Metabolic profiling for identification of potential biomarkers in potato tuber extracts associated with tolerance to *Spongospora subterranea* f. sp. *subterranea* infection.

By

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SUBMISSION DECLARATION

I, **Gontse Kehumile Joyce Modisane** declare that the thesis/dissertation, which I hereby submit for the degree at *Magister Scientiae* (Specialisation in Biochemistry) the University of Pretoria, is my work and has not previously been submitted by me for the degree at this or any other tertiary institution.

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DEDICATION

This research is dedicated to my pillar, the queen of my heart, my mother Helen Modisane, who always supported me throughout my studies and my life. I wouldn't have made it without your caring, love and guidance.
I love u mom.

ACKNOWLEDGEMENTS

Firstly and foremost, I would like to thank my creator, God the Father, for giving me life and strength for accomplishing my dreams to become a research scientist. Without Him, I wouldn't have made it.

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PREFACE

The research set out in this thesis was carried out to search for metabolic biomarkers in potato tuber extracts associated with tolerance to *Spongospora subterranea* f. sp. *subterranea* infection using UPLC-QTOF/MS-based untargeted metabolomics in combination with chemometrics methods. The thesis chapters' start with the introduction (**Chapter 1**), followed by the experimental work (**Chapter 2**), results (**Chapter 3**) and discussion (**Chapter 4**). The overall findings of the study have been summarized in a conclusion (**Chapter 5**) and lastly followed by limitations of the study and future perspectives (**Chapters 6 and 7**).

Symposia

The content of this dissertation was presented at the following Symposia:

Oral presentation at Biochemistry symposium, held at University of Pretoria (Groenkloof campus) which took place on 27 September 2017. Presentation by G.K.J Modisane, M. Lekota, J. van der Waals and Z. Apostolides, Metabolic profiling for identification of potential biomarkers in potato tuber extracts associated with tolerance to *Spongospora subterranea* f. sp. *subterranea* infection.

Poster presentation at Potatoes South Africa research symposium, held at Khaya Ibhubesi conference centre (Parys) which took place on 24-27 July 2018. Presentation by G.K.J Modisane, M. Lekota, J. van der Waals and Z. Apostolides, Metabolic profiling for identification of potential in potato tuber extracts associated with tolerance to *Spongospora subterranea* f. sp. *subterranea* infection.

Poster presentation at Department of Biochemistry, Genetics and Microbiology (BGM) research symposium, held at Future Africa UP Campus (Hatfield) which took place on 06 December 2018. Presentation by G.K.J Modisane, M. Lekota, J. van der Waals and Z. Apostolides, Metabolic profiling for identification of potential biomarkers in potato tuber extracts associated with tolerance to *Spongospora subterranea* f. sp. *subterranea* infection.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ADMIS	Automated mass spectral deconvolution and identification system
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BEH	Bridged ethylsiloxane hybrid
BLB	Bacterial leaf scourge
BPI	Base peak ion
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CE-MS	Capillary electrophoresis-mass spectrometry
CERK1	Chitin elicitor receptor kinase
CI	Chemical ionization
CID	Collision-induced dissociation
COW	Correlated Optimized Warping
DAMP	Damage-associated molecular pattern
DART	Direct analysis in real time
DI-MS	Direct infusion-mass spectrometry
EI	Electron impact ionization
ELISA	Enzyme-linked immunosorbent assay
ELRR	Elicitor recognition receptor
ESI	Electron spray ionisation
ETI	Effector-triggered immunity
FAB	Fast atom bombardment
FT-ICR	Fourier transformation-ion cyclotron resonance
GC	Gas chromatography
GI	Glycaemic index

GM	Genetically modified
GS	Glutamine synthetase
HCA	Hierarchical cluster analysis
HILIC	Hydrophilic interaction column
HPLC	High performance liquid chromatography
HR	Hypersensitive reaction
IR	Infrared radiation
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LDA	Linear discriminant analysis
MALDI	Matrix-associated laser desorption/ionization
MAMP	Microbe-associated molecular pattern
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotamide adenine dinucleotide phosphate
NBS-LRR	Nucleotide-binding site leucine-rich repeat proteins
NMR	Nuclear magnetic resonance
NPR1	Non-expressor of pathogenesis-related genes 1
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
PR	Pathogenesis related
PTI	PAMP- trigger immunity

RCBD	Randomized complete block design
RI	Retention index
RLK	Receptor-like kinase
ROS	Reactive oxygen species
RRM	Resistance related metabolite
RRP	Resistance related protein
RT-PCR	Real-time polymerase chain reaction
SA	Salicylic acid
SIM	Single ion monitoring
SMPDB	Small Molecule Pathway Database
SRM	Selected reaction monitoring
TMS	Trimethylsilyl
TOF	Time-of-flight
UHPLC	Ultra-high-pressure liquid chromatography
UPLC	Ultra-Performance liquid chromatography
QTOF	Quadrupole time-of-flight
UV	Ultra-violet
WAK	Wall-associated kinase

SUMMARY

Potatoes are popular and reliable crops all over the world. Unfortunately, its production is threatened by detrimental factors such as fungi, bacteria and viruses that attack potatoes during growth and storage. Among all the factors infecting the potato crops, the fungal disease is the most important diseases that cause severe damage in potatoes. *Spongospora subterranea* f. sp. *subterranea* (Sss) is one of the fungal pathogens of economic importance in potato production, causing a significant reduction in yield and quality of potato tubers globally. Currently, there are no effective control methods available for this pathogen, partly due to the longevity of the highly resistant sporosori surviving in the soil. The use of cultivars resistant to Sss is the most efficient and long-term strategy for preventing the build-up of field inoculum and the spread of the pathogen. However, classical breeding and selection methods are laborious, costly and time-consuming processes. Metabolomics, as a new advanced technology, was used to better understand the biochemical mechanisms of tolerance and to identify metabolic biomarkers that could distinguish between tolerant and susceptible cultivars with statistical significance at $p \leq 0.05$. Four susceptible and six tolerant cultivars of five biological repeats were each grown in the greenhouse inoculated with Sss pathogen-suspension and mock-solution. Based on the results, multivariate data analysis revealed different responses to Sss infection when comparing tolerant with susceptible cultivars. Most of the metabolites identified in the Sss-inoculated samples were abundant in tolerant compared to susceptible cultivars such as amino acids, phenylpropanoids, fatty acids, organic acids and alkaloids. These metabolites are known for their antimicrobial properties as well as cell wall thickening, used to suppress pathogen development in plants, leading to reduced susceptibility. In conclusion, palmitic acid, 3-indole acrylic acid and cuscohygrine were found as possible biomarkers for tolerance against Sss infection in potato tuber extracts. These biomarkers may play an important role in potato tolerance to Sss.

CHAPTER 1: INTRODUCTION

1.1. The host

Potato (*Solanum tuberosum* L.) is one of the most important staple food crop, with more than 300 million metric tons produced globally, while in South Africa about 2,4 million tons are produced (FAO., 2013). Furthermore, potatoes have higher phytonutrient content, which is modifiable to advancement through breeding and biotechnology approaches (Nzaramba *et al*., 2007). Potatoes are rich in carbohydrates, vitamins B1, B3 & B6, as well as, minerals such as potassium, phosphorus and magnesium. In addition to nutrients, potatoes are low in fat and protein, but high in essential amino acids and contain folate, riboflavin and pantothenic acid (Camire *et al*., 2009, Dias, 2012). Other than nutrients, potatoes are made of a number of bioactive compounds (primary and secondary metabolites), a few of which might be active in humans, and are collectively referred to as phytochemicals. These include carotenoids (lutein and zeaxanthin), phenolic acids (chlorogenic acid and caffeic acid), and flavonoids (quercetin and kaempferol), and other important groups such as alkaloids (chaconine and solanine), phytosterols, terpenoids, organosulfur and nitrogen-containing compounds (Liu, 2004). Among them, alkaloids are the most extensively studied with respect to their bioactivity, toxicology, and role in the plant's physiology (Friedman, 2006). These phytochemicals are highly desirable in the diet because of their beneficial effects on human health (Katan and ROOS, 2004). In this review, we discuss the phytochemicals present in potatoes and their health benefits in the following subtopics.

1.1.1. Phytochemicals in potatoes

Phytochemicals are chemical compounds produced naturally by plants that have protective/disease preventive properties. These compounds are non-nutritive chemicals found in fruits and vegetables that are unrecognized as macronutrients, such as protein and carbohydrates, or micronutrients, such as vitamins and minerals (Liu, 2004). They are not fundamental for short-term well-being, and much of the time, the human body lacks a mechanism for their accumulation. These compounds play an important role in a number of processes (Friedman, 1997), and researchers speculate that there might be many to be found in the food we eat. Recent studies demonstrate that they can also protect humans against diseases (Ezekiel *et al*., 2013, Tsao, 2009, Umaerus and Umaerus, 1994). In potatoes, only

carotenoids, alkaloid, phenolic compounds, and flavonoids are found in significant amounts (Tsao, 2009). Not all compounds in potato varieties have been reported in the studies as many compounds are unique or different. For instance, chlorogenic acid is the primary phenolic compound found in all potatoes; carotenoids are found in yellow and orange-fleshed potato; anthocyanin is only found in red and purple-fleshed potatoes (Andre *et al*., 2009, Barba *et al*., 2008, Brown, 2005). The phytochemical content of potatoes also depends on the type of the plant material used; for example, the phytochemicals present in the roots might differ from those in tubers or leaves and stems.

1.1.1.1. Phenolic compounds

Phenolic compounds are the products of plants secondary metabolism, which play an important role in plant defence against biotic and abiotic factors. In addition to that, it also plays a role in structural support and sealing of injured plant surface (Treutter, 2005). Moreover, phenolic compounds may play a part in enzyme-catalysed browning reactions that could detrimentally affect the colour, flavour and nutritional quality of potatoes *(*Ezekiel *et al*., 2013). These compounds are synthesized by the shikimic acid pathway, which is found in living organisms, such as plants and microorganisms, but not in animals, and are usually present as a bound form in foods, or attached to cell walls and proteins (Miller and Ruiz-Larrea, 2002). The primary phenolic acids found in potatoes are chlorogenic acid, sinapic acid, ferulic acid, p-coumaric acid, cinnamic acid and caffeic acid (Friedman, 1997), the chemical structures are shown in Figure 1.1. Chlorogenic acid is the ester of caffeic acid and quinic acid, and a major substrate for enzymatic oxidation leading to browning in plants, mainly potatoes and apples (Tomás- Barberán and Espín, 2001). Other phenolic acids reported in potatoes include gallic acid, protocatechuic, vanillic acid and salicylic acid, but, typically in smaller amounts (Schieber and Saldaña, 2009).

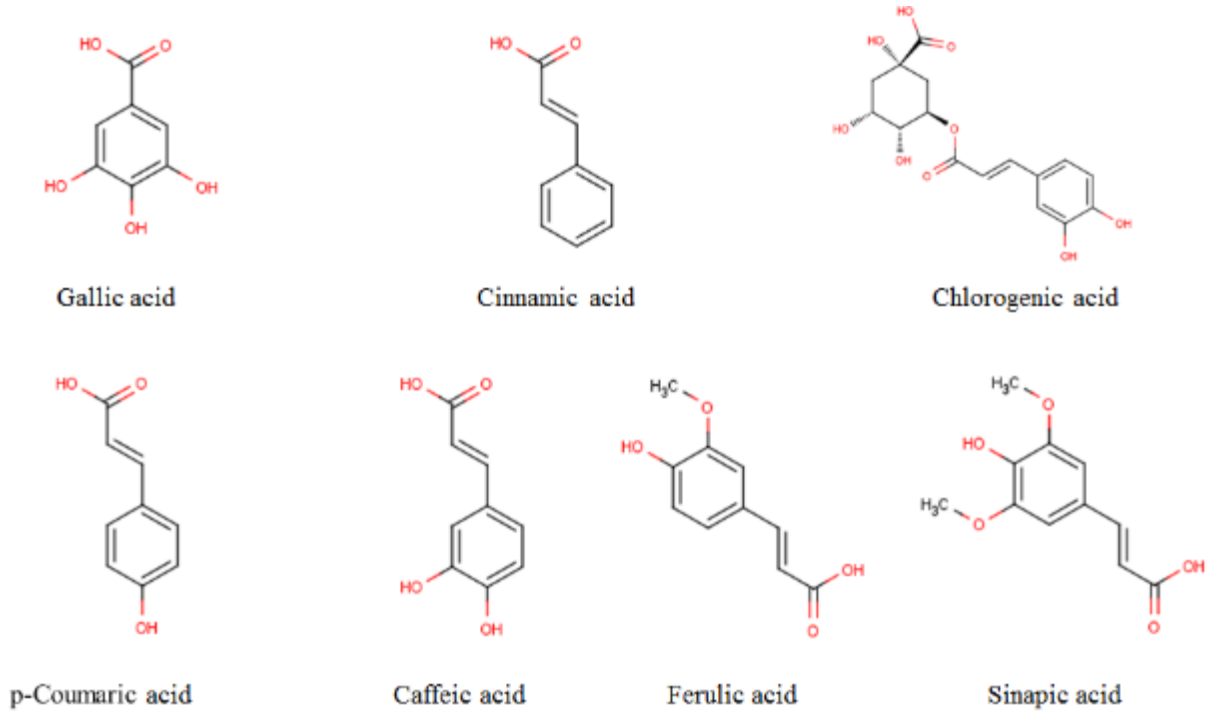
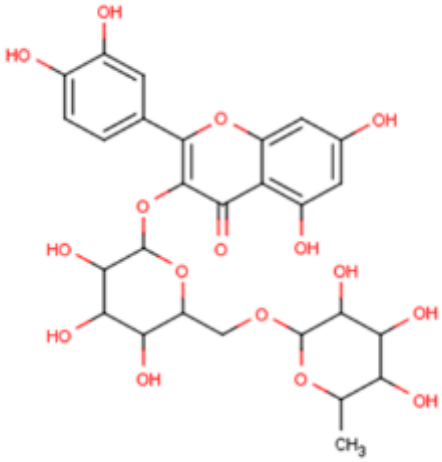
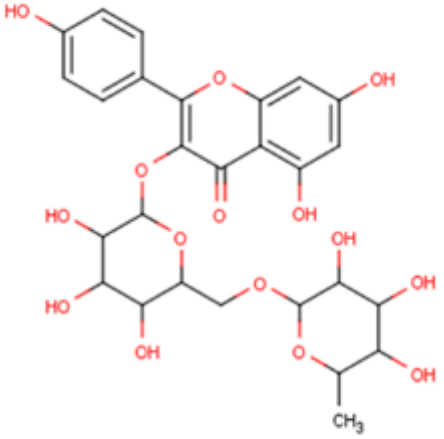


Figure 1.1: Major phenolic acids found in potatoes.

Flavonoids are other groups of phenolic compounds that are essential in our diets and nearly ubiquitous throughout the plant kingdom. These compounds are found in nature as conjugates, in glycosylated or esterified forms, but can be present as aglycones, in fruits or vegetables (Tomás- Barberán and Espín, 2001). The examples of flavonoids present in potatoes include quercetin and kaempferol glycosides, catechin and rutin (Lewis *et al*., 1998). Studies on flavonoids found in potato tubers are limited due to various cultivars used. For example, catechin, epicatechin, eriodictyol, kaempferol and naringenin were reported in skin extracts of coloured potato (Brown, 2005, Lewis *et al*., 1998). Andre *et al*. (2007) found rutin and kaempferol-3-rutinoside in 23 native Andean potato cultivars. Flavanol quercetin was reported to be three times more effective as an antioxidant activity than kaempferol and eriodictyol and was twice as effective as catechin (Tsao and Akhtar, 2005). Rutin is not the predominant polyphenol in potatoes, and not all reports from the literature have discovered this flavanol in all potato cultivars (Oertel *et al*., 2017). The structures of the major flavanols are shown in Figure 1.2.



Rutin-(quercetin 3-rutinoside)



Nicotiflorin-(Kaempferol-3-O-rutinoside)

Figure 1.2: The major flavanols: quercetin and kaempferol glycosides found in potatoes.

1.1.1.2. Carotenoids

Carotenoids are lipophilic compounds, which play an essential role in photosynthetic organisms. They perform an essential photoprotective role by quenching triplet state chlorophyll molecules and scavenging singlet oxygen and other toxic species derived from excess light energy, thus limiting membrane damage (Howitt and Pogson, 2006, Young, 1991). These compounds are synthesized in plastids from isoprenoid and are responsible for the yellow and orange colours of the flesh (DellaPenna and Pogson, 2006). The major potato carotenoids include neoxanthin, lutein, violaxanthin, zeaxanthin and β-carotene which are found in trace amounts. Carotenoids such as β-Carotene, α-carotene, and β-cryptoxanthin contain a pro-vitamin A activity which is converted to retinol (vitamin A) after digested in the human body (Brown, 2005). The chemical structures of carotenoids in potatoes are shown in Figure 1.3.

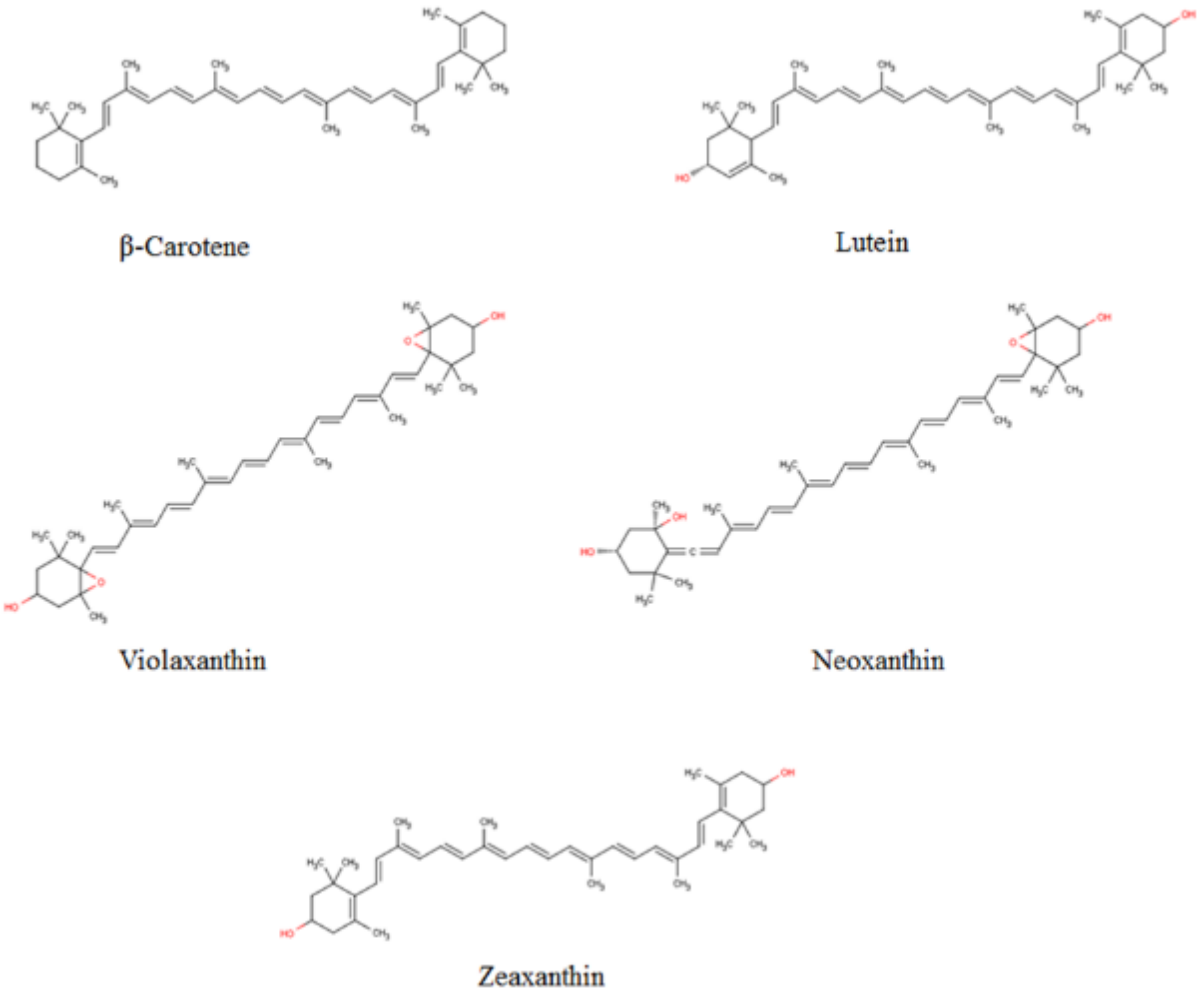


Figure 1.3: Carotenoids found in potatoes.

1.1.1.3. Steroidal glycoalkaloids

The steroidal glycoalkaloids (GAs) are natural toxins produced in all parts of potato plants, including foliage, cortex, periderm and parts of high metabolic activity followed by tubers and peels (Barceloux, 2009). Glycoalkaloids are synthesized as natural defence mechanisms against pests and diseases (Friedman, 2006). The two significant glycoalkaloids in cultivated potatoes are α-solanine and α-chaconine, both of which are derived from aglycones solanidine, but differ with respect to the composition of the sugars-side chain (Friedman and Dao, 1992, Sarquis *et al*., 2000). Moreover, these two compounds are held accountable for up to 95% of the total glycoalkaloid content in potato tubers (Jadhav *et al*., 1981). Removal of sprouts and peels of potatoes before processing (baking, boiling, frying, microwave) eliminates almost all glycoalkaloids (Friedman et al., 2003, Nema *et al*., 2008).

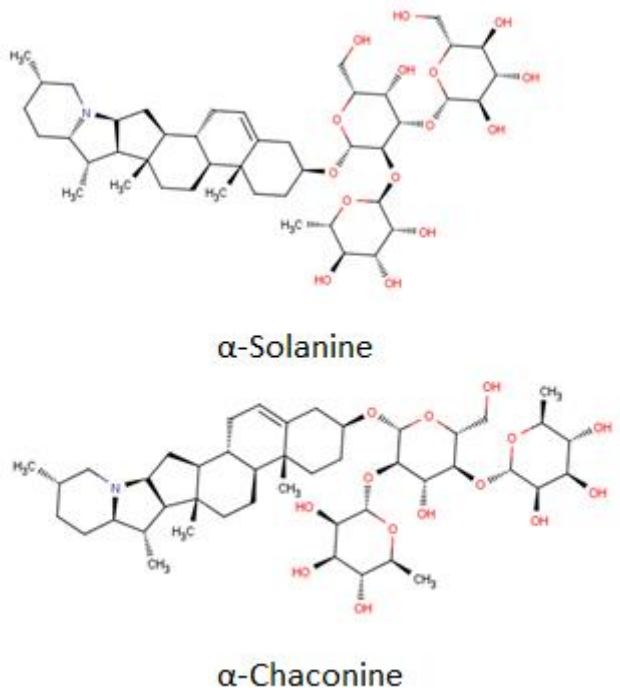


Figure 1.4: Main glycoalkaloids found in potatoes.

1.1.1.4. Terpenoids

Terpenoids are secondary metabolites, anti-microbial compounds naturally produced by plants in response to pathogen attack. Terpenes are categorized by the number of isoprene units (C5) in the molecule, such as monoterpene (C10), sesquiterpene (C15), diterpene (C20) and triterpene (C30) (Ashour *et al*., 2010). Sesquiterpene is one of the most significant compounds found in potatoes (Li *et al*., 2015, Lisker and Kuc, 1978). Examples of sesquiterpenes that accumulate in potatoes in response to stress are rishitin, lubimin, solavetivone, phytuberin, phytuberol, and anhydro-rotunol (Friend, 2012, Kuć, 1982, Kuc and Lisker, 1978, Stoessl *et al*., 1976, Stoessl *et al*., 1977). Rishitin, lubimin, and solavetivone generally comprise > 85% of the total sesquiterpenes (Li *et al*., 2015). However, rishitin is the predominant sesquiterpene compound, the other metabolites can be found in some pathogen interactions and under some environmental conditions, accumulate to higher concentrations (Price *et al*., 1976, Li *et al*., 2015). Rishitin was at first isolated from the Rishira cultivar of potatoes by Tomiyama *et al*. (1968) and was allocated the structure of a bicyclic norsesquiterpene alcohol (Katsui *et al*., 1968). The structures of rishitin, lubimin and phytuberin are shown in Figure 1.5**.**

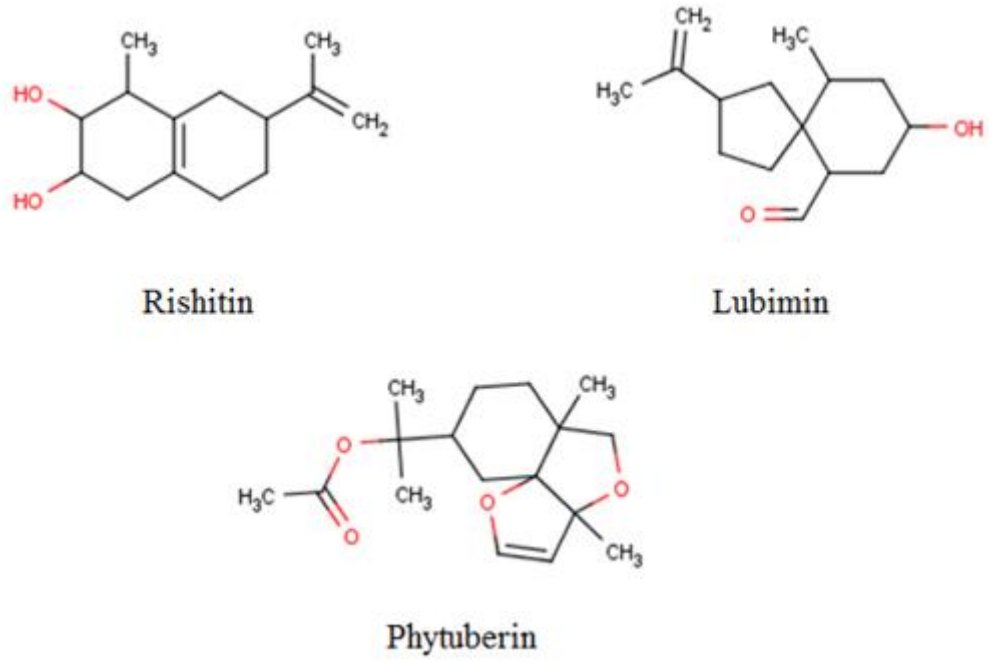


Figure 1.5: Major sesquitepernoids found in potatoes.

1.1.2. Health benefits of potato

As mentioned earlier, potato contains essential nutrients, whether as fresh or processed food, that can benefit human health in different ways. Many studies suggest that the health benefits of fruits and vegetables are attributed to the interactions of bioactive compounds, antioxidants, and other nutrients in the whole plant. Therefore, these compounds should be highly desirable in the diet and are known to reduce the risk of the development of multiple diseases. Diets rich in antioxidants flavonoids and carotenoids are associated with a lower incidence of atherosclerotic, heart disease, cancers, muscular degeneration and severity of cataracts (Wang *et al*., 1999) . Potato peels are a good source of natural antioxidants, which have been studied in various food systems (de Sotillo *et al*., 1994). These peels provide protection against acute liver injury (Singh *et al*., 2008) and oxidative damage to erythrocytes (Singh and Rajini, 2008). Thompson *et al*. (2009) have reported that the phytochemicals of freeze-dried powdered potato caused a 23% reduction in induced breast cancer in rats. Several other health benefits (longevity, heart and eye health) and therapeutic (antibacterial, anti-inflammatory, antimutagenic, antineoplastic, antiviral antiallergic, antithrombotic and vasodilator activity) of phenolic acids have been reported (Manach *et al*., 2004, Miller, 1996). Many of these effects result from powerful antioxidant and free radicals scavenging properties of phenolic compounds (Amakura *et al*., 2000, Rice-Evans *et al*., 1997).

Chlorogenic acid is well known for health-promoting effects, such as protection against degenerative diseases, cancer, heart disease (Nogueira and do Lago, 2007), hypertension (Yamaguchi *et al*., 2008) and viral and bacterial diseases. These compounds can slow down the release of glucose into the bloodstream (Bassoli *et al*., 2008); therefore, it might be helpful in lowering the glycaemic index (GI) of potatoes (Ezekiel *et al*., 2013). Hence, potatoes with a low GI are good for diabetic patients and may lower the risk of type 2 diabetes (Legrand and Scheen, 2007). Quercetin and Vitamin C have shown potential in cell membrane protection, as well as, anti-inflammatory activity in the vascular system by inhibiting the expression of molecules involved in the first phase of atherosclerosis (Kumar and Pandey, 2013). Quercetin phase 2 metabolites appear to inhibit the lung cancer cells proliferation (Kumar and Pandey, 2013). Anthocyanins have also been reported to prevent diseases, such as cardiovascular diseases, cancer and diabetes (Reddivari *et al*., 2007). Potato nutrients/bioactive compounds play an important role in fighting diseases in humans.

