC0003DMG400027369	PGSC0003DMG400006629
PGSC0003DMG400033570	PGSC0003DMG400006108	PGSC0003DMG400000360	PGSC0003DMG400016522
PGSC0003DMG401008784	PGSC0003DMG400006956	PGSC0003DMG400046688	PGSC0003DMG400022663
PGSC0003DMG400034395	PGSC0003DMG400006790	PGSC0003DMG400010554	PGSC0003DMG400038651
PGSC0003DMG400012089	PGSC0003DMG400015492	PGSC0003DMG400023437	PGSC0003DMG400029643
PGSC0003DMG401002685	PGSC0003DMG402007101	PGSC0003DMG400014428	PGSC0003DMG400037860
PGSC0003DMG400028480	PGSC0003DMG400010815	PGSC0003DMG401004358	PGSC0003DMG400006627
PGSC0003DMG400009388	PGSC0003DMG400021102	PGSC0003DMG400022405	PGSC0003DMG400018608
PGSC0003DMG400005091	PGSC0003DMG400001891	PGSC0003DMG401026940	PGSC0003DMG400003576
PGSC0003DMG400014603	PGSC0003DMG400016880	PGSC0003DMG400025117	PGSC0003DMG400025708
PGSC0003DMG400029492	PGSC0003DMG400023341	PGSC0003DMG400021074	PGSC0003DMG401022140
PGSC0003DMG400005299	PGSC0003DMG400029915	PGSC0003DMG400000614	PGSC0003DMG400003269
PGSC0003DMG400008344	PGSC0003DMG400027181	PGSC0003DMG400021690	PGSC0003DMG400019330
PGSC0003DMG400030804	PGSC0003DMG400029628	PGSC0003DMG400007627	PGSC0003DMG400025707
PGSC0003DMG400019770	PGSC0003DMG400012315	PGSC0003DMG400010132	PGSC0003DMG400029430
PGSC0003DMG400021823	PGSC0003DMG400026592	PGSC0003DMG400005216	PGSC0003DMG400015929
PGSC0003DMG403008349	PGSC0003DMG401030861	PGSC0003DMG400025878	PGSC0003DMG400017272

PGSC0003DMG400021056	PGSC0003DMG400023751	PGSC0003DMG400028352	PGSC0003DMG400040129
PGSC0003DMG400003360	PGSC0003DMG400025621	PGSC0003DMG400017582	PGSC0003DMG400018379
PGSC0003DMG400014246	PGSC0003DMG400007972	PGSC0003DMG400025772	PGSC0003DMG400002946
PGSC0003DMG400020205	PGSC0003DMG400000697	PGSC0003DMG400045812	PGSC0003DMG400020966
PGSC0003DMG400009359	PGSC0003DMG400000064	PGSC0003DMG400013101	PGSC0003DMG400021881
PGSC0003DMG400032162	PGSC0003DMG402008903	PGSC0003DMG400016985	PGSC0003DMG402015009
PGSC0003DMG400004474	PGSC0003DMG400030672	PGSC0003DMG400010009	PGSC0003DMG400000758
PGSC0003DMG400003802	PGSC0003DMG400007614	PGSC0003DMG400021596	PGSC0003DMG400008447
PGSC0003DMG400026554	PGSC0003DMG401030920	PGSC0003DMG400010611	PGSC0003DMG400027636
PGSC0003DMG400017637	PGSC0003DMG400010169	PGSC0003DMG400013549	PGSC0003DMG400018095
PGSC0003DMG400008043	PGSC0003DMG400001904	PGSC0003DMG400033945	PGSC0003DMG400021517
PGSC0003DMG400016967	PGSC0003DMG400026794	PGSC0003DMG400004979	PGSC0003DMG400030145
PGSC0003DMG400024364	PGSC0003DMG400007854	PGSC0003DMG400029416	PGSC0003DMG400025131
PGSC0003DMG400015354	PGSC0003DMG400016441	PGSC0003DMG400030990	PGSC0003DMG400019677
PGSC0003DMG400014576	PGSC0003DMG400011249	PGSC0003DMG400020937	PGSC0003DMG400024049
PGSC0003DMG400027031	PGSC0003DMG400011039	PGSC0003DMG400028388	PGSC0003DMG400001883
PGSC0003DMG400016971	PGSC0003DMG400008021	PGSC0003DMG400030154	PGSC0003DMG400018378
PGSC0003DMG400000919	PGSC0003DMG400022892	PGSC0003DMG400011944	PGSC0003DMG400016045
PGSC0003DMG400029461	PGSC0003DMG400001072	PGSC0003DMG400020082	PGSC0003DMG400011232
PGSC0003DMG400007645	PGSC0003DMG400006945	PGSC0003DMG400008269	PGSC0003DMG403026432
PGSC0003DMG400016599	PGSC0003DMG400016257	PGSC0003DMG400013212	PGSC0003DMG400010328
PGSC0003DMG400021688	PGSC0003DMG400016608	PGSC0003DMG400020377	PGSC0003DMG400004385
PGSC0003DMG400023406	PGSC0003DMG400016326	PGSC0003DMG400017891	PGSC0003DMG400017189
PGSC0003DMG400003444	PGSC0003DMG400005007	PGSC0003DMG400023345	PGSC0003DMG400030556
PGSC0003DMG400011365	PGSC0003DMG400010050	PGSC0003DMG400021843	PGSC0003DMG400045157
PGSC0003DMG400012785	PGSC0003DMG400013488	PGSC0003DMG400029780	PGSC0003DMG400002285
PGSC0003DMG400008982	PGSC0003DMG400007152	PGSC0003DMG402025391	PGSC0003DMG400030731
PGSC0003DMG400010984	PGSC0003DMG400005822	PGSC0003DMG402018044	PGSC0003DMG400017124
PGSC0003DMG400010924	PGSC0003DMG400004785	PGSC0003DMG400031494	PGSC0003DMG400027729
PGSC0003DMG400032121	PGSC0003DMG400010025	PGSC0003DMG400021680	PGSC0003DMG400007251
PGSC0003DMG402004193	PGSC0003DMG400008840	PGSC0003DMG400001211	PGSC0003DMG400016904

