# Citrus Rootstock Tolerance against *Phytophthora nicotianae*: a plant metabolomics approach

**By**

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## Declaration

I, Masiyiwa Ngoni Sakupwanya, declare that this thesis/dissertation, which I hereby submit for the PhD degree in Plant Pathology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE …….

DATE …….

## Abstract

The focus of this thesis is on the application of plant metabolomics methodologies to study citrus rootstock tolerance towards the root rot pathogen *Phytophthora nicotianae*. Initially, the tolerance of 16 citrus rootstocks towards the pathogen was assessed in greenhouse experiments. Rootstocks were categorised as tolerant, moderately tolerant and susceptible to the pathogen, during screening of pathogen inoculated *versus* healthy plants. The rootstocks Australian trifoliate, Benton citrange, Flying Dragon, Swingle citrumelo, Terra Bella citrumelo and Yuma citrange showed tolerance to *P. nicotianae* infection. Root materials from selected greenhouse experiments were subsequently used for the metabolomics studies where organic plant-root extracts were separated by means of UPLC/MS. In the first instance, MarkerLynx XS software was used to interrogate the citrus metabolome applying a metabolite fingerprinting analytical strategy. The markers associated with tolerant rootstocks included 259.0963, 313.1433, 327.1592 (*m/z*) putatively identified as Wyerone, 4'-prenyloxyresveratrol and Pulverochromenol respectively. This allowed us to find evidence from literature linking the markers with plant self defense in other crops, thus supporting the conception that they are related to tolerance in citrus rootstocks. The potential for acquiring resistance related metabolite markers was demonstrated over successive seasons. In the second instance a predictive model for rapid selection of tolerant rootstocks was developed. The predictive model formulation is a *de novo* and interesting outcome in this study especially for a non-model plant species such as citrus. To our knowledge this is the first report on the use of plant metabolomics to interrogate the citrus rootstock metabolome in association with *P. nicotianae* tolerance and for tolerance trait discovery for this plant-pathogen interaction. It is envisaged that these findings may enhance marker assisted selection for citrus rootstocks for local and international breeding programs. Proposed future work includes full annotation of the markers and working towards determining the function of the markers through biochemical pathway discovery.

**Keywords:** Biomarkers, Citrus rootstrock metabolome, Marker assisted breeding, Metabolite fingerprinting, *Phytophthora* root rot, Trait discovery.

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**Dedication**

For Myself, as I join the Doctors Urayayi and Tinoenda at the upper echelons of academic prowess.

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**List of Abbreviations**

| | |
|---|---|
| (*m*/*z*) | accurate mass (mass to charge) |
| ANOVA | analysis of variance |
| CV% | coefficients of variance |
| Da | Daltons |
| ESI | electrospray ionization |
| FAO | Food and Agriculture Organization |
| GC | gas chromatography |
| Ha | Hectare |
| HLB | Huanglongbing disease |
| HPLC | high performance liquid chromatography |
| ITS | internal transcribed spacer |
| LC | liquid chromatography |
| Mab | marker assisted breeding |
| Mg | Milligram |
| Mins | Minutes |
| mQTL | metabolite quality trait loci |
| MS | mass spectrometry |
| NMR | nuclear magnetic resonance |
| $^{O}C$ | degrees Celsius |
| OPLS DA | orthogonal partial least squared discriminant analysis |
| PCA | principle component analysis |
| PCNB | Pentachloronitrobenzene |
| QTL | quality trait loci |
| Rt | retention time |
| UPLC | ultra performance liquid chromatography |
| UPLC/MS-MS | ultra-high performance liquid chromatography-tandem mass spectrometry |
| v/v | volume/volume |
| Mg | Microgram |

**Statement**

During the course of this project, the author has presented oral presentations and has a published article.

**Presentation**: N.M. Sakupwanya, N. Labuschagne and Z. Apostolides 2014. Biochemical markers of *Phytophthora* tolerance in citrus rootstocks. 8th Citrus Congress, 17 to 20 August 2014, Champagne Sports Resort, Drakensberg, KwaZulu Natal, South Africa.

**Presentation**: Masiyiwa Ngoni Sakupwanya 5 July 2018. Prestige Seminar- Citrus rootstock tolerance against *Phytophthora nicotianae*: a plant metabolomics approach. SASPP Northern Brach Seminar, Plant Science Auditorium, University of Pretoria, Gauteng, South Africa.

**Publication**: Sakupwanya, N.M. Labuschagne, N, Loots, T., and Apostolides, Z. 2018. Towards developing a metabolic-marker based predictive model for *Phytophthora nicotianae* tolerance in citrus rootstocks. (Journal of Plant Pathology: Published article (https://doi.org/10.1007.s42161-018-0080-4).

# CHAPTER 1

## General Introduction

## 1.1 Background and motivation for the study

Citrus fruits have an important role in the human diet, which makes them a globally popular food commodity (Liu *et al.* 2012). Citrus is also widely utilized as a remedy for numerous ailments and is used for medical preparations more than the majority of other plants (Singh and Rajam, 2009). Commercial by-products resulting from citrus fruit production include pectin, oils and essences, as well as animal feed, lending to the commercial significance of the golden evergreen (Singh and Rajam, 2009; Liu *et al.*, 2012). Citrus fruit production is rated third most important after apples and banana fruits on a global scale (Singh and Rajam, 2009). The South African citrus industry plays a major and positive role in socio-economic enhancement of the nation. Up to 70 050 Ha of commercial tree production has resulted in a ranking of third largest global exporter of fruit (Citrus: World Markets and Trade 2018). Commercial citrus tree production is reliant on the graft union of two citrus species, scions and rootstocks (Saunt, 2000), of which the latter underpins profitable success (Castle, 2010; Albrecht *et al.*, 2016).

A major limiting factor to successful citrus fruit production comes about as a result of plant diseases affecting rootstocks (Matheron *et al.*, 1998; Graham and Feichtenberger, 2015). *Phytophthora* species cause several yield reducing diseases, wherever the commodity crop is commercially produced (Timmer and Menge, 1988; Saunt, 2000; Meitz-Hopkins *et al.*, 2014; Panabières *et al.*, 2016). *Phytophthora nicotianae* (Breda de Haan (syn. *P. parasitica* Dastur)), *P. palmivora* (E. J. Butler) and *P. citrophthora* (Smith & Smith) are primary infectious pathogens, responsible for damping off in citrus nurseries (Matheron *et al.*, 1998), and trunk, foot and root rot of trees in orchards around the world (Adaskaveg *et al.*, 2014; Meitz-Hopkins *et al.*, 2014; Graham and Feichtenberger, 2015).

Doidge (1925) provides the first information about *Phytophthora* associations with citrus in South Africa. *P. citrophthora* was the first among these pathogens reported on citrus, however today is mostly restricted to the Western Cape commercial citrus producing region of the country (Meitz-Hopkins *et al.*, 2014). *P. nicotianae* is the major causal factor in yield reduction with high percentage prevalence levels in orchards soils (Thompson *et al*., 1995; Burger, 2001; Meitz-Hopkins *et al*., 2014). It is the primary causal agent of fibrous root rot and tree decline in South African citrus nurseries and orchards (Thompson *et al.* 1995; Burger 2001; Meitz-Hopkins *et al.* 2014). The pathogen reduces tree vigor and health resulting in lowered fruit quality and yields which impact negatively on profits (Graham and Feichtenberger, 2015).

It is recognized and accepted that the use of pathogen tolerant rootstocks is the most sustainable means for *Phytophthora* disease management in the long term (Adaskaveg *et al.*, 2014). However the breeding and selection processes for developing new rootstocks with desirable agronomic traits is long and arduous (Castle, 2010). Furthermore, there is an increasing realisation within plant scientific communities, of an overall limited understanding of how plants as sessile organisms, actually defend themselves against stress factors (Hadacek, 2002; Steinberg, 2012; Pérez-Clemente *et al.*, 2013). In the case of the disease interaction between citrus rootstocks and *Phytophthora*, the previous point is further compounded by observations that no rootstocks are 100% immune to infection (Castle, 1987; Siviero *et al.*, 2006). Therefore, whilst citrus rootstocks are defined as tolerant as opposed to resistant (Graham 1990), the underlying factors that render greater tolerance to some rootstocks over others are not fully elucidated (Graham, 1995).

Greater efforts are therefore required to elucidate the mechanisms for tolerance in citrus rootstocks, using modern-day technologies to compliment traditional tolerance screening methods to better understand host-pathogen interactions (Talon and Gmitter, 2008; Singh and Rajam, 2009; Mochado *et al*., 2011). Talon and Gmitter, (2008) reported on the challenges associated with traditional breeding of citrus for disease tolerance, highlighting that advances in technology may alleviate these problems when correctly adapted. Further to the pathogenicity screening of citrus genotypes for the purposes of tolerance trait determinations in breeding programs, new technologies are at hand for exploration and exploitation by crop protection researchers (Talon and Gmitter, 2008; Machado *et al.*, 2011). The goal being to reduce the time required to develop new rootstock varieties with improved stress tolerance and desirable agronomic trials through trait discovery(Castle, 2010; Talon and Gmitter, 2008; Lucas, 2011).

Technological advances in mass spectrometry based chromatography instrumentation and online data analysis software, have resulted in new capacities for greater insights by crop protection specialists to better understand plant self defense (Sumner *et al.*, 2003; Hall, 2006; Fernie and Schauer, 2008; Allwood and Goodacre, 2010). These technologies have greatly increased the capacity of plant scientific communities to better interpret the flux of phytochemicals, culminating in the establishment of metabolome studies or plant metabolomics (Hadacek, 2002; Sumner *et al.*, 2003; Hall, 2006; Fernie and Schauer, 2008). For plant scientists, metabolomics tools are now available to holistically evaluate defense related phytochemicals both qualitatively and quantitatively (Sumner *et al.*, 2003; Kumaraswamy *et al.*, 2011). In this study, we investigate the possibilities for trait discovery and metabolite functionality elucidation through assessing the citrus rootstock metabolome applying plant metabolomics strategies. Changes in the metabolome occur as the end result of

changes in the transcriptome, that result from changes in the activities of enzymes, the proteome (Sumner *et al.*, 2003). Therefore, metabolome analysis is a valuable approach for inferring gene function for trait discovery in plant breeding (Sumner *et al.*, 2003; Fernie and Schauer, 2008; Evans *et al.*, 2009). Working backwards, metabolites that accumulate as a result of particular stress factors reveal the genes activated by the particular cell perturbation (Sumner *et al.*, 2003). In cases where the metabolites confer greater tolerance to the stress factor, scientists can identify biochemical markers, the pathways for tolerance and the genes responsible (Sumner *et al.*, 2003; Hamzehzarghani *et al.*, 2008; Kumaraswamy *et al.*, 2011). Furthermore, the concentration of particular metabolites in tolerant (resistant) cultivars over susceptible cultivars also points to better understanding of innate capacities to withstand biotic stress factors (Hamzehzarghani *et al.*, 2008; Kumaraswamy *et al.*, 2011). Figure 1.1, provides a visual schematic for plant metabolomics summarising the workflow pipeline.

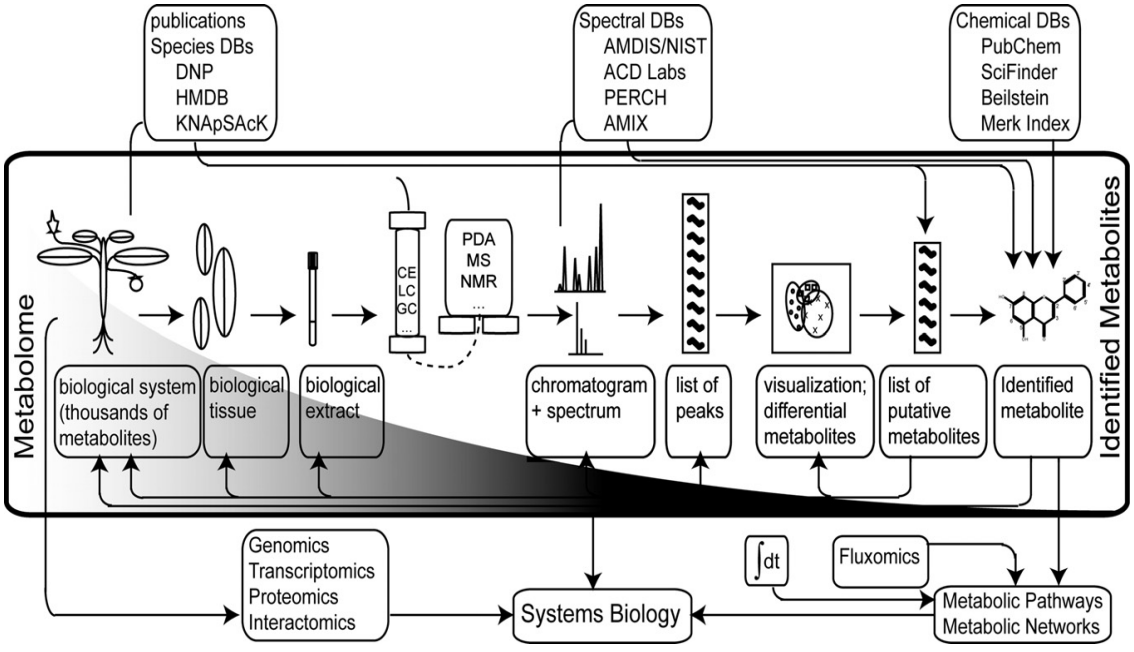


Figure 1.1: Metabolomics pipeline towards a systems biology approach: from the whole metabolome to identified metabolites. (Source: Moco et al., 2007).

Marshall and Powers, (2017) highlight the surge in scientific plant metabolomics investigations based on number of published research. Plant metabolomics approaches are being increasingly applied to investigate biotic factors negatively affecting plants (Tugizimana *et al.*, 2013), for example in wheat (*Triticum aestivum*) (Hamzehzarghani *et al.*, 2005) and in soya (*Glycine max*) (Sato *et al.*, 2013). Furthermore, profiling and fingerprinting analytical strategies of the plant metabolome are increasingly used in biomarker discovery, for example in potato (*Solanum tuberosum*) (Steinfath *et al.*, 2010) and in barley genotypes (Bollina *et al.*, 2011). In citrus pathology Albrecht *et al.*, (2016) used plant metabolomics applications to gain valuable information regarding Huanglongbing disease (HLB). As far as we know, there are no studies applying plant metabolomics studies to investigate the citrus rootstock metabolome in association with the root rot pathogen *P. nicotianae* or discover biomarkers for disease tolerance.

Several authorities on the study of plant metabolomics including Robert D. Hall, (Plant Research International, Wageningen University and Research Centre), credit Stephen G. Oliver with coining the term 'metabolome' for functional genomics (Hall, 2011). The metabolome consist of comprehensive data on quantification of metabolites extracted from plant materials (Sakurai *et al.*, 2014) Today metabolomics is the apogee of the Omics to follow the study of the genome - genomic, the transcriptome - transcriptomics and the proteome - proteomics (Patti *et al.*, 2013). The high specificity by which plants recognise pathogens and respond to suppress disease, implies that the small molecules which accumulate through constitutive or *de novo* synthesis, have a profound function in disease tolerance (Dixon, 2001; Garcion, *et al.*, 2007; Bednarek and Osbourn, 2009; Kumaraswamy *et al.*, 2011; Kushalappa and Gunnaiah, 2013). Studying the plant metabolome is therefore a modern means of identifying said phytochemicals, particularly defense related compounds.

Two categories of defense related metabolites proposed are pathogenicity and resistance related metabolites (Hamzehzarghani *et al.*, 2008; Kumaraswamy *et al.*, 2011). The identification of resistance related metabolites is punted to be a means of increasing the efficiency of crop breeding programs through biomarker discovery (Fernie and Schauer, 2008; Kumaraswamy *et al.*, 2011; Lucas, 2011). The identification of pathogenicity related metabolites will lead to greater understanding of resistance pathways (Kumaraswamy *et al*., 2011). We report on findings from our investigation satisfied through the following objectives.

### 1.2 Research Objectives

In this study, we outline a first attempt to gain a better understanding of the citrus rootstock-*P. nicotianae* interaction by studying the citrus metabolome as follows:

Phase 1: pathogenicity screening experiments, disease assessments and sample preparation. Phase 2: procedures entailing options for phytochemical extraction using organic solvent mixtures to produce crude extracts. Phase 3: options for extract separation methods and technologies, coupled to mass- spectrometry. Phase 4: data processing and analysis using online software leading to the identification of metabolites of interest. Each of the phases has its various options, and indeed limitations, all of which are dependent on the study objectives and hypotheses (Hall, 2006). More specifically our aims included analysing the citrus metabolome to detect small molecules potentially playing a role in disease tolerance against *P. nicotianae* in citrus rootstocks.

## 1.3 Project Outline

For this thesis, six chapters are presented summarized as follows.

Chapter 1 outlines a general introduction, in the form of background and motivation for the study.

Chapter 2 is an in depth review of the literature and touches on all important aspects regarding citrus, metabolomics for plant protection and the pathogen *P. nicotianae*.

Chapter 3 outlines the screening of a wide range of citrus rootstocks against the root rot pathogen under greenhouse conditions. The results inform us about the responses of the different rootstocks to root rot. Results highlight the rootstocks that are most tolerant as well as moderately tolerant rootstocks.

Chapter 4 describes, what is to the best of our knowledge, a first attempt to fingerprint the citrus metabolome to identify secondary metabolites related to plant self-defence. Metabolomics technologies and online software shed light on plant defense related secondary metabolites for this particular plant disease interaction.

Chapter 5 follows progress from the previous chapter to describe the development of a predictive model based on biomarkers calculated to discriminate between tolerant and susceptible citrus rootstocks.

Chapter 6 is a general discussion providing suggestions for future research resulting from the finding outline in this thesis.

## 1.4 Conclusion

The studies reported in this thesis provide information for the South African citrus industry in relation to *P. nicotianae* tolerance and significant new findings on the citrus rootstock metabolome. These findings have important implications for the citrus industry both locally and internationally regarding citrus root rot tolerance and marker assisted breeding/selection of rootstocks for *P. nicotianae* tolerance.