1.2. The pathogen

1.2.1. Pathogen occurrence, life cycle and epidemiology

Spongospora subterranea f. sp. *subterranea* (Sss) is an obligate biotrophic parasite, which needs a living host to reproduce and complete its life cycle, and cannot be cultured and grow on artificial media, and therefore, the Koch's postulates cannot be completed for the pathogen (Merz and Falloon, 2009). Furthermore, *Spongospora subterranea* f. sp. *subterranea* pathogen serve as a vector of potato mop-top virus (Kirk, 2008), causing severe blemishes on potato tubers to render them unmarketable (Hernandez Maldonado *et al*., 2013). These tubers are usually discarded before they reach the market, which is a great loss to the potato industry. The pathogen causes three diseases in potatoes, including powdery scab, root infection and root galling. The symptoms are shown in Figure 1.6. Severely infected potatoes shrink and lose weight during storage, and may become rotten, due to respiration losses through powdery scab lesions (Falloon, 2008). Furthermore, the powdery scab has an important effect on the potato seed industry, as seed tubers that are infected are undesirable for use in the establishment of the new crop as the pathogen can be transmitted from infected seed tubers to infect new potato seed (Harrison *et al*., 1997). The pathogen can infect other solanaceous plants such as tomatoes and some common weeds in the fields where potatoes are grown (Merz, 2008).

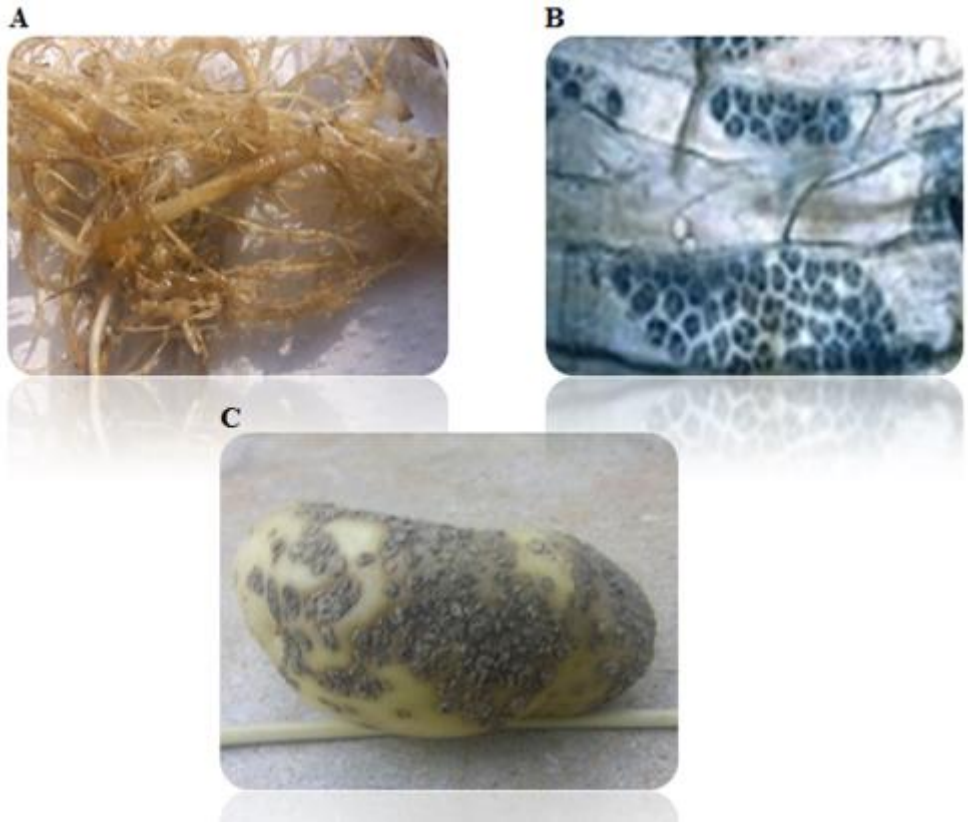


Figure 1.6: Three symptoms of powdery scab disease. a) Potato roots with milky white galls, b) potato roots showing zoosporangia (Hernandez Maldonado *et al*., 2013) and c) potato tuber with black powdery spore balls.

Powdery scab disease is widespread and occurs across major potato production regions and countries. The first reports of the disease were from Alaska (Carling, 1996), Pakistan (Ahmad *et al*., 1996). In 1997 the disease further spread in the USA reaching North Dakota (Draper et al., 1997), Costa Rica (Montera-Astua *et al*., 2002), South Korea (Kim *et al*., 2003). Surveys done in 2004 showed that powdery scab occurred commonly and severely in China (Christ, 2001), and Malta (Porta-Puglia and Mifsud, 2006). Furthermore, widespread distribution of powdery scab was found in Argentina (Clausen *et al*., 2005). In Germany, which has a long history of powdery scab, there is an increasing problem with the disease (Stachewicz and Enzian, 2002). The spread of powdery scab disease continued according to the national records in Bulgaria (Bobev, 2009), Iran (Norouzian *et al*., 2010), and Latvia (Turka and Bimšteine, 2011) as shown in Figure 1.7. Recently, the pathogen was detected in Sri Lanka (Babu and Merz, 2016), the island–nation of Cyprus (Kanetis *et al*., 2016), Colorado in the USA (Houser and Davidson, 2010), New Mexico (Mallik and Gudmestad, 2015), and in the Greek island of Crete (Vakalounakis *et al*., 2016). In South Africa, the powdery scab research has started recently (Figure 1.7), and the disease has been reported in the Limpopo province

due to the effect of different soil treatments on the development of *Spongospora subterranea* f. sp. *subterranea* pathogen in potato roots and tubers (Simango and Van der Waals, 2017).

Figure 1.7: Countries with a record of *Spongospora subterranea* f. sp. *subterranea*. Possible centre of the origin of Sss (●); the disease powdery scab has been recorded (•); long history of powdery scab research (▲); powdery scab research started more recently (■); potato mop-top virus research (◘), (Merz, 2008).

Spongospora subterranea f. sp. subterranea uses multiple mechanisms to infect, penetrate, colonize and induce cell death in host plant tissues. Infection typically occurs in two phases, the primary (inner circle) and secondary phases (outer circle), as shown in Figure 1.8. At the initial stage of the primary phase, one resting spore (cyst) germinates and releases a biflagellate primary zoospore that swims in moist soil to reach the host (Merz and Falloon, 2009). These primary zoospores swim to the roots, penetrate and infect epidermal cells or host roots hairs, and produce a multinucleate sporangial plasmodium, which develops within the root, and eventually become enclosed into multinucleate portions that finally develop to form zoosporangia (Falloon, 2008). The zoosporangia release secondary zoospores, which are of equivalent size to the primary zoospores and possess two anterior flagella, enabling them to swim and infect the cortical cells on roots, stolon and tubers of the host plant, resulting in the development of root galls and tuber lesions producing cystogenous plasmodia (Nitzan *et al*., 2008).

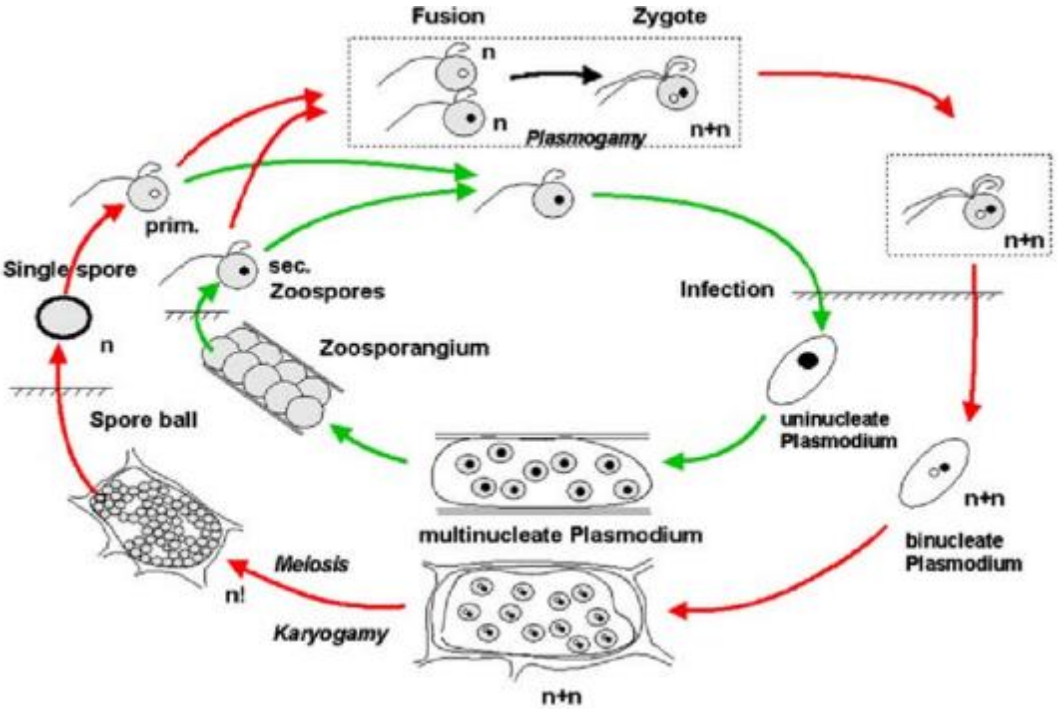


Figure 1.8: The life cycle of *Spongospora* species with an asexual phase (inner circle-green) and a sexual phase (outer circle-red) (Merz, 2008).

The pathogen is favoured by heavy soils with high water retention capacities that are thought to encourage the disease (Prentice *et al*., 2007). The pathogen generally requires cool temperatures between 9-17^{0}C and free water for infection (Wale, 2005). *Spongospora subterranea* f. sp. *subterranea* pathogen produces multiple resting spores, which can survive in the soil for 4-5 years (De Boer, 2000, Harrison *et al*., 1997) and are highly resistant to environmental stresses, which can spread through seed potatoes and contaminated soil (Merz, 2008). These resting spores can infect potato and many other plant hosts when releasing primary zoospores (Arcila Aristizabal *et al*., 2013, Jones and Harrison, 1969, Kole, 1954).

1.2.2. Control measures of *Spongospora subterranea* f. sp. *subterranea*

Powdery scab has become an extremely important disease in recent years and can infect a range of plants. Attempts have been made to reduce the spread of the disease using resistant cultivars, biological, chemicals and cultural controls. Cultural control methods centre on controlling the available water to prevent the zoospores from infecting include irrigating appropriately, appropriate soil used and ensuring proper drainage (Falloon, 2008). Avoidance of planting potatoes in fields that were planted with infected plants (Harrison *et al*., 1997) is one of the cultivation control measures for powdery scab disease. Control of powdery scab with soil application of chemicals is costly and harmful to the environment; and does not

offer complete control of the disease (Falloon, 2008). Use of resistant cultivars has been proven as the most significant tool for disease management and are an important objective of global potato breeding programs. Development of potato varieties with genetic resistance to powdery scab can be beneficial in control of the disease, as there is no single effective method of controlling the disease (Falloon, 2008).

1.2.3. Mechanism of resistance to *Spongospora subterranea* f .sp. *subterranea*

The mechanism of resistance to powdery scab is not yet known. However, Falloon *et al*. (2003) demonstrated that resistant cultivars have fewer *Spongospora subterranea* f. sp. *subterranea* zoosporangia root infections and tuber scabbing as well as fewer root galls when compared to susceptible cultivars. Furthermore, resistance differs among cultivars; some cultivars are resistant to tuber (stolon) infection, but may be severely susceptible to root infection and galling (Bus, 2000, Falloon *et al*., 2003, Fornier *et al*., 1996, Gans and Vaughan, 2000, Iftikhar, 2001, Lees, 2000); suggesting that the mechanisms of powdery scab resistance differ for the roots and tubers in different potato varieties (Falloon *et al*., 2003) and also indicating that host resistance is expressed at the sites of zoospore penetration of the root and stolon cells (Falloon *et al*., 2003). All these mechanisms of resistance are under the control of genes. Identification of these genes may be of great use to improve or develop more effective methods for controlling the disease (Laurentin *et al*., 2003). New technologies in plant molecular genetics have identified a few genes for resistance to potato diseases inside the germplasm pool accessible to potato breeders. Resistance to *Spongospora subterranea* f. sp. *subterranea* was observed to be inherited from one generation to the next (Harrison *et al*., 1997, Merz *et al*., 2004, Wastie *et al*., 1988). Moreover, it had been proposed by Falloon *et al*. (2003) that resistance among various potato cultivars ranges from highly resistant to highly susceptible cultivars in potatoes, suggesting existence of a quantitative type of resistance to powdery scab and it is considered to be under polygenic control (Falloon *et al*., 2003, Wastie, 1991), each gene with relatively small effect on the resistance of the cultivar (Bradshaw, 1994, Parlevliet, 1989).

1.2.4. Detection and quantification of *Spongospora subterranea* f .sp. *subterranea* infection

The detection of the pathogen in the soil provides a means of targeting the infected field with high disease pressure and applying appropriate control measures to reduce the development of the disease (Fornier *et al*., 1996). Visual inspection of seed tubers with powdery scab has

been commonly used; however, there are some disadvantages associated with this method of detection. For example, blemished tubers may be in contact with blemish-free tubers by contamination. In addition, this method may be inaccurate as it is challenging to identify powdery scab lesions distinguishing them from common scab lesions (Merz *et al*., 2012). Even though, molecular tests have proven to detect all forms of the pathogen (zoosporangia, plasmodia, spore balls) in tubers, roots, as well as, in the soil, (Bouchek-Mechiche *et al*., 2011) in order to avoid misidentification of the disease; several methods have to be used. Different methods have been developed for the detection and quantification of *Spongospora subterranea* f. sp. *subterranea* infection. These methods include enzyme-linked immunosorbent assay (ELISA) technique (Walsh *et al*., 1996), Polymerase Chain Reaction (PCR) (Bell *et al*., 1999, Bulman and Marshall, 1998, Qu *et al*., 2006), Real-time PCR technique (RT- PCR) (van de Graaf *et al*., 2003, Wale, 2005, Ward *et al*., 2004), as well as, bioassays (Walsh *et al*., 1996). All of the diagnostic tools are sensitive, specific and rapid; and can be applied for the detection of *Spongospora subterranea* f. sp. *subterranea* spore balls on tubers, roots and in-field soil for disease management strategy development and disease threshold determination (Merz *et al*., 2012).

The use of metabolomics tools to detect and identify metabolites associated with *Spongospora subterranea* f. sp. *subterranea* infection in potatoes, has until now been used to a limited extent. Recently, only a few studies by Bittara *et al*. (2013) and Balendres *et al*. (2016) have been published. Bittara *et al*. (2013) studied the molecular and phytochemical characterization of eight potato genotypes and its relationship with infection by *Spongospora subterranea* f. sp. *subterranea* using paper chromatography. Balendres *et al*. (2016) identified low molecular weight compounds in potato roots exudates that stimulate resting spore germination of the soil-borne pathogen *Spongospora subterranea* f. sp. *subterranea* using a UPLC/MS-based metabolomics study. Since very few metabolomics studies have been done, more research is needed to be done on this disease. Therefore, in the current study, the tolerant biomarkers were investigated in response to *Spongospora subterranea* f. sp. *subterranea* infection using metabolomics technique.

1.3. Plant-pathogen interaction

Plant-pathogen interaction is a relationship where the pathogen (parasite) depends on the plant (host) for food. Such relationships could be biotrophic, where the pathogen can infect and colonize a plant but not kill it; necrotrophic, where the pathogen can attack the host tissue

and feed on the dead tissue, or hemibiotrophic, where it kills the host in the later stage (Allwood *et al*., 2008). In general, hemibiotrophic and necrotrophic pathogens produce elicitors called PAMP/MAMP/DAMP which are recognized by receptor-like kinases (RLK), LysM domain, chitin elicitor receptor kinase (CERK1) and wall-associated receptor kinases (WAKs), leading to the activation of plant defence response through MAPK kinase kinase (MAPKKK) pathway (Kushalappa *et al*., 2016). In contrast, biotrophic produce effectors recognized by NBS-LRR proteins, leading to a hypersensitive response through MAPK/SA/NPR1 pathway (Kushalappa *et al*., 2016), shown in Figure 1.9. In response to pathogen attack, these elicitors/effectors recognized by plants are triggered by R genes such as elicitor recognition receptor (ELRR), effector recognition receptor (ERR), transcription factor (TF) and mitogen-activated protein kinase (MAPK), producing resistance-related metabolites (RRMs), proteins (RRPs) and phytohormones to suppress plant disease (Jones and Dangl, 2006). Concurrently, several metabolites such as specific ion channels, reactive oxygen species (ROS), phytohormones, phytoalexins, phytoanticipins, are activated following infection, which is also triggered by downstream genes leading to a hypersensitive response (Kushalappa *et al*., 2016).

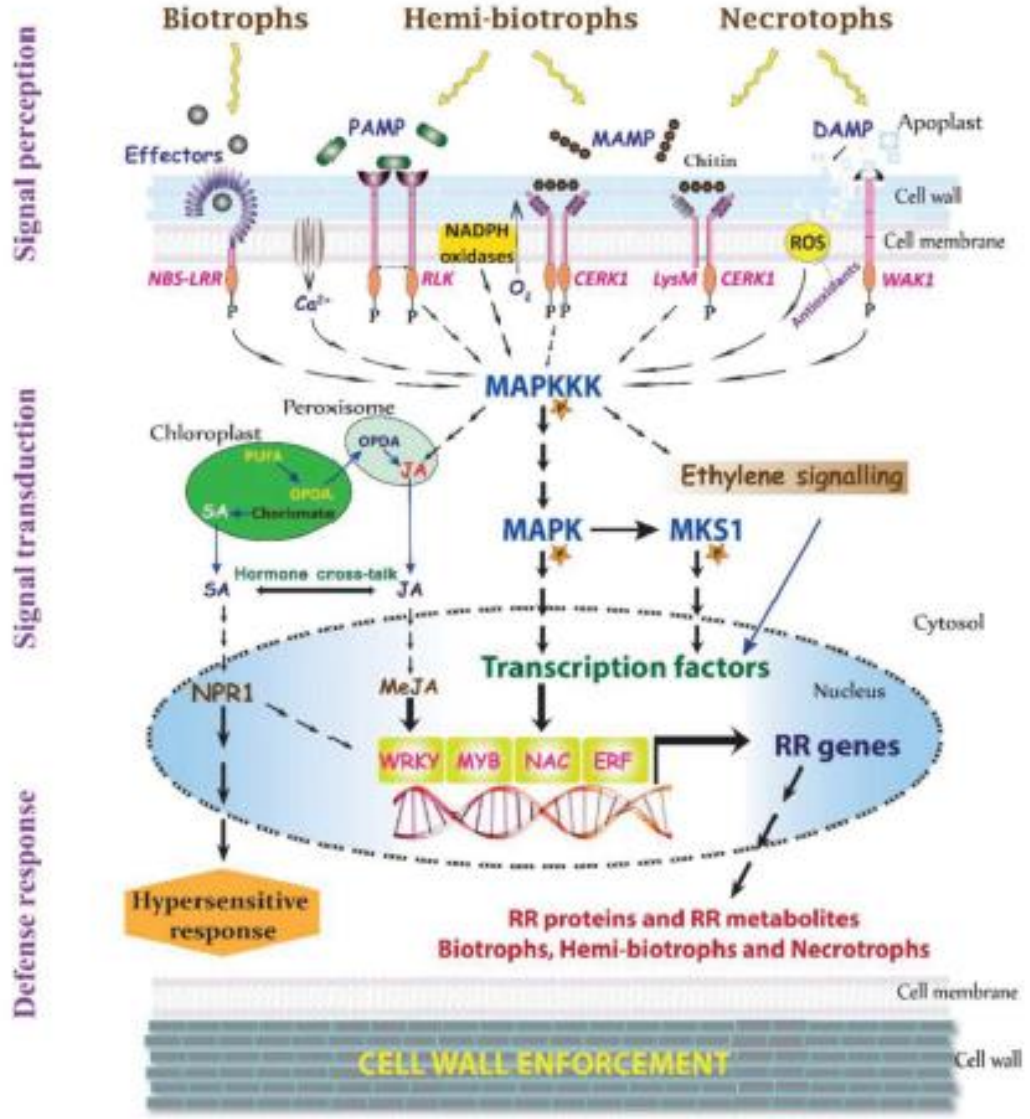


Figure 1.9: Snapshot of key players involved in plant-pathogen interaction. Plants are exposed to several biotrophs, hemi-biotrophs and necrotophs pathogens, which often attack and propagate in apoplastic space of plant tissues (Kushalappa *et al*., 2016).

1.3.1. How plants respond to pathogen attacks

When plants are exposed to pathogens, specific ion channels, reactive oxygen species (ROS), phytohormones, phytoalexins, phytoanticipins, primary and secondary metabolites are activated as shown in Figure 1.10 (Heuberger et al., 2014). A rapid generation of ROS is observed after stress sensing. ROS is produced in response to the biotic stresses during the oxidative burst. In addition, ROS serves as a signalling molecule inside the plant cells (Ebel and Cosio, 1994). Phytohormones, such as Ethylene (Ding *et al*., 2011, Lloyd *et al*., 2011), Salicylic acid (SA), Abscisic acid (ABA) and Jasmonic acid (JA) (Ding *et al*., 2011,

Kumaraswamy *et al*., 2011) are some of the signaling molecules, which play a crucial role in various aspects of plant defense against abiotic and biotic stress (Creelman and Mullet, 1997, Ecker, 1995). SA signalling is involved in resistance against biotrophic (Kushalappa *et al*., 2016) and hemibiotrophic pathogens, whereas JA and Ethylene signalling are significant toward necrotrophic (Figure 1.9) (Pieterse *et al*., 2009).

Primary metabolites involved in defence belong to carbohydrates, amines/amino acids, organic acids and lipids (Heuberger et al., 2014). As plants respond to pathogen infection, there is a shift in primary metabolite concentration, and this is associated with the changes in energy metabolism, nitrogen metabolism and cellular homeostasis as seen in Figure 1.10 (Heuberger et al., 2014). As mentioned before, secondary metabolites also play a part in the defence against pathogen attack and are classified into two groups as phytoanticipins and phytoalexins (Dixon, 2001, Heuberger et al., 2014). Phytoalexins are low molecular weight anti-microbial compounds that are only synthesized when the plant is under stress *(*Ahuja *et al*., 2012). Examples of phytoalexins include indoles, alkaloids, terpenoids, polyphenols, glucosinolates and other nitrogen and sulfur-containing molecules (Heuberger et al., 2014). Phytoalexins are also considered as molecular markers for disease resistance to pathogenic microbes in a wide range of plants (Ahuja *et al*., 2012). Phytoanticipins are anti-microbial metabolites, including cyanogenic glycosides and glucosinolates. Plant defence responses involve interactions between several groups of metabolites. The presence of these metabolites defines the resistant, tolerant or susceptible phenotype (Heuberger et al., 2014). Analysis of the metabolic pathways involved in disease tolerance and susceptibility should, therefore, cover both primary and secondary metabolic pathways (Sumner *et al*., 2003).

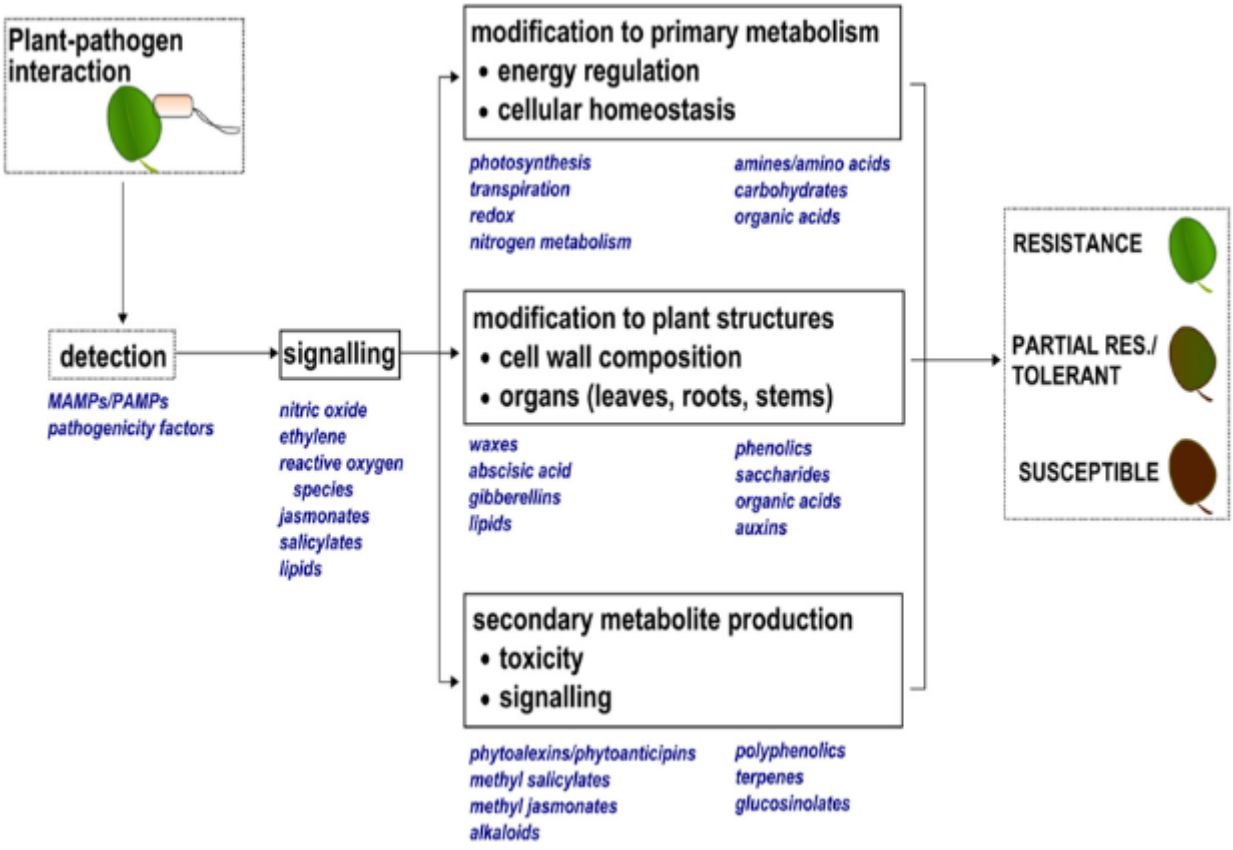


Figure 1.10: Molecular signalling cascades and physiological modifications that occur during plant-pathogen interaction (Heuberger et al., 2014).

1.3.2. Metabolomics analysis of plant-pathogen interactions

The study of metabolomics involves two organisms, plant and pathogen, with distinct metabolic systems. There is a complexity in the metabolic analysis of the plant-pathogen relationships with regard to a large number of compounds with different chemical classes and in identifying which metabolites responses are derived from the interacting pathogen, and which are from the plant (host) (Allwood *et al*., 2008). It is surprising that to date few metabolomics studies have targeted this area using different metabolic systems varying in complexity, due to the challenges encountered between the two organisms (plant and pathogen) (Kushalappa *et al*., 2016). For example, Narasimhan *et al*. (2003) used the *Arabidopsis thaliana* root leaches of the wild-type and mutant to detect the rhizosphere metabolome using HPLC-ESI-MS system. Choi *et al*. (2004) found an increase in phenylpropanoids and terpenoid indole alkaloids using 1H-NMR spectra coupled with multivariate analysis to profile *Catharanthus roseus* leaves associated with phytoplasma infection. Another study by Bednarek *et al*. (2005) used *Arabidopsis thaliana* wild-type and

cultured mutant roots infected by *Pythium sylvaticum* oomycetes, to search for aromatic metabolites using 1H-NMR for structural identification through APCI-MS and ESI-MS coupled with a Q-TOF-MS system. The level of indolics was up-regulated in resistance cultivars compared to susceptible cultivars, while the three phenylpropanoids were down-regulated following *Pythium sylvaticum* infection. Previously, the metabolomics analysis of plant-pathogen interactions mostly used 1H-NMR as a fingerprinting technology, even though some have used it for identifying targeted metabolites (Allwood *et al*., 2008). These highlight the use of metabolic fingerprinting, which gives a rapid analysis and searching for sources of varying metabolites within a set of samples (Allwood *et al*., 2008). For instance, William Allwood *et al*. (2006) used FT-IR fingerprinting, which offered a much higher sample number at lower costs, and skilled technical staff is not needed when compared with 1H-NMR (Dunn *et al*., 2005).