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PGSC0003DMG400025939	PGSC0003DMG400017060	PGSC0003DMG400016674	PGSC0003DMG403028300
PGSC0003DMG400011250	PGSC0003DMG400027804	PGSC0003DMG400027719	PGSC0003DMG400004458
PGSC0003DMG400028102	PGSC0003DMG400001422	PGSC0003DMG400016280	PGSC0003DMG400029178
PGSC0003DMG400023937	PGSC0003DMG400029989	PGSC0003DMG400007743	PGSC0003DMG400006678
PGSC0003DMG400025105	PGSC0003DMG400000513	PGSC0003DMG400003697	PGSC0003DMG400006644
PGSC0003DMG400029109	PGSC0003DMG400025560	PGSC0003DMG402002283	PGSC0003DMG402017076
PGSC0003DMG400014983	PGSC0003DMG400010439	PGSC0003DMG400017730	PGSC0003DMG402032501
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PGSC0003DMG400030905	PGSC0003DMG400013201	PGSC0003DMG401002283	PGSC0003DMG400007250
PGSC0003DMG401006676	PGSC0003DMG400003250	PGSC0003DMG400002847	PGSC0003DMG400022950
PGSC0003DMG400016793	PGSC0003DMG400008163	PGSC0003DMG402024195	PGSC0003DMG400031041
PGSC0003DMG400014561	PGSC0003DMG400008357	PGSC0003DMG400026880	PGSC0003DMG400025556
PGSC0003DMG400016866	PGSC0003DMG400014223	PGSC0003DMG401019771	PGSC0003DMG400020137
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PGSC0003DMG400042965	PGSC0003DMG400016525	PGSC0003DMG400018978	PGSC0003DMG400034859
PGSC0003DMG400019531	PGSC0003DMG400002937	PGSC0003DMG400006454	PGSC0003DMG400002321
PGSC0003DMG400025543	PGSC0003DMG400017918	PGSC0003DMG400009837	
PGSC0003DMG400000408	PGSC0003DMG400009115	PGSC0003DMG400022151	
PGSC0003DMG400025776	PGSC0003DMG400011745	PGSC0003DMG400028046	
PGSC0003DMG400010923	PGSC0003DMG400005036	PGSC0003DMG400002871	
PGSC0003DMG400008850	PGSC0003DMG400019824	PGSC0003DMG400010401	
PGSC0003DMG400015534	PGSC0003DMG400013886	PGSC0003DMG400044149	
PGSC0003DMG400027819	PGSC0003DMG400013798	PGSC0003DMG400002286	
PGSC0003DMG400004900	PGSC0003DMG400009591	PGSC0003DMG400020153	
PGSC0003DMG400015351	PGSC0003DMG400013604	PGSC0003DMG400010418	
PGSC0003DMG400007563	PGSC0003DMG400008484	PGSC0003DMG400025005	
PGSC0003DMG400004029	PGSC0003DMG400021877	PGSC0003DMG401028714	
PGSC0003DMG400010236	PGSC0003DMG400043403	PGSC0003DMG400018080	
PGSC0003DMG400015109	PGSC0003DMG400020174	PGSC0003DMG400002336	