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# CHAPTER 2

## Literature Review

## 2.1 Background on Citrus

By 2004, the literature on *Citrus* was considered to be in chaos dispite having been described in written accounts emanating from 2500 years ago, in records from the peoples of China (Southeast Asia) and the subsequent large amouts of work carried out on the cash crop (Mabberley, 2004). Nicolosi, (2007), outlines early reports of *Citrus* species and their use in regions of China, indicating that some of the ancient citron varieties were considered to be lucky talismans, and thus named 'the hand of Buddha'. Davies and Albrigo, (1994), Saunt, (2000), Gmitter *et al.*, (2009) and Liu *et al.*, (2012), provide comprehensive literature discussing the history, distribution and uses of citrus fruits into the modern era. Singh and Rajam, (2009), provide some literature on optimal climatic conditions for citrus production, pests and diseases affecting the crop as well as tree improvement through biotechnology (somatic hybridization and genetic transformation). While the taxonomy of *Citrus* is in flux, the fruits of cultivated varieties are easily identified and also popular food commodities around the world (Gmitter *et al.*, 2009; Liu *et al.*, 2012).

Saunt, (2000) indicates that the initiation of budding practices from the 1840's, were as a direct result of the devastation caused by root rot pathogens, later identified as *Phytophthora* species. Therefore citrus fruits are commercially produced from the budding union of two trees, the rootstock and the scion. Citrus fruit production boomed from the 1960s increasing from 30 million tons to over 105 million tons at the end of 2006, with half this production being orange fruits (Saunt, 2000; Liu *et al.*, 2012). Commercial citrus production expanded to over 140 countries in that time, rendering the fruit globally available and popular for uses beneficial to human nutrition, as animal feeds and other industries (Saunt, 2000; Gmitter *et al.*, 2009; Liu *et al.*, 2012).

Commercial citrus fruit production is rated at the third most important after apples and banana fruits on a global scale (Singh and Rajam, 2009). Brazil and the United States of America lead the world's orange fruit production figures (Gmitter *et al.*, 2009; Singh and Rajam, 2009; Liu *et al.*, 2012), with averages of 17, 813 and 8, 217 million metric tons recorded respectively between 2007 and 2012. During the same period the European Union and Russia were the world leaders of orange fruit importers, averaging 866.6 and 520.8 metric tons respectively, with the Republic of South Africa satisfying most of their requirements, reportedly exporting 968.4 metric tons of fruit annually (Fig. 2.1).

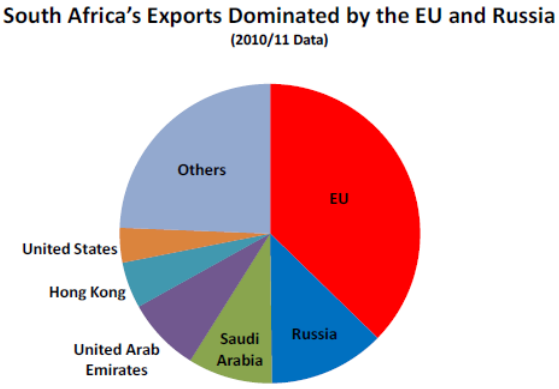


Figure 2.1. Dominance of South African orange fruit exports globally. Source: Foreign Agricultural Service, United States Department of Agriculture:- Citrus: World Markets and Trade 2013.

The South African export market is counted as the third largest worldwide, with up to 70 000 Ha planted to the crop (Citrus: World Markets and Trade 2018; Meitz-Hopkins *et al.*, 2014), with an emerging market in the USA through the Africa Growth Opportunities Act (AGOA). The nation is included among other major citrus fruit producing nations such as Argentina, China, Egypt, Mexico, Spain, and Turkey where tangerines, grapefruits, lemons and limes lead production over oranges (Gmitter *et al*., 2009; Liu *et al*., 2012). Web-based information provided by the Foreign Agricultural Service (FAS) on global citrus production is easily

accessible for example the United States Department of Agriculture:- Citrus: World Markets and Trade.

The importance and recognition of the range of citrus fruits particularly oranges, lemons, limes, grapefruits and tangerines is due to their characteristic aromatics and distinct juicy taste (Singh and Rajam, 2009; Gmitter *et al.*, 2009; Liu *et al.*, 2012) with the main production zones of the world indicated in Figure 2.2.

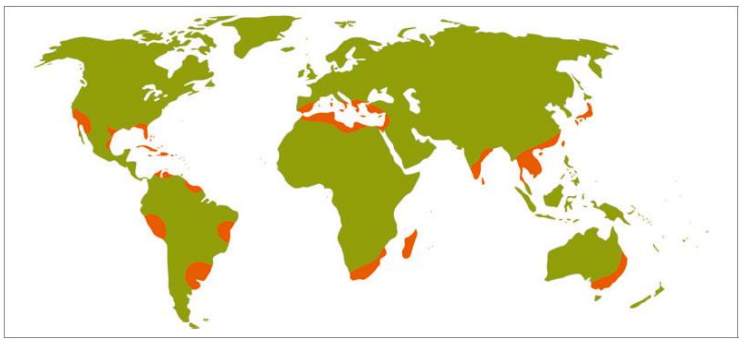

Figure 2.2. Highlighted in orange, the world leading citrus producing regions falling on either side of a belt around the equator covering tropical and subtropical areas of the world between 35◦N and 35◦S latitudes. Source: Liu *et al*., 2012.

The fruit are a form of specialized berry or hesperidia (Mabberley, 2004; Singh and Rajam, 2009), and are highly nutritious providing carbohydrates, dietary fibre, vitamins and antioxidants, all vital to human health with growing awareness as to the fruit's capacity to avert chronic ailments (Gmitter *et al.*, 2012; Liu *et al.*, 2012). The fruit can be eaten fresh once ripe or processed to prepare fresh or concentrated juices with orange juices constituting up to 85% of total processed production globally (Liu *et al.*, 2012). Commercial sub-products resulting from citrus fruit production include pectin, oils and essences, as well as animal feed, lending to the commercial significance of the golden evergreen (Davies and Albrigo, 1994; Singh and Rajam, 2009; Liu *et al.*, 2012). Mabberley, (2004) discuss use of citrus in human health matters for diseases suck as scurvy and AIDS.

As with most, if not all commercially produced agricultural plants, citrus has a plethora of biotic and abiotic factors negatively influencing profitable production. One genus of phytopathogenic organisms however, harbours species of pathogens that recurrently pose a very real threat to sustainable and profitable fruit production – the *Phytophthora* (Graham and Feichtenberger, 2015; Panabieres *et al*. 2016). The plant-pathogen interaction between citrus rootstocks and *Phytophthora nicotianae* will be the focus of the study and the following section provides a brief outline on scions and rootstocks that make up the bases of citrus fruit production under threat from *P. nicotianae*.

### 2.1.2 Citrus scions and rootstock

Citrus plants belong to the genus *Citrus L.*, subtribe *Citriae*, family *Rutaceae* and subfamily *Aurantioideae* (Saunt, 2000; Gmitter *et al.*, 2009). The economically important species of scions or fruiting citrus, cultivated around the world, include *Citrus sinensis* (L.) Osbeck (sweet orange), *C. reticulata* Blanko (mandarin), *C. x paradisi* Mac. f. (grapefruit), *C. limon* (L.) Burm. f. (lemon), *C. aurantiifolia* (Christm) Swing. (lime), *C. aurantium* (L.) (sour orange) and *C. grandis* (L.) Osbeck (pummelo) (Saunt, 2000; Mabberley, 2004; Singh and Rajam, 2009; Castle, 2010). In South Africa *Citrus sinensis* (Eureka lemons) and *Citrus reticulata* (Nules clementines) are the commonly cultivated fruiting citrus (Meitz-Hopkins et al., 2014).

Scions are budded to rootstock in nurseries, and Castle, (2010) highlights rootstocks that have formed the global portfolio for citrus industries over the last 100 years to date (Table 2.1).

**Table 2.4** Citrus rootstock world profile according to historic phases in research and development.

| | **Rootstock** |
|---|---|
| **Phase 1** **1900-1970** | Sour orange |
| **Phase 2** **1900-1970** | rough lemon, Volkamer lemon, sweet orange, Cleopatra mandarin |
| **Phase 3** **1970 – to date** | Trifoliate orange and hybrids including Benton, Carrizo, C-35 and Troyer citrange; Swingle citrumelo |
| ***Available here** **2011-2013** | Australian trifoliate & Flying dragon both trifoliate orange, Terra Bella, Esselen rough lemon, *Minneola* x *Trifoliate* hybrid; X639-hybrid |

**(* Rootstocks available for evaluation in this study. Phases 1, 2 and 3 Sourced from: Castle, 2010)**

Some important rootstock cultivars include *Citrus x aurantium* (L.) (sour orange), *C. jambhiri* (Lush) (rough lemon), Volkamer lemon, *C. sinensis* (L.) Osbeck (sweet orange), *C. reticulata* (Blanco) (Cleopatra mandarin), *Citrus* (*Poncirus*) *trifoliata* (Raf.) (trifoliate orange) and its hybrids the citranges and citrumelos, *Citrus macrophylla* and Rangpur lime (Davies and Albrigo, 1994; Castle, 2010; Roose, 2014). Other important citrus hybrids identified as potential rootstocks in research for screening and or commercial replacements include Tangor (sweet orange X tangerine) and Tangelo (tangerine X grapefruit) (Saunt, 2000; Singh and Rajam, 2009) and X639, a Cleopatra mandarin and trifoliate orange hybrid, produced in South Africa (Castle, 2010; Roose, 2014).

A surprising point in citrus breeding is that the majority of citrus planting material used commercially, did not arise as a result of systematic and targeted breeding strategies, but rather from bud sport mutations, spontaneously as seedling and/or by introduction and trials of materials from one location to another (Talon and Gmitter, 2008; Singh and Rajam, 2009).

Citrus is therefore counted among the most difficult plants to improve in terms of traits of agricultural importance, using conventional (Mendelian) methods (Talon and Gmitter, 2008; Castel, 2010). This results in a long and arduous breeding and selection period of between ten to twenty years for new line release for commercial use (Castle, 2010). This situation encourages citrus plant scientists to formulate cutting edge techniques to accelerate breeding efforts for improved cultivars (Talon and Gmitter, 2008; Albrecht *et al*., 2016). Plant pathologists increasingly investigate proteomics and metabolomics-based approaches as suggested by Talon and Gmitter, (2008) to improve citrus resistance towards pathogens while working in teams to select from a long list of desired agronomic traits (Table 2.2).

**Table 2.2** Summary of international rootstock-related citrus tree attributes sought after in citrus rootstock breeding.

| | Attribute | Remarks on attributes as a rootstock selection criterion |
|---|---|---|
| 1 | Yield | A major consideration |
| 2 | Precocity | A special factor as interest in higher-density plantings increases |
| 3 | Yield efficiency | Can be important, but not usually a selection criterion |
| 4 | Fruit quality | A major factor for fresh fruit growers |
| 5 | Fruit size | Important enough to be considered separately from quality |
| 6 | Juice quality | Brix and acid are affected; a major factor for growers of both fresh and processing fruit |
| 7 | Tree growth | Usually considered in terms of vigor and eventual tree size, a criterion that is increasing in importance |
| 8 | Compatibility | Scion-rootstock vegetative compatibility is often important |
| 9 | Ease of propagation | Largely a matter of seed production and degree of nucellar embryony |
| 10 | Shoot flushing | A new criterion related to Huanglongbing and spread by psyllids |
| 11 | Mineral nutrition | Not usually a selection criterion, but there are rootstock effects |
| 12 | Salinity | In some instances, an important selection criterion |
| 13 | Clay soil | High content or sol horizons can affect a rootstock decision |
| 14 | High soil pH | A very important factor with trifoliate orange-based rootstocks |
| 15 | Wet soil (flooding) | Not usually a selection factor but can be important |
| 16 | Drought | Modern irrigation methods usually preclude this as a selection factor |
| 17 | Cold (freezes) | Often considered in regions threatened by chronic cold |
| 18 | Citrus blight | At one time, a major consideration, but less so now in Florida |
| 19 | Phytophthora rots | Still an important factor in rootstock decisions |
| 20 | P. palmivora/root weevil complex | A problem specific to Florida involving a particular species of Phytophthora fungus and *Diaprepes* root weevil |
| 21 | Root weevils | A troublesome problem at times in some areas |
| 22 | Burrowing nematode | A problem in Florida for which specific rootstocks are used |
| 23 | Citrus nematode | A more universal problem with specific rootstock options |
| 24 | Tristeza virus | A serious threat with some rootstock options |
| 25 | Exocortis and xyloporosis viroids | Not generally a threat today with clean budwood |

**Source: Castle, 2010.**

Although both scions and rootstocks are critical to profitable fruit production, failure of rootstocks has been documented as having serious negative consequences, as they underpin successful citrus production (Castle, 2010; Albrecht *et al*., 2016). In our efforts to assess the host-pathogen interaction between a wide range of rootstocks and *P. nicotianae* the application of modern-day plant metabolomics technologies are investigated. The following

section in this chapter introduces the *Phytophthora* and outlines their importance as plant pathogens.

## 2.2 The Genus *Phytophthora*

### 2.2.1 Introduction

The genus *Phytophthora* has been at the forefront of plant disease interaction studies since the founding days of plant pathology by Heinrich Anton de Bary over 120 years ago (Laviola *et al.*, 1990; Kroon *et al.*, 2012; Meng *et al.*, 2014; Panabieres *et al*. 2016). Owing to their specialized biology, *Phytophthora* species are wide spread in soils and cause a wide variety of plant diseases on a tremendous number of hosts including herbaceous and woody species (Erwin and Ribeiro, 1996; Agrios, 1997; Judelson and Blanco, 2005; Nagel *et al.*, 2013). To date, the *Phytophthora* contain some 120 recognized member species, most of which are aggressive primary plant pathogens and this number is added to annually (Panabieres *et al*., 2016).

In trees and shrubs, the damage and subsequent losses caused by resultant root rot diseases are grave and often go undetected or unidentified (Tsao, 1990; Agrios, 1997). Because the pathogen affects the roots, plants typically exhibit symptoms of drought, with the diseases rendering infected plants more susceptible to colonization by secondary invaders, which are commonly mistaken as the cause of plant demise (Tsao, 1990; Agrios; 1997). Hardham, (2001) indicate that while some species such as *P. cinnamomi* have a broad host range, species such as *P. infestans* are pathogenic to a smaller range of host plants upon which they are regarded as highly destructive disease causing agents of economic importance (Erwin and Ribeiro, 1996; Andres *et al.*, 2006). In South Africa the root rot pathogen *P. nicotianae* is common focus for research groups investigating the myriad of threats the pathogen possesses.

### 2.2.2 *Phytophthora* classification

Commonly referred to as fungi (i.e. kingdom Fungi or Mycetae), the *Phytophthora* are actually in the domain Eukarya and more closely related to diatoms and brown algae (Fig. 2.3) which differ from true fungi in several biochemical, morphological and molecular characteristics (Hardham, 2001; Judelson and Blanco, 2005; Rossman and Palm, 2006; Blackman *et al.*, 2010; Meng *et al.*, 2014).

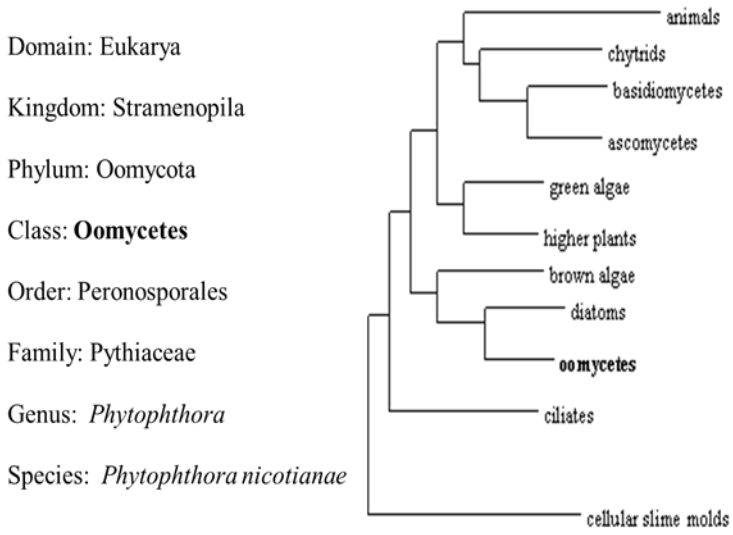


Figure 2.3. Taxonomic classification of *Phytophthora nicotianae* Breda de Haan 1896 and dendrogram of relationship with other Classes.

The genus *Phytophthora* falls under the Kingdom Straminipilia (Stramenopila); Phylum Oomycota, which are partially characterized by cell walls composed of small amounts of hydroxyproline, cellulose; a typical plant cell constituent and glucans (Judelson and Blanco, 2005; Rossman and Palm, 2006; Hardham and Blackman, 2010; Adaskaveg *et al.*, 2014; Meng *et al.*, 2014). Generally characterized as containing members that produce motile zoospores in zoosporangia and comprising sexual resting spores or oospores produced by the union of male (antheridia) and female (oogonia) fusion, these pathogens are highly adaptive

and successful (Judelson and Blanco, 2005; Narayan, 2010; Kroon *et al.*, 2012). The Family under which the genus *Phytophthora* belongs is the *Pythiaceae*, which harbors two notorious facultative parasites *Pythium* and *Phytophthora* falling under the Order Peronosporales (Fig. 2.3).

### 2.2.3 *Phytophthora* life cycle, biology and mode of infection

Figure 2.4 below, depicts a generic life cycle for the genus *Phytophthora*, including both sexual and asexual reproduction processes, and illustrates the terminologies associated with pathogen morphology.

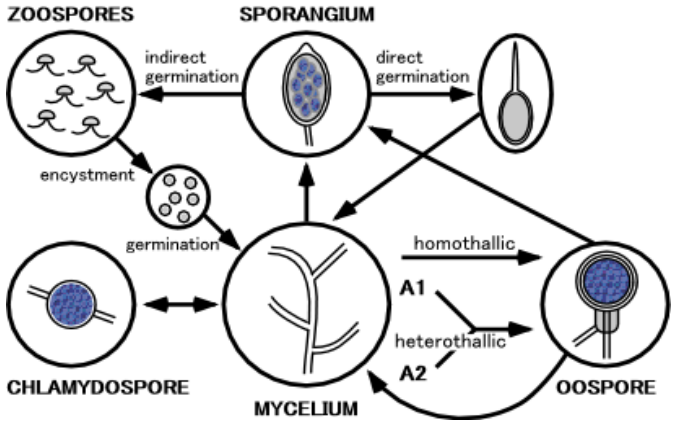


Figure 2.4. Generic *Phytophthora* life cycle: Also available in Meng *et al*., (2014). (Source: http://upload.wikimedia.org/wikipedia/commons/5/56/.).