According to Heuberger et al. (2014), a group of studies have used LC-MS platforms for targeted workflows and GC-MS for untargeted workflows to assist in answering key questions related to plant immunity. The numbers of accumulative metabolites identified in these platforms are regarded as biotic stress-tolerant or metabolic biomarkers/resistance-related metabolites. These metabolic markers or resistance-related metabolites are biosynthesised by plants in various metabolic pathways, shown in Figure 1.11 (Kushalappa et al., 2016). For instance, 16 unsaturated fatty acids together with amino acids were identified as major compounds in rice cultivars resistant to gall midge using gas chromatography coupled with mass spectrometry system (Agarrwal *et al*., 2014). When exposed to bacterial leaf scourge (BLB), caused by *Xanthomonas oryzae pv. oryzae*, tolerant and susceptible rice cultivars showed different changes in several metabolites, for example, lipids, carbohydrates, alkaloids, xanthophylls and acetophenone (Sana *et al*., 2010). Metabolomic analysis revealed similar metabolic changes in barley, rice, and purple false bromegrass, in which polyamines, quinate, malate, and non-polymerized lignin precursors increased during infection by *Magnaporthe oryzae* (Parker *et al*., 2009). Phenylpropanoid and phenolic compounds accumulation were also reported in wheat-related to *Fusarium graminearum* infection (Gunnaiah *et al*., 2012). These phenylpropanoid compounds constitute a significant mechanism of plant defensive component, that changes cell wall composition and stiffness in roots (Hong *et al*., 2016a). Therefore, the thickened cell wall may aid in plant defence against pathogen attack. Rapid changes also occurred in secondary metabolites, with the generation of phytohormones, for instance, SA and JA, changes in antioxidants activity, and production

of phenylpropanoids, hydroxycinnamic acids, flavonoids and monolignols (Dixon and Paiva, 1995). A number of sesquiterpenoids and diterpenoids were induced specifically with the elicitation of SA (Choi *et al*., 2006). As with primary metabolites, similar changes occurred in resistance and disease development; however, they were more rapid in the previous case (Tao *et al*., 2003). The content of the majority of carboxylic acids increased in potato sprouts under the influence of *Rhizoctonia solani*, and this caused a decrease of the protein amino acid pool of sprouts with the concomitant increase of that of non-protein amino acids (Aliferis and Jabaji, 2012). Steroidal glycoalkaloids, such as α-chaconine, and α-solanine were associated with resistance to *Pseudomonas infestans* and *Pectobacterium corotovorum susbsp. carotovorum* infection in five different potato species obtained from crosses of *Solanum tuberosum*, and accessions of *Solanum andigena*, *Solanum phureja*, *Solanum vernei* and *Solanum berthaultii* (Andrivon *et al*., 2003).

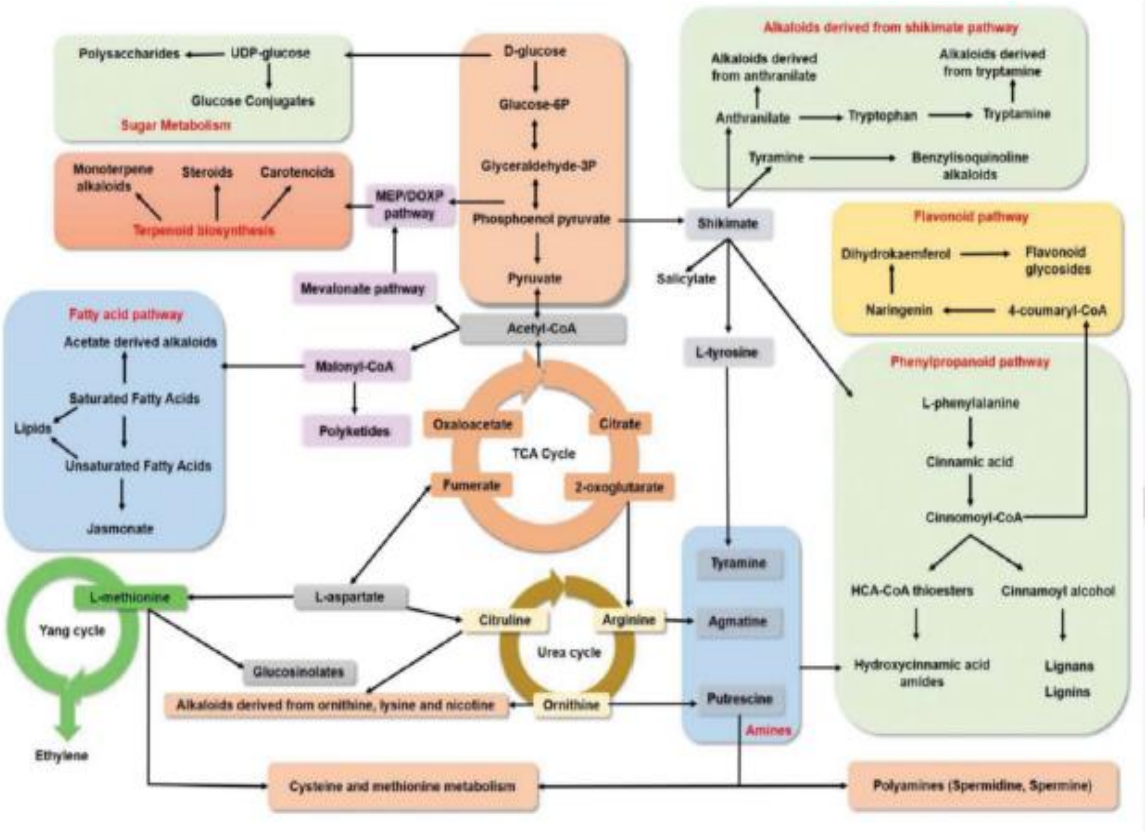


Figure 1.11: Metabolic pathway involved in the biosynthesis of related resistance metabolites (RRMs) by plants, in response to biotic stress. The conjugate complex metabolites, not degraded by the enzymes produced by most pathogens, are produced in the phenylpropanoid, flavonoid, fatty acid, sugars, terpenoids, and alkaloid metabolic pathways (shown in red text). These compounds play a significant role in plant defence against biotic stress. The biosynthesis of phenylalanine, tyrosine, and tryptophan leads to the production of phenylpropanoids, flavonoids, and some alkaloid (grey). The production of anthranilate leads to the production of some alkaloids. Acetyl-CoA leads to the production of terpenes, consisting of isoprene units forming mono- di-, tri-, and sesquiterpenes (purple). Acetyl-CoA produces malonyl CoA, which leads to the production of fatty acids and lipids (blue). The biosynthesis of arginine leads to the production of putrescine, spermidine, and spermine

(pink). Lastly, the biosynthesis of aspartate and methionine leads to the production of sulfur-containing compounds (green) *(*Kushalappa *et al*., 2016).

1.4. Metabolomics

1.4.1. What is metabolomics?

Metabolomics is a powerful technique that aims to provide an unbiased, comprehensive qualitative and quantitative overview of the metabolites present in an organism, cell, tissue or biological fluid (Hall, 2006). The number of metabolites in the plant kingdom is estimated to be around 200,000 (Dunn and Ellis, 2005). A comprehensive analysis of the metabolome should, therefore, at least cover the following chemical classes of compounds: carbohydrates, amino acids, organic acids, fatty acids, vitamins and secondary metabolites, such as phenylpropanoids, terpenoids, alkaloids and glucosinolates (Sumner et al., 2003). The application of metabolomics includes the determination of the effect of biochemical or environmental stresses on plants or microbes and genetically modified (GM) plants, (Le Gall *et al*., 2003), bacterial characteristics (Vaidyanathan *et al*., 2002), human health and nutrition assessment (Watkins and German, 2002) and metabolic engineering (Teusink and Westerhoff, 2000). This technology, therefore, is essential in understanding the molecular or metabolic basis of potato tubers that exhibit tolerance to *Spongospora subterranea* f. sp. *subterranea* infection. Plant metabolomics publications were not very common in the last decades, although it has now become increasingly widespread, as shown in Figure 1.12.

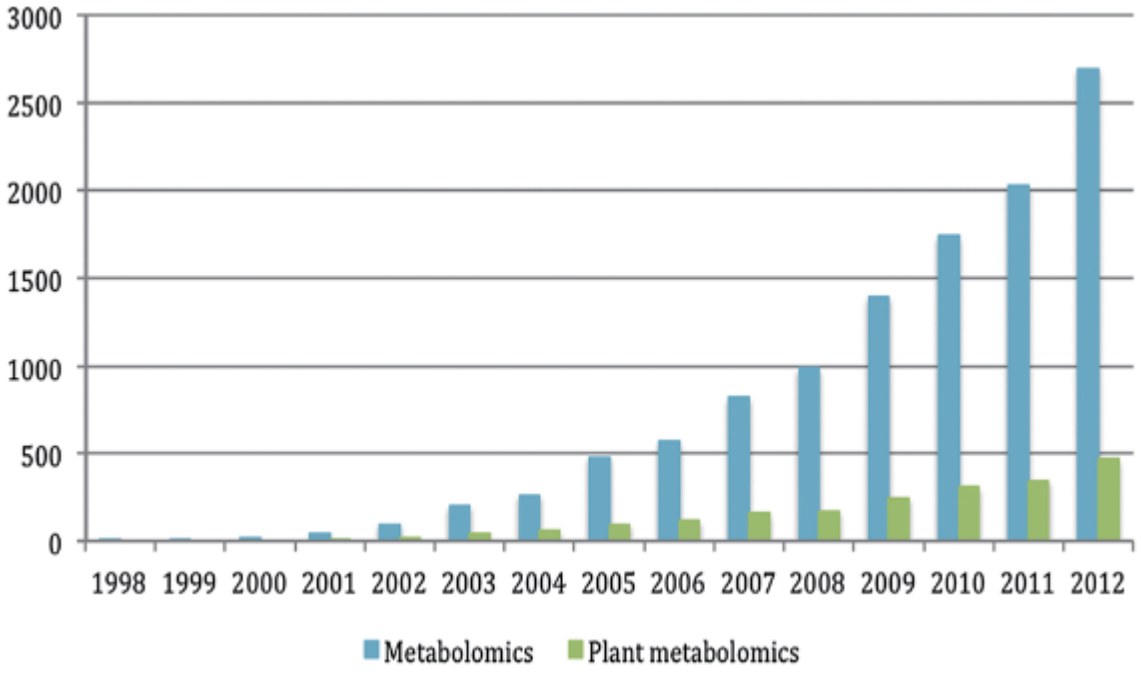


Figure 1.12: The number of metabolomics publications (y-axis) increased yearly (x-axis) from 1998 to 2012. The technique has become popular in the following years, particularly metabolomics as a whole (indicated in blue) (Patti *et al*., 2012).

Metabolomics is the youngest field in the omics family, which is closest to the phenotype and is connected to all other 'omes', to predict gene activity than the transcriptome and proteome approaches. It aims to capture the relative differences between biological samples based on their metabolite profile. Metabolomics provides an insight into the interconnection of metabolic pathways, and biomolecules present due to change in metabolism (Sumner *et al*., 2003)gt; this is very useful as a first step to better understanding the *Spongospora subterranea* f. sp. *subterranea* infection and its subsequent control. The metabolomics technique is beneficial because the quality of the crop plants is directly related to metabolite content; hence, knowledge of content can help decipher what gives the crop plant said quality (Hall, 2006). Other functional genomics techniques could have been used, such as transcriptomics and proteomics. However, these techniques do have some limitations. Transcriptomics deals with mRNA transcript profiling, while proteomics deals with protein profiling (Hall, 2006). The transcriptome represents a code on which protein synthesis is dependent upon; however, an increase in the mRNA levels does not necessarily correlate to an increase in cellular protein levels (Sumner *et al*., 2003). Another point to note is that translation does not ultimately produce an enzymatically active protein (Sumner *et al*., 2003). It can be seen that any change in the transcriptome or proteome does not directly correspond to a change in the metabolome (Sumner *et al*., 2003). Another shortcoming of transcriptomics and proteomics is the ability to identify mRNA and protein sequences only through database matching hence the quality of the sequence is extremely important in order to obtain good matches (Sumner *et al*., 2003). Despite the mentioned limitations, transcriptomics and proteomics are applicable in other situations and can also be integrated with metabolomics.

1.4.2. Workflow of a typical metabolomics study

Metabolic analysis can be classified as a targeted or untargeted approach. Targeted metabolomics approach focuses on identifying and quantifying a specific group of metabolites in the sample. In contrast, untargeted metabolomics focuses on the detection of many groups of metabolites to obtain fingerprints without identifying or quantifying specific compounds (Monton and Soga, 2007). Untargeted metabolomics has been studied in plant diseases to identify possible fingerprints of biological phenomena (Cevallos- Cevallos *et al*., 2009). This study, however, aims to use the untargeted metabolomics approach. The

metabolomics workflow for untargeted analysis can be seen in Figure 1.13. The first steps in metabolomics workflow would be the sample preparation, extraction, detection of metabolites and finally their identification, but not every step is always needed. Only detection, data processing and identification of metabolites are the essential steps in all reported metabolomics studies (Hall, 2006).

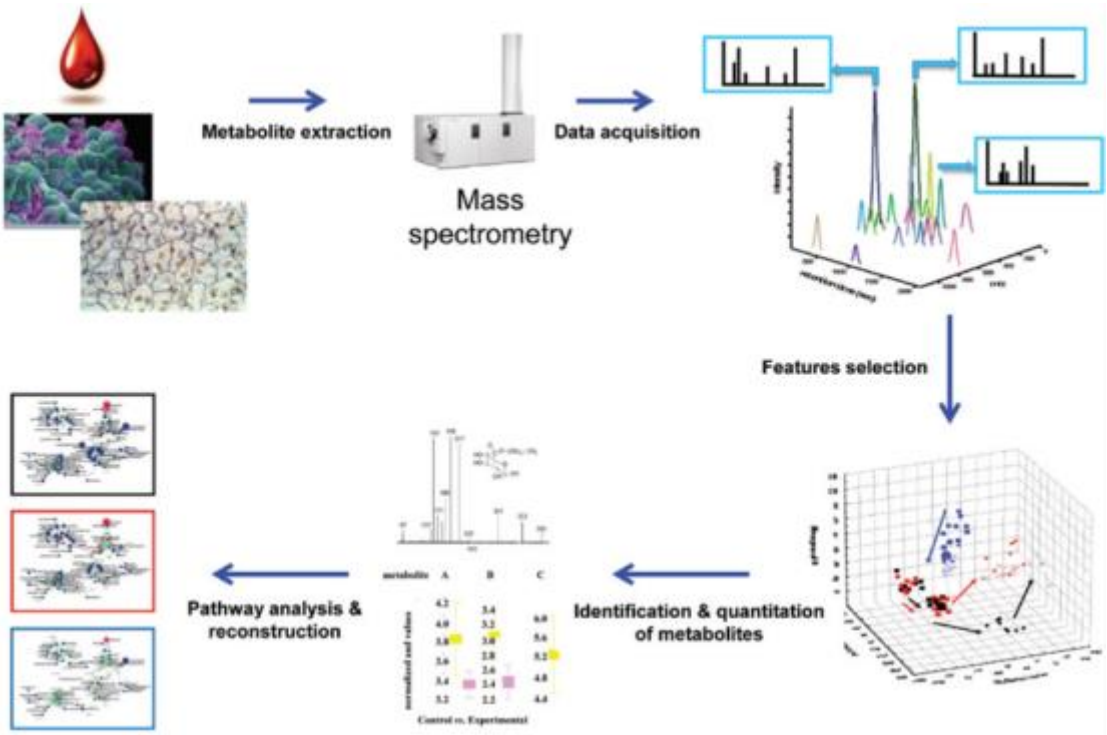


Figure 1.13: The untargeted metabolomics workflow (Mathew and Padmanaban, 2013).

1.4.2.1. Sample preparation for plant metabolomics

Metabolomics analysis contains three distinct experimental parts. The first step is the sample preparation, second is the data acquisition, and the last is the data analysis using suitable chemometrics methods. Sample preparation is a critical step with significant consequences for the isolated compounds and results accuracy. All practical considerations should be taken seriously from the start, because if an error occurs, the whole process, such as the view on the metabolome, might affect the samples. Generally, sample preparation for plant metabolomics study involves four steps: harvesting, drying, extraction of plant material and sample preparation for metabolite analysis, as shown in Figure 1.14. Some of these steps, for example, drying or extraction, may be avoided depending on the characteristic of the sample and the analytical technique used. The extraction methods used in metabolomics studies are the ones commonly used for phytochemical research (Huie, 2002).

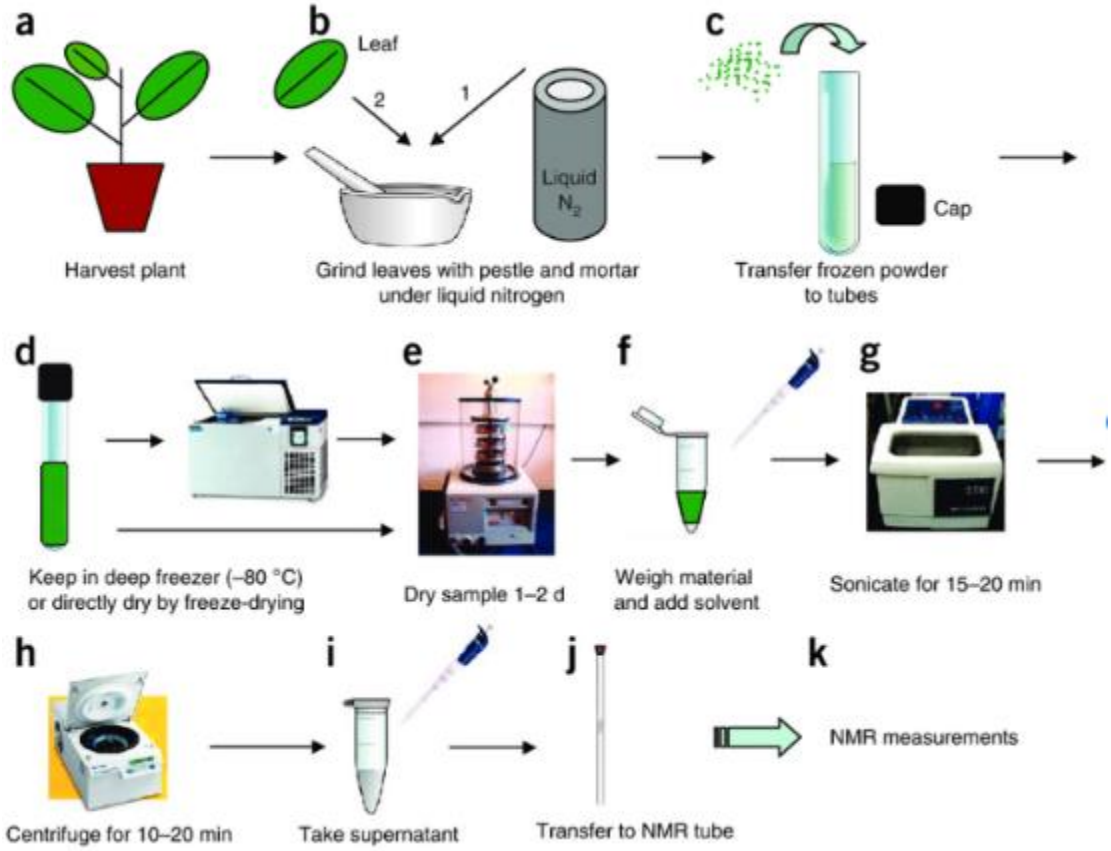


Figure 1.14: Representative diagram of sample preparation steps for NMR plant metabolomics studies *(*Kim *et al*., 2010).

Several aspects must be considered during extraction methods, such as the sample duration of extraction, solvent characteristics, ratio solvent and temperature. Metabolomics analysis consists of a large variety of metabolites at different intensities, with different polarities. However, there is no single solvent capable of dissolving the whole range of compounds. Therefore, the choice of solvent extraction is critical in metabolite analysis and is thus limiting the view on the metabolome of the sample (Kim and Verpoorte, 2010). In fact, one probably needs to do several extraction techniques, using different solvents to have a total view of the metabolome. Solvents may be chosen based on their physiochemical properties, such as selectivity, toxicity, polarity and inertness. When choosing a solvent for extraction method, specific requirements for the analytical tool to be used must be met. For LC-MS, organic solvents, such as Methanol (MeOH), Acetonitrile (ACN), chloroform, ethyl acetate, ethanol and formic acid (FA) (e.g. 0.1%) have been used in plant metabolomics studies, and aqueous MeOH has been utilized in NMR analysis (Kim and Verpoorte, 2010). In GC-MS, chloroform has been used as the range of possible solvents is limited to volatile and thermally stable compounds, and in case of polar compounds, derivatization is expected. In this study,

MeOH and H_2O mixture was used as the extraction procedure for metabolite analysis in potato tubers. MeOH-H_2O appeared to be the most suitable solvent because it brings out trace amounts of various substances such as phenolics, flavonoids, glucosinolates, lipids, sugars, amino acids terpenoids and alkaloids, moreover, is less toxic than chloroform and acetone. Furthermore, methanol has a low boiling point of 65 °C which can easily evaporate so it can be separated from the extract unlike ethanol and other solvents (Sultana *et al*., 2009). There are many publications regarding the comparison of different solvents, but most of them points to methanol, because many of the compounds can dissolve in it with great freedom, which is important for plant material (Jang *et al*., 2017). As mentioned in the introduction of sample preparation, most steps are comparable, regardless of the analytical technique used. In the end, it is imperative that all these steps in the metabolomics study be standardized in order to allow direct comparison of the data generated at different times and place (Kim and Verpoorte, 2010).

1.4.2.2. Data acquisition

There are several platforms available for data acquisition (measurements), and each of them has their own excellence. These include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), direct infusion-mass spectrometry (DI-MS), Fourier transform infrared spectroscopy (FT-IR), capillary electrophoresis-mass spectrometry (CE-MS) and nuclear magnetic resonance spectrometry (NMR) technologies. However, no single technique can separate all metabolites in the biological sample, as each technology has an unavoidable intrinsic bias toward certain metabolite groups (Dunn and Ellis, 2005). MS and NMR are the most commonly applied technologies used in plant metabolomics based on sensitivity, speed and broad application (Hall, 2006). NMR is a very powerful and useful tool for identification of bulk metabolites and is highly reproducible based on the energy absorption and re-emission of the atom nuclei to variations in an external magnetic field (Bothwell and Griffin, 2011), but its sensitivity is quite low compared to MS. In addition to NMR, the technique can also be used for structural identification. MS alone cannot differentiate between chemical isomers and is, therefore coupled to other separation techniques (Sumner *et al*., 2003) such as gas chromatography (GC-MS), liquid chromatography (LC-MS), ultra-Performance liquid chromatography (UPLC-MS) and Fourier transform ion cyclotron resonance (FTIR-MS) (Hall, 2006). Optical techniques, such as infrared radiation (IR) and ultraviolet (UV) spectrometry, can also be used to analyze plant metabolites (Ibáñez *et al*., 2010).

As mentioned previously, the choice of metabolic platform is determined by its selectivity, sensitivity and speed (Sumner *et al*., 2003). The NMR platform offers speed and selectivity, but has low sensitivity, whereas, GC/MS and LC/MS have higher sensitivity and selectivity, yet are long process compared to NMR (Sumner *et al*., 2003). Reverse phase column chemistry (C18/C8) is commonly used today in liquid chromatography, but for polar compounds injected on the LC column, the retention of the compound may be minimal, and they usually elute in or near the solvent front, thus the volume of interpretative data will be reduced (Lenz and Wilson, 2007, Swartz, 2005). To overcome this, other column chemistry may be applied, including hydrophilic interaction column (HILIC) (Tolstikov and Fiehn, 2002). High-pressure columns are also being developed (e.g. ultra-high-pressure liquid chromatography, UHPLC), or other columns, which can retain polar molecules (Plumb *et al*., 2006). Most plant metabolomics studies focus mainly on GC/MS and LC/MS which provide low-cost platforms compared to other methods; however, the major difference between the two technologies is that GC-MS separates volatile and thermally stable compounds, that need derivatization method (Sumner *et al*., 2003).

1.4.2.2.1. Mass spectroscopy (MS)

MS is a rapidly growing technology of small molecules (metabolite), such as sugars, lipids, amino acids, fatty acids, polyphenols, alkaloids, and macromolecules, such as proteins, nucleic acids and carbohydrates (Fiehn, 2002, Nielsen and Oliver, 2005, Oliver *et al*., 1998). Metabolite profiling, especially in the plant sciences, often employ MS and is usually combined with chromatography GC or LC-MS. In the MS system, sample ions are separated and detected by an electron multiplier tube or a micro-channel plate. The metabolites are then identified via comparison of exact mass ions (m/z), retention time (min) and fragmentation information with standards and spectral databases. A number of ion sources, such as electron impact ionization (EI) and fast atom bombardment (FAB), chemical ionization (CI), matrix-associated laser desorption/ionization (MALDI) or electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) and of mass analyzers, such as time-of-flight (TOF), quadrupole and ion trap are available (Xiao *et al*., 2012). Mass analyzer quadrupole and TOF are employed in the study and are more commonly used in plant metabolomics study. MS has a few advantages over other analytical methods, such as accuracy, rapidly and sensitivity and MS can measure a wide range of molecules and gives a relative molecular mass of each substance. In addition, MS can separate and identify similar compounds and can be useful when attempting to discern the identity of an unknown

compound, when used in conjugation with other identification technique, such as NMR. However, it is of course not useful if you try distinguishing between compounds, which have the same molecular formula.

a) Electrospray ionization (ESI) technique

ESI is one of the most common ionization technique used for metabolomics studies, particularly in LC-MS (Gaskell, 1997, Huhman and Sumner, 2002). To obtain a comprehensive profile, both positive and negative ionization modes must be performed where metabolites can be detected and ionized by the loss of an H1 (ESI-) or gain of an H1, an NH4 1, K1 or a Na1 (ESI+). However, the mass number (m/z) identification is more difficult with ESI1, as a result of potential adduct formation with any of four ions (Gaskell, 1997). During ESI, a high electric field is utilized to produce charged droplets from a liquid solution, lastly leading to the formation of gas-phase ions (Enke, 1997). The main advantages of the ESI ion source are soft ionization, no derivatization required, ionization of large mass range compounds, excellent quantitative analysis, high sensitivity, and suitability for non-volatile, and polar compounds. Nevertheless, ESI's shortcoming is that ion suppression is problematic due to competition effects during the ionization process. The effect of ESI+ and ESI- modes on intestinal fistula utilized for biomarker discovery were compared by Yin *et al*. (2006), and the markers that were selected from the ESI+ and ESI- scan mode of the patients, and controls were quite different. The main aim of combining the ESI+ and ESI- mode may provide us with more useful information about the samples. For instances, in the current study, both ESI ionization modes were used to obtain different markers in ESI+/ESI- that played a significant role in tolerance/susceptibility to *Spongospora subterranea* f. sp. *subterranea* infection. Other alternative ionization techniques used for LC-MS-based metabolomics, are nanoESI, APCI, and atmospheric pressure photoionization (APPI). Other important ionization techniques that are typically used for proteomics studies are direct analysis in real time (DART), and MALDI (Dally *et al*., 2003).

b) Triple-quadrupole (qQq)

MS system consists of many types of mass analyzers that are available for interfacing with liquid chromatography (LC). The most commonly used MS mass analyzers include single-quadrupole, triple-quadrupole (qQq), time of flight (TOF), ion trap, Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR). In addition, to mass analyzers, a number of hybrid systems increasing exist that combine two type of MS analyzers, such as quadrupole-

TOF (Q-TOF) instrument (Morris *et al*., 1996), quadrupole linear ion trap (Q-trap) (Hopfgartner *et al*., 2003, Le Blanc *et al*., 2003, Morris *et al*., 1996) and ion trap-Fourier transform (FT-trap) (Syka *et al*., 2004). MS triple-quadrupole is one of the methods used to obtain structural information or quantitation and is currently the most widely used instruments for MS/MS (Allwood *et al*., 2010). The instrument improves accuracy, measure a high-linear dynamic range and is capable of detecting metabolites at a range of m/z 50-4000 (Lu *et al*., 2008). Triple-quadrupole not only provides the m/z information but also generates the fragmentation ion information allowing different methods, such as full scan MS; single ion monitoring (SIM), neutral loss scan and product scan (daughter scan) and selected reaction monitoring (SRM) to be performed. During the triple-quadrupole process, mass ions are selected by the first quadrupole and are collisionally dissociated (CID) and analyzed by the third quadrupole. When using the process known as SRM, the 1st and the 3rd quadrupole monitor the parent and the daughter ion, in that order, of a fragmentation transition, which is specific for the target analyte (Lu *et al*., 2008). Therefore, it is commonly used for identification of compounds in qualitative analyses, but the neutral loss may also be applied to a compound of interest to identify the ions that have lost neutral moieties (Dettmer *et al*., 2007).

c) Time-of-flight (TOF)

TOF has been termed a powerful and robust instrument in plant metabolomics studies, used for the identification of metabolites in the biological system (Allwood *et al*., 2010, Wolfender *et al*., 2013). The main advantages of using TOF instrument are fast scanning capability, measuring a wide range of compounds, and high resolution (m/z 5000-20,000), improving mass accuracy (<5 ppm) and high sensitivity. TOF is commonly combined with electrospray ionization (ESI) and APCI (Mirivel *et al*., 2010). MS-MS experiments with a TOF instrument are also combined with a mass analyzer known as Q-TOF, where the last quadrupole of the triple-quadrupole configuration is substituted with a TOF analyzer. The system makes use of innovative Q-Tof technology, which functions by combining high field pusher and dual-stage reflectron designs. These incorporate high transmission parallel wire grids, which cause a reduction in ion turnaround times due to the pre-push kinetic energy generated and spread, this resulting in improved focusing of high-energy ions respectively. Q-TOF type of mass analyzer and ionization technique has a major impact, and detection limit, and the advantages, and disadvantages depend highly upon the chosen instrument type.