PGSC0003DMG400030925	PGSC0003DMG400029341	PGSC0003DMG400004315
PGSC0003DMG400005892	PGSC0003DMG400026683	PGSC0003DMG400001596
PGSC0003DMG400027483	PGSC0003DMG400030244	PGSC0003DMG400003143
PGSC0003DMG401013923	PGSC0003DMG400000386	PGSC0003DMG400011906
PGSC0003DMG400000520	PGSC0003DMG400021574	PGSC0003DMG400016947
PGSC0003DMG400023004	PGSC0003DMG400026382	PGSC0003DMG400018377
PGSC0003DMG400025099	PGSC0003DMG400029371	PGSC0003DMG400019774
PGSC0003DMG400029472	PGSC0003DMG400041402	PGSC0003DMG400018484
PGSC0003DMG400018015	PGSC0003DMG400028846	PGSC0003DMG400017125
PGSC0003DMG400003499	PGSC0003DMG400014791	PGSC0003DMG401020908
PGSC0003DMG400029799	PGSC0003DMG400001924	PGSC0003DMG400011340
PGSC0003DMG400030486	PGSC0003DMG400014774	PGSC0003DMG400023572
PGSC0003DMG400017398	PGSC0003DMG400008569	PGSC0003DMG401001926
PGSC0003DMG400001348	PGSC0003DMG400002350	PGSC0003DMG402020126
PGSC0003DMG401022285	PGSC0003DMG400001333	PGSC0003DMG400022426
PGSC0003DMG400009555	PGSC0003DMG400005101	PGSC0003DMG400010582
PGSC0003DMG400016764	PGSC0003DMG400028303	PGSC0003DMG402005881
PGSC0003DMG400008934	PGSC0003DMG400010022	PGSC0003DMG400012851
PGSC0003DMG400023502	PGSC0003DMG400017053	PGSC0003DMG400002221
PGSC0003DMG400017897	PGSC0003DMG400006462	PGSC0003DMG400018176
PGSC0003DMG401015792	PGSC0003DMG400022775	PGSC0003DMG400010882
PGSC0003DMG400027814	PGSC0003DMG401008903	PGSC0003DMG400028491
PGSC0003DMG400031236	PGSC0003DMG400001448	PGSC0003DMG400027715
PGSC0003DMG400015650	PGSC0003DMG400022063	PGSC0003DMG400031795
PGSC0003DMG400031476	PGSC0003DMG400030134	PGSC0003DMG400023882
PGSC0003DMG400018483	PGSC0003DMG400019285	PGSC0003DMG400002198
PGSC0003DMG400012805	PGSC0003DMG400025214	PGSC0003DMG400028251
PGSC0003DMG401009742	PGSC0003DMG400009635	PGSC0003DMG400027677
PGSC0003DMG400017614	PGSC0003DMG400025897	PGSC0003DMG400027876
PGSC0003DMG400016949	PGSC0003DMG400027631	PGSC0003DMG400030402
PGSC0003DMG400028021	PGSC0003DMG400002697	PGSC0003DMG400011328

PGSC0003DMG400025591	PGSC0003DMG400032792	PGSC0003DMG400008878
PGSC0003DMG400013106	PGSC0003DMG400003065	PGSC0003DMG400020424
PGSC0003DMG402002919	PGSC0003DMG400020139	PGSC0003DMG400022996
PGSC0003DMG400001823	PGSC0003DMG400004872	PGSC0003DMG402025430

INV-Innovator, VDP-Vanderplank

Supplementary Table 5.2. List of genes down-regulated in tolerant and moderately susceptible potato cultivars inoculated/not-inoculated with *Spongospora subterranea* f. sp. *subterranea.*