The presence of mating types, results in the development of different races within a species, as with the races 0 & 1 of *P. nicotianae* (Kroon *et al.*, 2012; Meng *et al.*, 2014; Panabieres *et al*. 2016). *P. nicotianae* falls under clade 1 with the review of clade formation among the *Phytophthora* discussed by Kroon *et al.*, (2012). The asexual life cycle of *Phytophthora* may be summarized as starting from non-septate mycelia, which grow into papillae shaped sporangia (Judelson and Blanco, 2005; Meng *et al*., 2014). Asexual sporangia can germinate via two different pathways 1- direct germination and 2- zoosporogenesis. Direct germination

involves the emergence of plant infecting hyphae through the sporangium wall. Sporangia that do not germinate directly are zoosporangia, and contain plant infecting, motile zoospores that are released through the papilla (Judelson and Blanco, 2005). The asexual zoospores are motile in water and typically become encysted upon contact with host roots, where germination occurs, penetrating and infecting host plants (Judelson and Blanco, 2005; Raftoyannis and Dick, 2006; Hardham and Blackman, 2010).

During the sexual reproduction cycle, the fusion of mating types A1 and A2 of heterothallic species like *P. nicotianae* occurs (Graham and Timmer, 2004; Panabieres *et al*. 2016). This results in production of resting sexual spores called oospores which are capable of long periods of survival in soils (Graham and Timmer, 2004; Judelson and Blanco, 2005; Kroon *et al.*, 2012). Oospores can thus persist, typically forming within rotting plant tissues in soils to later germinate and produce either hyphal tubes or germ sporangia (asexual spores). The germ sporangia are borne on sporangiophores, emerging on the surface of plant debris or soils, develop into zoosporangia (Judelson and Blanco, 2005). As they mature, sporangia form an apical papilla from which the motile zoospores are released to seek-out fresh hosts to infect. Along with oospores, *P. nicotianae* can also produce another form of survival structures called chlmydospores, which can persist in plant debris and soils for years at a time under unfavorable environmental or plant infection conditions (Tsao, 1990; Erwin and Ribeiro, 1996; Panabieres *et al*. 2016).

*Phytophthora* translates to ‘plant destroyer’ and can be recognized by their typical oval or lemon shaped zoosporangia and well developed nonseptate-branching mycelia (Erwin and Ribeiro, 1996; Kroon *et al.*, 2012; Meng *et al.*, 2014). The spores of *Phytophthora* are

appropriately dubbed ‘the weapons’ of the plant destroyer by Judelson and Blanco, (2005) and all propagules are capable of host plant penetration leading to infection (Fig. 2.5).

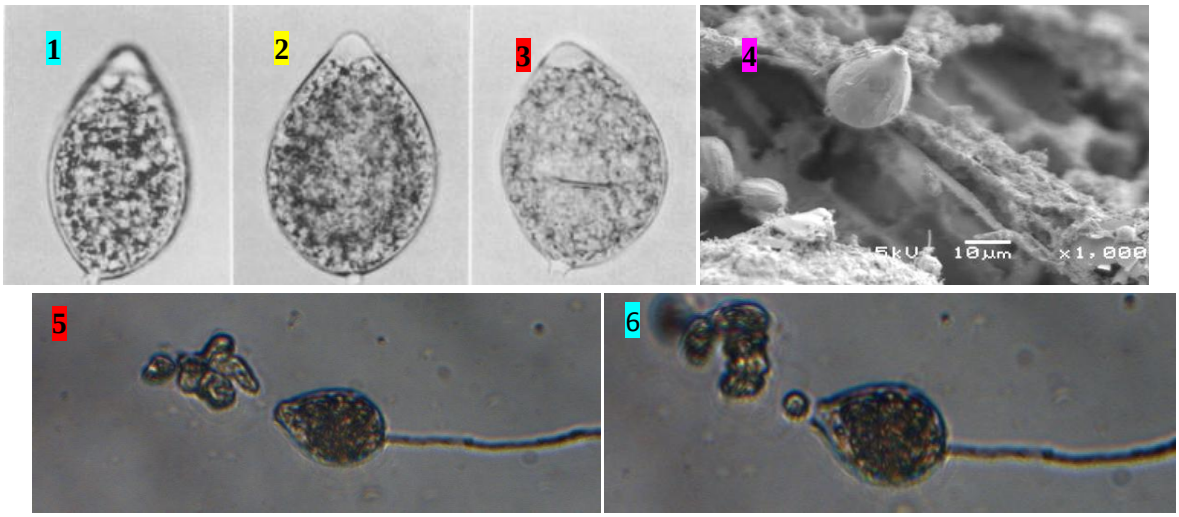


Figure 2.5. *Phytophthora nicotianae* sporangia. Top left (1, 2, 3), Source: Trichilo and Aragaki, 1982. Top right (4), Scanning Electron Micrograph of sporangium attached to citrus root. Below (5 and 6) zoospore-genesis (zoospore release), Allan Hall Microscopy Center University of Pretoria 2013.

The uni-nucleate zoospores of polycyclic *Phytophthora nicotianae* are counted as the principal dispersive propagule owing to their capacity for mobility, driven by two flagella which propel the infectious spores through soil and water (Hardham, 2001; Judelson and Blanco, 2005; Raftoyannis and Dick, 2006; Hardham and Blackman, 2010). Furthermore, zoospores exhibit two phenomena which add to their potency as motile infectious agents:

1. their capacity to detect gradients of a variety of compounds emanating from host roots including ions, amino acids and sugars resulting in their ability to be chemotactically and electrotactically attracted towards the source (Raftoyannis and Dick, 2006; Hardham and Blackman, 2010) and
2. their capacity for autoattraction or autoaggregation where motile spores move towards each other increasing infections frequencies (Hardham, 2007; Narayan *et al.*, 2010).

A further facet to the success of the *Phytophthora* is their capacity to first establish within host tissues as biotrophs by means of a network of haustoria but then adapting to a more necrotrophic type of growth or mode of action as the host triggers the hypersensitive response (Judelson and Blanco, 2005; Hardham and Blackman, 2010; Boava *et al.*, 2011). This form of trophism is referred to as hemibiotrophic and presents a different set of challenges for researchers investigating diseases caused by pathogen in the genus *Phytophthora* (Hardham and Blackman, 2010; Boava *et al.*, 2011). Figure 2.6 below, (viewing from top right), illustrates the mode of infection of motile zoospores, which are chemotactically attracted to the root surface where they settle and encyst with their ventral surface facing the root (Judelson and Blanco, 2005; Hardham and Blackman, 2010; Narayan *et al.*, 2010).

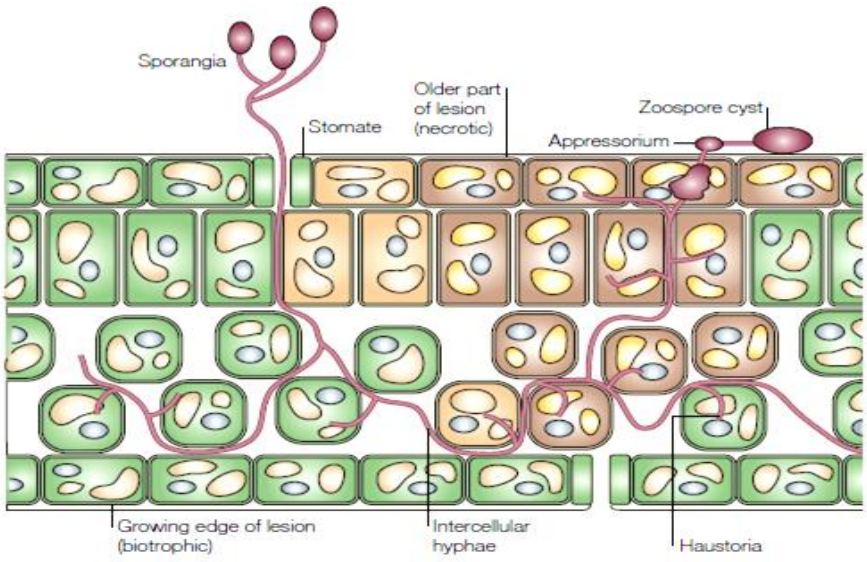


Figure 2.6. Schematic illustration showing the infection cycle for a soil borne *Phytophthora* species establishing disease in a root of a susceptible plant. (Source: Judelson and Blanco, 2005).

Adhesive material is secreted during the first few minutes of encystment (Judelson and Blanco, 2005; Meng *et al.*, 2014). The cyst germinates and the germ tube penetrates the epidermis intercellularly or intracellularly (Fig. 2.6) (Judelson and Blanco, 2005). Within 2–3

days under optimal temperature conditions, multinucleate sporangia develop on the root surface and cleave to form uninucleate zoospores that are released through an apical pore in the sporangium (Judelson and Blanco, 2005; Siviero *et al.*, 2006). Ultimately the *Phytophthora* have evolved the means to suppress or evade the hypersensitive response, countering the plant's defense mechanisms (Jones and Dangl 2006; Hardham and Blackman, 2010; Boava *et al.*, 2011).

### 2.2.4 *Phytophthora nicotianae* root rot in citrus

Citrus trees within the different global regions of citrus production are attacked by different species of the pathogen alone, in a complex with another *Phytophthora* or other pathogens (Matheron *et al.*, 1998; Graham and Feichtenberger, 2015). *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur), *P. palmivora* (E. J. Butler) and *P. citrophthora* (Smith & Smith) are primary pathogens responsible for damping off in citrus nurseries and trunk, foot and root rots of trees in orchards around the world (Widmer *et al.* 1998; Graham and Timmer, 2004; Savita *et al.*, 2012; Adaskaveg *et al.*, 2014; Meitz-Hopkins *et al.*, 2014; Graham and Feichtenberger, 2015). *P. nicotianae* is reported to be more common in the tropical and subtropical citrus regions such as the citrus growing regions of Brazil (Mourao *et. al.* 2008; Boava *et al.*, 2011; Graham and Feichtenberger, 2015), Florida (Graham, 1990; Timmer *et al.*, 1991; Graham and Feichtenberger, 2015), Egypt (Ahmed *et al.*, 2012) and South Africa (Burger, 2001; Thompson *et al.*, 1995; Nagel *et al.*, 2013; Meitz-Hopkins *et al.*, 2014) causing root rot and tree die-back.

*P. nicotianae* has adapted to such warmer conditions where its optimal temperatures for growth prevail and allow it to grow, out competing other *Phytophthora* species (Siviero *et al.*, 2006; Panabières *et al.*, 2016). The early infection stages of root rot in feeder or fibrous roots

of citrus rootstocks, is characterized by soft water-soaked lesion formations followed by disintegration of the outer cortex or sheath of the roots which impairs uptake of water and nutrients (Graham and Timmer, 2004; Adaskaveg *et al.*, 2014; Graham and Feichtenberger, 2015). Rot results in white thread-like stele protruding from decaying tissues characterizing the latter stages of the disease (Adaskaveg *et al.*, 2014). In mature trees, root rot results in tree decline characterized by foliage yellowing, leaf drop and die-back of twigs and branches (Graham and Timmer, 2004; Adaskaveg *et al.*, 2014; Graham and Feichtenberger, 2015). The pathogen reduces tree vigour and health resulting in lowered fruit quality and yields that impact negatively on profits (Graham and Feichtenberger, 2015). *Phytophthora* often go unidentified as primary causal agents of tree decline disease allowing propagule build up and dissemination (Tsao, 1990).

Pathogenic *Phytophthora* species are the most important primary causal agents of rot in citrus causing several diseases, of which trunk rot (gummosis) and root rots are considered to be the most relevant from the perspective of both research and industry (Laviola *et al.*, 1990; Mourao *et. al.* 2008; Castle, 2010; Adaskaveg *et al.*, 2014; Graham and Feichtenberger, 2015). Through scientific observations and grower feedback, researchers respond and focus their efforts to select the most desirable citrus rootstocks from the range of planting material being developed for their prevailing environment (Castle, 2010; Roose, 2014).

### 2.2.5 Management of *Phytophthora* root rot diseases in Citrus production

The world-wide nature and economic importance of citriculture and its by-products, has resulted in well-established lists of recommendations for the management of gummosis and root rot caused by the *Phytophthora* as outlined for example by Matheron *et al.*, (1998) and Gade, (2012) for nursery disease management. Many modern approaches rely on integrated management strategies including biological controls and other soil augmentations (Gade, 2012) and highlight the importance of water management to restrict the *in situ* spread of infectious spores (Panabières *et al*., 2016). Owing to the dispersal of *P. nicotianae* zoospores in water, irrigation management is highlighted as an important means for disease mitigation (Adaskaveg *et al.*, 2014; Graham and Feichtenberger, 2015; Panabieres *et al*. 2016).

The use of systemic fungicides applied as either soil or foliar treatments is effective at controlling *Phytophthora* root rot and gummosis (Adaskaveg *et al.*, 2014). It has been established that in citrus groves treated with long term fungicide applications against *P. nicotianae*, fruit yield and quality improved, with increased fibrous root densities compared with untreated groves (Timmer *et al.*, 1991; Graham, 1995; Colburn and Graham, 2007). Fungicide applications therefore significantly reduce populations of the pathogen in soil, inferring that fibrous root rot does indeed result in yield reduction in citrus (Graham, 1995; Colburn and Graham, 2007). In cases where susceptible rootstock are planted and in situations with poor drainage, fungicide applications may become uneconomical (Adaskaveg *et al.*, 2014; Panabieres *et al*. 2016). The use of synthetic agrochemicals to mitigate the effects of plant disease agents is the standard 'silver-bullet' for large-scale producers, however the practise is expensive and environmentally damaging in the mid to long term (Panabieres *et al*. 2016). Graham and Feichtenberger, (2015) place emphasis on management approaches that also consider disease complexes between the *Phytophthora* and other pests

and disease. Furthermore, because *P. nicotianae* favours warmer climatic conditions compared to *P. citrophthora*, it is most likely to better adapt to global warming worsened by the anthropogenic activities of human-kind and cause new plant disease problems (Panabieres *et al*. 2016). This adds great impetus to plant scientists for the discovery and development of feasible disease mitigation strategies from applied and fundamental research perspectives.

Global biological control and integrated-pest management research programs have developed commercial products to meet the challenges of an over reliance on synthetic crop protection products. However, identifying more sustainable, long term or durable resistance in crop cultivars requires more diligently approaching plant disease management. Adaskaveg *et al.*, (2014) note that "the first line of defense against root rot caused by *P. nicotianae* is the use of more tolerant citrus rootstocks". The following section outline new technologies and strategies to better elucidate the mechanisms involved in plant defense against plant pathogens.

### 2.3 Metabolomics for Plant Protection

Studies are continually revealing that in plants, innate immunity is based on a surprisingly complex response that is highly flexible in its capacity to recognize and suppress different invaders or stress factors driven by metabolism (Dixon, 2001; Sumner *et al.*, 2003; Jones and Dangl, 2006; Pieterse and Van Loon, 2007; Duque *et al.*, 2013;). The detectible changes of small molecules can be measured on multiple levels using omics strategies, which are helping to better elucidate the complex phenomena of plant immunity (Hall, 2006; Patti *et al*., 2013; Johnson *et al*., 2016).

In metabolomics, technological advances in liquid and gas chromatography coupled with mass spectrometry detectors, accompanied by online data analysis software (Carreno-

Quintero *et al*., 2012), have allowed plant pathologists to collect and analyse metabolite profiles resulting from particular stress factors and work backwards to reveal the genes activated by the specific cell perturbation agent (Sumner *et al.*, 2003; Duque *et al.*, 2013; Kell and Oliver, 2016). From plant-pathogen interaction studies, researchers can now determine discriminating characteristics from metabolome fingerprints between plant genotypes, aiming to identify defense related metabolites (Kumaraswamy *et al*., 2011; Albrecht *et al.*, 2016).

Defense related metabolites can be either

i) pathogenicity related: measured through detectable changes (relative abundance/fold change) in disease suppression related compounds found to accumulate in tolerant plants over susceptible plants after inoculation (Bollina *et al.*, 2010; Kumaraswamy *et al.* 2011; Chamarthi *et al.*, 2014) or

ii) resistance related: measured through detectable changes (relative abundance/fold change) in metabolites unique to or upregulated in healthy tolerant cultivars as opposed to susceptible cultivars (Bollina *et al.*, 2010; Kumaraswamy *et al.* 2011; Chamarthi *et al.*, 2014).

Identification of such metabolites can therefore be used to advance our knowledge regarding plant resistance mechanisms or as biological markers for the accelerated selection of genotypes (Hamzehzarghani, *et al*. 2005; Fernie and Schauer, 2008; Steinfath *et al.*, 2010; Wolfender *et al.*, 2013; Johnson *et al*., 2016). Of major importance within the plant science community are the exciting prospects to reduce time consuming components of crop improvement through application of metabolomics strategies (Fernie and Schauer, 2008) (Fig. 2.7).

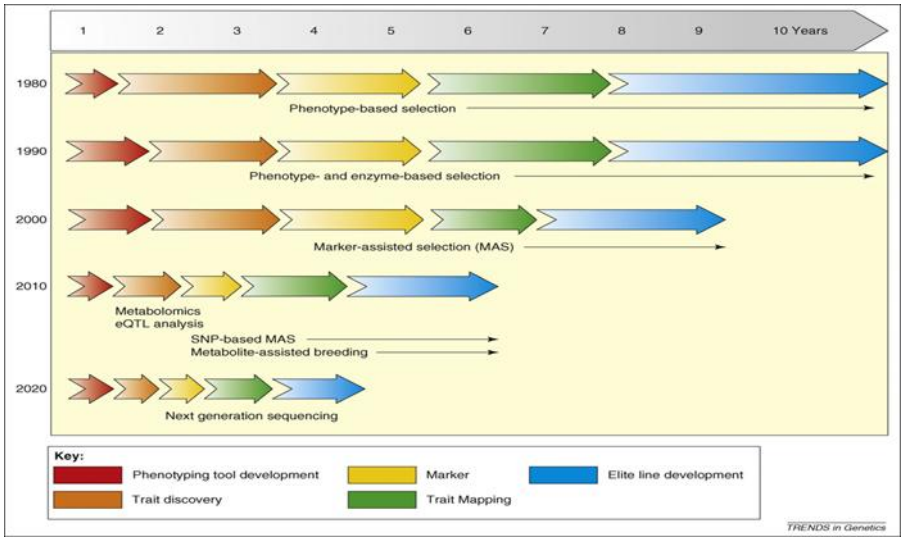


Figure 2.7. Breeding technology pipeline from past to present to future. The breeding pipeline from 1980 to that envisaged in 2020. In the past, trait discovery was mainly based on phenotypic observations, whereas marker development was restricted to phenotypic or enzymatic or protein markers. Thus, trait mapping and elite line development was a laborious task. The technological advances of molecular biology in the 1980s and 1990s enabled the application of molecular markers and improved the speed of trait mapping and commercial material development. Today, the application of marker assisted selection in combination with new -omics approaches, such as metabolomics or transcriptomics enabled rapid discovery of new traits and allelic variation and, thus, improves the time to market by several years. (Source: Fernie and Schauer, 2008).