1.4.2.2.2. Gas chromatography-mass spectrometry (GC-MS)

GC-MS is a combined system where volatile (e.g. alcohol, esters and terpenes), thermally stable non-polar compounds are first separated and detected by electron impact mass spectrometry (Dunn and Ellis, 2005). Chemical derivatization is used in non-volatile compounds (e.g. sugars, sugars alcohol, amino acids, nucleotides, sterols, and organic acids) prior GC separation to permit analysis of compounds with low volatilities (Sumner *et al*., 2003). The derivatization method usually involves two stages, starting first with oximation, followed by silylation process, whereby O-alkylhydroxylamine converts sample carbonyl groups to oximes for thermal stabilization (Roessner *et al*., 2000). The second stage involves the formation of volatile trimethyl esters (TMS) with a silylating compound (N-methyl-N-(trimethylsilyl)trifluoroacetamide- BSTFA) to replace exchangeable protons with TMS groups (Roessner *et al*., 2000). BSTFA and methoxyamine hydrochloride in pyridine were reported as the most appropriate derivatization reagent for oximation and silylation (Gullberg *et al*., 2004). These two reagents have shown to improve GC separation of metabolites in food analysis such as potato (Beckmann *et al*., 2007) and other products (Cevallos-Cevallos *et al*., 2009).

GC has become a standard approach for many metabolomics analyses due to its ability to produce high reproducible separation and fragmentation pattern of metabolites, coupling it with TOF producing quantitative data for hundreds of compounds involved in the biological system (Roessner *et al*., 2000). GC-MS faces several technological limitations that include the dynamic range of MS-based instruments, limiting a large variety of metabolites detected on a single technique, longer run times (higher than 60 min) and deconvolution of overlapping peaks (Roessner *et al*., 2000). These challenges may be reduced by employing a combination of fast acquisition rate TOF instruments coupled with deconvolution software. The deconvolution software, automated mass spectral deconvolution and identification system (ADMIS) is performed by using mass spectra to define chromatographic peaks (including overlapping peaks), and allows reduction of the run time from 60 minutes to less than 15 minutes as full chromatographic separation is not required (Halket *et al*., 1999). Although GC-TOF-MS data has been used best for deconvolution of peaks, however in LC-MS deconvolution, significant efforts are still required (Dunn and Ellis, 2005).

1.4.2.2.3. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

Recently, UPLC-MS has become a valuable tool, which provides better resolution and high peak capacity (Lenz and Wilson, 2007, Wilson *et al*., 2005). In fact, UPLC-MS has been shown to detect up to 20% more compounds with metabolomics applications compared to HPLC (Unger *et al*., 2008). UPLC as compared to HPLC technology takes full advantage of chromatographic principle to analyze samples with small particles column (1.7 µm) at a high flow rate for increased speed, with superior resolution and sensitivity (Swartz, 2005). However, HPLC lacks the horsepower to take full advantage of sub 2µm particles. The comparison between HPLC and UPLC are shown in Figure 1.15 below. UPLC-MS increased resolution in shorter run times, which could generate more information faster than HPLC-MS without making any sacrifices.

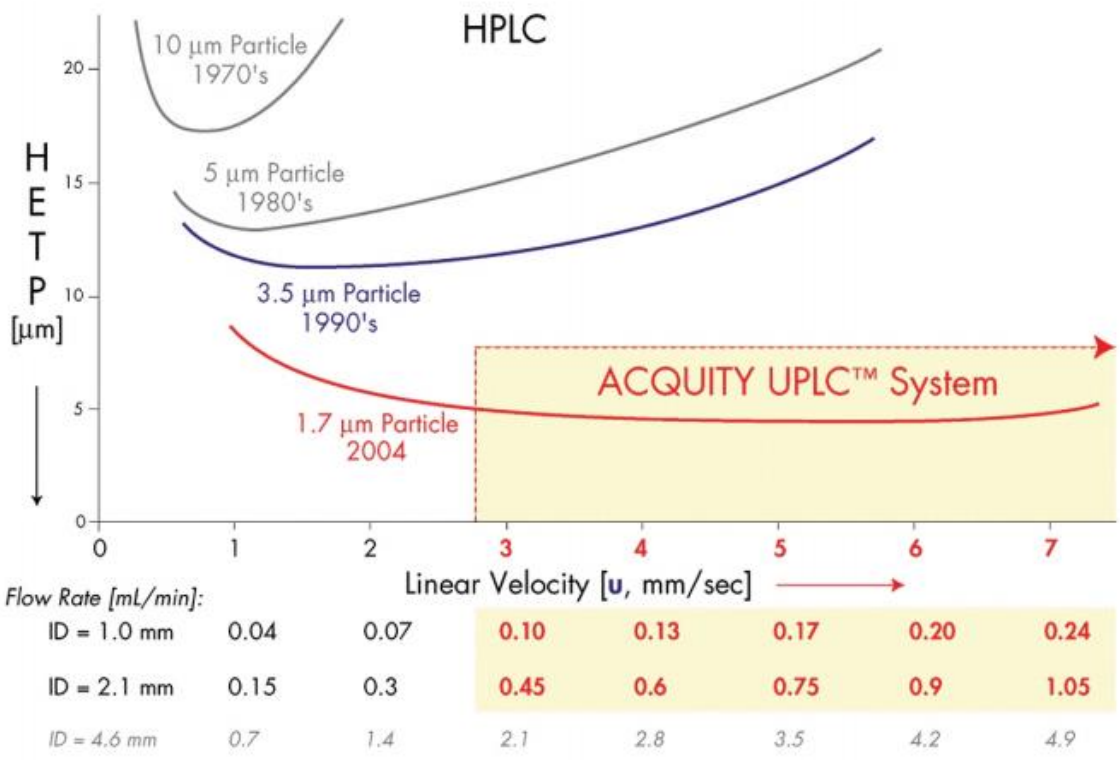


Figure 1.15: Van Deemter plot illustrating the evolution of particle size over the last three decades. Van Deemter equation describes the relationship between linear velocity [flow rate (x-axis)] and plate height [height equivalent to a theoretical plate- HETP or column efficiency (y-axis)]. Particle size is one of the variables, a Van Deemter equation can be used to investigate chromatographic performance (Swartz, 2005).

In 2004, ACQUITY UPLC BEH (bridged ethylsiloxane hybrid) 1.7 μm C18 column was introduced as the best column of choice for UPLC separation to provide a wide pH range (Swartz, 2005). Silica-based particles were employed in 2000 on HPLC system before the use of BEH particles, which had an excellent mechanical strength, with high efficiency, and operated over extended pH range (Swartz, 2005). However, silica-based particles suffered from numerous disadvantages, including pH range limitation and tailing of basic analytes. The polymeric columns were introduced to overcome pH limitations, but they had their own issues, including low efficiencies, smaller capacities and weaker mechanical strength than silica columns (Swartz, 2005). To overcome this problem and to provide enhanced mechanical stability required for the UPLC system, BEH was the solution. There are several column chemistries that may be applied in liquid chromatography, including hydrophilic interaction columns (HILIC) (Tolstikov and Fiehn, 2002) and UHPLC, or other columns which can retain polar molecules (Plumb *et al*., 2006). HSS T3 column was used in this study, which can separate both non-polar and polar compounds. Based on the optimized methods for each column chemistry, the results obtained by New and Chan (2008) indicated that HSS T3 column gave the best retention and separation of analytes (polar and non-polar) when running on the UHPLC system.

1.4.2.3. MS data pre- and post-processing

After data acquisition, a large volume of data obtained from a single sample needs to be processed and analyzed. These steps are crucial in metabolomics studies, mainly plant metabolomics, as plants have a large metabolome and are chemically diverse (Katajamaa and Orešič, 2005, Katajamaa and Orešič, 2007). The main aim of data processing is to transform the raw data into a form that can be easily used in the data analysis step. Data processing for LC-MS-based metabolic profiling can be further broken down into several stages, including filtering, peak detection, alignment and normalization (Katajamaa and Orešič, 2005, Katajamaa and Orešič, 2007). A typical LC/MS data file is comprised of histograms with recorded peaks that show different ions with their *m/z* and peak intensity that was obtained within a small time frame (Katajamaa and Orešič, 2007). Data processing makes the following characteristics of the observed ions easily accessible: *m/z*, retention time and ion intensity (Katajamaa and Orešič, 2007).

Several popular online software tools are used in LC-MS and GC-MS for peak detection, noise filtering, peak alignment, deconvolution, baseline correction and normalization include

MZMINE (Katajamaa *et al*., 2006, Katajamaa and Orešič, 2005), MetaboAnalyst (Xiao *et al*., 2012) and XCMS (Broeckling *et al*., 2006, Smith *et al*., 2006). List of tools available for metabolomics spectral processing and data analysis are reviewed here (Alonso *et al*., 2015). Metabolomics software that is commonly used is MarkerLynx (Waters) and Mass Hunter (Agilent). For any statistical analysis, it is essential that the data is aligned and variances between samples that are not attributed to true differences are reduced. In LC-MS, the use of chemometrics methods become a significant impediment on the data due to the differences in the temperature, gradient reproducibility, column variability and drift in the mass to charge direction corrected by the mass adjustment. Since variable in a data table ought to characterize the similar property over all samples, variability in retention time-drift cause issues for statistical modelling.

1.4.2.3.1. Filtering

The first step in data processing starts with filtering, and this aims to eliminate noise or baseline effects from a full scan metabolic profiling (Katajamaa and Orešič, 2007). There are two types of noises that can be found in the LC/MS data, namely chemical noise and random noise; the former is caused by molecules in the buffers and solvents, and the latter is caused by the detector(Katajamaa and Orešič, 2007). To eliminate the noise, signal processing techniques are used. Some signals processing techniques employed are median filtering in specified window sizes, Savitzy-Golay local polynomial fitting and wavelet transformation (Katajamaa and Orešič, 2007, Smith *et al*., 2006). (Katajamaa and Orešič) briefly described a two-step process to remove the baseline: the first step was to determine the shape of the baseline and the second step is to subtract the shape from the raw signal (Katajamaa and Orešič, 2007).

1.4.2.3.2. Peak detection

After filtering, the peak detection step follows. Peak detection aims to identify all the signals caused by true ions, and in the process, avoid the signals from false positives (Katajamaa and Orešič, 2007). This step is important; however, it is not typically performed in metabolomics, as it is not generally appropriately done (Katajamaa and Orešič, 2007). Due to the challenges of this step, gave two alternatives, with the first involving direct correlations of the raw data, and this should be possible by comparing data points directly (Katajamaa and Orešič, 2007). The second alternative involves performing the alignment step before feature detection, and

for this situation, peak detection is performed on the merged raw data from sample pairs (Katajamaa and Orešič, 2007).

1.4.2.3.3. Chromatogram alignment

The alignment step is utilized to correct retention time differences between sample data and to combine data from various samples (Katajamaa and Orešič, 2007). Alignment is necessary because peaks from the same sample usually have close m/z values, but these values can drift slightly between LC/MS runs (Katajamaa and Orešič, 2005). To conquer the issue of the drift in retention time between the sample, internal standards are added to each sample before the LC/MS run (Smith *et al*., 2006). During processing, the peaks that correspond to the internal standards are used to align the retention times between samples. This procedure, however, assumes that deviation in the retention times follows a linear pattern, which is not valid, and it makes the sample preparation time longer. The additional standards also have a chance of concealing other metabolites (Smith *et al*., 2006). Other alignment methods have been used, which do not require standards and allow nonlinearities, such as the Correlated Optimized Warping (COW) method (Smith *et al*., 2006). This method finds possible sets of segmented warnings that can be used to align one chromatogram to another (Katajamaa and Orešič, 2007, Smith *et al*., 2006). Another alignment approach is clustering of chromatographic peaks without correcting retention times as described by Katajamaa and Orešič (2007). The alignment depends on the proximity of *m/z* and retention times. To summarize, there are three strategies that can be used for alignment process; firstly, the alignment of chromatograms utilizing internal standards, for instance, secondly, peak detection, and thirdly the matching between samples and lastly, the summation of chromatographic data (Katajamaa and Orešič, 2007). At the end of the day, the choice of alignment technique directs the downstream data analysis required.

1.4.2.3.4. Normalization

The final step of data processing is normalization. This involves a reduction of systematic error by adjusting the intensities within each sample run (Katajamaa and Orešič, 2005, Katajamaa and Orešič, 2007). To accomplish this, the ion intensities between data or runs need to be reduced accordingly (Lu *et al*., 2008). Katajamaa and Orešič described two approaches for normalization; the first uses statistical models, and the second uses internal or external standards to normalize the data based on empirical rules, like specific regions of the retention time (Katajamaa and Orešič, 2007). After the metabolomics data has undergone processing, it is then analyzed. Some of the main goals of data analysis are the interpretation of the processed data involved in the identification of similarities and differences between

data, classification of samples and identification and quantification of metabolites (Lu *et al*., 2008). The data analysis follows in the next topic.

1.4.2.4. Chemometrics and bioinformatics

Bioinformatics uses a computational technique to develop a method (e.g. database design, data mining, explanation of data clustering) and a software tool for understanding biological data (Luscombe *et al*., 2001). Many software tools are available for biological data manipulation, and more are yet to be developed. However, these tools do not equate to improve analysis of biological data. Metabolomics studies depend on bioinformatics for data processing and analysis. This study used software tools such as MassLynx v4.1 and JMP Pro 14 for data processing and statistical analysis. The use of bioinformatics is vital as it uses databases from which metabolites are identified for biological data interpretation.

Metabolomics studies require a large volume of data with a specific end goal to perform statistics which can unquestionably give an account for metabolite levels, trends, or responses and controls (Villas-Boas *et al*., 2007). The enormous data generated by metabolomics studies are analyzed using chemometrics. Chemometrics are the chemical discipline that uses mathematics and statistical methods to extract relevant information analyzed on instrument data (Wold, 1995). These chemometrics methods produce output as projected spectra or chromatograms (Lin *et al*., 2011, Villas-Boas *et al*., 2007). This study used the chemometrics technique for comparisons between the treatment and the control samples. It is, therefore, a powerful and useful technique that can be used due to the identification of metabolites being limited by the availability of few libraries (Plumb *et al*., 2005, Wilson *et al*., 2005). The process of compound identification based on MS data is time-consuming and riddled with pitfalls. Using a chemometrics method instead of libraries is exceptionally effective because it depends mainly on the control sample collection or preparation and the comparisons to the subjects at hand (Villas-Boas *et al*., 2007).

1.4.2.4.1. Univariate data analysis

Univariate is a typical data analysis used for interpretation of metabolomics data. However, univariate data does not involve the interactions between different metabolic features. There are several univariate methods that have been used for metabolomics data analysis, although multivariate analysis is mostly used. This is because univariate analysis only focuses on one variable at a time, while multivariate concentrate on two or more variables. However, it is

recommended that both univariate and multivariate statistics should be used for data analysis to increase the information generated by the metabolomics study.

a) Analysis of variance (ANOVA)

ANOVA is one of the univariate methods that is commonly used in metabolomics study, which tests the statistical significance of the differences in means between two or more groups of data (Axelson, 2010, Bartlett *et al*., 2000). As defined by Altman (1990), the principle behind ANOVA is that the total variability of the datasets is partitioned into components due to different sources of variation. The hypothesis is assessed based on the fact that all samples have an equal mean against the mean difference (Axelson, 2010). The premise of the analysis is that the populations from which the samples are derived are regularly disseminated containing a similar standard deviation (Altman, 1990). Another generalized form of univariate ANOVA has been employed and is called multivariate analysis of variance (MANOVA) (Tabachnick and Fidell, 1996). Its purpose differs a little from ANOVA as it tests the difference between two or more vectors of means.

1.4.2.4.2. Multivariate data analysis

As opposed to univariate methods, multivariate analysis methods involve all the metabolomics feature at once and, consequently, identify the relationship patterns between them. These pattern- recognition groups are divided into two groups: supervised and unsupervised methods. The main difference between supervised and unsupervised methods is that the latter requires only the original data and no added information, while the former needs a calibration based on a set of observations classified by independent means (Sumner *et al*., 2003). Identifications and quantification of metabolites between sample groups (e.g. treatment vs. control) usually uses various multivariate techniques, such as principal component analysis (PCA), hierarchical cluster analysis (HCA), linear discriminant analysis (LDA) orthogonal partial least square discriminant analysis (OPLS-DA) or correlation analysis (Gika *et al*., 2007, Stenlund *et al*., 2008).

a) Principal component analysis (PCA)

PCA is one of the most widely unsupervised methods since it provides a quick overview of the information concealed in the data (Lu *et al*., 2008). The uses of PCA include investigation of clustering tendencies, detection of outliers and visualization of data structures (Lu *et al*.,

2008). The main aim of PCA is to describe the variance in a set of multivariate data in terms of principal components or underlying orthogonal variables (Sumner *et al*., 2003). According to Sumner *et al*. (2003), PCA is described as a linear additive model, because each principal component of the data represents a portion of the total variance of the data set. This is useful because two or three of the most significant principal components can represent over 90% of the total variance, meaning that the data resynthesized from a few principal components, thereby reducing the dimensions of the data set (Sumner *et al*., 2003). This allows for quick and easy visualization of similarities and differences in the data set. PCA gives a simplified representation of the information contained in the spectra, and cannot generally use additional information on the data, for example, class information (Lu *et al*., 2008). Therefore, a supervised analysis, such as OPLS-DA, can be utilized.

b) Partial least square discriminant analysis (PLS-DA)

PLS is one of the supervised methods that is commonly used in multivariate analysis (Lu *et al*., 2008). PLS involves relating a data matrix containing independent variables (X matrix) from samples to a matrix containing dependent variables (Y matrix) for those samples (Lu *et al*., 2008). The use of PLS-DA is to improve the separation between observation groups. Orthogonal partial least square discriminant analysis **(**OPLS-DA), which is the latest propelled advancement of PLS-DA, can facilitate the separation and interpretation of different types of variations in two distinct samples (Trygg and Wold, 2002). It comprises a single component that predicts class contrast, and variety is determined by the second orthogonal component with respect to the first principal component (Westerhuis *et al*., 2010). OPLS-DA scores plot usually goes together with S-plot for searching for metabolic biomarkers. The OPLS-DA loading S-plot plays a vital role in the determination of metabolic biomarkers that differentiate between two groups (Wiklund, 2008). However, S-plot loadings are challenging to interpret with more than two groups. This is an advantage as some markers are present only in one sample groups or two samples groups, but not the entire three experimental groups (Westerhuis *et al*., 2010, Wiklund, 2008). The markers in the S-plot with large differences between groups are found at the bottom left or top right quadrant of the 'S'-shape, and the least differences are found at the middle (centre) of the S-plot. Variables at the S-tails represent potential biomarkers that are unique to the treatment or control samples. Each feature is identified by retention time underscore accurate mass.

1.4.2.5. Metabolite identification and spectral database

Once data processing and statistical analysis are completed, identification of metabolites or potential biomarkers follows. Metabolite annotation involves combining different MS data, for instance, accurate mass, fragmentation patterns and isotopic pattern with parameters, such as retention time and spectral matching information to the in-house chemical library or external metabolite databases of authentic standards (Heuberger et al., 2014, Moco et al., 2007). An in-house spectral database may seem ideal. However, plenty of resources are needed to develop, frequently to set up, and there might be difficulty in obtaining authentic standards as there is a large volume of metabolites produced in plant species (Heuberger et al., 2014). Therefore, it is better to use external databases instead of in-house databases because it does not require any previous knowledge of the metabolome under investigation (Heuberger et al., 2014). There are several metabolomics databases currently available to identify compounds in the biological systems, but they do not contain a reference for all plant metabolites. To provide a good enough reference for metabolites, more than one database can be used (Sumner *et al*., 2003). Databases such as KEGG, Human Metabolome database, Massbank, Golm and METLIN have been used to identify the candidate molecules (Heuberger et al., 2014). In addition, a library search is used for metabolites identification. The standard spectra library search for GC-MS are the National Institute of Standards and Technology (NIST) (~200.000 spectra) and Wiley (~400.000 spectra) (Lu *et al*., 2008). A more detailed list of available spectral databases for metabolite identification is published here (Alonso *et al*., 2015). For LC-MS, few spectral libraries have been developed compared to GC-MS libraries.

Ionization techniques, such as ESI and APCI, comprise of molecular ions depending on the chemical property of the sample, solvent composition, and nature of the matrix. The fragmentation information of the metabolite is provided by collision-induced dissociation (CID) product ion mass spectrum. However, the fragmentation pattern differs when using different types of MS mass analyzers (triple quad and ion trap) and even utilizing different instruments of similar mass analyzer type but different company brands (Bristow *et al*., 2004, Jansen *et al*., 2005). The retention time is always included in the library when increasing the specificity of the library search. In addition, Kovats retention index (RI) system is a standardized parameter used to align chromatograms and identify compounds from

metabolomics data. It depends on many parameters and conditions of the GC-MS analysis. However, this system has been used in GC-MS analysis, but has not been demonstrated in LC/MS analysis (Evans *et al*., 2009). However, using RI libraries is an expensive and time-consuming effort. In a case where a perfect match is not found between the queried data and the database, at that point, partial matches demonstrate the same molecular structure to the unknown compound in question (Heuberger et al., 2014). In addition to spectral matching, individual masses within a spectrum can be manually interpreted to inform on the structure of the precursor molecule. A manual interpretation usually requires high-resolution mass information and may depend on identifying patterns in the data to identify the molecular weight of the target compound (Heuberger et al., 2014). The external chemical databases are used to search molecular weight and are the next-best option method where confident spectral matching or interpretation is impossible. This method is restricted by an instrumental error in finding the mass and error in the manual interpretation of the mass spectrum (Heuberger et al., 2014).

As mentioned at the beginning of this review, powdery scab caused by *Spongospora subterranea* f. sp. *subterranea* has become a vital disease in recent years due to the increased damage it causes in potatoes (Falloon, 2008). However, there is no effective method of controlling the disease due to the persistence of its survival structure, the spore ball and the ability of its zoospores to infect and multiply within the roots of a susceptible host, making control and eradication of the disease problematic (Harrison *et al*., 1997). On the other hand, several methods have been tested for control of the disease. The best form of control would be planting of tolerant cultivars which have been shown as effective for reducing the incidence and severity of the disease in potato crops (Harrison *et al*., 1997). The use of metabolomics to find possible biomarkers provides a means to quickly identify tolerant and susceptible powdery scab potatoes. This will prove beneficial as other means of controlling *Spongospora subterranea* f. sp. *subterranea* infection are an expensive and time-consuming process. By identifying the metabolites that show a significant difference in abundance between the tolerant and susceptible groups and identifying the possible biomarkers will aid in the control of *Spongospora subterranea* f. sp. *subterranea* infection in potato tubers. To date, the study of plant-pathogen interaction in metabolomics studies has been limited over the years. Only a small number of groups have done research on systems of varying complexity, and those studies are reviewed in Allwood *et al*. (2008). Technological advances in GC-MS and LC-MS accompanied by statistical data analysis software have resulted in new

capacities for plant breeding and protection (Heuberger et al., 2014). Metabolomics as a powerful tool may be used to characterize the interaction between the plant and pathogen, which significantly broadens our knowledge of the metabolic and molecular mechanisms regulating plant growth, development and stress responses, and the improvement of crop yield and quality. The objective of this study was to search for metabolic biomarkers in potato tuber extracts inoculated with *Spongospora subterranea* f. sp. *subterranea* and mock-solution using UPLC-QTOF/MS based metabolomics, coupled with multivariate statistical data analysis.

Null Hypothesis

H_0: There is no statistically significant difference in the metabolite peak areas between tolerant and susceptible potato cultivars, before and after infection by *Spongospora subterranea* f. sp. *subterranea*, at the 95% level of confidence.

Research Objectives

- To establish a global metabolite profile for both tolerant and susceptible potato cultivars using untargeted UPLC-QTOF/MS.
- To identify metabolic markers for tolerance/susceptibility to *Spongospora subterranea* f. sp. *subterranea* infection using univariate and multivariate analysis.
- To link the identified metabolites with their metabolic pathway for identification of the mechanism of action.

CHAPTER 2 MATERIALS AND METHODS

2.1. Chemical solvents

All chemicals for UPLC-QTOF/MS experiments were of ultra-pure UPLC-MS grade. Methanol and Acetonitrile were purchased from ROMIL Ltd (Cambridge, United Kingdom), while Formic acid (99% purity) was obtained from MERCK (Darmstadt, Germany). UPLC de-ionised water (H_2O) was purified using a purification system (ELGA PURELAB Ultra, Labotec (PTY) Ltd, Midrand, SA).

2.2. Potato plant production

Sprouted mini tubers of ten potato cultivars with different levels of susceptibility to *Spongospora subterranea* f. sp. *subterranea* infection (Table 2.1) were grown in the Plant pathology greenhouse at University of Pretoria under temperature-controlled conditions (22 ± 2°C), within a 16-hour photoperiod for 100 days. The tubers were planted in plastic pots (13.5 cm height X 15 cm diameter) filled with pasteurized sandy loam soil. The pots were irrigated every second day with 200 ml of sterile distilled water and were fertilized every second week with 100 ml solution per pot of Dr Fisher's Multifeed® Classic (NPK 19:8:16 [43]) (Nulandis Ltd) containing trace elements.

Table 2.1: List of ten potato cultivars differing in tolerance and susceptibility to *Spongospora subterranea* f. sp. *subterranea*.

No. of cultivars	Cultivar	Maturity group[a]	Skin and flesh colour	Powdery scab susceptibility & tolerance level
1	BP1	Medium to late	White	Susceptible[*]
2	Lanorma	Early	White	Susceptible
3	Up-to-date	Medium to late	Cream white	Susceptible
4	Valor	Medium to late	Cream white	Susceptible
5	Avalanche	Medium to late	White	Tolerant
6	Fiana	Medium to late	Light yellow	Tolerant
7	Innovator	Early to medium	Brown/russet	Tolerant
8	Mondial	Late	White	Tolerant
9	Ronaldo	Medium to late	Red	Tolerant
10	Sifra	late	Light yellow	Tolerant

Maturity group[a]: early = less than 90, medium = 90-110 and late = 110-150 days (Potato Seed Production, 2013). *Potato cultivars were arbitrarily categorised according to their tuber infection susceptibility levels in previous pot trials: Susceptible (1 to 4), Tolerant (5 to 10) (Lekota *et al*. manuscript in preparation).

2.3. *Spongospora subterranea* f. sp. *subterranea* inoculum preparation

Spongospora subterranea f. sp. *subterranea* (Sss) inoculum was obtained by removing powdery scab lesions from severely infected field-grown tubers of BP1 cultivar. The inoculum was prepared as described by Van de Graaf *et al*. (2007). Dried powdery scab lesions were ground to a powder using sterile pestle and mortar. The resulting powder was sieved through a sterile 75 µm mesh sieve. One week after emergence, five plants of each cultivar were inoculated with four grams of Sss inoculum suspended in 50 ml of distilled water. The Sss inoculum concentration was equivalent to 5 x 10^4 sporosori per gram of soil determined using a hemocytometer. The other five plants per cultivar were treated with 50 ml of sterile distilled water as a control. The experiment was conducted in a randomized complete block design (RCBD), consisting of two treatments (Sss-inoculated and mock-inoculated plants) with five biological replicates per cultivar (100 plants = 50 inoculated + 50 un-inoculated plants).