INV 1	VDP 1	INV 2	VDP 2	Common INV/VDP 1	Common INV/VDP 2
PGSC0003DMG400020017	PGSC0003DMG400023070	PGSC0003DMG400021796	PGSC0003DMG400030007	PGSC0003DMG400015495	PGSC0003DMG400025774
PGSC0003DMG400028388	PGSC0003DMG401018696	PGSC0003DMG400025501	PGSC0003DMG400033607	PGSC0003DMG400003043	PGSC0003DMG400003219
PGSC0003DMG400032810	PGSC0003DMG400019173	PGSC0003DMG400012453	PGSC0003DMG400020698	PGSC0003DMG400006367	PGSC0003DMG400001528
PGSC0003DMG400015832	PGSC0003DMG400000263	PGSC0003DMG401032501	PGSC0003DMG400010148	PGSC0003DMG400016931	PGSC0003DMG400020216
PGSC0003DMG400019415	PGSC0003DMG401005959	PGSC0003DMG400022859	PGSC0003DMG400003530	PGSC0003DMG400011102	PGSC0003DMG400026855
PGSC0003DMG400026692	PGSC0003DMG400011662	PGSC0003DMG400022483	PGSC0003DMG400007815	PGSC0003DMG400011949	
PGSC0003DMG400000247	PGSC0003DMG400004575	PGSC0003DMG400025451	PGSC0003DMG400007552	PGSC0003DMG400005339	
PGSC0003DMG401028714	PGSC0003DMG400020751	PGSC0003DMG400020870	PGSC0003DMG400014651	PGSC0003DMG400024870	
PGSC0003DMG400027539	PGSC0003DMG400017701	PGSC0003DMG400013814	PGSC0003DMG400028426	PGSC0003DMG400032247	
PGSC0003DMG400029752	PGSC0003DMG401027371	PGSC0003DMG400021216	PGSC0003DMG400014310	PGSC0003DMG400023700	
PGSC0003DMG400008433	PGSC0003DMG400003397	PGSC0003DMG400001407	PGSC0003DMG400004693		
PGSC0003DMG400011830	PGSC0003DMG400028654	PGSC0003DMG400027857	PGSC0003DMG400033078		
PGSC0003DMG400006207	PGSC0003DMG400022366	PGSC0003DMG400008794	PGSC0003DMG400002123		
PGSC0003DMG400028538	PGSC0003DMG400004694	PGSC0003DMG400024062	PGSC0003DMG400014309		
PGSC0003DMG400017728	PGSC0003DMG402016183	PGSC0003DMG400027358	PGSC0003DMG400033900		
PGSC0003DMG400027180	PGSC0003DMG400042218	PGSC0003DMG400039633	PGSC0003DMG400005925		
PGSC0003DMG400000155	PGSC0003DMG402010883	PGSC0003DMG400017185	PGSC0003DMG400022674		
PGSC0003DMG400011777	PGSC0003DMG400004142	PGSC0003DMG400005730	PGSC0003DMG400003916		
PGSC0003DMG400011929	PGSC0003DMG400006759	PGSC0003DMG400002186	PGSC0003DMG400000620		
PGSC0003DMG400026049	PGSC0003DMG400000339	PGSC0003DMG400016068	PGSC0003DMG400011284		

PGSC0003DMG400024049	PGSC0003DMG400005436	PGSC0003DMG400013124	PGSC0003DMG400020215
PGSC0003DMG400029316	PGSC0003DMG402012656	PGSC0003DMG400005809	PGSC0003DMG402006167
PGSC0003DMG400008932	PGSC0003DMG400031731	PGSC0003DMG400010170	PGSC0003DMG400010031
PGSC0003DMG400027181	PGSC0003DMG400030638	PGSC0003DMG400017913	PGSC0003DMG400013017
PGSC0003DMG400040471	PGSC0003DMG400015895	PGSC0003DMG400046798	PGSC0003DMG400028331
PGSC0003DMG400006692	PGSC0003DMG400026969	PGSC0003DMG400002582	PGSC0003DMG400002417
PGSC0003DMG400025876	PGSC0003DMG401008890	PGSC0003DMG400002414	PGSC0003DMG400015275
PGSC0003DMG401002869	PGSC0003DMG400008348	PGSC0003DMG400019106	PGSC0003DMG401031759
PGSC0003DMG400017103	PGSC0003DMG400027546	PGSC0003DMG400018630	PGSC0003DMG400002581
PGSC0003DMG400022063	PGSC0003DMG400011177	PGSC0003DMG400029099	PGSC0003DMG400012454
PGSC0003DMG400004458	PGSC0003DMG400015652	PGSC0003DMG400014836	PGSC0003DMG400008128
PGSC0003DMG400020377	PGSC0003DMG402010374	PGSC0003DMG400024786	PGSC0003DMG400023777
PGSC0003DMG400020809	PGSC0003DMG400030649	PGSC0003DMG400006389	PGSC0003DMG400007734
PGSC0003DMG400026543	PGSC0003DMG402002551	PGSC0003DMG400006922	PGSC0003DMG402004193
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PGSC0003DMG400015289	PGSC0003DMG400003057	PGSC0003DMG400022846	PGSC0003DMG400009095
PGSC0003DMG400005580	PGSC0003DMG400034963	PGSC0003DMG400033348	PGSC0003DMG401004193
PGSC0003DMG400023220	PGSC0003DMG400012826	PGSC0003DMG400013210	PGSC0003DMG400016533
PGSC0003DMG400013939	PGSC0003DMG400022131	PGSC0003DMG400031742	PGSC0003DMG400003802
PGSC0003DMG401018257	PGSC0003DMG400018575	PGSC0003DMG400010807	PGSC0003DMG400015642
PGSC0003DMG400010134	PGSC0003DMG400020787	PGSC0003DMG400045182	PGSC0003DMG400011406
PGSC0003DMG400030413	PGSC0003DMG403029631	PGSC0003DMG400014603	PGSC0003DMG400009866
PGSC0003DMG400028543	PGSC0003DMG400008620	PGSC0003DMG402016602	PGSC0003DMG400011073
PGSC0003DMG400002937	PGSC0003DMG400029780	PGSC0003DMG400031535	PGSC0003DMG400026176
PGSC0003DMG402025391	PGSC0003DMG401010883	PGSC0003DMG400015129	PGSC0003DMG400019531
PGSC0003DMG400033563	PGSC0003DMG400028445	PGSC0003DMG400012852	PGSC0003DMG400021688
PGSC0003DMG400011232	PGSC0003DMG400010240	PGSC0003DMG401023186	PGSC0003DMG400010193