Both primary and secondary plant metabolites hold promise, whether using gas or liquid chromatography, when exploring phenotype resistance (Kumaraswamy *et al*., 2011; Carreno-Quintero *et al*., 2012). Some platforms include both LC and GC instrumentation linked to MS detectors, while some approaches use nuclear magnetic resonance (NMR) either as stand-alone or merged with mass spectrometry (Ernst *et al*., 2014; Marshall and Powers, 2017). While chromatography technologies continue to evolve, so too do mass spectrometry and computational data analysis technologies which remain an integral component in plant metabolomics (Evans *et al*., 2009; Allwood and Goodacre, 2010; Tautenhahn *et al*., 2012; Wolfender *et al*., 2013; Gowda *et al*., 2014; Marshall and Powers, 2017). All expanding the

capacity for plant scientists to better use and understand biochemical information to interrogate the plant phenotype (Carreno-Quintero *et al*., 2012; Kell and Oliver, 2016).

Hamzehzarghani *et al*. (2005) were among the first to apply plant metabolomics technology for the disease interaction between wheat and the Fusarium head blight pathogen, *Gibberella zeae* (anamorph *Fusarium graminearum* Schw.). Their experimental designs allowed for the capture of information on metabolites unique to particular treatments; making it possible to discriminate between cultivars, between tolerant and/or susceptible wheat cultivars of uninoculated plants, as well as induced metabolites, when comparing the metabolome of inoculated *versus* uninoculated plants using untargeted metabolomics approaches (Hamzehzarghani *et al*. 2005). Resultant GC/MS analysis also revealed novel metabolites and metabolites that were up or down regulated during the host-pathogen interaction, further highlighting the complicated response by plants to pathogen stress (Hamzehzarghani *et al*. 2005). Trait discovery highlighted the importance of metabolomics applications to advance scientific understanding of the disease interaction (Hamzehzarghani *et al*. 2005).

Major findings from their research demonstrated the efficacy of plant metabolomics approaches to reduce the time consuming components of screening trials, through identification of metabolite quality trait loci (mQTLs) (Hamzehzarghani *et al*., 2005; (Hamzehzarghani *et al*., 2008; Fernie and Schauer, 2008). Such markers are envisaged as a means to minimize the time required to develop elite lines through biomarker discovery (Hamzehzarghani *et al*. 2008; Lucas, 2011). Fernie and Schauer, (2008) highlight the surge of marker assisted trait discovery applying omics approaches in crop protection and importantly, their potential to vastly improve on the time to market periods currently faced by plants breeders (Fig. 2.7). The potential for elucidating biochemical mechanisms for resistance in plants through metabolomics applications is considerable (Johnson *et al*., 2016; Marshall and

Powers, 2017). Scientific capabilities to interpret and utilize the large data generated from LC/MS untargeted plant metabolome studies are strengthened through a growing list of online processing software (De Vos *et al*., 2007; Carreno-Quintero *et al*., 2012).

Approaches to data analysis are therefore added facets to plant metabolomics that continue to see a growing demand for improvement and easy accessible software online owing to the large size of metabolomics data sets which can only be addressed through computational statistics (Tugizimana *et al*. 2013; Witzel *et al*. 2015). A growing arsenal of statistical tools and options are available online with Tautenhahn *et al*., 2012 and Gowda *et al*., (2014) discussing the web-based platform for XCMS, which was designed to process and visualise metabolomics data. Tugizimana *et al*., (2016) discuss MarkerLynx XS, the data processing packed deliver through MassLynx (ver 4.1, Waters). They provide vital review on pre-processing and pre-treatment steps for large metabolomics data during data mining. There are generally two steps to approaching MS metabolomics data sets; the first requires initial data processing and the second requires data analysis (Sakurai *et al*., 2014; Witzel *et al*., 2015 Tugizimana *et al*., 2016). Data processing involves alignment, feature or variable detection, filtering and normalization, while data analysis involves algorithm selection, evaluation and model examination (Witzel *et al*., 2015; Kumar *et al*., 2017). Mahieu *et al*., (2016) note the importance of the evolving bioinformatics software and compatibility with other metabolomics technologies for data processing. Figure 8 summarises the data mining strategy to analyse metabolomics data where:

a. initial pre-processing from data acquisition to generation of metabolome data in the form of sample list in MarkerLynx XS for example (Sakurai *et al*., 2014; Tugizimana *et al*., 2016)
b. data mining, hypothesis generation and results visualization (Sakurai *et al*., 2014)

c. sorting and dissemination of the data for further analysis including annotation, further profiling and or fingerprinting (Sakurai *et al*., 2014)

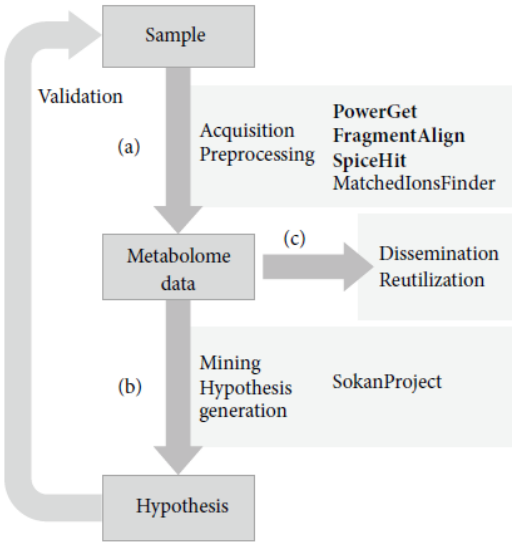


Figure 3.8. Typical workflow pipeline for plant metabolomics data handling (modified from Sakurai *et al*., 2014).

Various data analysis techniques including *t* tests, univariant (one or two factor ANOVA) and multivariate are used during data analysis to fingerprint metabolites (De Vos *et al*., 2007; Fernandez *et al*., 2016). Data are mean-centred to put all data on equal footing and Pareto scaled to adjust for measurement errors prior to multivariate analyses (Tugizimana *et al*., 2016). Multivariate approaches to statistics include unsupervised principal component analysis (PCA) which provide an initial snapshot of variances in the data (Worley *et al*., 2013). For plant pathologists value derived from PCA and loadings plots offers relevant visual evidence for marker discovery by discriminating between treatments. Followed by supervised methods including partial least square discriminant analysis (PLS-DA) and orthogonal partial least square (OPLS) which provide variables or features of importance to a study (Fernandez *et al*., 2017; Marshall and Powers, 2017). Along each step however,

researchers are encouraged to select the best options, including the various online options for statistics, to fulfil their experimental goals and hypotheses (Sakurai *et al*., 2014; Witzel *et al*., 2015; Tugizimana *et al*., 2016; Fernandez *et al*., 2017; Kumar *et al*., 2017). This involves investing time to learn the various options available. It is therefore imperative to understand what information in the form of data, can be extracted from the application of the different analytical platforms and workflow pipelines, and how the data can help to better comprehend the nature of plant phenotypes (Arbona *et al*., 2013; Ernst *et al*., 2014).

To my knowledge, no information regarding LC/MS metabolomics research investigating the citrus rootstock-*P. nicotianae* disease interaction has yet been published, although secondary metabolite accumulation and functionality has been studied. In citrus rootstock research, work to understand the role of phytoalexins was conducted by Khan *et al.*, (1985) who identified the phytoalexin xanthyletin as a likely secondary metabolite involved in the response of citrus rootstocks to *Phytophthora citrophthora*. The coumarin xanthyletin, was found to have inhibitory activity against the pathogen *in vitro* (Khan *et al.*, 1985). Afek and Sztejnberg, (1988) used thin layer chromatography (TLC) to investigate aspects of the same host-pathogen interaction. Results revealed the early accumulation of the phytoalexin scoporone in pathogen inoculated plant roots of both tolerant and susceptible rootstocks. However, the concentration of the metabolite was higher and increased more rapidly measured over 1-8 days in the pathogen resistant rootstocks compared with susceptible rootstocks (Afek and Sztejnberg, 1988). The researchers went on to determine the *in vitro* toxicity of scoporone against the citrus pathogen *Phytophthora citrophthora* and the spores of fungi including *Verticillium dahliae* and *Penicillium digitatum*, among others (Afek and Sztejnberg, 1988). The bioassay results showed the potent nature of the phytoalexin, which

has the function of suppressing plant pathogens and suggests its role or involvement in the resistance of certain citrus rootstocks against *P. citrophthora* (Afek and Sztejnberg, 1988).

Aucamp *et al*., (2000) confirmed scoporone as a secondary metabolite in citrus and that its levels increased upon activation of the defense response in different plant organs such as the bark and roots, using micellar electrokinetic capillary chromatography and TLC. Fourie, (2004) used a TLC approach to determine whether scoporone was involved in the tolerance of citrus rootstocks against *P. nicotianae* but could not conclusively show that the phytoalexin plays a role in this plant-pathogen interaction. The prominence of phytoalexins in citrus defense response warrants the application of metabolomics approaches in modern-day citrus research because the technology is ultimately designed for their identification and annotation. Wolfender *et al*., (2015) outline analytical strategies in the form of metabolite fingerprinting, metabolite profiling and metabolite target analysis used to assess the rich biochemistry of plant metabolites. Strategy options are based of project objectives with all outcomes aiming to better understand the phytochemical flux in response to and in defense from stress factors.

The applicability of plant metabolomics approaches to investigate tolerance of citrus rootstocks against *P. nicotianae* is therefore highly warranted, as observed by research into other citrus diseases and abiotic stresses (Desta *et al*., 2016). Juan Cevallos-Cevallos and colleagues published research on GC-MS based metabolomics to differentiate sensitivity of citrus varieties to Huanglongbing (HLB) disease in 2012. More recently Albrecht *et al*., (2016) also working on HLB disease affecting citrus, used a hyphenated gas chromatography platform approach combining a time of flight (TOF) component to their extract separation procedure. Work by these research groups reveals new information on the metabolome flux

as a result of pathogen infection, on disease tolerant and sensitive citrus varieties. Abiotic stresses research on citrus was conducted by Arbona *et al*., (2013) and more recently, Matsukawa *et al*., (2017) published results from their research to better understand the effects of wound stress on citrus using plant metabolomics applications. Shiratake and Suzuki (2016) review the various Omics studies of citrus, grape and rosaceae fruit trees, highlighting the very firm embrace of metabolomics in fruit tree breeding. These examples are among a growing list of information published by citrus scientists relating to observations that metabolomics approaches have great potential as a means to improve and accelerate citrus breeding (Desta *et al*., 2016). Unfortunately none of the rootstocks that make up the global portfolio today are 100% immune to infection by the *Phytophthora* (Castle, 1987), maintaining the threat this genus of plant pathogens has on citrus fruit production (Talon and Gmitter, 2008).

## 2.4 Conclusion

The need to continually and successfully evaluate new and existing citrus rootstocks according to their tolerance against *P. nicotianae* is essential. However, methods to reduce the time in tolerant cultivar identification are still required. The advent of plant metabolomics applications allows plant scientists to better reimagine crop protection strategies (Fernie and Schauer, 2008; Kell and Oliver, 2016). Plant biologists have broadly embraced the study of plant secondary metabolites even prior to the modern-day application of metabolomics technologies. Here we investigate metabolomics strategies and apply them to a well research host-pathogen interaction in the hope of discovering new insights. The following chapters outline steps taken through conventional screening of rootstocks to LC/MS data analysis to uncover new insight into the citrus metabolome.

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# CHAPTER 3

**Screening of citrus rootstocks for tolerance against the root rot pathogen *Phytophthora nicotianae* under green-house conditions**

**Abstract**

*Phytophthora nicotianae* is responsible for fibrous root rot and tree decline of citrus. The pathogen has a negative impact on profitable fruit production. In the current study, citrus rootstocks grown from seed were screened with the objective of assessing root rot caused by *P. nicotianae* in the greenhouse. Sixteen citrus rootstocks were evaluated and our findings indicate several promising rootstocks exhibiting tolerance to the pathogen and may be used as replacement rootstocks for *P. nicotianae* tolerance in South Africa. Five experiments were conducted over 3 seasons, exposing citrus rootstocks to the pathogen in either sand/peat or soil/sand potting mixtures for 60 days. After the exposure period, plants were assessed according to a root rot rating scale: Australian trifoliate, Benton citrange, Flying dragon, Swingle citrumelo, Terra Bella citrumelo and Yuma citrange were consistently shown to be tolerant to *P. nicotianae* infection. These rootstocks scored low (0-1) root rot scores on a scale of 0 to 4, (i.e low percentage disease severity). The tolerant rootstocks have a greater capacity to withstand the pathogen reflected by the good state of their root systems. Susceptible rootstocks (Cairn rough lemon, Carrizo citrange, Sunki x Benece, Troyer citrange, Volkamer lemon and X639) received high root rot scores (3-4) (i.e disease severity between 83 and 91%) observed as roots exhibiting symptoms of extensive fibrous root rot. The collection of rootstocks screened also included varieties that exhibited moderate responses to the pathogen with disease severity not exceeding 65%. It remains important to recurrently screen citrus rootstocks for root rot tolerance owing to the specialised biology of *P. nicotianae*, particularly in light of global warming and warnings that anthropogenic activities of humankind favour *P. nicotianae* as global temperatures rise.

## 3.1 Introduction

In South African citriculture, *Phytophthora nicotianae* (van Breda de Haan) is the primary causal agent of fibrous root rot and associated tree decline, resulting in fruit yield reductions and tree death (Thompson *et al.*, 1995; Burger, 2001; Fourie, 2004; Meitz-Hopkins *et al.*, 2014;). The hemibiotrophic *Phytophthora* species, *P. citrophthora* and *P. nicotianae*, are the predominant soilborne pathogens of significant agronomic and scientific importance in the country (Meitz-Hopkins *et al.*, 2014; Panabières *et al.*, 2016). *P. nicotianae* has been recorded as the more wide spread of the two pathogens in the various citrus production areas of South Africa, followed by *P. citrophthora* which was reported as being restricted to the Western Cape production areas (Thompson *et al.*, 1995; Meitz-Hopkins *et al.*, 2014).

The citrus rootstocks used in South Africa for commercial fruit production include Carrizo / Troyer citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf); Cairn rough lemon (*Citrus jambhiri* (Lush)); and Swingle citrumelo (*Citrus paradisi "Duncan" Macf* × *Poncirus triofoliata*) (Meitz-Hopkins *et al.*, 2014). Other rootstocks used to a lesser extent include C-35 hybrid (*C. sinensis* (L.) Osbeck. x *P. trifoliata*) and Volkamer lemon (*Citrus volkameriana* L.) (Burger, 2001; Meitz-Hopkins *et al.*, 2014). These rootstocks underpin the production of citrus fruits which are increasingly being planted, and gaining greater export market share (Meitz-Hopkins *et al.*, 2014). No commercial citrus rootstocks are 100% immune to *Phytophthora* infection (Castle, 1987; Siviero *et al.*, 2006) increasing the threat these pathogens pose to the fruit production industry. However, several rootstocks are reported to better withstand pathogen infection (Graham, 1990; Graham, 1995; Burger, 2001). Although control of *Phytophthora* root rot of citrus by means of synthetic fungicides is effective, it is expensive and the sustainability of such practises is low in the mid to long-

term (Gade, 2012; Panabières *et al*., 2016). The financial costs, negative environmental impact, including the deleterious effects on non-target organisms and the phenomenon of pathogen resistance build-up towards active ingredients, all add to the problem of chemical control measures (Sandler *et al.*, 1989; Burger, 2001; Gade, 2012; Panabières *et al*., 2016). The use of resistant rootstocks is the most effective, long term method of managing this problem (Castle 2010; Adaskaveg *et al*., 2014; Albrecht *et al*., 2016). A major objective within citrus breeding programs is to sustainably manage plant pathogens by developing new rootstocks that can better withstand pathogens (Grosser *et al.*, 1995). This requires and includes recurrent screening of newly developed lines or genotypes, including somatic hybrids to assess their tolerance /performance compared with commercial rootstocks (Grosser *et al.*, 1995; Bowman *et al*., 2003; Bright *et al*., 2004; Mourao *et al*., 2008; Gmitter *et al*., 2012). Such screening is an integral part of cultivar improvement strategies to enhance citrus production (Grosser *et al*., 1995).

Burger, (2001) evaluated citrus rootstocks in mini field plots under South African conditions and was able to rank the rootstocks according to their tolerance towards root rot. Similar evaluations on the plant-pathogen interaction conclude that different rootstocks respond differently to infection by *P. nicotianae* as shown by Graham, (1995) and Graham, (1990). In the current study citrus rootstocks (Table 3.1) grown from seed obtained from Citrus Research International's Foundation Block (Uitenhage, South Africa), were screened with the objective of assessing and confirming their tolerance towards *P. nicotianae* in the green-house. Tolerance is defined as the capacity of a plant to withstand pathogenic infection without serious damage (Shurtleff and Averre, 1997). Our findings confirmed that several citrus rootstocks are indeed tolerant to *P. nicotianae* and therefore have potential as

replacement planting material against *Phytophthora* root rot. A selection of these rootstocks were included in the metabolomics studies described in Chapters 4 and 5.

## 3.2 Materials and Methods

### 3.2.1 Citrus rootstocks

Table 3.1 shows the rootstocks evaluated in the current study. Seed of the various rootstocks were obtained from Citrus Research Internationals' Foundation Block (Uitenhage, South Africa) and were germinated in trays (44 x 190 x 20 cm) filled with heat sterilized vermiculite at 28$^{O}$C in growth cabinets with a 12 hour light/dark cycle (Conviron- Winnipeg, Montabo, Canada). The vermiculite growth medium was kept moist by watering with heat sterilised water. Seedlings were fertilized fortnightly with water soluble fertilizer (6-1-4, N-K-P, 1 g/L, Hygrotech). Two months post sowing, all plants were moved from the growth cabinets to a greenhouse. Seedlings were transplanted singly into 16 cm diameter plastic pots containing steam pasteurized sand/peat potting mixture (3:1 v/v) and maintained under greenhouse conditions with natural light until inoculation with the pathogen. Plants were watered twice weekly with sterile water with the inclusion of fertilizer once a fortnight.