2.4. Sample collection and preparation

Potato plants (n=100) were harvested during the last week of August from the 28th to 30th of August 2017. One week after harvesting, tubers were rinsed in sterile water to remove sand and stored at 4°C in Ziploc plastic bags until further analysis. The sample preparation was conducted by collecting tubers (n=10) from each plant (n=5) per treatment. Entire tubers (skin and flesh) were cut in small cubes with a sterile scalpel and immediately frozen in liquid nitrogen to avoid metabolite changes caused by enzymatic reactions connected to the handling and wounding of the tubers. The small cubes were then transferred to 50 ml conical tubes (four tubers per tube) and immediately freeze-dried for 4-5 days (de Sotillo *et al*., 1994). The freeze-dried tubers were then ground into a fine powder using a coffee grinder to increase surface area for maximum extraction efficiency. The tuber dry matter content was determined and kept in 50 ml conical tubes at -80ºC until further analysis.

2.5. Metabolite extraction

Metabolites were extracted from collected, frozen powdered tuber materials to evaluate the response of metabolic profiles in 10 potato cultivars under the two treatments (un-inoculated and inoculated samples). The powdered tuber material (10 mg per tube) obtained from 10 potato cultivars was mixed with 1 ml of methanol:water (70:30 v/v) solution (Lachman *et al*., 2012) in 2 ml sterile Eppendorf tubes. The mixtures were immersed in an ultrasonic bath (UMCS (Pty) Ltd, Kenmare, Krugersdorp, SA) for 15 minutes and then centrifuged at 14 000g for six minutes to remove particles, using a MiniSpin® micro-centrifuge (Eppendorf AG, Germany). The supernatants were transferred to new sterile Eppendorf tubes and evaporated in a dry bath heater (Labnet International, Inc. USA) at 60°C for 24 hours to remove the excess solvent. The dried extracts were cooled at room temperature for five minutes and reconstituted in 1 ml of acetonitrile:water (50:50 v/v) solution and vortexed for 30 seconds. The tuber extracts were centrifuged at 14 000g for four minutes, and the supernatant was filtered through an ultra-performance liquid chromatography (UPLC) certified clear glass (12 x 32 mm, 1 ml) screw neck total recovery vial, with cap and PTFE/silicone septum (Waters Corporation, Milford, MA, USA). Tuber extracts were then stored at 4°C prior to UPLC-QTOF/MS analysis.

2.6. UPLC–QTOF/MS

All chromatographic and mass spectrometric analyses were performed in the Department of Chemistry, University of Pretoria. A Waters Acquity UPLC system coupled to an SYNAPT G2 high definition QTOF mass spectrometer (MS) instrument (Waters Corporation, Milford, MA, USA) was used to evaluate the metabolic markers present in the potato extracts. The UPLC system was equipped with electron spray ionization (ESI) source used to acquire negative and positive ion data. Chromatographic separation of potato tuber methanol extracts was achieved on a Waters Acquity HSS T3 UPLC column (100 mm × 2.1 mm, 1.8 µm) (Waters Corporation, Milford, MA, USA). The mobile phase consisted of solvent A: 0.1% Formic acid with 99.9% de-ionized water (H_2O) and solvent B: 100% acetonitrile. The gradient elution for ESI positive and negative ion mode was performed as described in Table 2.2. The injection volume was 5 µl, and the flow rate was 0.2 ml/min. The total run time was 30 min, the column temperature was 40ºC, and the sample temperature was 4ºC.

Table 2.2: UPLC-MS gradient elution for ESI positive and negative ionization mode.

Time (min)	Flow rate (ml/min)	Solvent A (%): 99.9% dddH_2O + 0.1% FA	Solvent B (%): 100% ACN + 0.1% FA
0.0	0.2	99	1.0
0.20	0.2	99	1.0
16.00	0.2	0.0	100
20.00	0.2	0.0	100
20.00	0.2	99	1.0
30.00	0.2	99	1.0

2.6.1. Mass spectrometry acquisition parameters

For MS detection, an electrospray ionization quadrupole time of flight mass spectrometry (ESI-QTOF/MS) detector was used in both positive and negative mode. Sodium formate solution (5 mM) was utilized to calibrate the instrument, and leucine encephalin (2 ng/µl) was used as reference material for tuning and calibration. The mass range was set to scan from 50-1200 Da. The MS optimum experimental conditions were set as follows for both positive and negative mode: the capillary voltage was 2.5 kV, sampling cone voltage at 25 V, source temperature set at 120 °C, desolvation temperature at 400 °C, cone gas flow of 10 1\h and desolvation gas flow of 500 l\h. The metabolomics data was acquired in MS^E mode,

consisting of a scan with low collision energy ramp of 10-30 eV and a high collision energy ramp of 15-60 eV.

2.7. Multivariate statistical data analysis and biomarker identification

Data analysis was performed on MassLynx V 4.1 software (Waters, Milford, MA, USA) to process the UPLC-QTOF/MS raw data files and the MarkerLynx Extended Statistics (XS) EZInfo 2.0 was then used to analyze the data for multivariate statistical analysis. The software performed peak integration and alignment, as well as, background noise elimination yielding data in an appropriate format for statistical analysis. The raw data was exported into an Excel sheet and further analyzed using univariate statistical analysis. The univariate analysis was performed using JMP Pro 14.1.0 software (SAS Institute Inc., Cary, NC, USA) to indicate the significance based on one-way analysis of variance (ANOVA). Student's t-test, with the alpha level (significance level) set to 0.05 was used to determine the significance of the relative abundance of metabolites between the susceptible and tolerant cultivars for both the inoculated and un-inoculated samples. Multivariate statistics in the form of principal component analysis (PCA) & orthogonal partial least square discriminant analysis (OPLS-DA) scores plots and S-plots was done using MarkerLynx software to search for metabolic biomarkers in different sample groups (tolerant/susceptible groups). The MarkerLynx parameters of multivariate data matrices were set as follows for both positive and negative mode: retention time (Rt) from 0-30 min, mass range from 100-900 Da, mass window of 0.05 Da, Rt window of 0.10 min and marker intensity threshold (counts) of 3 000 (positive) and 1 000 for negative ion mode. The template applied to the data was Pareto-scaling, as it worked well with an inherently wide dynamic range, such as data acquired by mass spectrometry. This was accompanied by additional noise filtering after template application to strengthen the signal response compared to noise. The accurate mass (m/z), retention times (min) and mass spectral peak intensity of the potato tuber samples were utilized to produce a score plot and S-plot from OPLS-DA model.

For biomarker identification, the process was carried out as follows: selected markings of the loadings data from the S-plot that was accompanied by OPLS-DA scores plot was transferred to the MarkerLynx data viewer with metabolite features of retention times and m/z ratios. The following identification parameters were applied using display options: mass search window was set at 0.1, and maximum hits were set at 50 to search for a molecular formula of the pseudo-molecular ions data [$(M-H)^-$ or $(M+H)^+$]. Metabolic biomarkers were identified

using MarkerLynx XS online databases and libraries [NIST, KEGG, ChemSpider, BioCyc, PubChem, Plantcyc, Golm metabolome, and Chebi et cetera]. The searching of these molecular formulas was restricted to C, H, N, O, S, and P elementary compositions.

2.8. Data analysis for powdery scab disease incidence and index

Powdery scab severity scores and indices data from the two greenhouse experiments were combined and analysed using SAS 9.4 (SAS Institute, Cary, NC) (Lekota *et al*. manuscript in preparation). The LSD extended student t-test was also calculated and was used to compare the means of two or more varieties. Powdery scab disease incidence was calculated as the percentage of tubers with symptoms out of the total number of tubers assessed per treatment (Baldwin *et al*., 2008). Powdery scab index was also calculated, by multiplying incidence (% plants with infection) by the mean severity scores (Gau *et al*., 2015). The disease severity scores were arbitrarily classified into tolerant (0.0-0.5) and susceptible (0.6-3) for the powdery scab disease (Lekota *et al*. manuscript in preparation).

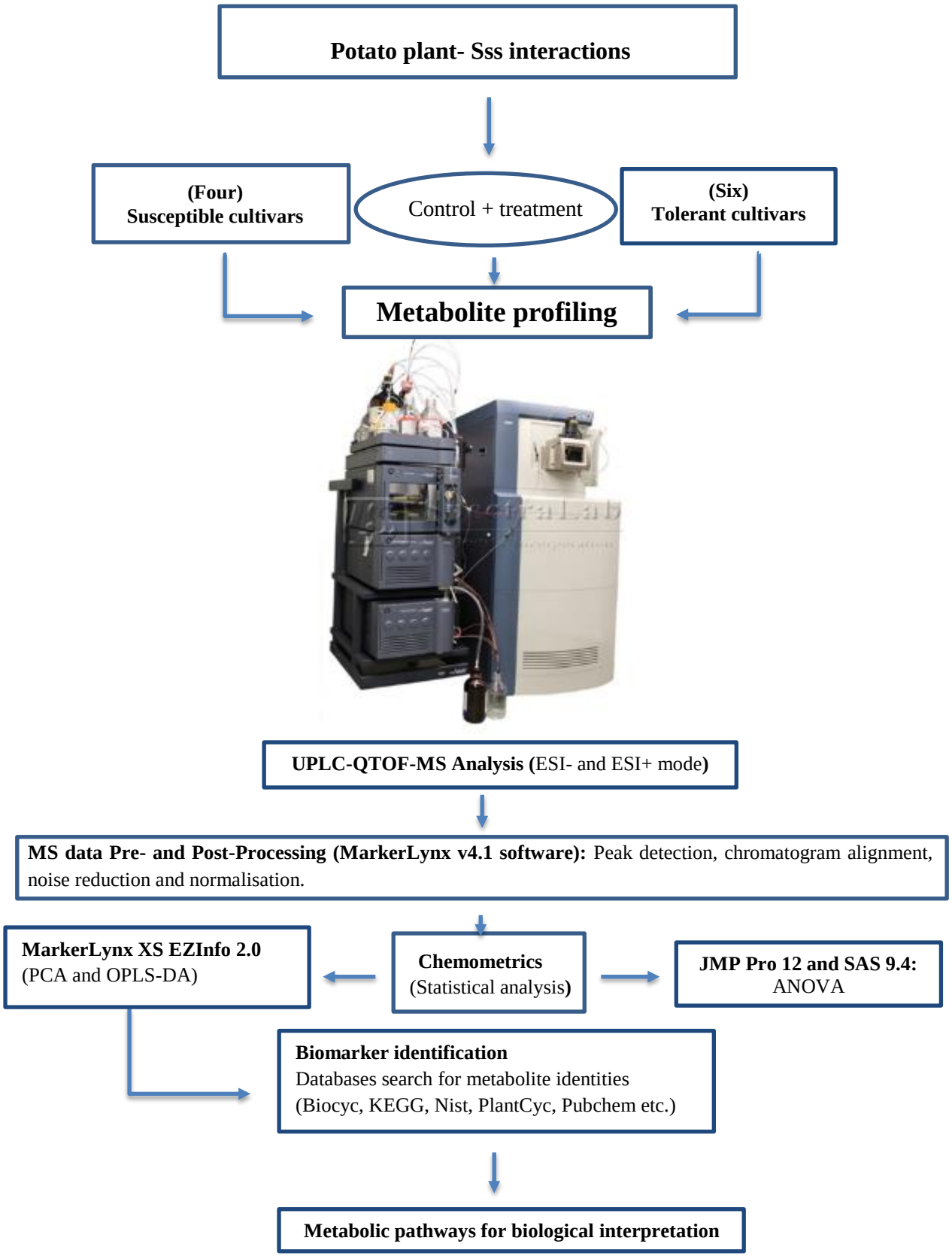


Figure 2.1: Summary of metabolomics workflow for this study.

CHAPTER 3 RESULTS

3.1. Evaluation of susceptibility level to *Spongospora subterranea* f. sp. *subterranea* infection

Table 3.1. Mean powdery scab severity, disease incidence and disease index in twelve potato cultivars obtained from the two greenhouse experiments analyzed by SAS 9.4 statistical software.

No. of cultivars	Cultivar	Disease index	Disease incidence (%)	Powdery scab severity
1	Innovator	0.0 c[y]	0.0 e	0.0 g
2	Sifra	3.2 c	10.7 de	0.17 efg
3	Fianna	3.3 c	11.4 de	0.07 fg
4	Avalanche	7.6 bc	18.2 cd	0.88 ab
5	Ronaldo	12.2 bc	17.9 cde	0.33 cdefg
6	Mondial	26.0 bc	23.8 bcd	0.19 cdefg
7	Lanorma	15.8 bc	25.0 bcd	0.59 bcdef
8	Valor	20.2 bc	27.4 bcd	0.59 bcdef
9	FL2108	26.0 bc	32.9 abc	0.64 bcde
10	Up-to-date	28.6 bc	36.9 ab	0.72 bcd
11	BP1	36.6 b	40.3 ab	0.79 abc
12	Vanderplank	74.7 a	48.8 a	1,26 a
	P-Value	**0.0022**	**0.0001**	**<0.0001**
	LSD [x]	**0.6644**	**0.5165**	**0.5457**

[y]Means followed by the same letter (s) in a column are not significantly different at P ≤ 0.05.

[x]LSD = least significant difference.

Based on cultivar's disease severity score, cultivars were arbitrarily classified into tolerant (0.0-0.5) and susceptible (0.6-3) for the powdery scab disease (Lekota *et al*. manuscript in preparation).

Powdery scab incidence and index for all 12 cultivars were consistent in two trials. In Table 3.1, the tubers of the 12 cultivars showed a range of susceptibility and tolerance to Sss infection. The LSD was not significantly different among the tolerant and susceptible cultivars. Based on the results, there was a significant difference at ($p \leq 0.05$) in powdery scab disease incidence and disease index between the 12 potato cultivars. The highest mean powdery scab disease index was observed in cultivar Vanderplank (74.66) followed by BP1

with the disease index of 36.6 (Table 3.1). There were no visible powdery scab lesions on Innovator (Table 3.1). Based on the powdery scab results shown in Table 3.1, only 10 cultivars were used in the current study and were therefore classified as tolerant (n=6) and susceptible (n=4) to *Spongospora subterranea* f. sp. *subterranea* tuber infection, as seen in Figure 3.1 below.

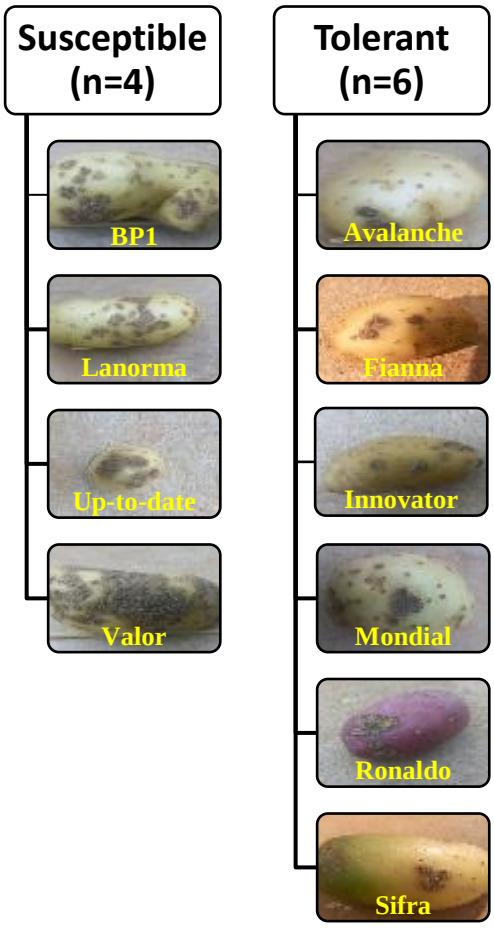


Figure 3.1: A hierarchy diagram illustrating the symptom of powdery scab disease showing black powdery spore balls on the skin of potatoes. Four susceptible cultivars showing a severe damage of disease (left) and six tolerant cultivars with a little damage (right).

3.2. The comparison of the freeze-dried tuber masses between *Spongospora subterranea* f. sp. *subterranea* -inoculated and un-inoculated samples

The means of the dry tuber masses were calculated together with the standard deviation (SD), coefficient of variation (CV) and the student t-test. The coefficient of variation is the ratio of the standard deviation and the mean. The aim was to compare the two different treatments which have different dry tuber mass values. In Table 3.2, Cultivar 1 has a CV of 27%, and Cultivar 5 has a CV of 60% in inoculated samples. Thus, Cultivar 5 has more variation

relative to its mean (average). A paired t-test was performed to compare two treatments mean having 100 samples in which observation of 50 samples (inoculated) can be paired with the observation in the other 50 samples (un-inoculated). Based on the student t-test results, there was no significant difference between the inoculated and the un-inoculated samples. However, the dry mass was a bit higher in inoculated compared to the un-inoculated samples, as seen in Table 3.2. For instances, the dry weight of Avalanche was observed as 4.6 g in un-inoculated samples, and the inoculated samples dropped to 1.8 g.

Table 3.2. Mean of the dry tuber weights determined in both inoculated and un-inoculated samples.

No of cultivars	Cultivar	Powdery scab	Average dry mass (Inocu)	SD	CV	Average dry mass (Un-ino)	SD	CV	P-value (Inocu vs Un-ino)
1	Avalanche	Tolerant	1.8 g	0.49	0.27	4.6 g	3.91	0.85	0.211314
2	Fianna	Tolerant	6.2 g	2.01	0.32	6.5 g	2.74	0.42	0.826027
3	Innovator	Tolerant	3.5 g	1.31	0.37	3.4 g	0.90	0.26	0.757708
4	Mondial	Tolerant	3.8 g	1.64	0.43	4.3 g	1.37	0.31	0.68525
5	Ronaldo	Tolerant	2.6 g	1.62	0.60	3.8 g	1.74	0.45	0.890371
6	Sifra	Tolerant	3.7 g	1.14	0.31	4.3 g	1.94	0.44	0.534856
7	BP1	Susceptible	4.4 g	2.15	0.48	4.5 g	1.67	0.37	0.933681
8	Larnoma	Susceptible	4.2 g	1.49	0.35	4.5 g	1.61	0.38	0.987682
9	Up-to-date	Susceptible	2.2 g	1.25	0.56	0.9 g	0.45	0.52	0.178241
10	Valor	Susceptible	5.1 g	1.45	0.28	4.8 g	1.81	0.38	0.809590

SD= Standard deviation.CV= Coefficient variation.

3.3. Ultra- performance liquid chromatography-high definition mass spectrometry

UPLC-QTOF/MS was employed to distinguish the metabolite changes between tolerant and susceptible cultivars under two treatments. Methanolic tuber extracts (un-inoculated and inoculated samples) was analyzed on a UPLC-QTOF/MS system in both positive and negative ion mode. Different modes of ionization were used to result in different fragmentation patterns. However, the analytes ionised better in the positive mode because it showed a better resolution with multiple peaks compared to the negative mode. Only the ESI positive mode data is further presented; the negative mode is displayed in the appendix file (Figure 1A). The representative base peak intensity (BPI) chromatograms display the complexity of the tuber extracts obtained from susceptible (Figure 3.2) and tolerant cultivars

(Figure 3.3) for both un-inoculated and inoculated samples. Visual inspection of the BPI chromatograms shows a clear difference in peak intensities for each sample and the presence or absence of some of the ion features. Such differences indicate changes in the metabolic level related to *Spongospora subterranea* f. sp. *subterranea* infection.

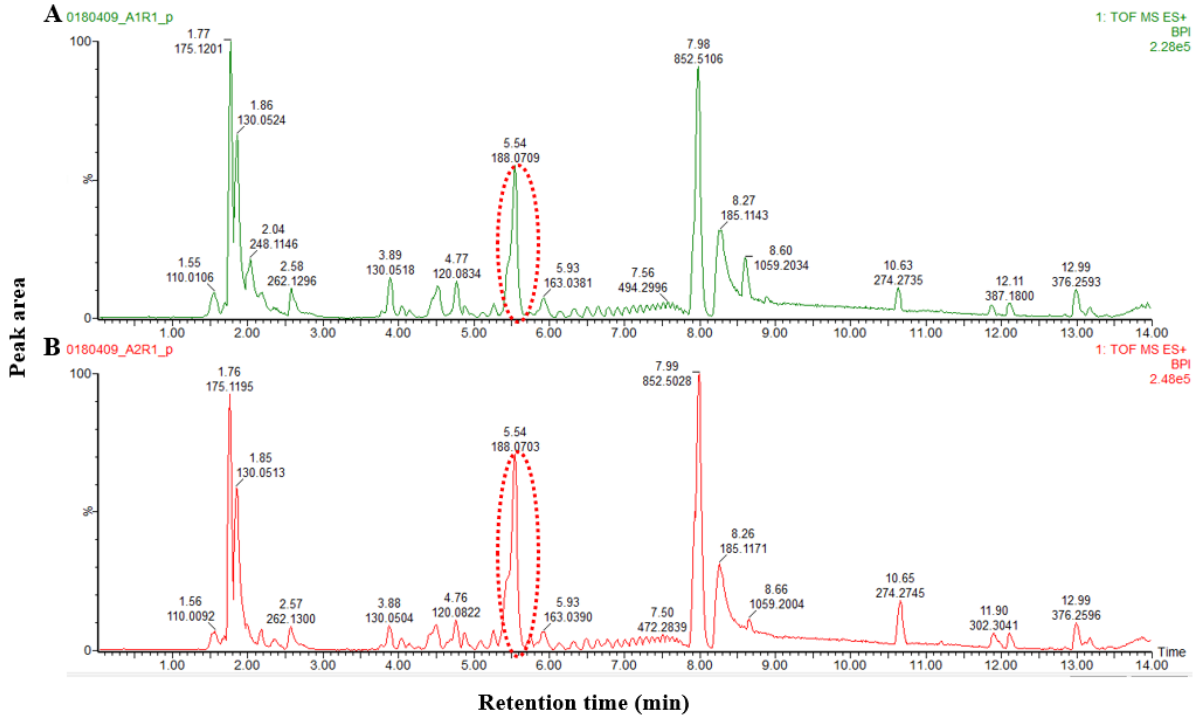


Figure 3.2: Base peak ion (BPI) chromatogram of methanolic tuber extracts obtained from *Spongospora subterranea* f. sp. *subterranea* inoculated and un-inoculated samples analyzed by ESI positive mode. The chromatograms show a clear difference in peak intensities of selected cultivar between (A) Up-to-date (susceptible) inoculated and (B) Up-to-date (susceptible) un-inoculated samples. The red dotted circles show the metabolic differences between the two samples. The tuber samples were aligned on the UPLC system according to retention time (min) and mass to charge ratio (m/z).

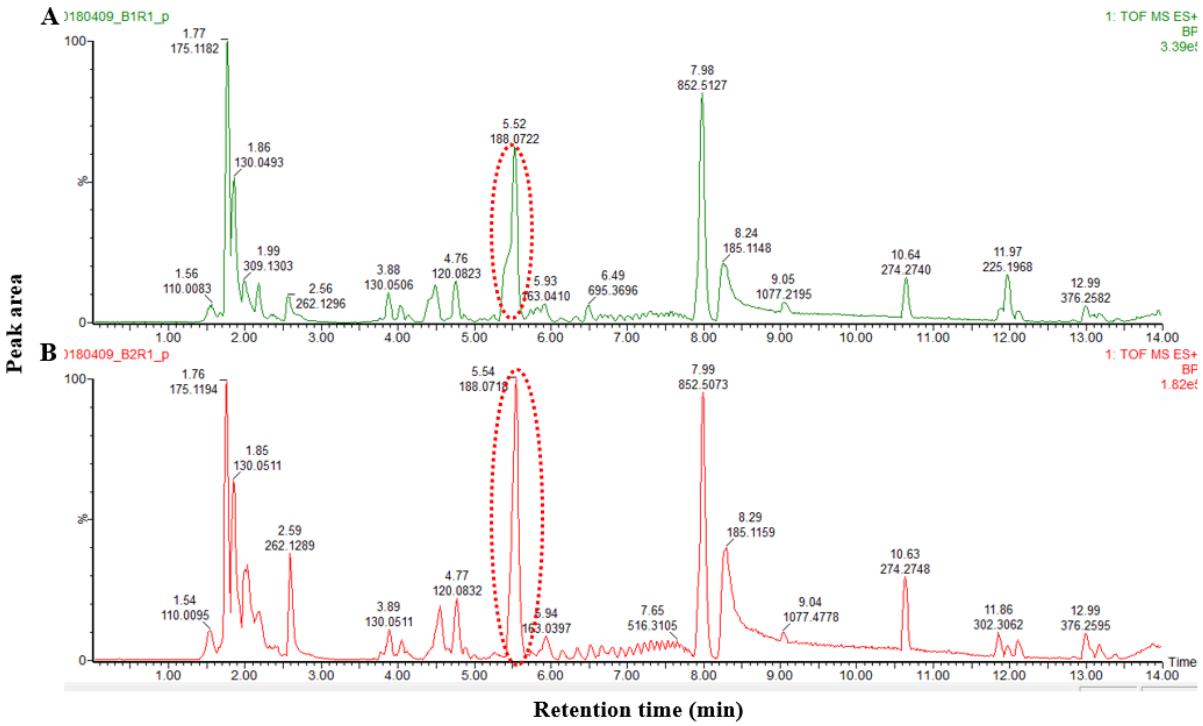


Figure 3.3: UPLC-TOF/MS (ESI+) Base peak ion (BPI) chromatograms of tuber extracts inoculated with *Spongospora subterranea* f. sp. *subterranea* pathogen and mock-solution. The chromatogram represents the inoculated sample (A) Mondial (tolerant) inoculated and (B) Mondial (tolerant) un-inoculated samples.

3.4. Multivariate statistical analysis

Due to the large volume of data generated from UPLC-QTOF/MS spectra, multivariate statistical methods were used to classify the metabolic phenotype and identify the differentiating metabolites. Multivariate statistics in the form of principal component analysis (PCA) & orthogonal partial least square discriminant analysis (OPLS-DA) scores plots and S-plots were performed on data sets obtained from the UPLC-QTOF/MS spectra of potato tuber extracts in both positive and negative ion mode. Moreover, the score plots were achieved on MarkerLynx software to search for metabolic biomarkers in different cultivar groups (tolerant/susceptible groups). The data sets were analyzed on MarkerLynx XS EZInfo 2.0 and were first visualized using PCA followed by OPLS-DA modelling. The PCA score plots of inoculated and un-inoculated samples are shown in (Figure 3.4), and the OPLS-DA score plots are shown (Figure 3.5 and 3.6) acquired in ESI positive mode. PCA uses a few principle components to get the best overview of the grouping trends and outliers of raw data (Lu *et al.*, 2008). OPLS-DA shows which variables are responsible for class discrimination. Furthermore, OPLS-DA differs from PCA by facilitating the separation and interpretation of different types of variations in two distinct samples and searching for metabolic biomarkers (Trygg and Wold, 2002).

3.4.1. PCA modelling

Multivariate analysis revealed distinct metabolic profiles and biomarkers when comparing susceptible and tolerant cultivars. PCA score plots, constructed using the first two components of this model, exhibited a clear separation and clustering between and within susceptible and tolerant cultivars in both inoculated (Figure 3.4A) and un-inoculated tuber samples (Figure 3.4B). Such differential sample clustering, shown by PCA score plot, indicate *Spongospora subterranea* f. sp. *subterranea* caused metabolic changes in the potato samples. For group discrimination and biomarker discovery, a supervised model, OPLS-DA, was used (Figure 3.5).