PGSC0003DMG400026682	PGSC0003DMG400013599	PGSC0003DMG400046823	PGSC0003DMG400009847
PGSC0003DMG400007496	PGSC0003DMG400019270	PGSC0003DMG400023237	PGSC0003DMG400011035
PGSC0003DMG400024478	PGSC0003DMG400006603	PGSC0003DMG400030078	PGSC0003DMG400011013
PGSC0003DMG400014211	PGSC0003DMG400003551	PGSC0003DMG400018069	PGSC0003DMG400012538
PGSC0003DMG400007743	PGSC0003DMG400014449	PGSC0003DMG400016848	PGSC0003DMG400018170
PGSC0003DMG400008591	PGSC0003DMG400018766	PGSC0003DMG400010605	PGSC0003DMG400042482
PGSC0003DMG400000866	PGSC0003DMG401015451	PGSC0003DMG400024364	PGSC0003DMG400000816
PGSC0003DMG400016653	PGSC0003DMG400031841	PGSC0003DMG400011569	PGSC0003DMG400028021
PGSC0003DMG401025831	PGSC0003DMG400012118	PGSC0003DMG400007005	PGSC0003DMG400043830
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PGSC0003DMG400019542	PGSC0003DMG400002824	PGSC0003DMG400014034	PGSC0003DMG400031479
PGSC0003DMG400007887	PGSC0003DMG400020164	PGSC0003DMG400012805	PGSC0003DMG400005528
PGSC0003DMG400003548	PGSC0003DMG400011420	PGSC0003DMG400020588	PGSC0003DMG400025547
PGSC0003DMG401024842	PGSC0003DMG400026221	PGSC0003DMG400015351	PGSC0003DMG400002217
PGSC0003DMG400001914	PGSC0003DMG400024157	PGSC0003DMG400006248	PGSC0003DMG400017766
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PGSC0003DMG400028931	PGSC0003DMG400004735	PGSC0003DMG400003099	PGSC0003DMG400039149
PGSC0003DMG400000397	PGSC0003DMG400014384	PGSC0003DMG400029462	PGSC0003DMG400005521
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PGSC0003DMG400031768	PGSC0003DMG400023441	PGSC0003DMG400000919	PGSC0003DMG400024366
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PGSC0003DMG400011649	PGSC0003DMG400027505	PGSC0003DMG400008982
PGSC0003DMG400004800	PGSC0003DMG400024357	PGSC0003DMG400016452
PGSC0003DMG400000719	PGSC0003DMG400009967	PGSC0003DMG400026191
PGSC0003DMG400013099	PGSC0003DMG402023760	PGSC0003DMG400014056
PGSC0003DMG400012017	PGSC0003DMG400007346	PGSC0003DMG400024363
PGSC0003DMG400007803	PGSC0003DMG402010918	PGSC0003DMG400020053
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PGSC0003DMG402002349	PGSC0003DMG400020449	PGSC0003DMG400026607
PGSC0003DMG400004275	PGSC0003DMG400017707	PGSC0003DMG400005170

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