### 3.2.2 Pathogen isolation & identification

Isolates of the pathogen were obtained from soil in infested citrus orchards (Mbombela, Limpopo province, South Africa) by means of the citrus leaf-disk method (Grimm and Alexander, 1973). Briefly, soil slurries of orchard soils were prepared using sterile distilled water in sterile 90mm diameter Petri dishes ensuring a layer of water over the soil. Ten citrus leaf-disks from mature rough lemon trees were floated on the surface of each dish and

incubated in the dark at room temperature for 72 hours. Subcultures of *P. nicotianae* were obtained by submerging citrus leaf-disks into selective media prepared from 1 L potato dextrose agar augmented with 200 mg ampicilin, 20 mg pimaricin, 20 mg rifampicin, 50 mg hymexazol and 100 mg pentachloronitrobenzene (PCNB) (Maseko *et al.*, 2007). Pure cultures were obtained by transferring hyphal tips to fresh V8 juice agar plates and incubating in the dark at $25^{O}$C (Maseko *et al.*, 2007). Pathogen virulence was confirmed by infecting citrus seedlings and re-isolating the pathogen from infected roots, fulfilling Koch's Postulates. Morphological and molecular identification of the isolate was conducted by Dr W. Botha (Agricultural Research Council, Plant Protection Research Institute, Pretoria, South Africa) Sequence analysis on the isolate of the internal transcribed spacer (ITS), spacers 1 and 2 confirmed the isolate as *P. nicotianae*.

### 3.2.3 Plant inoculation & experimental design

Millet seed inoculum of *P. nicotianae* was produced as previously described by Fourie (2004). Briefly, autoclave bags containing 200 g millet seed were moistened with 100 ml distilled water, sealed and triple autoclaved. Bags containing sterile millet seed were then inoculated with twenty, 6 mm diameter plugs of *P. nicotianae* growing on V8 juice agar (10-12 days old cultures) and incubated for 4 weeks at room temperature in the dark. Mock inoculations comprised millet seed not inoculated with the pathogen. Five greenhouse experiments were conducted over three seasons to evaluate the tolerance of citrus rootstocks to *P. nicotianae*. Two experiments were conducted over two seasons in a sand/peat potting mixture and three experiments were conducted over three seasons in a soil/sand potting mixture as listed below:

Sand/peat potting mixture experiments: Experiment 1 Season 1: - seedlings 7 months post sowing;

Experiment 3 Season 2: - seedlings 7 months post sowing.

Soil/sand potting mixture experiments: Experiment 2 Season 1: - seedlings 9 months post sowing;

Experiment 4 Season 2: - seedlings 11 months post sowing;

Experiment 5 Season 3: - seedlings 9 months post sowing.

It was however not always possible to acquire sufficient numbers of all rootstocks throughout all experiments, but each of the test rootstocks were evaluated at least over two seasons. Citrus seedlings were transplanted into either sand/peat (3:1 v/v) or soil/sand (2:1 v/v) potting mixture. The potting mixtures were augmented with either *P. nicotianae* inoculated millet seed or sterile millet seed (negative control) to produce a 5% (v/v) inoculum. For each cultivar an uninoculated control (negative control) was included as well as a pathogen inoculated treatment with at least four replicates where one pot containing one plant represented one replicate. The experimental design was a completely randomised (block less) design. Soils were kept moist throughout the experiment to provide favourable conditions for infection. Greenhouse temperatures were maintained at $28^{O}C\pm2$.

### 3.2.4 Disease assessment

Plants were harvested 8 weeks after inoculation by gently removing them from their pots, rinsing the soil from the roots in running tap water. Root rot severity was assessed by means of a rating scale of 0 to 4 where 0 = no root rot; 1 = 25% root rot; 2 = 50% root rot; 3 = 75% root rot and 4 = 100% root rot (Fourie, 2004; Burger, 2001). Percentage root rot was calculated and the data were rank transformed prior to one-way analysis of variance (ANOVA) using JMP Pro 11 (SAS Statistical package). Post ANOVA means separations were made with Tukey-Kramer HSD procedure and this was the criteria used to assign

rootstocks into tolerance categories. Plant roots were then excised from the stems and stored as frozen samples at -20$^{O}$C prior to biochemical analysis of root extracts.

### 3.3 Results and Discussion

A total of sixteen citrus rootstocks were evaluated over three seasons. It was possible to categorise the rootstocks as tolerant, moderately tolerant or susceptible according to percentage root rot recorded (Table 3.2). The trend for disease tolerance was the same for the citrus rootstocks whether the experiments were in sand/peat or soil/sand potting mixtures. Trifoliate orange cultivars Australian trifoliate (AT) and Flying dragon (FD) and the trifoliate orange hybrids Tera Bella citrumelo (TB) and Yuma citrange (YC) were categorised as tolerant in all experiments (root rot below 20%). Rootstock such as SwC, TB, AT and FD were previously reported as tolerant by Burger, (2001). Reports from Florida show that trifoliate orange and Swingle citrumelo are regarded as moderately resistant to *Phytophthora* (Graham and Timmer, 2004; Graham and Feichtenberger, 2015) which concurs with our findings, although in our context the term tolerant is preferred to the terms 'moderately resistant' because the tolerant rootstock were infected. Bright *et al.*, (2004) conducted a broad trial investigating how soil, rootstock and climatic factors influence populations of *P. nicotianae*. They reported that SwC, a rootstock cultivar typically tolerant to *P. nicotianae*, responded poorly in heavy soils with high clay content and poor drainage (Bright *et al.*, 2004).

A moderately tolerant response was observed for trifoliate hybrids C35 citrange (C35) and Minneola tangelo x trifoliate orange (MxT), a similar result as observed by Burger (2001), as well as in Esselen rough lemon, a previously untested rootstock. These rootstocks had between 27 and 53% root rot in both potting mixtures across all seasons. Disease severity was

highest for Cairn rough lemon (CRL), Carrizo citrange (CC), Troyer citrange (TC), Sunki mandarin x Benece trifoliate (SxB), Volkamer lemon (Volk) and X639 hybrid, which consistently showed root rot above 70%. These rootstocks have been documented as susceptible to root rot in South Africa (Burger, 2001). Graham and Feichtenberger, (2015) noted that in Florida Carrizo citrange and Volkamer lemon are tolerant towards *P. nicotianae*. It is therefore important to screen citrus rootstocks for root rot tolerance under the prevailing climatic conditions for the different fruit producing regions. Our findings further indicate that there were significant ($P<0.05$) differences between the tolerant and susceptible groups of rootstocks (Table 3.2). In South African orchards the main commercial rootstocks include Carrizo and Troyer citrange, Cairn rough lemon and Swingle citrumelo (Meitz-Hopkins *et al.*, 2014).

### 3.5 Conclusion

Traditionally the importance of screening assessment cannot be under stated, as they allow plant pathologists opportunities to evaluate locally produced rootstocks with stains of locally obtained pathogens. Our findings indicate several promising rootstocks for possible use as replacement rootstocks for *P. nicotianae* tolerance in South Africa and importantly concur with previous findings. It remains important to screen rootstocks for root rot tolerance owing to the specialised biology of *P. nicotianae*. Panabières *et al*., (2016) warn that anthropogenic activities of humankind favour *P. nicotianae* in particular as global temperatures rise, therefore meeting the challenges it presents to agricultural crop protection must include ongoing screening and use of modern-day technologies. Root materials from Experiments 4 and 5 were stored as frozen samples for further plant metabolomics work to fingerprint the citrus rootstock metabolome. The following chapter outlines progress towards enhancing greenhouse screening experiments with LC/MS based analytical approaches.

### 3.6 References


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**Table 3.1** List of Citrus Rootstocks Evaluated in Screening Experiments.

| Rootstock cultivar common name | Citrus Species (parental cross) |
|---|---|
| Australian trifoliate | *Poncirus trifoliata* (L) Raf. |
| Benton citrange | *Citrus sinensis* (L.) Osbeck. X *P. trifoliata* |
| Cairn rough lemon[(C)] | *Citrus jambhiri* (Lush) |
| Carrizo citrange[(C)] | *C. sinensis* (L.) Osbeck. X *P. trifoliata* |
| C-35 citrange[(SC)] | *C. sinensis* (L.) Osbeck. X *P. trifoliata* |
| CM x SwC | Cleopatra mandarin X Swingle citrumelo |
| Esselen rough lemon | *Citrus jambhiri* (Lush) |
| Flying Dragon | *P. trifoliate* |
| Minneola x Trifoliate | *C. reticulata* Blanco X *C. paradise* X *P. trifoliata* |
| Swingle citrumelo[(C)] | *Citrus paradise* X *P. trifoliata* |
| Sunki mandarin and Benece trifoliate | *C. sunki* (Hort. etTanaka) X *P. trifoliata* |
| Tera Bella citrumelo | *Citrus sinensis* X *P. trifoliata* |
| Troyer citrange[(C)] | *Citrus sinensis* (L.) Osbeck. X *C. trifoliata* |
| Volkamer lemon[(SC)] | *Citrus volkameriana* |
| Yuma citrange | *Citrus sinensis* X *P. trifoliata* |
| X639 hybrid[(LR)] | *Cleopatra mandarin* X *P. trifoliata* |

C = commercial rootstock, SC semi-commercial replacement rootstock; LR = locally developed rootstock.

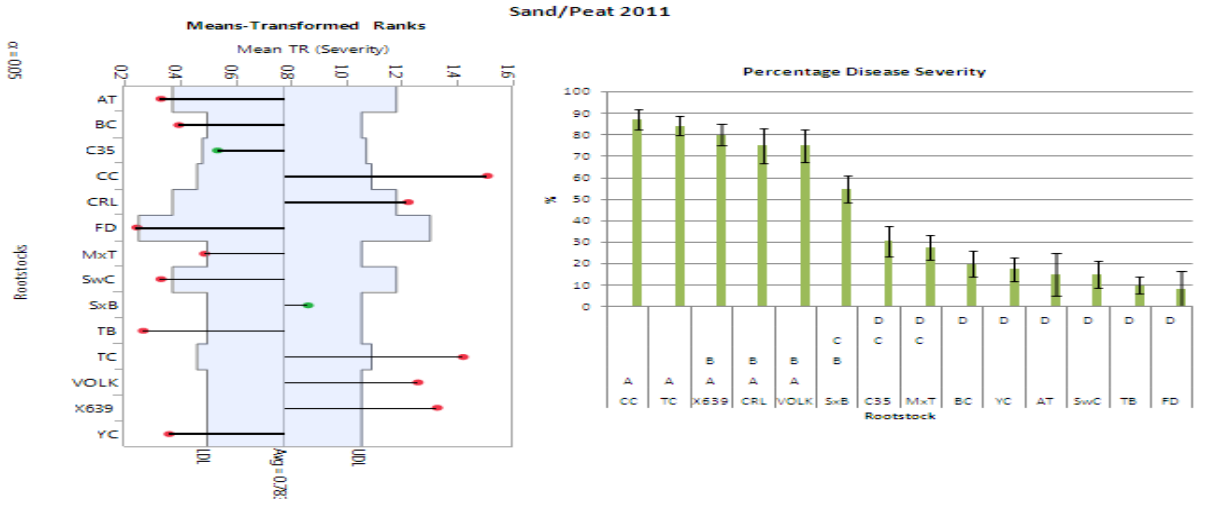
**Table 3.5** Summary of screening trials evaluating citrus rootstocks for tolerance against *Phytophthora nicotianae* root rot: percentage root rot in citrus rootstock seedlings artificially inoculated with *P. nicotianae* in the greenhouse.

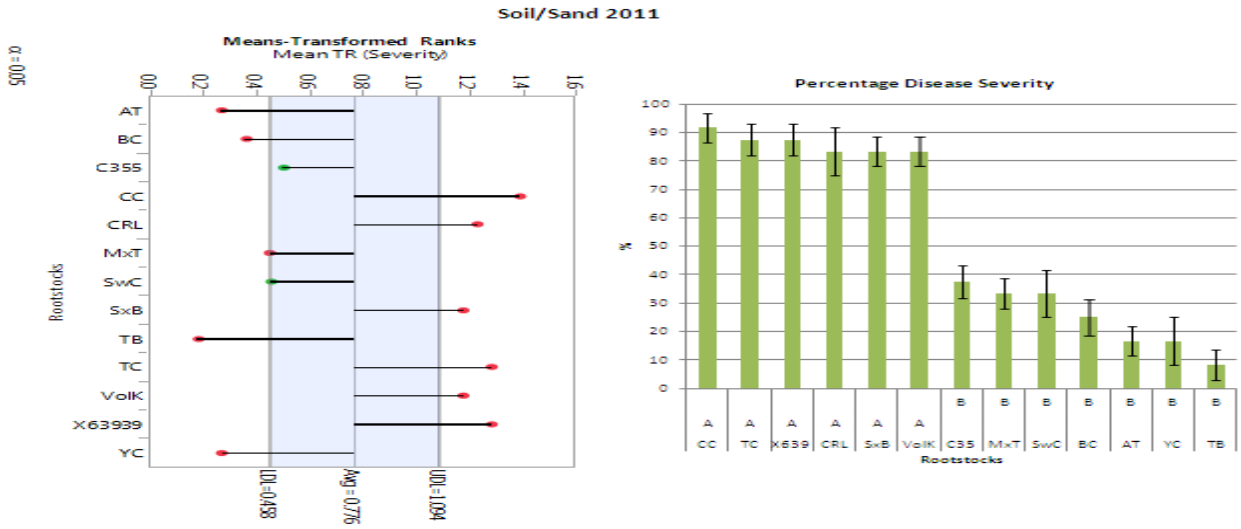
| | **Expt.1** | **Expt.2** | **Expt.3** | **Expt.4** | **Expt. 5** | |
|---|---|---|---|---|---|---|
| **Rootstocks** | Root rot (%) | Root rot (%) | Root rot (%) | Root rot (%) | Root rot (%) | **Category** |
| CC – Carrizo citrange | 87.5 $^{a}$ | 87.5 $^{a}$ | 91.6 $^{a}$ | 85 $^{ab}$ | - | S |
| TC – Troyer citrange | 84.3 $^{a}$ | 82.5 $^{a}$ | 87.5 $^{a}$ | - | - | S |
| X639 – X639 hybrid | 80 $^{a}$ | 85 $^{a}$ | 87.5 $^{a}$ | 85 $^{ab}$ | - | S |
| CRL – Cairn rough lemon | 75 $^{a}$ | 72.5 $^{ab}$ | 83.3 $^{a}$ | 87.5 $^{a}$ | 72.5 $^{a}$ | S |
| VOLK – Volkamer lemon | 75 $^{a}$ | 59.5 $^{bc}$ | 83.3 $^{a}$ | 80 $^{ab}$ | - | S |
| SxB – Sunki x Benece | 55 $^{b}$ | 60 $^{bc}$ | 83.3 $^{a}$ | 75 $^{ab}$ | 62.5 $^{a}$ | S |
| C35 – C35 hybrid | 30.5 $^{c}$ | 31.5 $^{def}$ | 37.5 $^{b}$ | 45.8 $^{cd}$ | - | M |
| MxT – Minneola x Trifoliate | 27.5 $^{c}$ | 40 $^{d}$ | 33.3 $^{bc}$ | 53.1 $^{cd}$ | - | M |
| ERL – Esselen rough lemon | - | 35 $^{de}$ | - | 35.7 $^{de}$ | - | M |
| C+S – C. mandarin x S. citrumelo | - | 18.7 $^{efg}$ | - | 64.2 $^{bc}$ | - | M |
| BC – Benton citrange | 20 $^{cd}$ | 10 $^{g}$ | 25 $^{bcd}$ | 21.8 $^{ef}$ | 12.5 $^{b}$ | T |
| YC – Yuma citrange | 17.5 $^{cd}$ | - | 16.6 $^{cd}$ | - | - | T |
| AT – Australian trifoliate | 15 $^{cd}$ | - | 16.6 $^{cd}$ | - | 15 $^{b}$ | T |
| SwC – Swingle citrumelo | 15 $^{cd}$ | 17.5 $^{fg}$ | 33.3 $^{bc}$ | 14.2 $^{f}$ | 22.7 $^{b}$ | T |
| TB – Terra Bella citrumelo | 10 $^{d}$ | 7.5 $^{g}$ | 8.3 $^{d}$ | 8.3 $^{f}$ | 9.3 $^{b}$ | T |
| FD – Flying dragon | 8.3 $^{cd}$ | 12.5 $^{g}$ | - | 18.7 $^{ef}$ | 7.5 $^{b}$ | T |

Root rot (%) determined according to a rating scale of 0-4. Within column mean values followed by the same letter are not significantly different (Tukey-Kramer test $P<0.05$). - = no rootstocks. S = susceptible; M = moderately tolerant; T = tolerant.