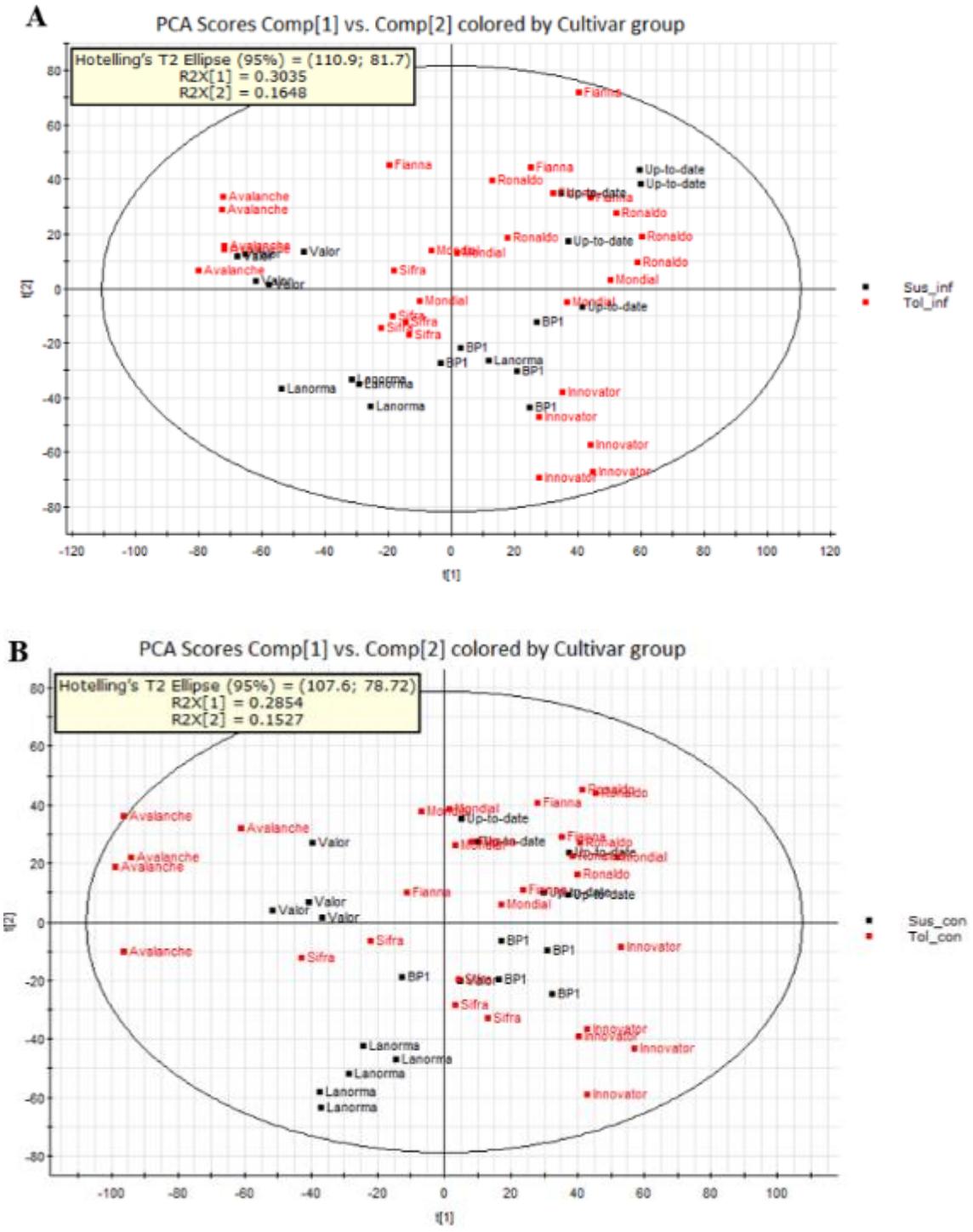


Figure 3.4: PCA clustering of tuber extracts analyzed under two treatments (*Spongospora subterranea* f. sp. *subterranea*- inoculated and mock-inoculated samples) detected in positive ion mode. A: PCA score plots showing susceptible (black) and tolerant (red) cultivars. The R^2X (cum) and Q^2 values of this model were 0.473 and 0.406, respectively. B: PCA scores of un-inoculated tuber samples analyzed in positive mode. The R^2X (cum) and Q2 values of this model were 0.479 and 0.426, respectively. The ellipse shows the 95% confidence interval using Hotelling T2 statistics. The score plot shows the clustering of the differences between groups along t[1] and differences within groups along t[2]. There are five biological repeats (n=5) for each cultivar. MarkerLynx XS EZInfo 2.0 was used to obtain the score plots.

3.4.2. OPLS-DA modelling

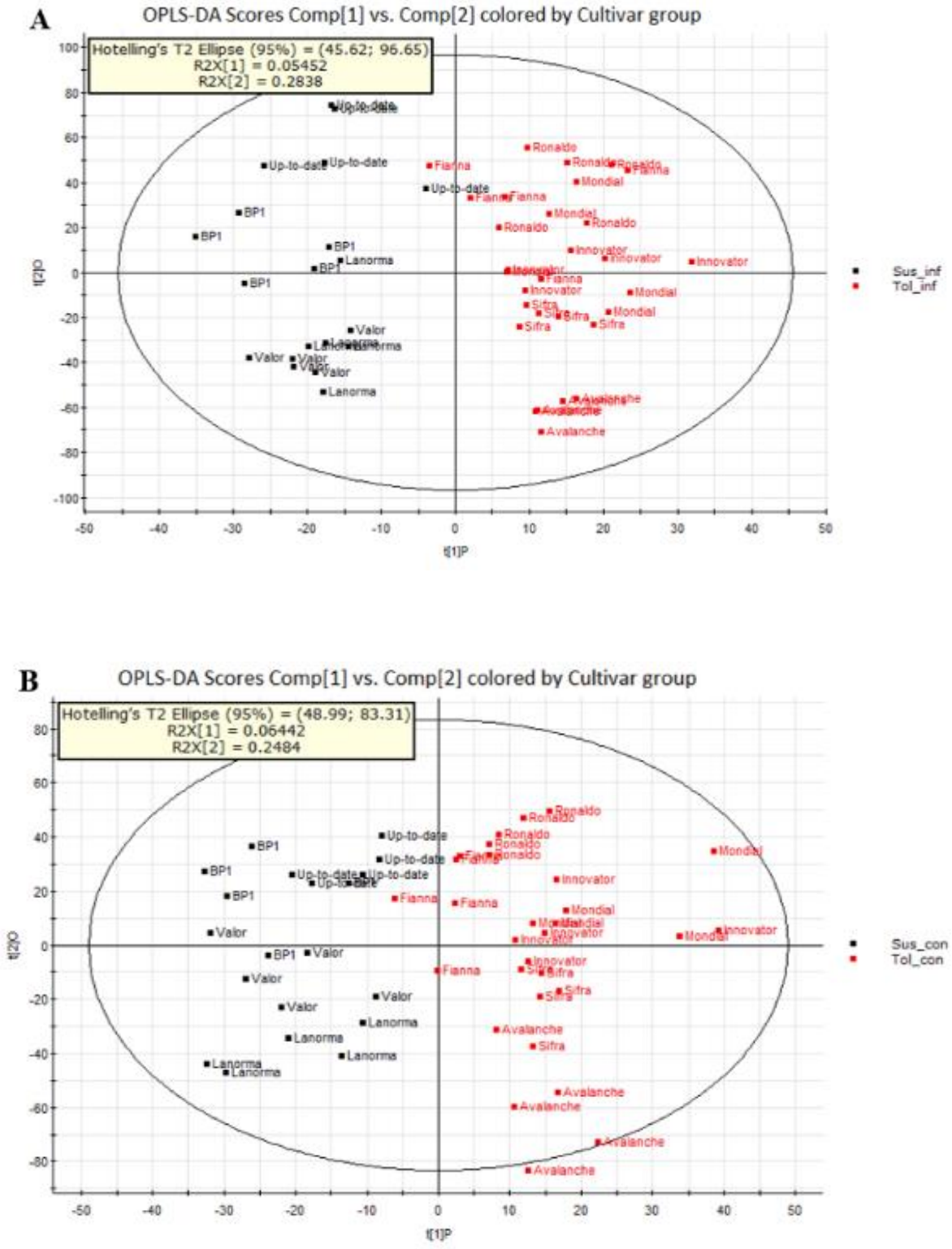


Figure 3.5: OPLS-DA score plots showing the different clustering and separation patterns between susceptible (black) and tolerant (red) cultivars from inoculated (A) and un-inoculated (B) tuber samples generated by UPLC-QTOF/MS in positive ion mode.

In Figure 3.5, the OPLS-DA summarise the relationship among different datasets to visualize group clustering between two cultivar groups. The two treatments (inoculated and un-inoculated samples) of 10 potato cultivars were compared, with the OPLS-DA scores plot showing distinct sample clustering and clear treatment separation. The validation of the OPLS-DA model was presented as follows, Figure 3.5A: R^2X (cum), R^2Y (cum) and Q^2 values of this model were 0.493, 0.806 and 0.586, respectively. Figure 3.5B: R^2X (cum), R^2Y (cum) and Q^2 values of this model were 0.462, 0.756 and 0.636, respectively.

3.4.3. OPLS-DA loading S-plot

The evaluation of OPLS-DA loadings S-plot permitted the extraction of statistically and potentially biochemically significant mass ions (biomarkers) in the samples. The corresponding loadings S-plot (Figure 3.6), whose loadings are situated away from the origin (shown by red boxes) was used to select discriminating feature ions between the inoculated and un-inoculated samples. The selected ion features are based on their contribution to the variation (x-axis) and correlation (y-axis) within the data sets. The ion features at the bottom left (x= -1) (y= -1) are stronger indicators for susceptible samples, and biomarkers at the top right of the curve (x=1) (y=1) are stronger indicators for tolerant samples. Biomarkers near the origin are not useful in distinguishing the sample groups. These selected ion features are further identified in Table 3.3, respectively.

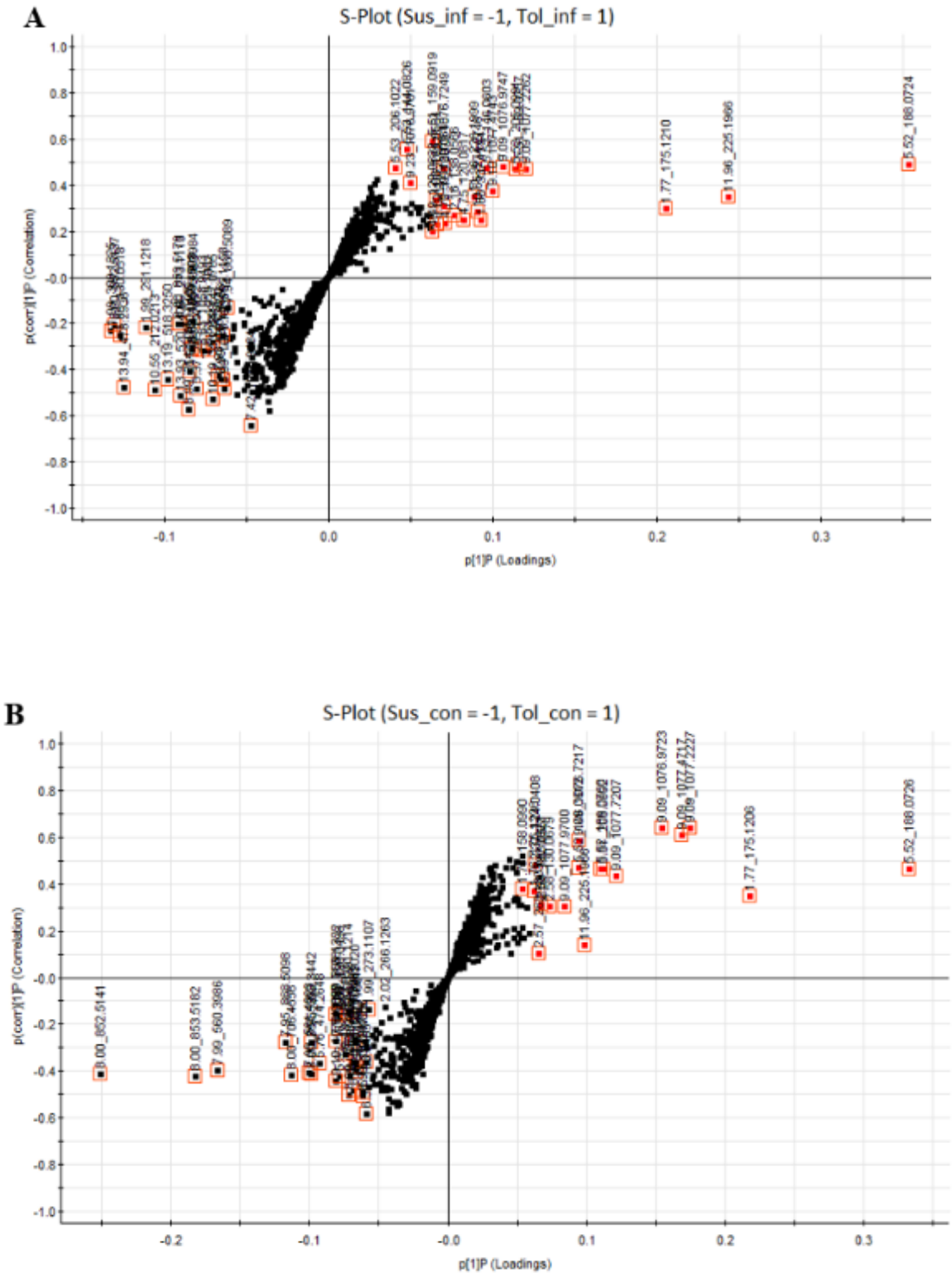


Figure 3.6: OPLS-DA loadings S-plot of inoculated (A) and un-inoculated (B) tuber samples analyzed in positive ion mode of UPLC-QTOF/MS showing group differences of susceptible at the lower left quadrant (negative correlation) and tolerant at the upper right quadrant (positive correlation). Features at the head and tail of the S-plot (red boxes) are unique to the tolerant (black) and susceptible (red) cultivars respectively. The features in the middle are common to both cultivar groups. MarkerLynx XS EZInfo 2.0 was used to obtain the score plots.

3.5. Metabolic signature of potato tuber extracts affected by *Spongospora subterranea* f. sp. *subterranea*

3.5.1. Biomarker identification

From the OPLS-DA Loadings S-plot model (Figure 3.6), feature ions from the tuber extracts of two treatments (Sss-inoculated and un-inoculated samples) were further analysed. Only the Sss-inoculated samples are presented in Table 3.3. Thus, Table 3.3 shows a total of 17 metabolites identified as known compounds (amino acids, fatty acids, organic acids, phenylpropanoids, and alkaloids), and 17 as unknown compounds. The p-value, as well as fold changes, were calculated, and the identity of the metabolites was categorised according to their metabolite classes. The chemical structures of these annotated biomarkers are shown in (Figure 3.7). The magnitude of change in abundances of identified biomarkers/metabolites varied among the tolerant cultivars relative to susceptible. Most of the metabolites detected in the inoculated tuber extracts were the amino acids such as phenylalanine, proline, tryptophan, tyrosine, arginine, glutamine and pyro-glutamic acid. However, there were no significant differences in these markers between susceptible and tolerant cultivars. Their relative abundance in tolerant cultivars was equal to/ greater than 1.2-fold that in the susceptible cultivars, but glutamine and pyro-glutamic acid fold changes were less than 0.9 in tolerant cultivars. The abundance of palmitic acid and cuscohygrine was greater than 15.7 and 15.9 fold change in inoculated samples. Palmitic acid, cuscohygrine and 3-indole acrylic acid significantly induced metabolites/biomarkers in tolerant cultivars following *Spongospora subterranea* f. sp. *subterranea* infection. The identity of several metabolites present in tuber extracts, putatively identified in Table 3.3, have been previously reported in the literature (Aliferis and Jabaji, 2012, Brechenmacher *et al*., 2010, Chitarrini *et al*., 2017, Yogendra *et al*., 2015b).

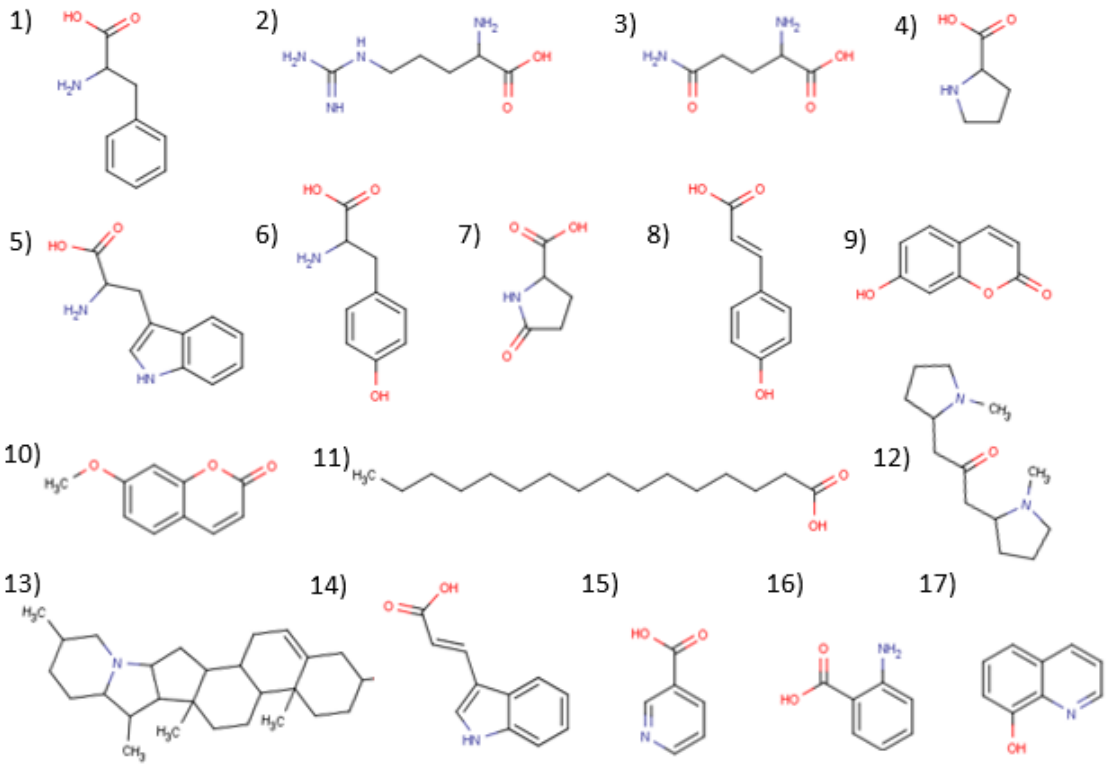


Figure 3.7: Structures of metabolites putatively identified in potato tuber extracts associated with *Spongospora subterranea* f. sp. *subterranea* infection. 1) Phenylalanine, 2) Arginine, 3) Glutamine, 4) Proline, 5) Tryptophan, 6) Tyrosine, 7) Pyroglutamic acid, 8) P-coumaric acid, 9) Umbelliferone, 10) Herniarin, 11) Palmitic acid, 12) Cuscohygrine, 13) Solanidine, 14) 3-indole acrylic acid, 15) nicotinic acid, 16) Anthranilic acid, 17) 8- quinolinol.

Table 3.3: Metabolite identities of potato tubers treated with *Spongospora subterranea* f. sp. *subterranea* analysed in both ESI positive and negative mode. Metabolites present in potato cultivars as discriminant ions were identified by the OPLS-DA model. The differences in group mean of these biomarkers were calculated by ANOVA. Fold change was calculated based on the peak area of metabolites ($\frac{tolerant}{susceptible}$). The metabolites are categorised according to their metabolite classes.

Feature no	Metabolite Identity #	ESI[x]	Treatment[y]	RT (min)	m/z	Molecular formula	p-value*	Fold change	Correlation
	Amino acids								
1	Arginine	+	Tol-inf	1.77	175.1210	C6H14N4O2	0.620506	1.2	Up
2	Glutamine	+	Sus-inf	1.84	147.0785	C5H10N2O3	0.031597*	0.4	Down
3	Pyro-glutamic	+	Sus-inf	3.87	130.0507	C5H7NO3	0.998394	0.9	Down
4	Phenylalanine	+	Tol-inf	4.49	166.0877	C9H11NO2	0.778419	1.2	Up
5	Proline	-	Tol-inf	2.13	116.0709	C5H9NO2	0.739185	1.2	Up
6	Tryptophan	+	Tol-inf	5.52	205.0990	C11H12N2O2	0.320394	1.4	Up
7	Tyrosine	+	Tol-inf	4.02	182.0829	C9H11NO3	0.525758	1.2	Up
	Alkaloids								
8	Cuscohygrine	+	Tol-inf	11.96	225.1966	C13H24N2O	0.015059*	15.9 (**1**)	Up
9	Solanidine	+	Sus-inf	7.95	398.3439	C27H43NO	0.525367	0.8	Down
	Fatty acids								
10	Palmitic acid	+	Tol-inf	10.65	274.2746	C16H32O2	0.001340**	15.7 (**2**)	Up
	Phenylpropanoids								
11	p-Coumaric acid	-	Tol-inf	4.03	165.0561	C9H8O3	0.493466	1.2	Up
12	Herniarin	-	Tol-inf	6.25	177.0551	C10H8O3	0.067174	1.2	Up
13	Umbelliferone	+	Sus-inf	6.33	163.0407	C9H6O3	0.359109	0.7	Down

	Other compounds								
14	Anthranilic acid	+	Tol-inf	2.16	138.0566	C7H7NO2	0.620506	1.2	Up
15	8-Quinolinol	+	Tol-inf	5.52	146.0604	C9H7NO	0.322838	1.4	Up
16	3-indoleacrylic acid	+	Tol-inf	5.29	188.0724	C11H9NO2	0.006669**	1.7	Up
17	Nicotinic acid	+	Tol-inf	2.36	124.0410	C6H5NO2	0.360793	1.2	Up
18	Biomarker 1	+	Tol-inf	5.52	367.1585		0.025044*	1.8	Up
19	Biomarker 2	+	Tol-inf	1.80	337.1754		0.009317**	1.7	Up
20	Biomarker 3	+	Tol-inf	5.29	349.1444		0.035020*	1.8	Up
21	Biomarker 4	+	Sus-inf	1.75	208.976		0.010356*	0.7	Down
22	Biomarker 5	+	Sus-inf	5.75	474.2644		0.033588*	0.7	Down
23	Biomarker 6	+	Sus-inf	12.94	518.3245		0.035387*	0.6	Down
24	Biomarker 7	+	Sus-inf	13.94	478.2937		0.033805*	0.6	Down
25	Biomarker 8	-	Tol-inf	5.39	203.1042		0.005555**	1.8	Up
26	Biomarker 9	-	Tol-inf	5.40	374.2456		0.000786**	1.5	Up
27	Biomarker 10	-	Tol-inf	2.27	464.1981		0.000185**	1.5	Up
28	Biomarker 11	-	Sus-inf	12.49	395.2455		0.015345*	1.9	Down
29	Biomarker 12	-	Sus-inf	12.84	721.3626		0.006206**	0.7	Down
30	Biomarker 13	-	Sus-inf	13.73	723.3791		0.007841**	0.7	Down
31	Biomarker 14	-	Sus-inf	1.939	245.0745		0.008144**	0.7	Down
32	Biomarker 15	-	Sus-inf	13.74	724.3857		0.01927*	0.7	Down
33	Biomarker 16	-	Sus-inf	13.73	677.3736		0.002371**	0.6	Down

34	Biomarker 17	-	Tol-inf	13.25	311.223	0.008892**	0.5	Up

[x] Electron ionisation mode.
+The compound was present in the negative/ positive ion mode.
[y] The compound was abundant in the tolerant/susceptible cultivars.
*Biomarkers found to be statistically different ($p \leq 0.05$) between the susceptible (n=4) and tolerant (n=6) inoculated samples.
**Biomarkers found to be statistically significant different ($p \leq 0.01$).
(1) Excluding mean results of Fianna replicate 3 (Figure 5A).
(2) Excluding mean results of Avalanche replicate 5 (Figure 8A).
Biomarkers identified from the Markelynx online databases (KEGG, CHEBI, Plantcyc, NIST, Chemspider, Pubchem and Golm database).

3.4.2. Univariate statistical analysis

The metabolite profile (abundances of metabolites) of tuber extracts was subjected to univariate statistical analysis using JMP Pro 14.1.0 software (SAS Institute Inc., Cary, NC, USA). Univariate analysis was used to determine the significant differences in the relative abundances by comparing tolerant and susceptible cultivars under two treatments (Sss-inoculated and un-inoculated). Profiles of the relative quantities identified in Table 3.3 are presented in Figure 3.8A-F for both inoculated and un-inoculated samples. Metabolites showing a significant difference at ($p \leq 0.05$) in tolerant cultivars was defined as a biomarker for tolerance against *Spongospora subterranea* f. sp. *subterranea* infection.

In Figure 3.7A, most of the amino acids such as phenylalanine, tryptophan, arginine, tyrosine and proline were increased in tolerant compared to susceptible cultivars before and after *Spongospora subterranea* f. sp. *subterranea* inoculation, but, they were not significantly different at $p \leq 0.05$. Only glutamine was significantly higher in susceptible compared to tolerant cultivars, in both inoculated and un-inoculated samples. Pyro-glutamic acid level was also increased in susceptible relative to the tolerant cultivars upon pathogen infection. Comparison of fatty acids abundance of tolerant versus susceptible cultivars revealed distinct responses to *Spongospora subterranea* f. sp. *subterranea* infection (Figure 3.8B). Palmitic acid was significantly higher in tolerant compared to susceptible cultivars for both inoculated and un-inoculated samples. The level of p-coumaric acid (Figure 3.8D) and herniarin (Figure 3.8E) was increased in tolerant compared to susceptible cultivars in both inoculated and un-inoculated samples. In contrast, the level of umbelliferone was decreased in the tolerant as compared to the susceptible cultivars before and after *Spongospora subterranea* f. sp. *subterranea* infection. Based on the results, umbelliferone (Figure 3.8D) showed a significant difference of ($p \leq 0.05$) between the two cultivar groups (susceptible_un-inoculated vs tolerant_un-inoculated) and herniarin (Figure 3.8E) between (tolerant_inoculated vs susceptible_un-inoculated) cultivar groups. Solanidine, as one of the alkaloids detected in UPLC-QTOF/MS system, showed an insignificant difference between the tolerant and susceptible cultivars before and after *Spongospora subterranea* f. sp. *subterranea* infection (Figure 3.8C). In contrast, cuscohygrine was significantly high in tolerant compared to susceptible cultivars for both inoculated and un-inoculated samples. *Spongospora subterranea* f. sp. *subterranea* inoculation revealed metabolic differences in other groups of secondary metabolites, such as 8-quinolinol, nicotinic acid (vitamin B3), 3-indole acrylic acid

and anthranilic acid (vitamin L1). All the metabolites were induced in tolerant as compared to susceptible cultivars in both inoculated and un-inoculated samples (Figure 3.8F). However, 3-indole acrylic acid only showed a significant difference of $p \leq 0.05$ between tolerant and susceptible cultivars in both inoculated and un-inoculated samples.

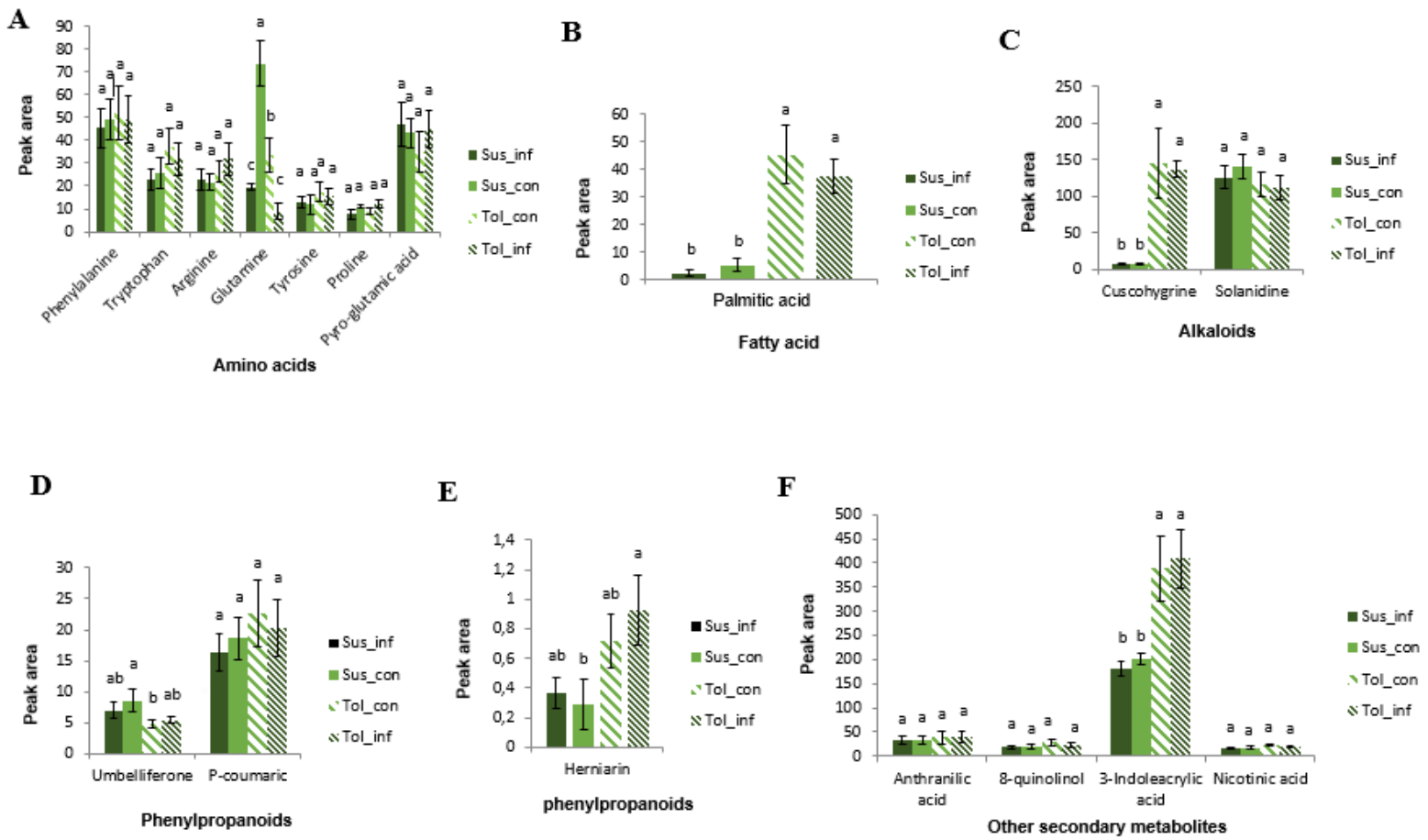


Figure 3.16: Differences in relative abundances of metabolites/biomarkers analyzed by JMP Pro 14.1.0 software under two treatments (*Spongospora subterranea* f. sp. *subterranea*-inoculated and mock-inoculated samples). A one-way ANOVA of data was conducted to determine the significant difference of up/down-regulation of each biomarker. The metabolites are represented as amino acids (A), fatty acids (B), alkaloids (C), phenylpropanoids (D&E) and other secondary metabolites (F). The susceptible cultivars are presented by the plain dark green (inoculated) and light green (un-inoculated) while tolerant are indicated by the dark green stripes (inoculated) and light green (un-inoculated) respectively. Error bars represent the means ± S.E.M. with n=4 (susceptible) and n=6 (tolerant). Histograms with different letters are statistically different with $p \leq 0.05$. SEM: (Standard error of the mean).