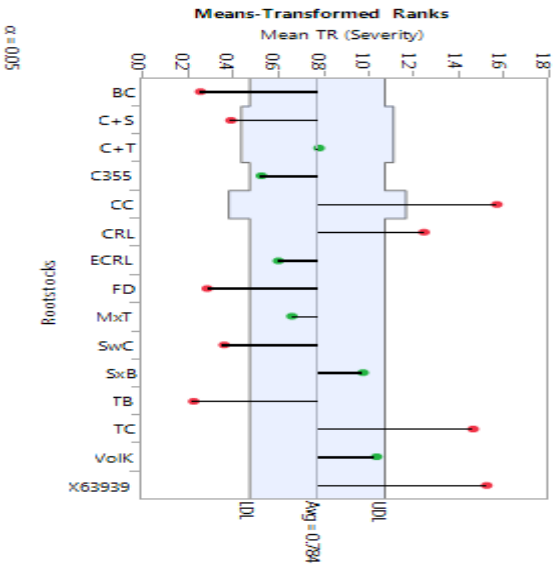
Graphic Results (Supplementary Information)

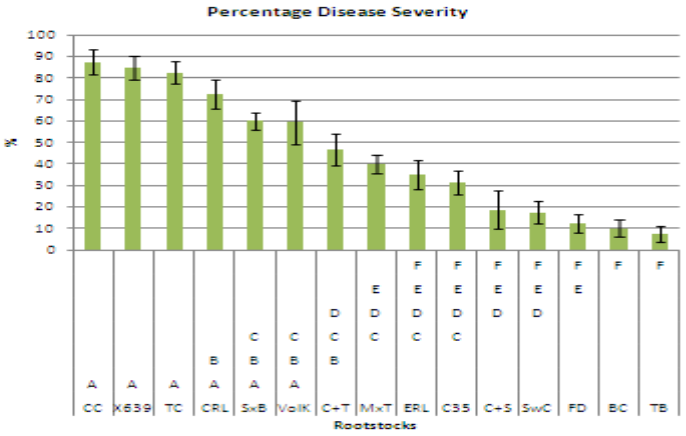
*Disease assessment*



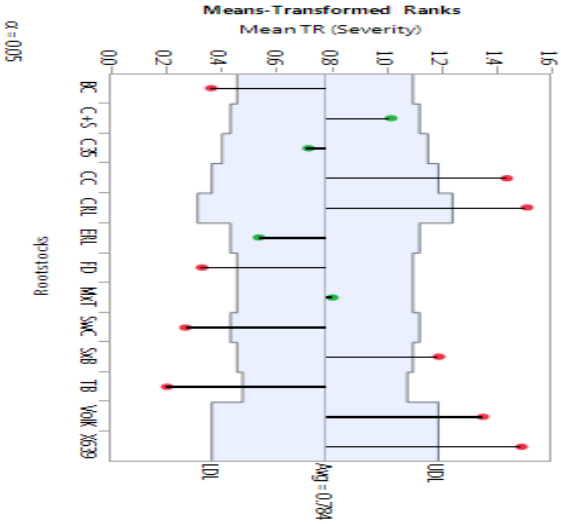



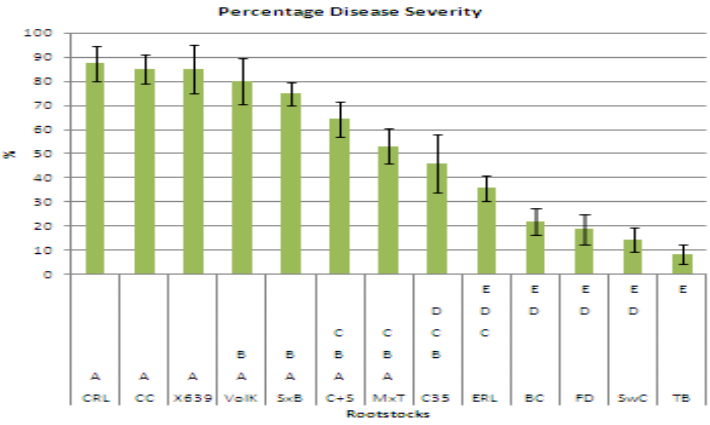
## Sand/Peat 2012



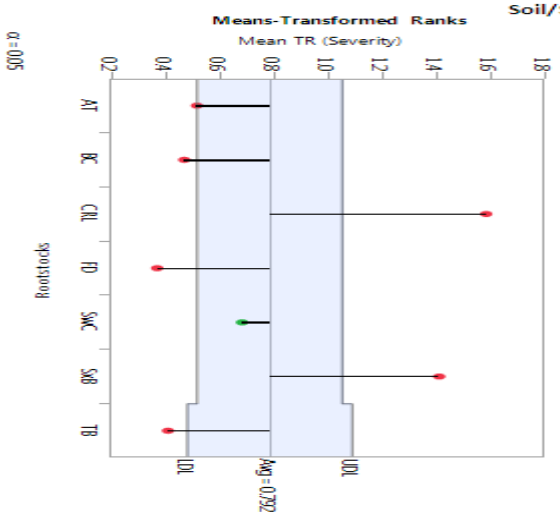



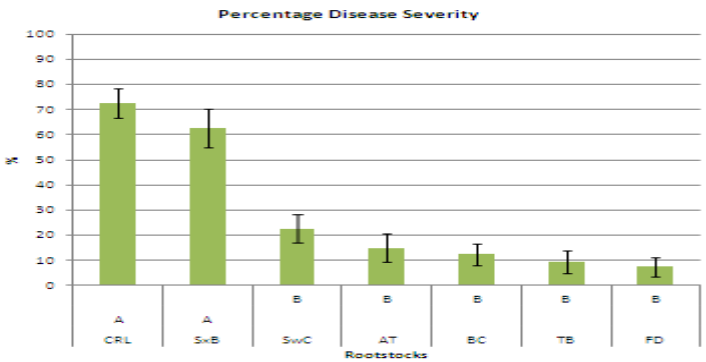

## Soil/Sand 2012

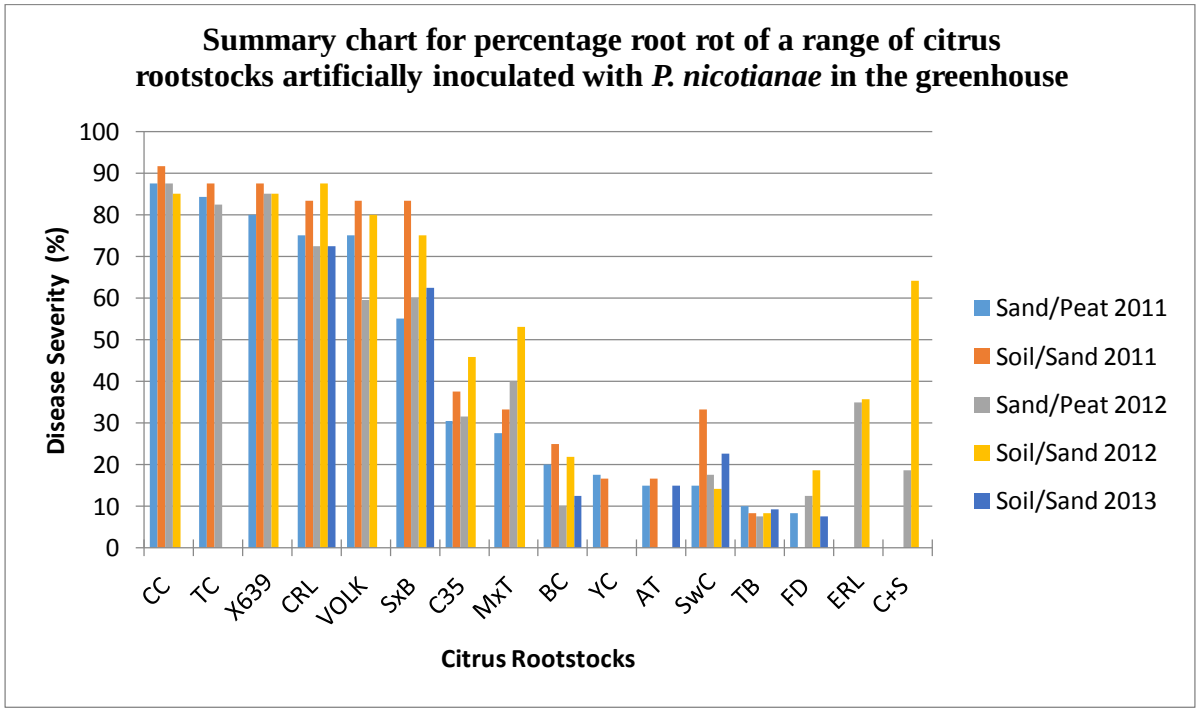



## Soil/Sand 2013




Summary chart for percentage root rot of a range of citrus rootstocks artificially inoculated with P. nicotianae in the greenhouse
Disease Severity (%)
100
90
80
70
60
50
40
30
20
10
0
CC
TC
X639
CRL
VOLK
SxB
C35
MxT
BC
YC
AT
SwC
TB
FD
ERL
C+S
Citrus Rootstocks
Sand/Peat 2011
Soil/Sand 2011
Sand/Peat 2012
Soil/Sand 2012
Soil/Sand 2013

# CHAPTER 4

## Metabolite fingerprinting of Citrus rootstocks for disease tolerance related biomarkers against *P. nicotianae*.

**Abstract**

Plant metabolomics allows for the quantitative analysis of secondary metabolites in plants. Historically phytochemicals associated with suppression of plant pathogen invasion, have been implicated in plant self defense. However, the mechanisms that are responsible for tolerance are not fully understood in plants. This first insight to the citrus metabolome applying metabolite fingerprinting analytical strategies has yielded promising results for tolerance trait discovery in the citrus rootstock- *P. nicotianae* interaction. The online software package MassLynx (4.1, Waters) was used to analyse LC/MS data. Results demonstrate effects of parameter adjustment as part of initial data mining processes for MarkerLynx XS, the data processing software delivered by MassLynx. The initial data mining allowed for hypothesis generation. Results from hypothesis driven metabolite fingerprinting of tolerant *versus* susceptible sample groups show clear separation observed visually in principle component analysis (PCA) score plots. S-plots of orthogonal partial least squared discriminant analyses (OPLS-DA) models and trend line plots results of tolerant *versus* susceptible citrus rootstock sample groups from two seasons, revealed recurring metabolite patterns highlighting potential metabolite markers associated with tolerance. The metabolites are constitutive or innate at higher concentrations (% intensity) in tolerant rootstocks as opposed to susceptible rootstocks, and this feature is a key aspect in discovering tolerance traits in plants. Furthermore the compounds proposed here as having a role in rendering tolerant rootstocks greater capacity to withstand *P. nicotianae* were previous linked to plant self defense through published reports in other plants. Future studies to annotate the potential markers discovered here are required.

## 4.1 Introduction

Citrus rootstocks underpin successful fruit production (Castle, 2010; Albrecht *et al*., 2016). A major threat to citrus rootstocks is the root rot pathogen *Phytophthora nicotianae*, which negatively affects plants in both nurseries and orchards (Matheron *et al.*, 1998; Graham and Feichtenberger, 2015; Panabieres *et al*. 2016). There are no commercial rootstocks that are 100 % immune to *P. nicotianae* infection (Castle, 1987; Siviero *et al.*, 2006) however it has been shown that some rootstocks, better withstand "tolerate" infection (Graham, 1990, Burger, 2001). *Poncirus trifoliata* (raf.) or Trifoliate orange species possesses traits of great scientific and economic value in citrus breeding (Talon and Gmitter, 2008). For increased disease tolerance, citrus plant breeders rely on hybrids developed from *P. trifoliata* (Talon and Gmitter, 2008). However, the biochemical mechanisms responsible plant disease response are still misunderstood (Goellner and Conrath, 2008; Hall, 2011; Pérez-Clemente *et al.*, 2013).

Previous studies have highlighted the importance of secondary metabolites in plant self defense during citrus root rot disease. Khan et al., (1985), identified the coumarin Xanthyletin as having a role in *Phytophthora* disease suppression. Scoparone (6, 7-Dimethoxycoumarin) was reported as an important disease suppressing phytochemical in the interaction between citrus rootstocks and *P. citrophthora* by Afek *et al.*, (1986). Today the biochemistry of secondary metabolites is best studied by applying and implementing strategies of plant metabolomics (Sumner *et al*., 2003; Hall, 2006). There is growing scientific interest into secondary metabolite analyses to evaluate plant-pathogen interactions for major crops including barley (Bollina *et al*., 2011), and tomato (Lopez-Gresa *et al*., 2012). Growing numbers of scientific publications are increasing highlighting the significance in

metabolomes studies (Marshal and Powers, 2016). Metabolite fingerprinting strategies are untargeted analytical approaches, important in the rapid classification of samples and apply multivariate discriminant analyses to determine differences between extracts (Tugizimana *et al*., 2013; Wolfender *et al*., 2015). The goals of such analyses are to compare the 'patterns' of metabolites in any given biological sample, however not specifically to identify individual metabolites (Tugizimana *et al*., 2013; Wolfender *et al*., 2015). In our case, we did however investigate the online data analysis package MassLynx (ver 4.1) which provides database searches for putative naming of top metabolites. Metabolite fingerprinting also helps in hypothesis generation, as new discoveries are made from data mining steps for metabolome fingerprinteing (Sakurai *et al*., 2014; Wolfender *et al*., 2015). As far as we know, no study on metabolite fingerprinting of the citrus metabolome in association with the root rot pathogen has been described. Therefore, we aim to provide new insight into the citrus metabolome using extracts of experimental plants from two independent seasons.

Biologists have come to benefit immensely from the developments in chromatography technologies for systems biology analysis and plant systematics (Sumner *et al.*, 2003; Hall, 2006; Allwood and Goodacre, 2010). Plant metabolomics approaches have resulted in great strides currently being made in metabolite discovery studies for citrus Huanglongbing (HLB) (Cevallos-Cevallos *et al*., 2009; Albrecht *et al*., 2016). Other citrus plant stress factors such as wound stress (Matsukawa *et al*., 2017) have also been assessed with the inclusion of metabolomics strategies. The aim of applying metabolomics is to gain a more fundamental understanding of the role secondary metabolites play in plant stress for crop improvement through trait discovery (Sumner *et al.*, 2003; Hall, 2006; Fernie and Schauer, 2008).

In this chapter we investigate the metabolome of citrus rootstock using a mass spectrometry based liquid chromatography approach for extract separation, and MassLynx (4.1 software, Waters) for large data analyses. We commenced the analyses with a data driven approach, which allowed for the measurement of metabolites without any preconceptions (Tugizimana *et al*., 2013). We outline metabolite fingerprinting in the form of preliminary training sample lists, which were prepared in MarlerLynx XS, the data processing package delivered with MassLynx 4.1, to develop an overview. MarkerLynx algorithms search for unique molecular markers in large data sets (Tugizimana *et al*., 2016) and allow for visualisation of results. To avoid problems with processing parameter settings, (of the numerous combination available on MarkerLynx XS (Tugizimana *et al*., 2016)), we focused on adjusting two essential parameters for our analyses namely Intensity threshold (counts) and Mass tolerance (Da) (Tugizimana *et al*., 2016).

Results from those initial data driven analyses showed no pattern for disease interaction, because the presence of unique compounds for citrus rootstocks exposed to the pathogen for 60 days were not detected. However, in the case of detecting secondary metabolites that demarcate tolerant and susceptible rootstocks, initial analyses showed promising results in the form of principle component analysis (PCA) score plots. This approach to data mining processes allowed sufficient insight into the citrus metabolome. Focusing on citrus species and applying hypothesis driven data analyses methods (Sakurai *et al*., 2014), the six data analyses steps required for MarkerLynx XS, were followed. We highlight results from methods applied to showcase the trend of metabolic markers considered here, to have a role in conferring *P. trifoliata* greater innate capacity to withstand root rot. This was followed up by analysing tolerant citrus rootstock *versus* susceptible rootstocks including hybrids. A

similar trend or pattern was observed over 2 seasons confirming that the markers are constitutively present and statistically associated with tolerant rootstocks. Putative identification of two selected markers suggests the compounds are related to tolerance owing to their association with disease suppression in other plants. These finding provide the local and international citrus breeding communities with insight to the potential of discovering tolerance traits through metabolome fingerprinting.

## 4.2 Materials and Methods

### 4.2.1 Plants and Extraction of metabolites

Frozen citrus root materials from the rootstock tolerance screening experiments 4 and 5 (Chapter 3 materials and methods section 3.2.3) (from here on experiment 4 = Season 1 and experiment 5 = Season 2), were used as the source of fresh plant material for preparation of crude extracts (Table 4.1). Using five replicates (where one plant represented one replicate) per treatment (either inoculated or uninoculated), roots were individually excised, frozen in liquid nitrogen and crushed to a fine powder using a mortar and pestle. One gram powdered root material was transferred to glass tubes and 3 ml cold ethyl acetate-ethanol (1:1) (Merck, HPLC grade) mixture was added for overnight extraction (Chong *et al*., 2006). Tubes were capped, vortexed for 30 seconds and allowed to settle for extraction overnight in the dark at room temperature. After 24 hours, extracts were recovered from the tubes and transferred to clean, labelled glass tubes before being evaporated to dryness in a fume hood. Resultant residues were suspended in 0.5 ml methanol (Merck® HPLC grade) to produce crude extracts. Aliquots of 50 µl for each rootstock extract were then transferred to Eppendorf tubes and stored at -20 ℃ until biochemical analyses.

### 4.2.2 Mass Spectrometry base analyses.

UPLC/MS-MS on root extracts were performed by the Central Analytical Facility at the University Stellenbosch, South Africa. Their procedure briefly: samples were randomised in the sample manager and analysed over a period of five consecutive days to minimize process variance. Two cocktail mixtures of commercially (Sigma) available flavonoid standards were prepared. The cocktails were injected at the beginning and after every eight samples, as technical repeats to confirm the stability of the UPLC/MS-MS system. Metabolites were separated using the Central Analytical Facilities standard procedure; briefly- 0.1% formic acid (solvent A) to acetonitrile (solvent B) gradient, at flow rate of 0.4 ml/min on a Waters BEH C18, 2.-1x100 mm column for a 25-minute run time. The extracts from Season 2 had an adjusted run time of 14 minutes (See Supplementary information – Chromatograms). Mass spectrometry readings were generated on a Waters SYNAPT™ G2 MS (Manchester, England) instrument using electron spray ionization (ESI) in positive mode with a cone voltage of 15 V.

### 4.2.3 Data Analysis

#### 4.2.3.1 Chromatograms and initial data driven analyses:

Chromatograms are presented in supplementary information below. The initial data driven analyses processes allowed for software familiarisation and provided an early overview of the metabolome under review. We used the MarkerLynx XS data processing package delivered with MassLynx (ver. 4.1, Waters) software to analyse the UPLC/MS-MS raw data and view chromatograms. Centroid electrospray ionization (ESI) positive ion mode raw data were used in this study. MarkerLynx data processing steps from data acquisition, sample list set up, method development and data processing enable data set familiarisation in data pre-processing. These initial steps help create dataset matrices and formulate hypotheses.

The following steps in MarkerLynx data processing are statistical analyses and metabolite identification steps, which enable visualisation of unsupervised and supervised multivariate analysis results. MarkerLynx uses advanced statistical processes to identify potential biomarkers developed from results matrices built through accurate mass (*m*/z), chromatographic retention time (rt), and peak-feature profiling (Tugizimana *et al*., 2016). For our initial analyses MarkerLynx Method editor parameter settings were adjusted for initial and final retention times between the 2 seasons (Season 1 – 1 to 23 mins; Season 2 - 1 to 12 mins) and for lower and higher masses for secondary metabolite detection (between 153-600) for both seasons. Results are presented as score plots of PCA and OPLS-DA models.

#### 4.2.3.2 Parameter adjustment in MarkerLynx

There are numerous options for parameter adjustment available for method development and data processing (Tugizimana *et al*., 2016). To examine and determine the effects of parameter adjustment on our data, we focus on the essential parameters (Tugizimana *et al*., 2016) of intensity threshold (counts) and mass tolerance (Da). Sample lists to explore the effects of inoculation *versus* no pathogen on *P. nicotianae* tolerant rootstocks were prepared to fingerprint the results matrix. Processing parameters varied in these limits- Intensity thresholds (counts) (10 and 100) and Mass tolerance (Da) set at 0.01 and 0.05 Da. Data were analysed to determine the effects of the adjustments on X Variable and noise levels (%). This was repeated for sample lists of data collected over two consecutive seasons (Table 4.1) and tabulated results are presented along with score plots of PCA models. PCA scores and loadings plots are the most relevant way to collect information in metabolite fingerprinting (Scholz *et al*., 2004).

#### 4.2.3.3 Hypothesis driven analyses and putative feature identification

Working on the hypothesis that tolerant rootstocks will yield resistant related metabolites, dataset matrix for the citrus species Australian trifoliate (*P. trifoliata*), Flying Dragon (*P. trifoliata*), Cairn rough lemon (*Citrus jambhiri*), Volkamer lemon (*Citrus volkameriana*) were prepared in MarkerLynx. The data processing parameters were adjusted to improve quality of the model depending on the experiment (Season 1 = intensity threshold (100 counts) and mass tolerance (0.01 Da) or Season 2 intensity threshold (100 counts) and mass tolerance (0.05 Da) (Table 2). Initial and final retention times and mass detection levels were maintained as noted above (4.2.3.2). Multivariate analyses comprising principle component analyses (PCA) was conducted on the data to obtain an initial overview. OPLS-DA on Pareto scaling data was performed to highlight metabolites that distinguish between pathogen tolerant rootstock species and pathogen susceptible species. The features of importance from S-plots were further analysed so as to showcase the potential of trait discovery through online database searches. MarkerLynx allows for integrated searches on online databases such as BioCyc, KEGG, Life Chemicals, NIST, PubChem among others. The same analyses were conducted including citrus hybrids to further confirm that the markers are present in tolerant over susceptible rootstocks.

## 4.3 Results and Discussion

### 4.3.1 Data Driven Analyses

#### 4.3.1.1 Chromatograms and initial data driven analyses:

We did not aim to replicate the chromatograms from season to season by changing LC runtimes as shown in supplementary information below (Appendix 1). Initially from the

dataset matrices developed, we contrasted- pathogen inoculated *versus* uninoculated rootstocks and- pathogen tolerant *versus* susceptible rootstocks. The patterns in metabolites and their clustering behaviour for the citrus metabolome are shown as PCA plots in Figures 4.1a Season 1 Inoculated *versus* Control sample; Figure 4.1b Season 1 Tolerant *versus* Susceptible samples; Figure 4.1c Inoculated *versus* Control sample; and Figure 4.1d Tolerant *versus* Susceptible samples. There is no separation or clustering between inoculated *versus* control rootstocks (Fig 4.1a and 4.1c) hinting that the pathogen effect was no longer affecting the plant metabolome at 60 days after inoculation. This point is strengthened by observations of the clustering patters of the rootstocks by variety whether pathogen treated or not. There is interesting separation of pathogen tolerant and pathogen susceptible rootstocks for both seasons (Fig 4.1b and 4.1d). For plant scientists the PCA plot provides the first relevant and indicative signs for interpreting large data (Scholz *et al*., 2004). Although the plots are low dimensional visual representations of large data sets, they are commonly used by experts in metabolomics (Worley *et al*., 2013), they find the maximum variation within the data (Albercht *et al*., 2016) and they provide early-stage insight of relevant biological information visually (Scholz *et al*., 2004). In our case the variance within the data is towards high percentage $R^2X$ values (Fig 4.1 b and 4.1d).

#### 4.3.1.2 Parameter adjustment

To explore collection parameters in MarkerLynx XS (as part of initial training and data driven analyses) we observed reductions in X Variable particularly as we adjusted intensity threshold from 10 counts to 100 counts (Table 4.2). Noise levels were also affected by parameter adjustments (Table 4.2). Of the tolerant rootstocks evaluated (BC, FD, SWC and TB) the two PCA plots (Fig 4.2a and 4.2b) show no separation between pathogen inoculated

and control rootstocks. A difference in $R^2X$ values was however observed, in relation to the parameter adjustments. This point highlights the importance of intensity threshold (counts) and mass tolerance (Da) for experimental design using MarkerLynx software with implications for improving model quality (Tugizimana *et al*., 2016). Adjusting the intensity threshold between 10 and 100 counts (Season 1) resulted in a total of 10019 metabolites being excluded for the analyses and noise levels reduction from 36 to 33% (Table 4.2). There were no changes in PCA clustering patterns in cases where intensity threshold was set to 10 or 100. However the $R^2X$ parameters did change between PCA plots (Fig 4.2a = $R^2X$ 0.3903 and 4.2b = $R^2X$ 0.4158) which has implications for model quality (Tugizimana *et al*., 2016). Although there was only a 3 % reduction in noise level, the resultant raise in $R^2X$ from 0.3903 to 0.4158 improves model quality. Understanding the collection parameters and the effects of adjusting them, provided insight into defined features assessed and how much data may be potentially lost through adjustments (Tugizimana *et al*., 2016). Furthermore, hypothesis generation was enhanced through mining the metabolome in this manner (Sakurai *et al*., 2014). In their overview of the citrus metabolome in association with HLB Albrecht *et al*., (2016) observed separation of rootstock into varieties/treatment from interpreting clusters in PCA models. While they used a different disease interaction the information gained from PCA models is of considerable importance and highly valuable.

In our case, the results suggest that there were no unique metabolites distinguishing inoculated *versus* control tolerant citrus rootstocks whether intensity thresholds were adjusted or not. However, our plants were exposed to the pathogen for 60 days as previously described (Chapter 3). Afek *et al*., (1986) studied the interaction between *P. citrophthora* and citrus rootstocks with varying tolerances to infection. In later work by Afek and Sztejnberg, (1988),

results indicated a rapid pattern for defense related metabolite accumulation. The accumulation pattern for scoparone in pathogen inoculated tolerant plants increased from below 50 µg fresh weight at 2 days of infection, to peak at levels of 400 µg fresh weight after 4 days, then subsided to levels between 200 and 300 µg fresh weight after 8 days (Afek and Sztejnberg, 1988). In uninoculated and susceptible plants, scoparone levels remained unchanged below 50 µg fresh weight while lesion length continued to increase at 8 days after infection in susceptible plants (Afek and Sztejnberg, 1988). Their results showed the disease mitigating effects of the coumarin, which was not induced in susceptible or uninoculated plants. Their work also provides insight into the reduction of scoparone at 8 days suggesting its rapid induction upon infection followed by a decrease in concentration. Kumar *et al*., (2015) observed the varying concentrations of induced defense related metabolites in the chickpea Fusarium disease interaction, at different times during early disease progression over 12 days. These examples illustrate the need to test for pathogen-induced secondary metabolites during the early stages of the infection process. In the current study, we can postulate that long (60 days) exposure of plants to the pathogen results in stabilisation of the metabolic status. Time course studies may therefore provide more detailed information regarding immediate pathogen effects on secondary metabolite flux and identification of pathogenicity related metabolites. In this case, having used roots from experiments where plants were exposed to the pathogen for 60 days, no markers for disease diagnostics (ie pathogenicity related metabolites) could be detected.

In the absence of pathogen induced secondary metabolites (pathogenicity related metabolites), citrus rootstock tolerance to *P. nicotianae* may be further hypothesized upon from the perspective of constitutive metabolites (resistance related metabolites)

(Kumaraswamy *et al.* 2011; Chamarthi *et al.*, 2014). Constitutive metabolites may be used as biomarkers in trait discovery and will be detectible through observing differences in their concentrations (% intensity levels) between tolerant *versus* susceptible rootstocks. Such markers are termed resistance related biomarkers (Kumaraswamy *et al.* 2011). In our study, the initial data driven analytical approach provided sufficient information for hypothesis generation focusing on tolerant *versus* susceptible citrus rootstocks.

### 4.3.2 Hypothesis driven data analyses

Working hypotheses were generated through interpretations of the citrus metabolome so far as part of the data mining process (Sakurai *et al*., 2014). Table 4.3 shows the processing parameters applied (counts & Da), and the resultant number of X Variables, percentage noise levels and $R^2X$ values, calculated during online mathematical procedures to contrast the metabolome of *P. nicotianae* tolerant rootstocks and *P. nicotianae* susceptible rootstocks. Figures 4.3 shows complete separation between tolerant and susceptible rootstocks for both seasons. PCA scores and loadings plots are the most relevant means of collecting information in metabolite fingerprinting (Scholz *et al*., 2004). In the first instance for the citrus species alone PCA was carried out resulting in - Season 1: $R^2X$ 1= 0.518; Season 2: $R^2X$ 1 = 0.505 (Fig 4.3a and 4.3c respectively). The quality of PCA models is validated by significantly higher % parameter $R^2X$ (Kumar *et al*., 2015). The loadings plots of the PCA model visually point to potential tolerance markers responsible for the traits represented by outliers that fall within corresponding quadrants of the PCA plot (Fig. 4.3a-1 and 4.3b-1). A similar pattern was observed during analyses that included citrus hybrids Benton citrange (BC), Swingle citrumelo (SWC) and Terra Bella (TB) *versus* Carrizo citrange (CC) and Volkamer lemon (Volk) - Season 1: $R^2X$ 1 = 0.3464; Season 2: $R^2X$ 1 = 0.248 (Fig. 4.3c and 4.3c-1; Fig. 4.3d

and 4.3d-1). Such clear separations are indicative of unique metabolite discovery as shown by Kumar *et al*., (2015), who observed clear separation in all PCA and OPLS-DA models during metabolite discovery in the chickpea-Fusarium disease interaction. In cases where clear separation was observed in their models, unique (induced) secondary metabolites were detected and reported on as features responsible for the separation (Kumar *et al*., 2015).

These preliminary observations from unsupervised multivariate analyses highlight significant changes in the metabolite fingerprint between pathogen tolerant and pathogen susceptible citrus rootstocks from samples originating from two seasons. The loading plots show these changes and indicate metabolites responsible for the variation, observed as features furthest from the point of origin (Fig. 4.3a-1; 4.3b-1; 4.3c-1 and 4.3d-1). The loading plots are important in visualising metabolites responsible for group separation (Albrecht *et al*., 2016). The models provided overview and perspective on the variables responsible for clustering observed in the loadings plots representing unique ion features that have potential use as biomarkers. Similarly, with the PCA plots with clear separation between treatment groups, clear separation was observed in the follow-up supervised multivariate analyses visualised as OPLS-DA plots. In MarkerLynx XS these plots are generated by using the Group Differences option as part of data processing steps, to generate multivariate models.

In the current study, the S-plots reveal the individual features from the data set visually (Figures 4.4a-1; 4.4b-1; 4.4c-1 and 4.4d-1). These features reliably distinguish between the sample groups and were selected according to their position on the S-plot in MarkerLynx XS (Tugizimana *et al*., 2016). The S-plot is divided into quadrants so that markers that fall close

to (x=1) (y=1) away from the axis are stronger indicators of potential markers for tolerance and those that fall close to (x=-1) (y=-1) away from the axis are strong indicators of susceptibility (Figures 4.4a-1; 4.4b-1; 4.4c-1 and4. 4d-1). In plant metabolomics, such variables or features are statistically significant and represent a high potential for tolerance trait discovery (Tugizimana *et al.*, 2016). A pattern emerged from the fingerprinting processes of citrus rootstock metabolome data in the current study; we observed a similar trend for several relevant features in both seasons. Although metabolite fingerprinting does not typically aim to identify markers (Scholz *et al*., 2004; Tugizimana *et al*., 2016), MarkerLynx XS offers online database searches for further trend line visualisation and putative identification of potential markers. Importantly for the online database searches in the current study, we aimed to link the top recurring markers identified in the S-plots to compounds related to plant self-defence.

The features highlighted in Table 4.4 and in the subsequent trend line plots (Fig. 4.5) produce the same 'patterns' across the 2 seasons by consistently showing increased or higher % intensity in the tolerant rootstock species over the susceptible citrus rootstock species. These observations suggest the potential of trait discovery, with Appendix 2 providing a brief tutorial to interpret trend line data. Figure 4.5a is a visual representation of MarkerLynx putative assignment window modified with the top marker trend line plots for Season 1: 1= 21.85_259.0972; 2= 22.15_324.1241; 3= 22.22_314.1497 and 4= 22.22_313.1445 (rt_*m/z*). Figure 4.5b highlights top marker trend line plots for Season 2: 1= 6.94_259.0962; 2= 8.73_313.1435; 3= 9.68_327.1592 and 4= 9.67_328.1624.

For plant pathologists assisting plant breeders, it is important to report on the nature of potential metabolites proposed as biomarkers. In the current study, this has been achieved in part through presenting visualisations in the form of trend line plot results produced in MarkerLynx XS. The capacity of the software to deliver the complex scientific information from LC-MS data in these plots is decidedly convenient. Trend line plots, developed from S-plot peripheral features using MarkerLynx XS software, best illustrate the difference (% intensity) of the detectible top metabolites in comparison with opposing treatments. The trend line plots are also important for rapid visual analysis in metabolite fingerprinting and are used in conjunction with the S-plot and loadings plots of the OPLS-DA models. They provide a clear picture highlighting the importance of the potential resistance related markers. Figure 4.6a shows trend lines plots from Season 1 citrus rootstock hybrids, and Figure 4.6b shows trend line plots from Season 2 citrus rootstock hybrids. The plots clearly illustrate how particular metabolites are detected at greater levels (% intensity) in pathogen tolerant rootstocks *versus* pathogen susceptible rootstocks. The online database searches also provided top hits for putative metabolite identification of which four are listed in Table 4.4.

These putative assignments are based on online database searches to link the metabolites (Table 4.4) with previous work associating them with plant self-defence. Wyerone is a phytoalexin associated with conferring greater tolerance in broad beans (*Vicia faba* L.) following infection by *Botrytis fabae* (Letcher *et al*., 1970; Fawcett *et al*., 1971). The ion features for this suspected compound from season 2 were 6.93_259.0963 and 7.57_259.0964 (Table 4.4). 4'-prenyloxyresveratrol is a secondary metabolite previously reported to have anti-microbial properties and has been extracted from mulberry (*Morus spp.*) and bread fruit (*Artocarpus incises*) plants (Likhitwitayawuid and Sritularak, 2001). It is associated with

Stilbenoid biosynthesis via the mixed phenylpropanoid/polyketide biosynthetic pathway (Likhitwitayawuid and Sritularak, 2001). In plants, the defence response to pathogens is increasingly better linked or associated with the production and accumulation of phytoalexins, through activation of the general phenylpropanoid pathway (shikimate-phenylpropanoids-flavonoids pathways) (Bennett and Wallsgrove, 1994; Shulaev *et al.*, 2008; Pérez-Clemente *et al.*, 2013).

The association of these compounds with plant self-defence, renders these findings significant, however further research is required to confirm the putative annotation of these compounds. It is however important to fully verify our findings based on the information in Figure 4.6a and Figure 4.6b when hybrids are included in the analyses. It appears that several top markers are still prominent in the case of tolerant hybrids compared with citrus rootstock species analyses. Challenges with online database searches may also result in limitations for metabolite identification. The following chapter aims to develop a predictive model to examine whether potential biomarkers can be used in plant breeding to reduce the time required to select new rootstocks.

## 4.4 Conclusion

This first insight into the citrus metabolome applying metabolite fingerprinting analytical strategies has yielded promising results for tolerance trait discovery in the citrus rootstock- *P. nicotianae* interaction. No pattern describing differences between inoculated and control plants were observed when plants were assessed after 60 days of pathogen exposure. This suggests that any metabolites responsible for initial symptom suppression in tolerant

rootstocks may have decreased to normal levels long after initial infection (Afek and Sztejnberg, 1988). Very clear separation observed from PCA plots of tolerant *versus* susceptible rootstocks was evident from initial data mining processes. Metabolite fingerprinting showed several ion features (Table 4.4) recurrently present in 2 sets of data from 2 successive seasons. The association of these metabolites with the trait of *P. nicotianae* tolerance renders them of significant scientific importance.

These metabolites are constitutive or innate at higher concentration (% intensity) in tolerant rootstocks, opposed to susceptible rootstocks, and this feature is a key aspect in discovering tolerance traits in plants (Kumaraswamy *et al*., 2011). Although the primary aim of metabolite fingerprinting is not to identify individual compounds (Wolfender *et al*., 2015), in the current study we have taken a step toward determining whether the top markers are associated with plant self defense. From the initial literature survey, it is evident that our potential metabolite markers have been reported to have an association with plant stress defense responses in other plants. It is however necessary to confirm these findings through further work, fully annotating the top markers and better linking their function to citrus root rot tolerance.

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**Table 4.1** Citrus rootstocks that were included in the metabolite fingerprinting study

| Rootstock Varieties Season 1 | Rootstock Varieties Season 2 |
|---|---|
| Benton citrange *C. sinensis* X *P. trifoliate* (BC) | Australian trifoliate *P. trifoliate* (AT) |
| Cairn rough lemon *Citrus jambhiri* (RL) | Benton citrange *C. sinensis* X *P. trifoliate* (BC) |
| Carrizo citrange *C. sinensis* X *P. trifoliate* (CC) | Cairn rough lemon *Citrus jambhiri* (CRL) |
| Flying Dragon *P. trifoliate* (FD) | Flying Dragon *P. trifoliate* (FD) |
| Sunki mandarin and Benece trifoliate *C. sunki* X *P. trifoliate* (SXB) | Sunki mandarin and Benece trifoliate *C. sunki* X *P. trifoliate* (SXB |
| Swingle citrumelo *C. paradise* X *P. trifoliate* (SWC) | Swingle citrumelo *C. paradise* X *P. trifoliate* (SWC) |
| Tera Bella citrumelo *C. sinensis* X *P. trifoliate* (TB) | Tera Bella citrumelo *C. sinensis* X *P. trifoliate* (TB) |
| Volkamer lemon *Citrus volkameriana* (Volk) | |
| X639 hybrid *Cleopatra mandarin* X *P. trifoliate* (X639) | |

**Table 4.2** Parameters associated with the different datasets generated from MarkerLynx XS processing. (Corresponds with Figure 2).

| Experiment Sample list | Mass Tolerance (Da) | Intensity Threshold (counts) | X-Variables | Noise Levels (%) |
|---|---|---|---|---|
| Season 1 Tol-Con v Inoc | 0.01 | 10 | 18403 | 36 |
| Season 1 Tol-Con v Inoc | 0.01 | 100 | 8384 | 33 |
| Season 2 Tol-Con v Inoc | 0.01 | 10 | 26336 | 62 |
| Season 2 Tol-Con v Inoc | 0.01 | 100 | 11829 | 56 |
| Season 2 Tol-Con v Inoc | 0.05 | 100 | 8239 | 52 |

Tol= tolerant; Con= control; Inoc= inoculated.