CHAPTER 4 DISCUSSION

To cope with biotic stress, such as pathogen attacks by fungi, bacteria and viruses, plants utilise a multicomponent complex defence mechanism that is classified as either constitutive or inducible (Pieterse *et al*., 2009). These defence mechanisms are linked to resistance related metabolites/ defence markers and may have antimicrobial activity, contributing to a plant's defence, which will determine whether the plant exhibits a resistant, tolerant or susceptible phenotype (Heuberger et al., 2014). In this study, the metabolomics approach was used as a tool to phenotype ten potato cultivars differing in tolerance and susceptibility to *Spongospora subterranea* f. sp. *subterranea* infection. The untargeted metabolic profiling of tuber extracts was examined in negative and positive ion mode of UPLC-QTOF/MS system. UPLC-QTOF/MS analysis as a system of separation and detection of metabolites simultaneously detected a wide range of polar and non-polar compounds in the methanol extract. Several mass spectrometry ion features were found to be significantly different between the tolerant and susceptible cultivars. Some metabolites were however statistically not significant, but their levels showed higher accumulation in tolerant compared to susceptible cultivars. The reason behind this might be due to the small sample sizes, and in future, larger sample sizes should be used for valid statistical results. The metabolites putatively identified in Table 3.3 are shown in both positive and negative ion mode which belongs to the phenylpropanoid pathway, amino acids metabolism, fatty acids metabolism, organic acids and alkaloids biosynthesis. Moreover, these metabolites are considered as metabolic markers for disease resistance to pathogenic microbes in a wide range of plants (Ahuja *et al*., 2012).

Amino acids such as phenylalanine, tryptophan, arginine, tyrosine and proline were increased in tolerant compared to susceptible cultivars for both inoculated and un-inoculated samples (Figure 3.8A). The increase of the amino acids in tolerant cultivars may act as a defence against *Spongospora subterranea* f. sp. *subterranea* infection through the activation of the salicylic acid (SA)-mediated pathway. Several studies have also shown an increase of phenylalanine, threonine, and glutamine during plant-pathogen interactions. Rojas *et al*. (2014) proposed that the increase of the amino acids was associated with the activation of a signal transduction cascade that leads to plant defense responses. Phenylalanine and glutamine were identified as major compounds in rice cultivars resistant to gall midge using GC-MS (Agarrwal *et al*., 2014). Another study reported an accumulation of seven amino acids (phenylalanine, tryptophan, proline, glycine, serine, threonine, and isoleucine) in citrus plants against Huanglongbing bacterium (*Candidatus* Liberibacter asiaticus) (Killiny and

Nehela, 2017). According to Heuberger *et al*. (2014), there is a shift in primary metabolite concentration, and this is related to the physiological/morphological modifications that prevent the growth of pathogens due to structural modifications in plants. Glutamine is a nitrogen supply to plnts and donates nitrogen to numerous compounds including amino acids, nucleic acids, and other nitrogen-containing compounds (Tripathy and Pattanayak, 2012). The biological functions of glutamine are regulation of plant growth and stress response (Kan *et al*., 2015). Glutamine synthetase (GS) is an enzyme that plays a significant role in nitrogen metabolism and plant defence (Pageau *et al*., 2005). The biosynthesis of glutamine is formed by the conversion of glutamate and ammonia to form glutamine whereby ATP phosphorylates glutamate to form ADP and acyl-phosphate intermediate, γ-glutamyl phosphate reacts with ammonia to form glutamine and inorganic phosphate (Eisenberg *et al*., 2000). In this study, glutamine and pyroglutamic acid were the only metabolites differently accumulated in response to *Spongospora subterranea* f. sp. *subterranea* infection as compared to the other amino acids (Figure 3.8A). *Spongospora subterranea* f. sp. *subterranea* infection caused a reduction of glutamine in tolerant compared to susceptible cultivars relative to the increase of several amino acids (phenylalanine, tryptophan, proline, arginine and tyrosine). As explained by (Pageau *et al*., 2005) the decrease of glutamine in tolerant cultivars before and after infection was associated with the deficiency of nitrogen in the host leading to an increase in susceptibility.

The reduction of nitrogen causes an increase in susceptibility resulting in cell death through the activation of the cell death process called hypersensitive response (HR) (Pageau *et al*., 2005). The effect of nitrogen intake on disease resistance in plants may be influenced by the type of pathogens used (e.g. bacteria, fungi or viruses) (Hoffland *et al*., 2000). When pathogens enter the host, they are exposed to the new and changing environment that leads them to survive or adapt to that particular environment. Moreover, when fungal or bacterial pathogens attack the plants, the size of the affected areas is variable depending on the plant nutrients (Hoffland *et al*., 2000, Long *et al*., 2000). Nitrogen has a significant impact on the growth and development of plant pathogens (Pageau *et al*., 2005). The reduced availability of nitrogen also affects the nitrogen nutrients in the plant and the severity of the disease caused by pathogens can also be affected (Solomon *et al*., 2003). The results also showed a decreased level of Pyro-glutamic acid in the tolerant compared to susceptible cultivars for both inoculated and uninoculated samples (Figure 3.8A). This result coincides with the results of Aliferis and Jabaji (2012), where *Rhizoctonia solani* infection caused a decrease of

the pyro-glutamic acid in resistant potato sprouts relative to the increase of protein amino acids (phenylalanine, tryptophan, proline, glycine, serine, threonine and tyrosine). The reason behind this was associated with the activation of PR proteins. PR proteins are a group of proteins that play an important role in plant defence against fungi, bacteria and viruses (Stintzi *et al*., 1993). More work is however required, to understand why some amino acids are reduced or accumulated compared to others in terms of the type of pathogen used (such as virus, fungi and bacteria).

Fatty acids are hydrophobic compounds that are essential components of membrane lipids and are an important source of reserve energy. In most plants, fatty acids play a key role in plant defence against pathogens (Kachroo and Kachroo, 2009). Fatty acids are derived from phytohormones (such as SA, Ethylene, JA), which are commonly known for their role in wound responses and plant defence against pathogens (Pollard *et al*., 2008). In the present study, palmitic acid was the only compound identified in the fatty acids class. A significant increase in the peak intensity of this compound was observed in the tolerant cultivar compared to susceptible, which was involved in the synthesis of signalling cascades or acting as a membrane secondary messenger (Figure 3.8B). This observation is in agreement with the results obtained in other studies, for example Agarrwal *et al*. (2014) identified palmitic acid, stearic, oleic acid, linoleic acid, and stearic acid as the most significant compounds in rice cultivars resistant to gall midge. Another study has reported a higher concentration of palmitic acid related to resistance in *Vitis vinifera* (Chitarrini *et al*., 2017). According to Aliferis and Jabaji (2012), unsaturated fatty acids (16:0,18:0) were highly increased compared to saturated ones (18:1) after infection. As explained by Kachroo *et al*. (2001) the was a deficiency of suppressor of SA insensitivity (SSi2) mutant, compared to the wild-type. According to Kolattukudy (1981), palmitic acid and 16-hydroxy palmitate are monomers of cutin, deposited on the cuticle, which acts as a physical barrier against invading pathogens. In many plants, fatty acids and lipids serve as mediators in signal transduction, membrane trafficking and cytoskeletal rearrangement (Wang, 2004). Therefore, fatty acids may play an essential role in potato resistance to *Spongospora subterranea* f. sp. *subterranea*, mainly as an antimicrobial compound and act as physical barriers for pathogen invasion.

Molecular classes of secondary metabolites include phenylpropanoids, flavonoids, alkaloids, terpenoids, glucosinolates and other nitrogen and sulfur-containing groups (Heuberger *et al*., 2014). Phenylpropanoids are the precursors of lignin which plays a vital role in plant stress defence mechanisms, which change cell wall composition and stiffness in roots (Hong *et al*.,

2016a). A study by Ali *et al*. (2012), previously identified phenylpropanoid metabolites in response to *Plasmopara viticola* which were responsible for distinguishing resistant cultivars from the susceptible ones. There was an increase of these phenylpropanoid metabolites in tolerant compared to susceptible cultivars in both inoculated and un-inoculated samples (Figure 3.8D &E). However, umbelliferone was lower in tolerant than in susceptible cultivars (Figure 3.8D). P-coumaric acid serves as one of the hydroxycinnamic acids amides (HCCAs) highly abundant in food (Yogendra *et al*., 2015b). Hydroxycinnamic acid amides are usually produced in plants as polymers of amides and hydroxyphenols, in several mixtures (Dong *et al*., 2015, Kusano *et al*., 2015, Wen *et al*., 2014, Yogendra *et al*., 2015a). In response to pathogen stress, HCCAs are deposited as secondary cell walls to protect plants from invading pathogens, as proved in wheat against *Fusarium graminearum* (Gunnaiah *et al*., 2012), in tomato against *Pseudomonas syringae* (López-Gresa *et al*., 2011), in Arabidopsis against *Botrytis cinerea* (Lloyd *et al*., 2011), and in potato against late blight (Yogendra *et al*., 2015a). The increased content of phenylpropanoids in response to fungal attack was well known for strengthening the cell wall (Walter, 1992). Umbelliferone is one of the major hydroxylated coumarins, ubiquitously produced in plants (Bourgaud *et al*., 2006). Coumarins are secondary compounds contributing to the environmental adaptation of plants and are involved in plant defence and regulation of oxidation stress (Bourgaud *et al*., 2006). Among coumarins, umbelliferone occupies a vital role in the plant phenylpropanoid network. Umbelliferone is formed after ortho-hydroxylation of p-coumaric acid that leads to the synthesis of 2,4-dihydroxycinnamic acid (Bhattacharya *et al*., 2010). Herniarin was also accumulated at a high level in tolerant cultivars in both inoculated and un-inoculated samples (Figure 3.8E). Moreover, is a precursor of coumarin derived from umbelliferone catalyzed by *O*-methyl transferase, found in plants such as *Matricaria chamomile* (Ahmad and Misra, 1997, Ma *et al*., 2007) and *Lavandula angustifolia* (Brown, 1963).

Alkaloids are a broad group of natural toxic substances produced by a large variety of organisms, including bacteria, fungi, plants, and animals (Martins *et al*., 2013). Moreover, alkaloids are synthesized as natural defence mechanisms against invading pathogens and are most extensively studied with respect to their bioactivity, toxicology, and role in the plant's physiology (Friedman, 2006). Mevalonate pathway played a central role in potato plant defense by regulating the biosynthesis of steroidal and pyrrolidine alkaloids before and after *Spongospora subterranea* f. sp. *subterranea* infection. Cuscohygrine significantly increased in tolerant cultivars compared to the susceptible cultivars relative to the decrease of

solanidine before and after *Spongospora subterranea* f. sp. *subterranea* inoculation (Figure 3.8C). The increase of cuscohyrine suggests its involvement in the defence capability of the tolerant cultivars against *Spongospora subterranea* f. sp. *subterranea* infection. Cuscohygrine is one of the pyrrolidine alkaloids found mainly in plants known as *Erythroxylum coca,* and its biosynthesis is derived from ornithine and arginine (Leete, 1983). A number of reports found cuscohygrine in a variety of plant systems, including *Atropa belladonna* (deadly nightshade), Datura species; *Datura inoxia* (thorn apple), *Datura stramonium* (jimson weed), *Datura metel, Helleborus niger (*black henbane*), Mandrogora officinaru (*Mandrake*), Scopolia carniolica,* and *Withania somniferum* (O'Donovan and Keogh, 1969, Ionkova *et al*., 1994). The role of cuscohygrine in tolerant cultivars as a plant defence against *Spongospora subterranea* f. sp. *subterranea* infection should be further investigated; however, for now, we can identify this compound as a putative biomarker. Only solanidine was identified as steroidal glycoalkaloids in the tuber extracts for both inoculated and un-inoculated samples. However, several glycoalkaloids such as α-solanine, α-chaconine, swainosine and solasodine were absent in the samples, this may be due to the insufficient amount of sample analyzed. Aliferis and Jabaji (2012) have also reported a relative abundance of several glycoalkaloids in potato sprouts related to *Rhizoctonia solani* infection, including solanidine, solasodine, solasodenone, α-solanine, α-chaconine and solasonine using FT-ICR/MS system. As explained by Fewell and Roddick (1997), steroidal glycoalkaloids such as α-solanine, α-chaconine and β-chaconine, inhibit spore germination and hyphal elongation in fungi.

Other secondary metabolites such as 8-quinolinol, nicotinic acid (vitamin B3), 3-indole acrylic acid and anthranilic acid (vitamin L1) were induced in the tolerant compared to susceptible cultivars (Figure 3.8F). However, these metabolites showed an insignificant difference between tolerant and susceptible cultivars before and after *Spongospora subterranea* f. sp. *subterranea* infection. Only 3-indole acrylic acid showed a significant difference of $p \leq 0.05$ between tolerant and susceptible cultivars. Vitamins such as thiamine (Vitamin B1), riboflavin (Vitamin B2), nicotinic acid (Vitamin B3), menadione (Vitamin K3) and pyridoxine (Vitamin B6) are important compounds which can reinforce plant resistance and environmental stress tolerance as well as acting directly as an antimicrobial activity (Hong *et al*., 2016b). Nicotinic acid is one of the B vitamins known to elevate plant innate immunity against fungal attack and other B complexes (Ahn *et al*., 2005). Biosynthesis of nicotinic acid from tryptophan in higher plants via kynurenine and quinolic acid requires both Vitamin B2 and B6 (Uhlik and Gowans, 1974). Moreover, nicotinic acid plays a vital

role as a component of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which occupies a key position in cellular energetics and metabolisms (Uhlik and Gowans, 1974). The accumulation of nicotinic acid has been reported by Aliferis and Jabaji (2012) in resistant potato sprouts after *Rhizoctonia solani* infection. Anthranilic acid is an intermediate in the indole biosynthesis, as well as, in the tryptophan degradation in bacteria (Kurnasov *et al*., 2003). This compound is often found in plants and bacteria, and is formed by a series of enzymatic reactions from phosphoenol pyruvic acid and erythrose-4-phosphate (Boyle, 2005, Wiklund and Bergman, 2006). Furthermore, anthranilic acid can be integrated into many alkaloids isolated from plants (Wiklund and Bergman, 2006). A similar study has reported up-regulation of anthranilic acid in resistance cultivars in response to bacterial pathogen (*Pseudomonas syringe*) infiltration of Arabidopsis leaves (Niyogi and Fink, 1992, Niyogi *et al*., 1993). 8-quinolinol is derived from quinoline, which is found in plants, as well as, from synthesis, and is used as a fungicide in agriculture (Ingole *et al*., 2013). Moreover, this metabolite was demonstrated as a phenolic allelochemical compound that has displayed a strong antibacterial and antifungal activity against important pathogenic microbes. This includes bacterial pathogens such as *Xanthomonas compestris,* oomycete *Phytophthora infestans* and fungi such as *Aspergillus niger, Rhizoctonia solani and Fusarium oxysporum.* Another study reported 8-quinolinol in *Centaurea diffusa* root exudates as an antimicrobial and phytotoxic compound (Li *et al*., 2010). 3-indole acrylic acid, which is a member of indole family derived from acrylic acid was also accumulated higher in tolerant relative to susceptible cultivars before and after *Spongospora subterranea* f. sp. *subterranea* infection (Figure 3.8F). A similar study by Sade *et al*. (2015), reported 3-indole acrylic acid as highly increased in resistant compared to susceptible *Solanum lycopersicum* cultivars in response to yellow leaf curl virus infection.

CHAPTER 5 CONCLUSION

Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) together with chemometrics, has successfully differentiated metabolic profiles of tolerant and susceptible potato tubers in the inoculated and un-inoculated samples. The use of the metabolomics approach may be a powerful tool to identify metabolic biomarkers present in tuber extracts tolerance to *Spongospora subterranea* f. sp. *subterranean* infection. Additionally, it provides a more meaningful understanding of the plant-pathogen interaction of this important pathogen. The present study has identified several metabolites in potato belonging to phenylpropanoids, fatty acids, amino acids, organic acids and alkaloids, which play an important role in plant defence against invading pathogens. Most of the biomarkers significantly different at $p \leq 0.05$ were identified as unknown compounds. Based on the findings, palmitic acid, 3-indole acrylic acid and cuscohygrine could act as possible biomarkers for tolerance against *Spongospora subterranea* f. sp. *subterranea* infection.

Interestingly, we reported the presence of cuscohygrine, nicotinic acid (vitamin B3), anthranilic acid (vitamin L1), 8-quinolinol, 3-indole acrylic acid, and herniarin for the first time in potato tubers related to *Spongospora subterranea* f. sp. *subterranea* infection. These findings may provide potato breeders with information for the development of potato cultivars tolerant to *Spongospora subterranea* f. sp. *subterranea*. This work has provided new knowledge on the potato metabolome, and the biochemical pathways involved in potato tolerance to *Spongospora subterranea* f. sp. *subterranea* pathogen. Finally, to our knowledge, this work demonstrated for the first time that a metabolomics approach could be used successfully to identify metabolic biomarkers in potato tuber extracts that exhibit tolerance to *Spongospora subterranea* f. sp. *subterranea* infection.

CHAPTER 6 LIMITATIONS

- **Insufficient sample size for statistical measurement**

Using a small sample size was one of the significant limitations in this study because it was difficult to find significant differences from the collected data. Based on the research findings, it is essential to have a sufficient sample size to conclude valid results. If a larger sample size is used, it is easier to get more precise results. Therefore, statistical analysis requires a large sample size to ensure that the statistics results are valid and apply to the larger population.

- **Technique/method used to analyze a massive metabolomics data**

Metabolomics studies face several unique challenges that make the field particularly demanding. Some of these limitations are the analytical platform used, type of column and handling of the massive multi-dimensional data generated in metabolomics study. How we analyzed the metabolomics data or measured the variables using single software (MassLynx V4.1) for metabolomics spectral processing and data analysis has limited us in our ability to get all the valuable information available in the sample results. For instances, this software can do data processing, multivariate statistical analysis and identification. However, it could not identify all the unknown compounds detected in the tuber extracts with the available online databases. The fact that some of the noise peaks were eliminated during the method development processes, had an impact on the results because they may have caused a loss of some of the important metabolites. Therefore, it would have been useful to compare the results obtained using other metabolomics software. For multivariate statistical analysis, software such as SIMCA and MetaboAnalyst could be used since they have a hierarchical cluster analysis (heat map). Furthermore, MetaboAnalyst is an online tool that does multiple tests including data processing, statistical analysis, functional enrichment analysis and metabolic pathway analysis. The type of column used in different platforms can also affect the number of metabolites found within the biological system. Therefore, the right platform and column used must be taken into consideration, depending on which compounds one is looking for in that sample. For instance, ACQUITY UPLC has several columns that can be used to separate polar, mid-polar, polar and non-polar metabolites.

- **Limitations of the analytical platform used**

Since metabolite analysis of small molecules in complex plant matrices is usually a difficult task to conduct, several platforms such as GC-MS, LC-MS or NMR are required for the identification and quantification of these analytes. The analytical method used was chosen based on sensitivity and selectivity. However, this technique could not separate all the metabolites within the tuber extracts. Thus, other platforms are required such as GC-MS which separates volatile compounds and thermally stable compounds using gas chromatography. NMR is also a powerful technique that can determine the structure of the unknown compounds and is used to identify unknown compounds by matching metabolites with the spectral libraries or infer the basic structure directly. In the current study, only a single platform (UPLC-QTOF/MS) was used to separate and detect polar and non-polar compounds in the potato tuber extracts. Therefore, using one platform was one of the limitations of the study as it has limited the other metabolites we wanted to investigate in potato plants. For instances, many sugar molecules, alkaloids and lipids are expected to be found using GC-MS platform. The unknown compounds may be further identified using NMR.

- **Lack of previous research studies on the topic**

Literature reviews are an essential part, as it helps to identify the work that has been done. Lack of literature findings on some metabolites identified in the current study was one of the possible limitations, as we could not find the biological importance of some of these markers. Therefore, when there is very little research on the specific research topic, new methods/techniques may need to be developed. The study of potato-Sss interactions, particularly metabolomics has been limited, and in similar studies, there are few publications focused in this area. The reason behind this is due to the complication of enormous diversity of different chemical classes and in identifying which metabolites are derived from the plants, and which are from the interacting pathogen.

CHAPTER 7 FUTURE PERSPECTIVES

Further studies are required to improve the metabolic platform for potato extracts to facilitate accurate and valid identification of the markers within the sample. NMR may further identify the unknown compounds found to be significantly different in tolerant versus susceptible potato cultivars, in response to *Spongospora subterranea* f. sp. *subterranea* infection. A targeted metabolomics approach is necessary for the following step to quantify as many as possible metabolites present in the potato. Since there are no single techniques available to separate all metabolites within a biological system, other platforms such as GC-MS and NMR can be applied for the identification of other classes of metabolites such as carbohydrates, flavonoids and terpenoids as they also play an important role in plant defence. More research is required to consider palmitic acid, 3-indole acrylic acid and cuscohygrine as biomarker for *Spongospora subterranea* f. sp. *subterranea* tolerance.

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CHAPTER 9 APPENDIX

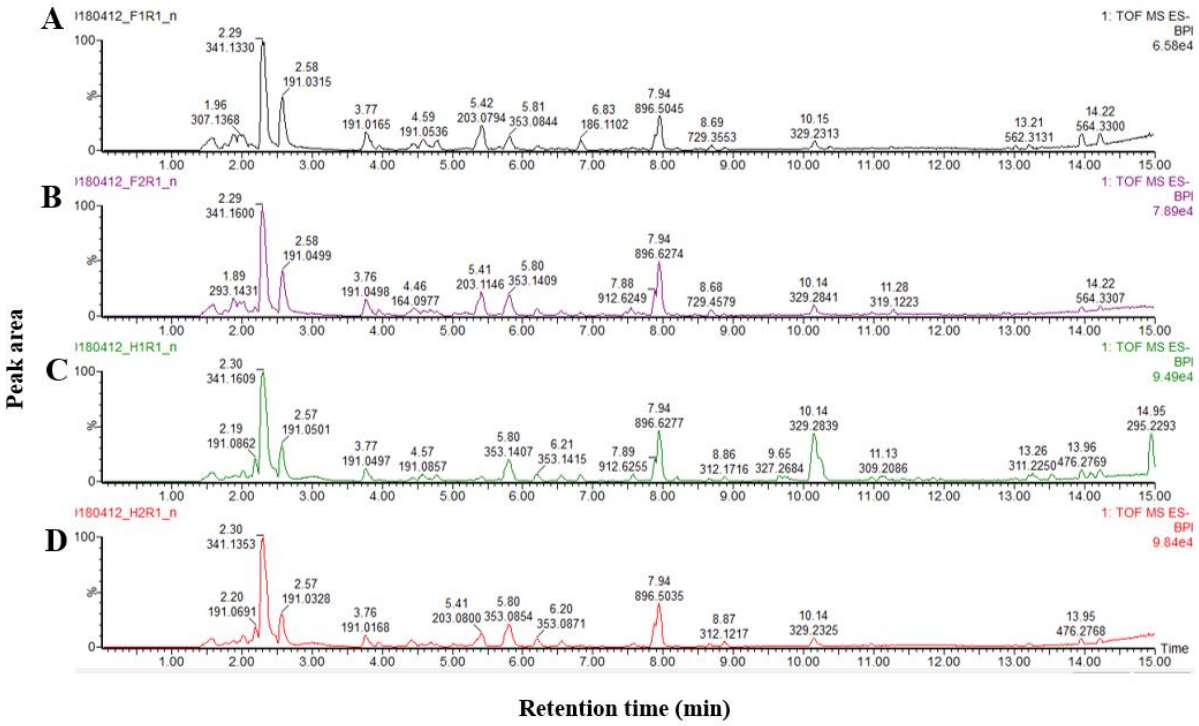


Figure 1A: Overlaid, representative, base peak intensity (BPI) UPLC mass chromatograms of tuber extracts obtained from *Spongospora subterranea* f. sp. *subterranea* inoculated and un-inoculated samples analysed in negative ion mode. A: Up-to-date (susceptible) inoculated, B: Up-to date un-inoculated samples, C: Mondial (tolerant) inoculated and D: Mondial un-inoculated samples.

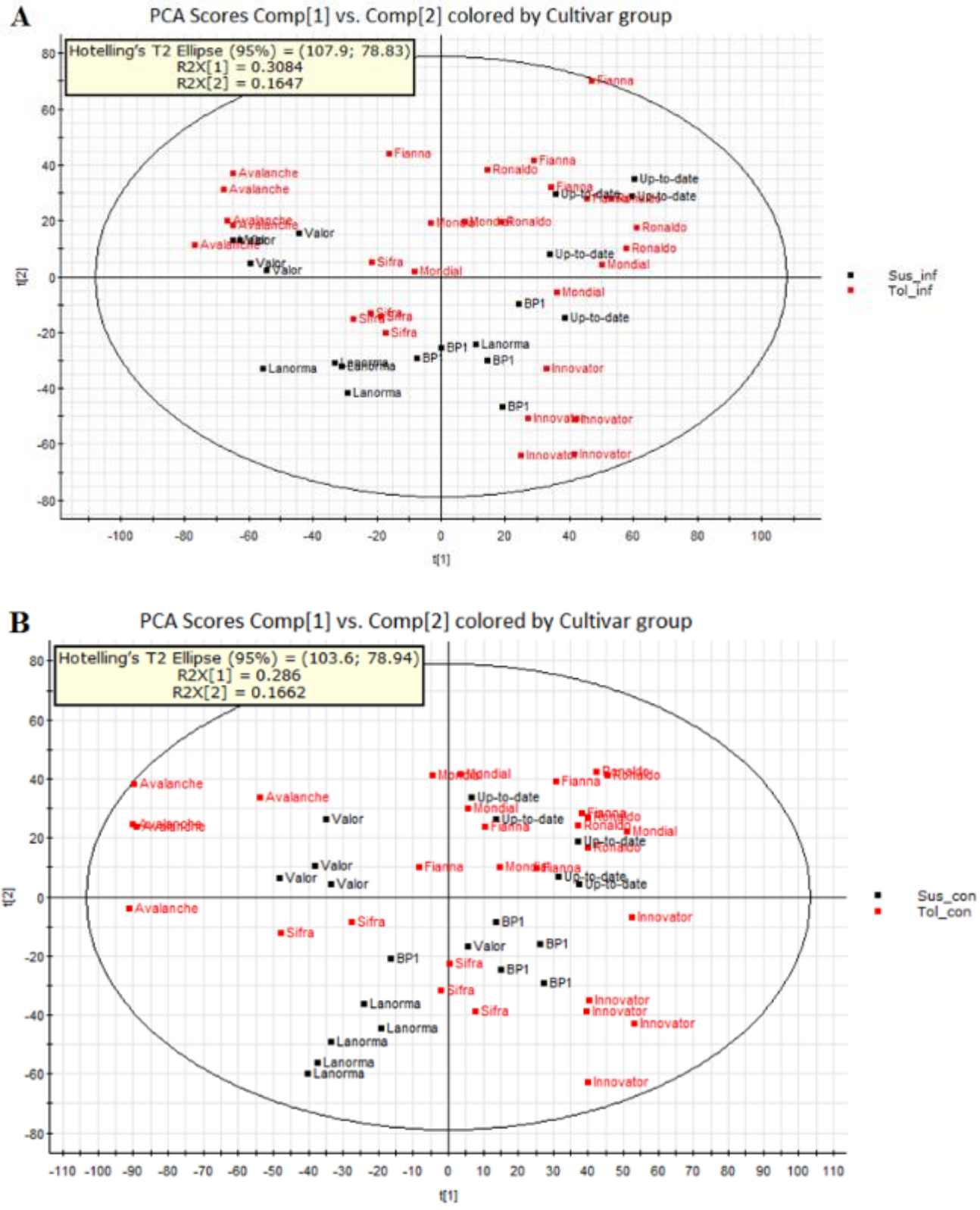


Figure 2A: PCA score plot of potato tubers analyzed in negative ion mode of UPLC-QTOF/MS inoculated with *Spongospora subterranea* f. sp. *subterranea* (A) and mock inoculated (B) analysed in negative mode showing susceptible (black) and tolerant (red) cultivars.

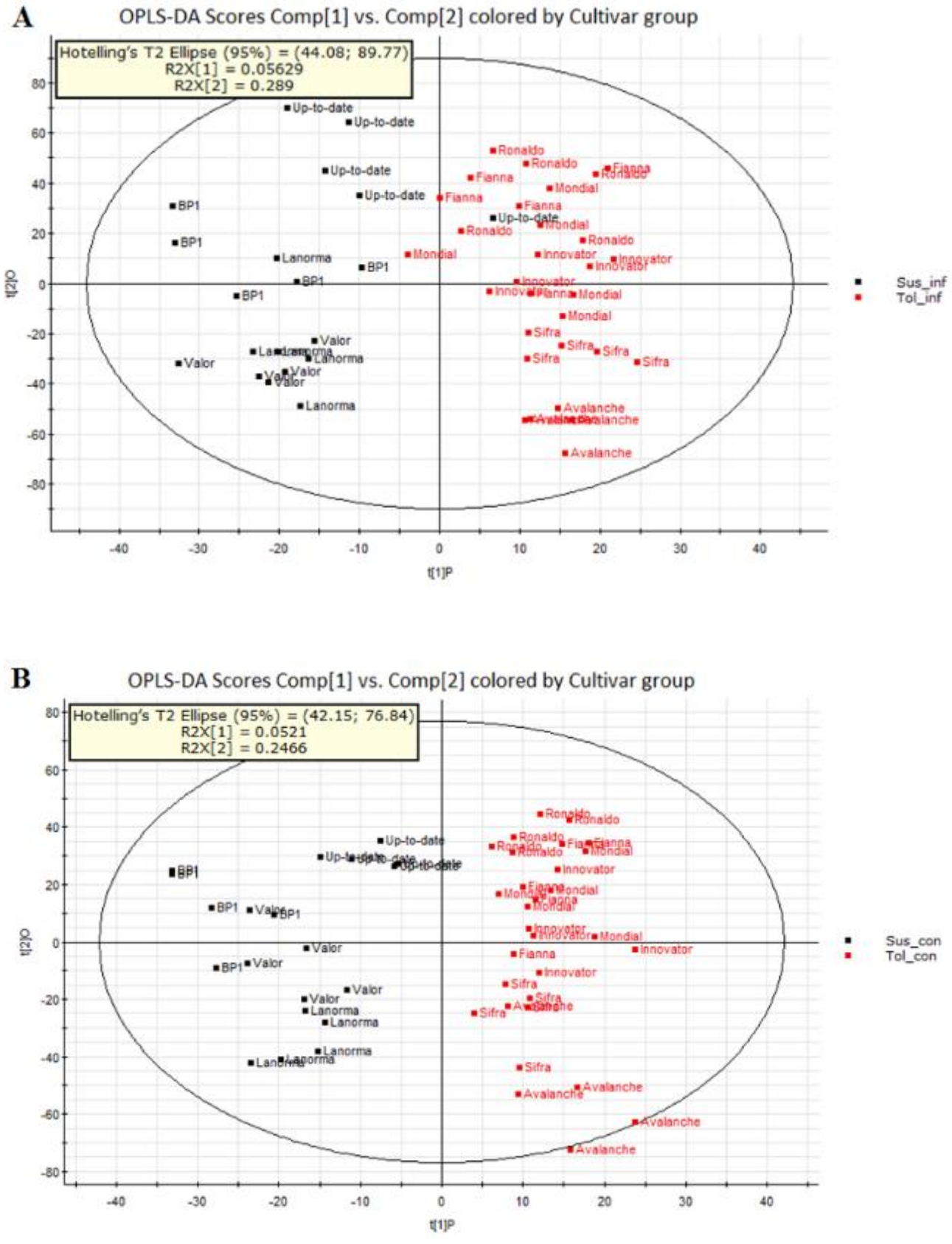


Figure 3A: OPLS-DA score plot of tuber extracts perfomed on negative ion mode, under two treatments (inoculated –A and un-inoculated –B) showing susceptible (black) and tolerant (red) cultivars.

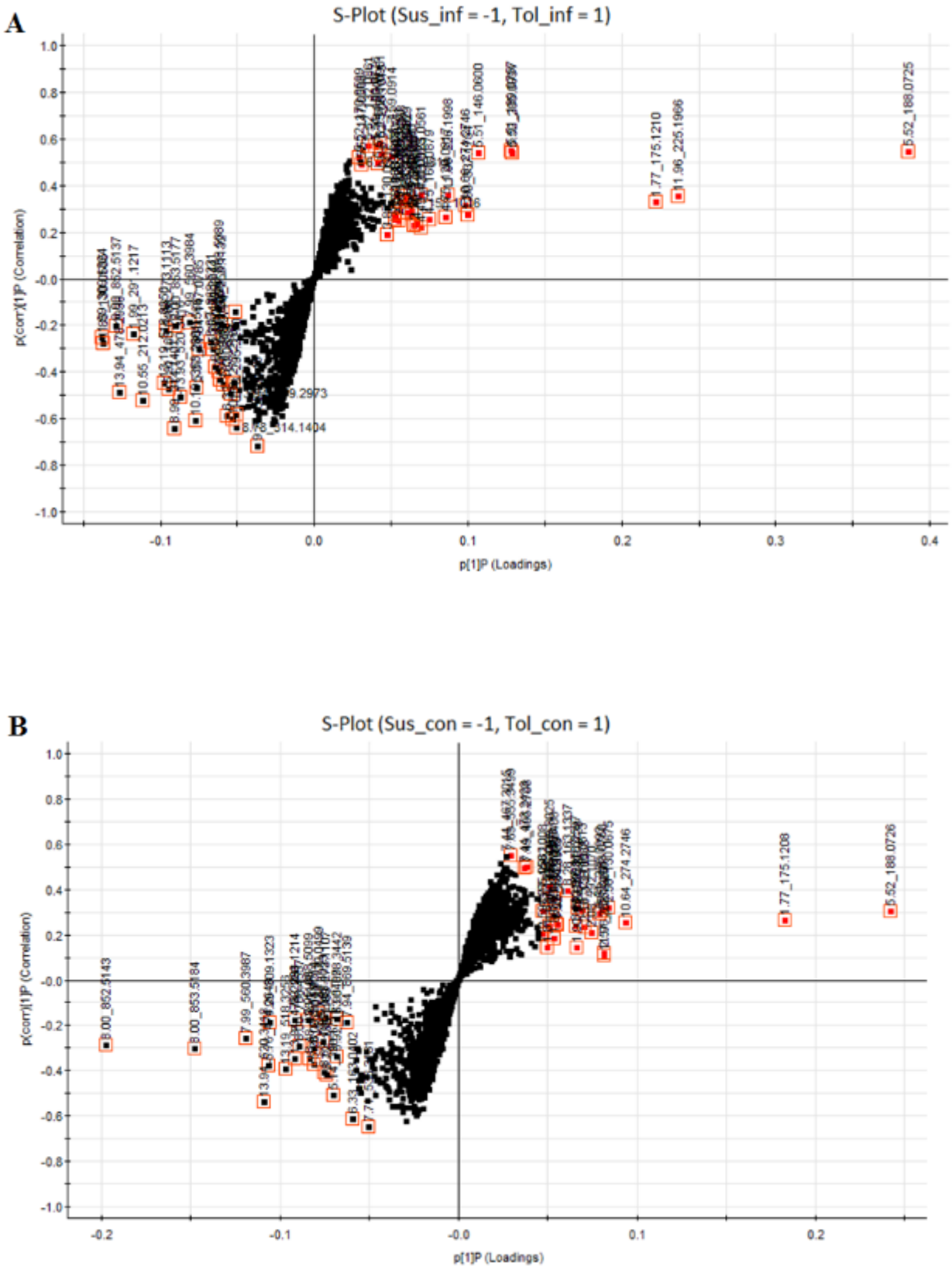


Figure 4A: OPLS-DA –based identification of discriminating biomarkers responsible for sample clustering perfomed on negative ion mode under pathogen treated and watered conditions showing susceptible (black) and tolerant (red) cultivars.

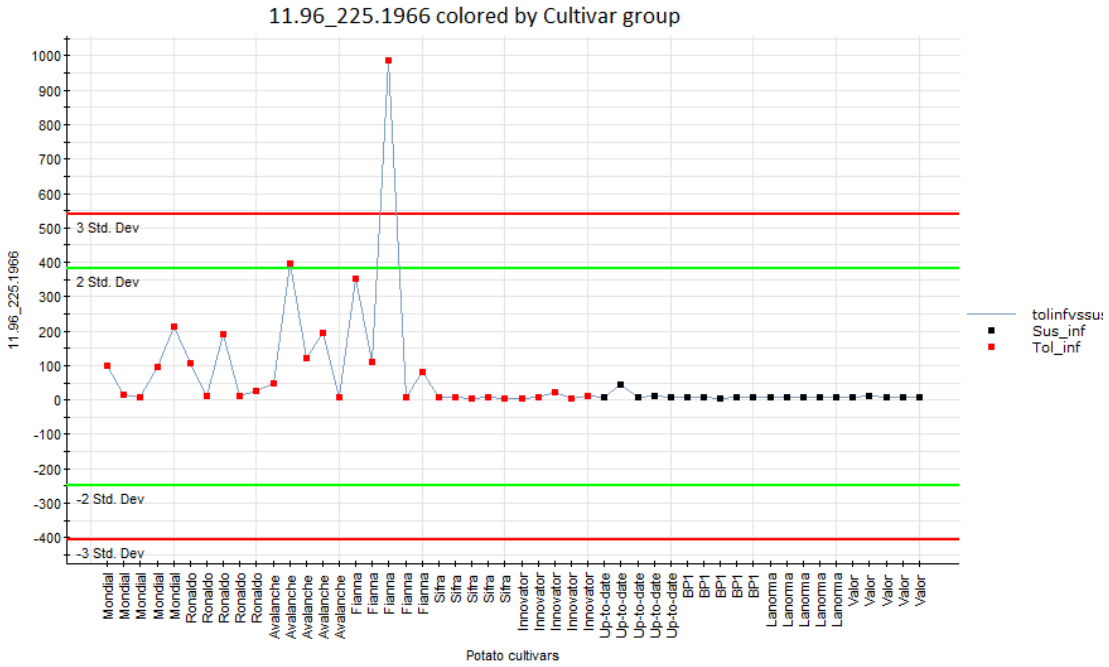


Figure 5A: Trend plot of Cuscohygrine obtained from inoculated samples analysed by (ESI+) UPLC-QTOF/MS. The plot displays the relative abundance of cuscohygrine found in the samples groups [tolerant (red) and susceptible (black)] included in the data sets. The marker with a retention time, 11.96 min and mass ion, 225.1966 m/z represents cuscohygrine. Hotteling's T2 range showing outliers grouping outside the 95% confidence interval.

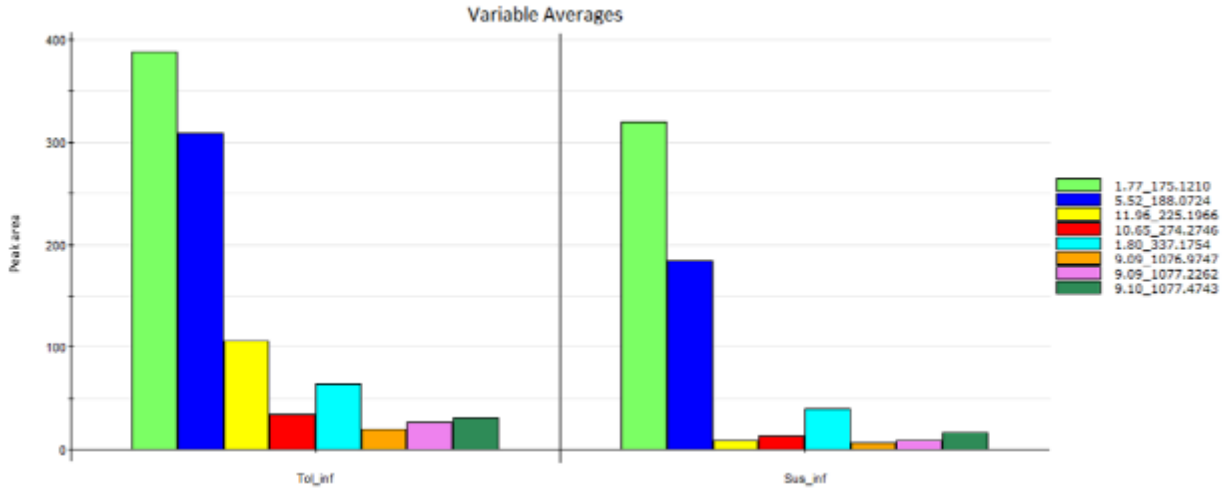


Figure 6A: Variable averages of group differentiating (Tol_inoculated and Sus_inoculated) potato tuber extracts. The different colours representing the unique features of retention time and mass ions generated from the susceptible and tolerant infected potato samples.

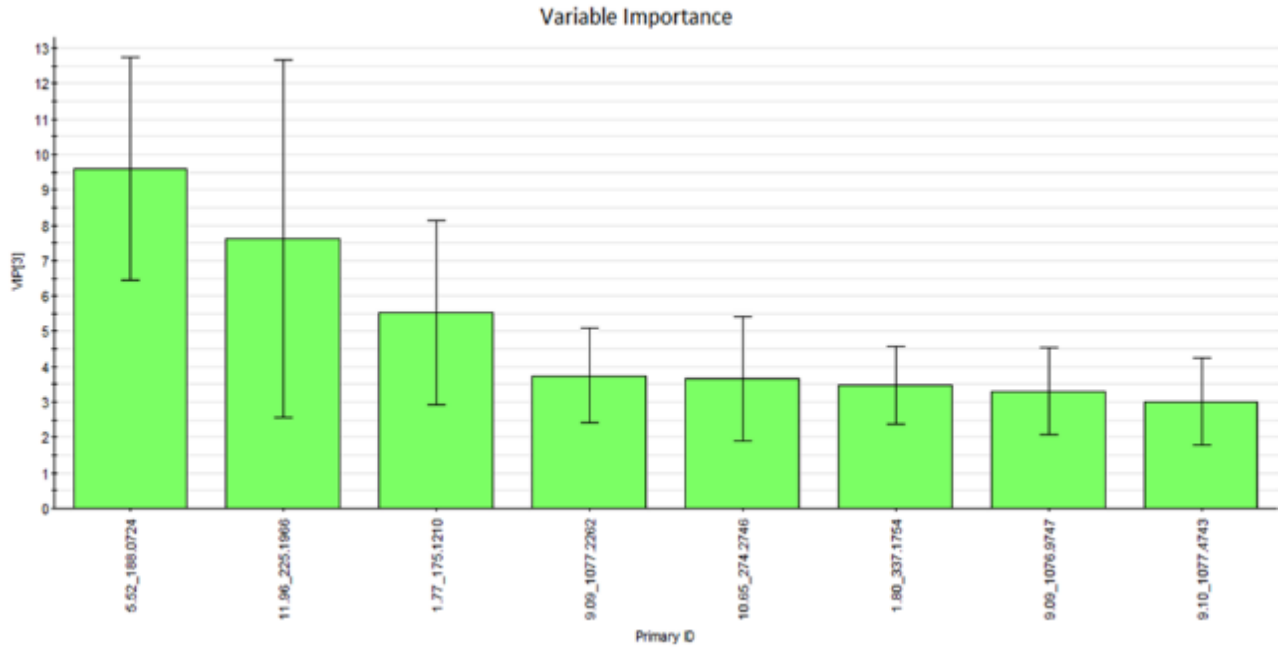


Figure 7A: Variable importance projection (VIP) plot of group differentiating (Sus_inf and Tol_inf) potato tuber extracts metabolites detected in positive mode of UPLC-TOF/MS. VIP variables of all metabolites were >1. The Error bars represents the mean ± S.E.M. with n=4 (susceptible) and n=6 (tolerant).

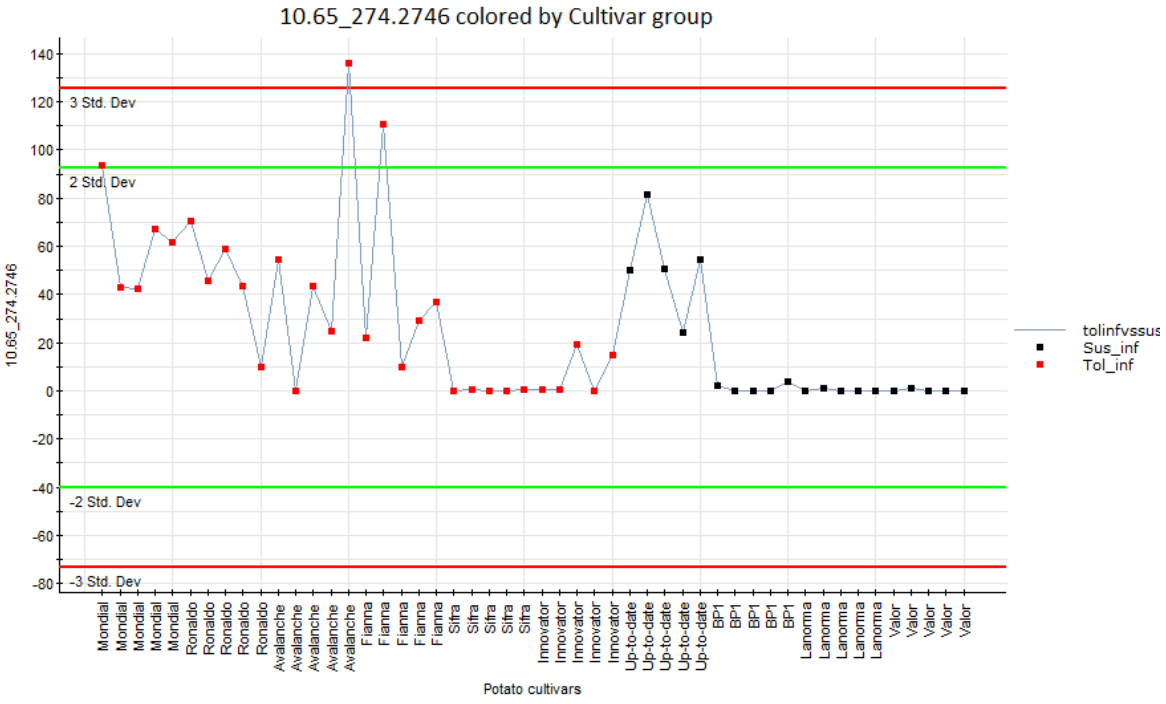


Figure 8A: Relative abundances of palmitic acid with the retention time of 10.65 min and mass ion of 188.0724 m/z, that differentiate between susceptible and tolerant cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea* infection analyszed by UPLC-QTOF/MS in positive ion mode.

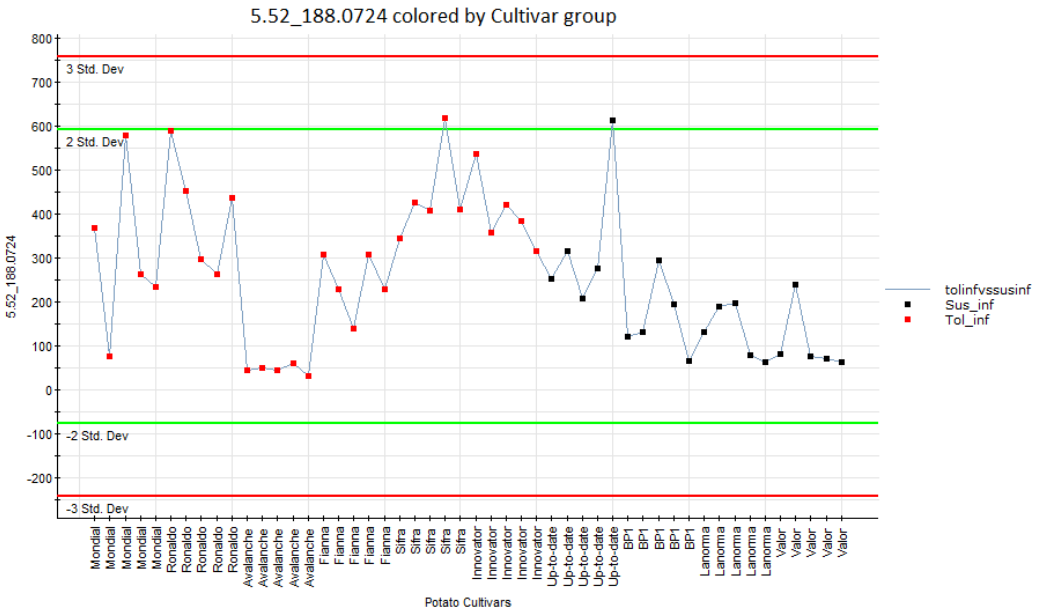


Figure 9A: Trend plot of Indole-3 acrylic acid with the retention time of 5.52 min and mass ion of 188.0724 m/z obtained from inoculated samples cultivars acquired in positive ion mode.

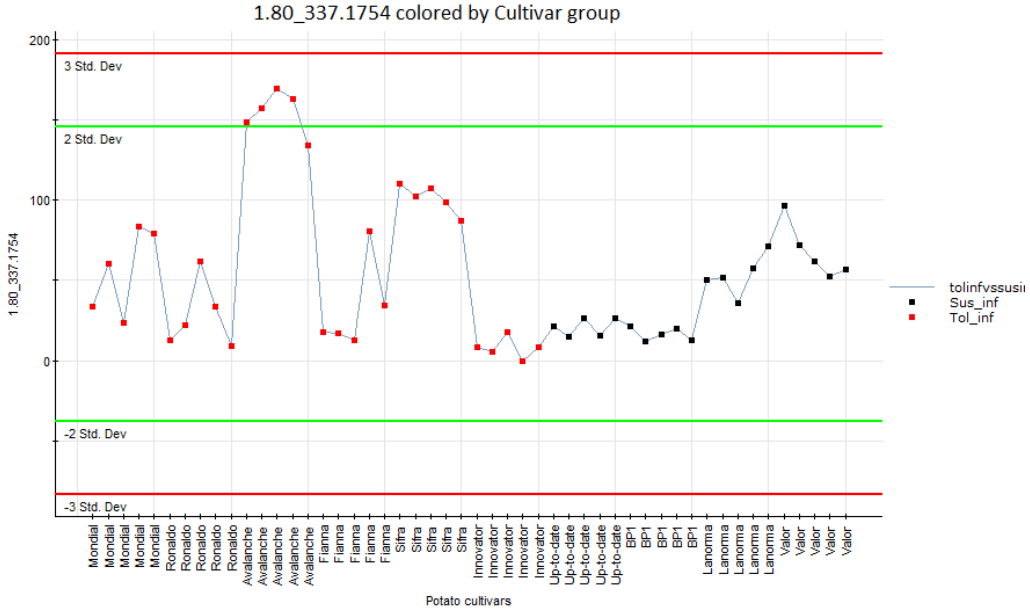


Figure 10A: The relative abundances of the unknown biomarkers with retention time of 1.80 min and mass ion of 337.1754 m/z identified in inoculated samples analysed by UPLC-QTOF/MS of positive ion mode.

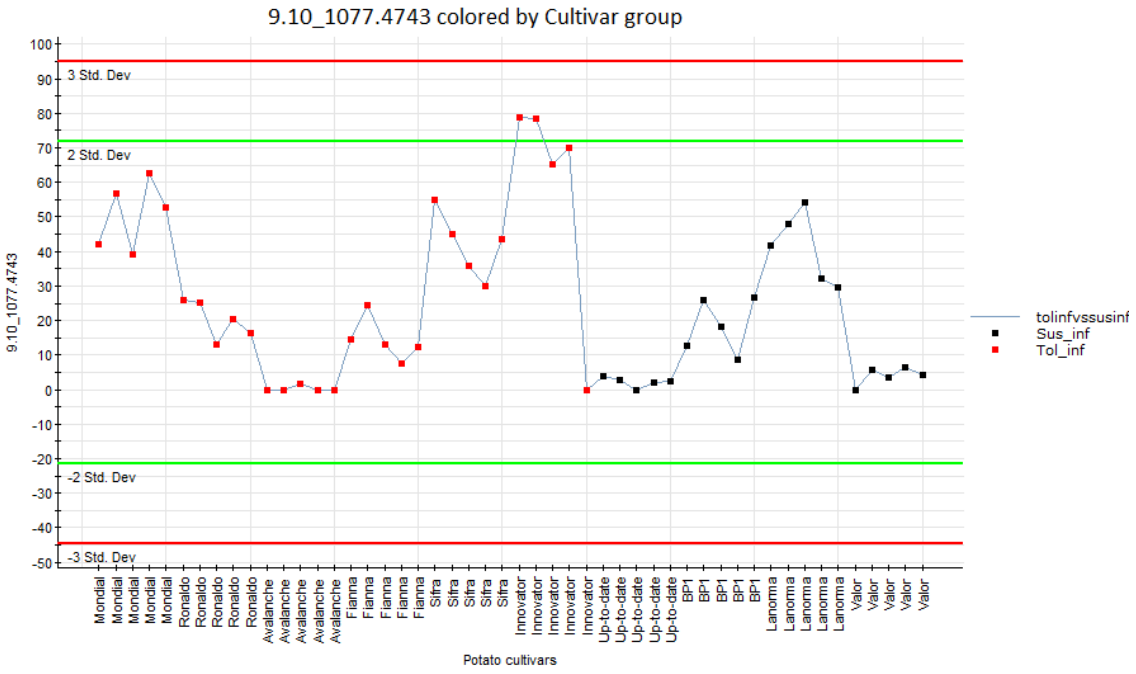
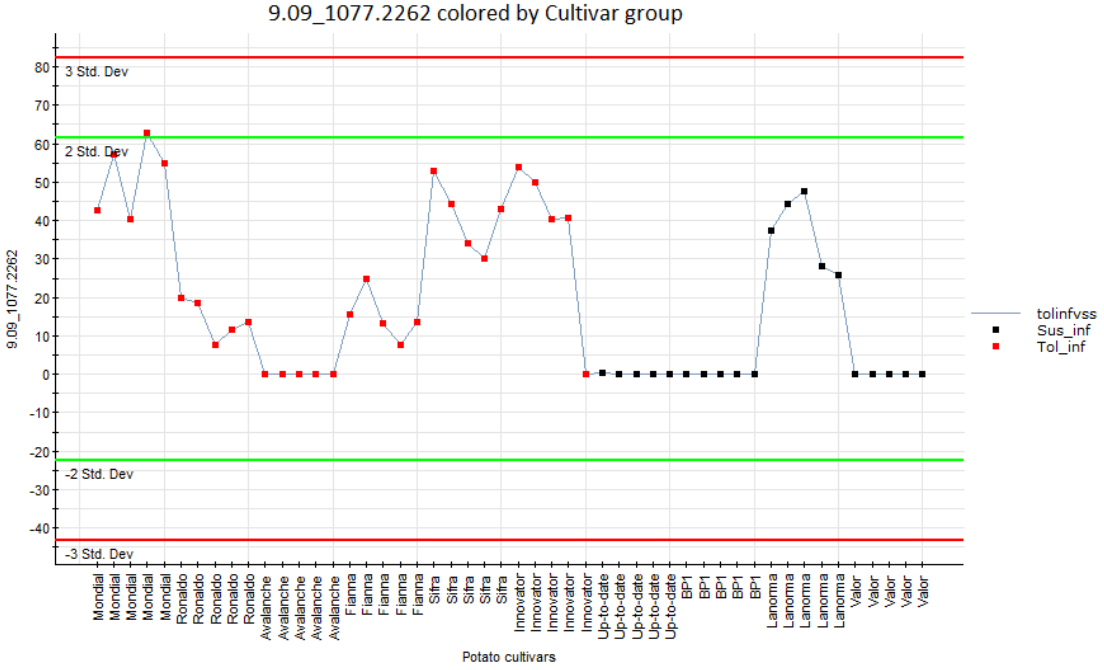


Figure 11A : The relative abundances of the unknown biomarkers with retention time of 9.10 min and mass ion of 1077.4743 m/z identified in inoculated samples analysed by UPLC-QTOF/MS of positive ion mode.



Figur 12A: The trend plot showing the relative abundace of the unknown biomarker with retention time of 9.09 min and mass ion of 1077.2262 m/z detected in positive ion mode.