**Table 4.3** Parameter settings and results from hypothesis driven data analysis approach generated on MarkerLynx XS software. (Corresponds with figure 4.3).

| Experiment Sample list | Mass Tolerance (Da) | Intensity Threshold (counts) | X-Variables | Noise Levels (%) | PCA $R^X2$-1; $R^X2$-2 |
|---|---|---|---|---|---|
| Season 1 Tol Spp. v Sus Spp. | 0.01 | 100 | 6746 | 37 | 0.518;0.1232 |
| Season 2 Tol Spp. v Sus Spp. | 0.01 | 100 | 9572 | 48 | 0.4923;0.0909 |
| *Season 1 Tol v Suc | 0.01 | 100 | 9617 | 36 | 0.3464;0.1951 |
| *Season 2 Tol v Sus | 0.05 | 100 | 9339 | 47 | 0.248;0.1283 |

*including hybrids. Tol= tolerant; Sus= susceptible.

**Table 4.4** Putative assignment of top markers.

| Feature (m/z_rt) | Calculated mass/ Da | Accurate mass/ Da | Mass difference/ Da | Putative Identification |
|---|---|---|---|---|
| 327.1592_9.67 | 326.1514 | 326.1518 | 0.0004 | Pulverochromenol |
| 259.0963_6.93 | 258.0885 | 258.0892 | 0.0007 | Wyerone |
| 259.0964_7.57 | 258.0886 | 258.0892 | 0.0006 | Wyerone |
| 313.1433_8.72 | 312.1355 | 312.1362 | 0.0007 | 4’-prenyloxyresveratrol |

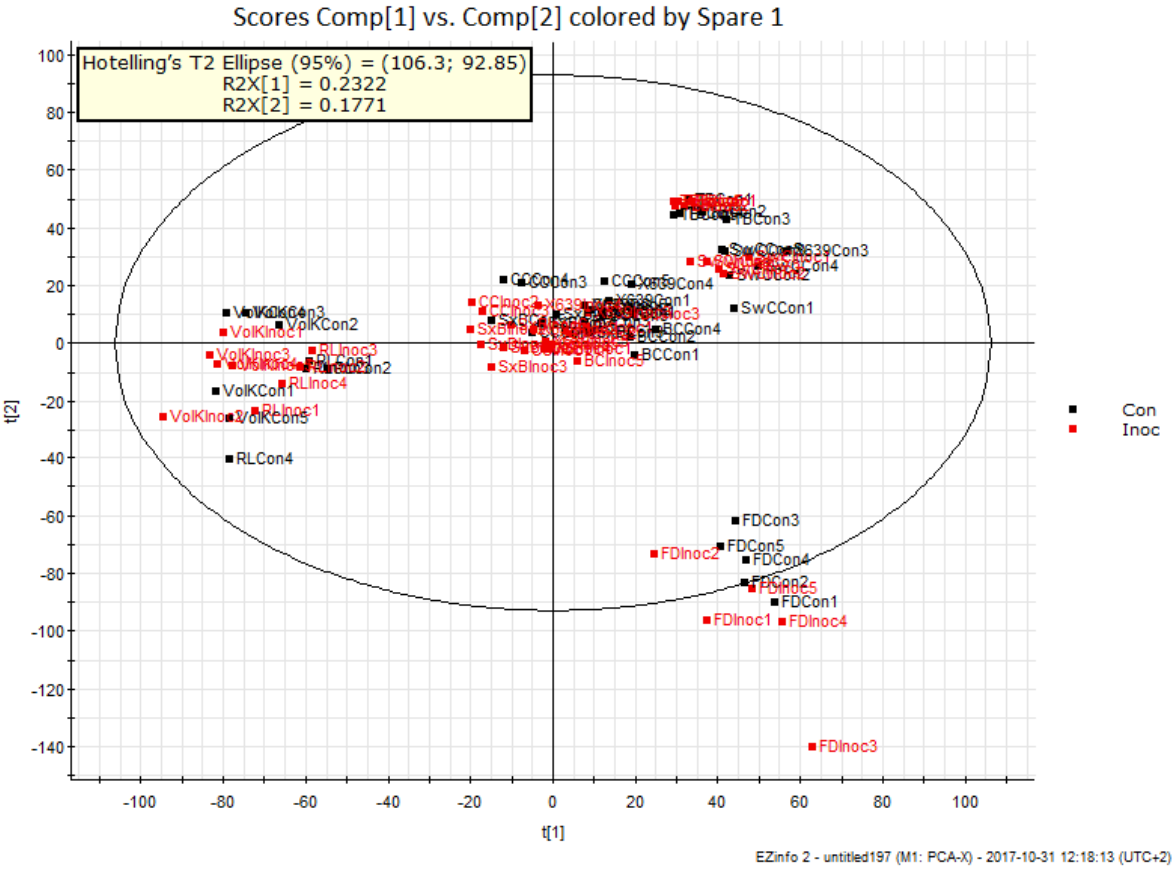



Figure 4.1a. Score plot of principal component analysis (PCA) based on LC/MS citrus metabolome treatment Initial data driven **Season 1** = Control *versus P.nicotianae* inoculated samples. The rootstocks are represented by their abbreviations e.g. FD – Flying dragon (Table 4.1). No separation between inoculated (red) and control (black) samples indicating no variables of interest detected due to pathogen effect.

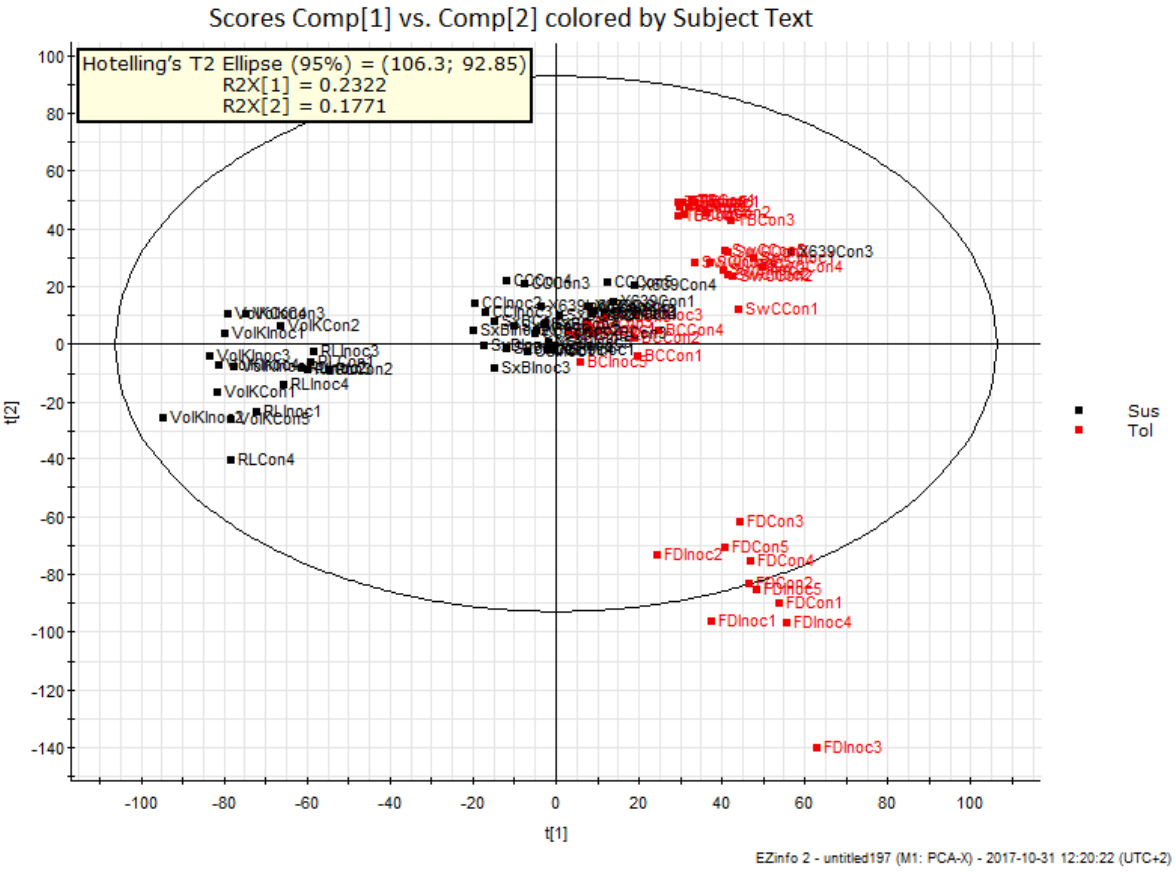



Figure 4.1b. Score plot for principal component analysis (PCA) based on LC/MS citrus metabolome treatment Initial data driven **Season 1** = Tolerant *versus* Susceptible. The rootstocks are represented by their abbreviations e.g. FD – Flying dragon (Table 4.1). Separation between tolerant (red) and susceptible (black) samples and good model quality ($R^2X$ 1=0.2322). This clustering between tolerant and susceptible rootstocks suggests potential variables of interest as trait markers.

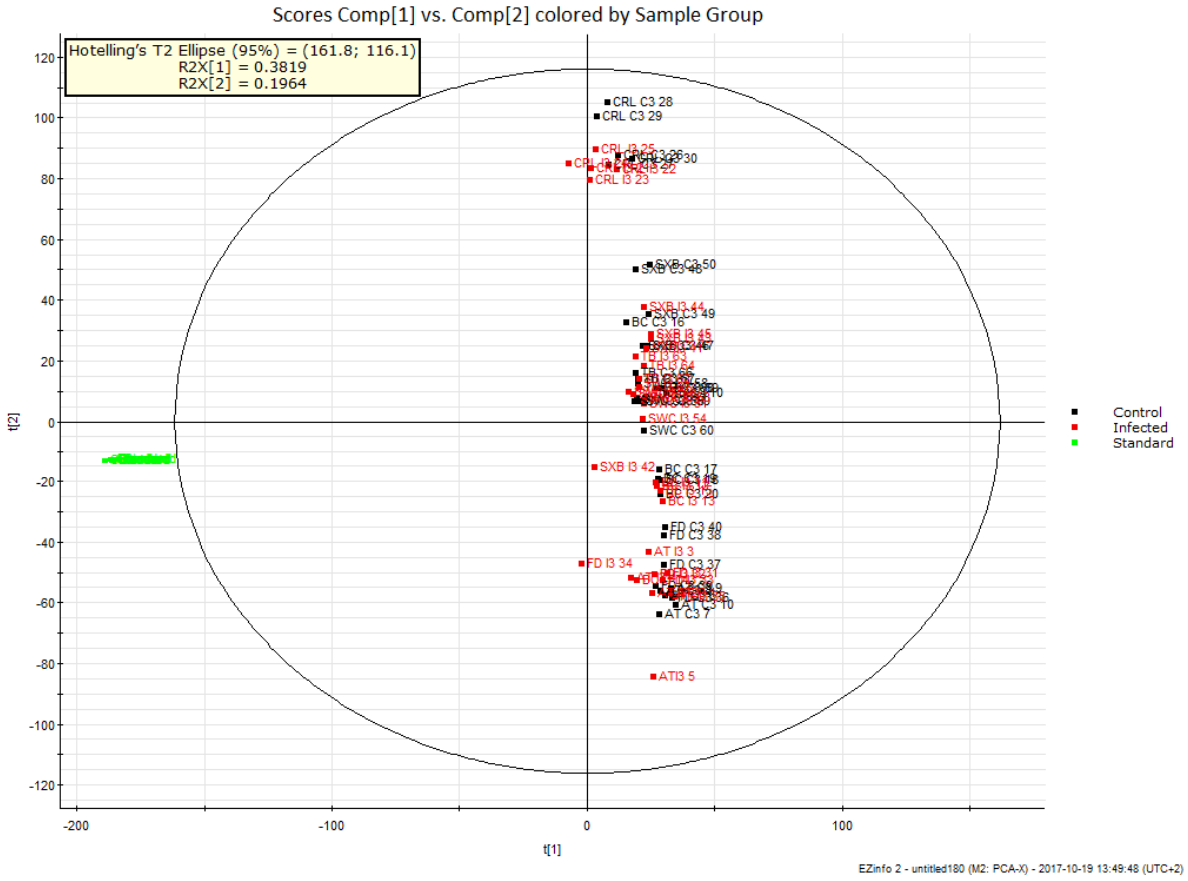



Figure 4.1c. Score plot for principal component analysis (PCA) based on LC/MS citrus metabolome treatment Initial data driven **Season 2** = Control *versus* Inoculated samples. The rootstocks are represented by their abbreviations e.g. FD – Flying dragon (Table 4.1). No separation between inoculated (red) and control (black) samples indicating no variables of interest for pathogen effect. PCA a = label by Control v Inoculated. (Standards (green) left in to show efficient LC/MS through their clustering pattern). Typically, the standards are removed from analyses however, for the purposes of this initial section, they are included.

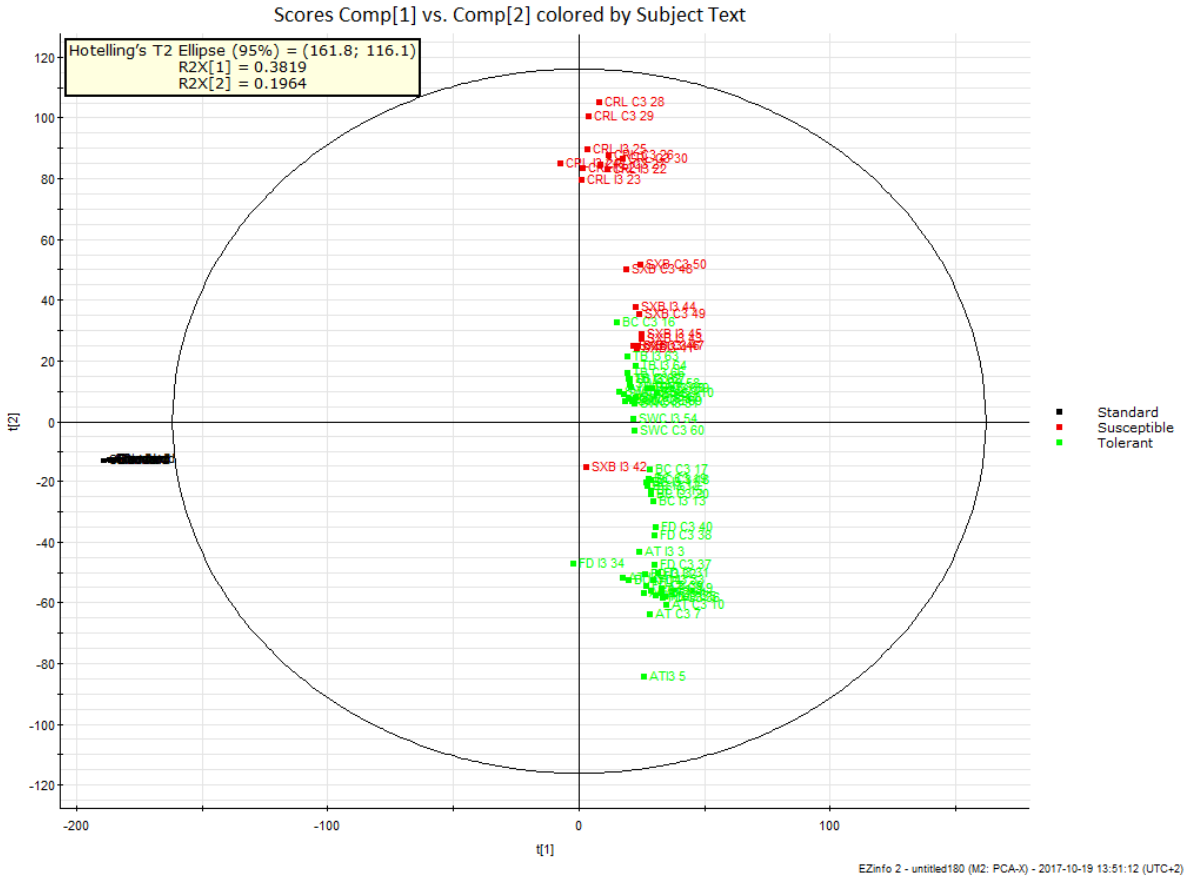



Figure 4.1d. Score plot for principal component analysis (PCA) based on LC/MS citrus metabolome treatment Initial data driven **Season 2**= Tolerant *versus* Susceptible citrus rootstocks. The rootstocks are represented by their abbreviations e.g. FD – Flying dragon (Table 4.1). Separation between tolerant (green) and susceptible (red) samples with $R^2X$ 1 value= 0.3819. Although the standards affect the results the clustering between tolerant and susceptible rootstocks is evident (also observed for season 1 Figure 4.1b)

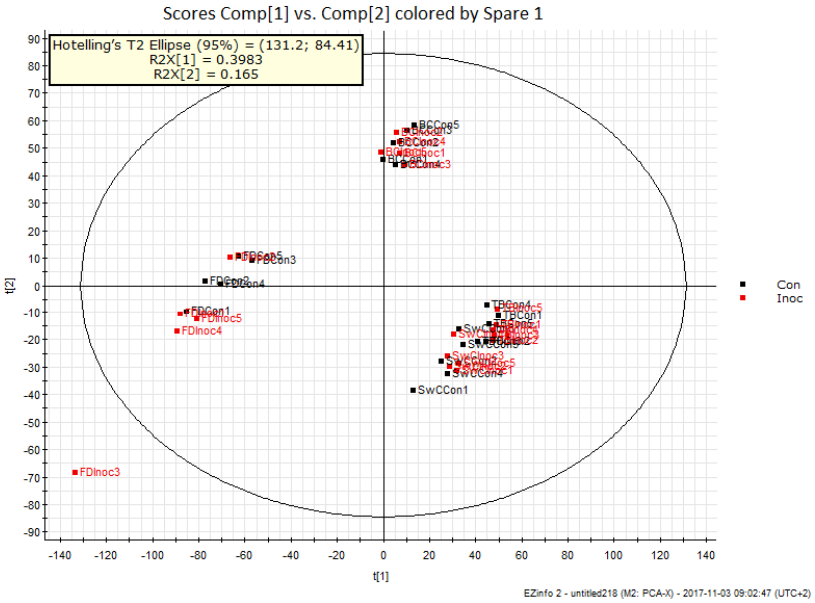



Figure 4.2a. Score plots of principal component analyses (PCA) for parameter adjustment based on LC/MS citrus metabolome **Season 1** Intensity threshold – 10 counts: (Corresponds with Table 4.2). No separation between inoculated (red) and control (black) samples.

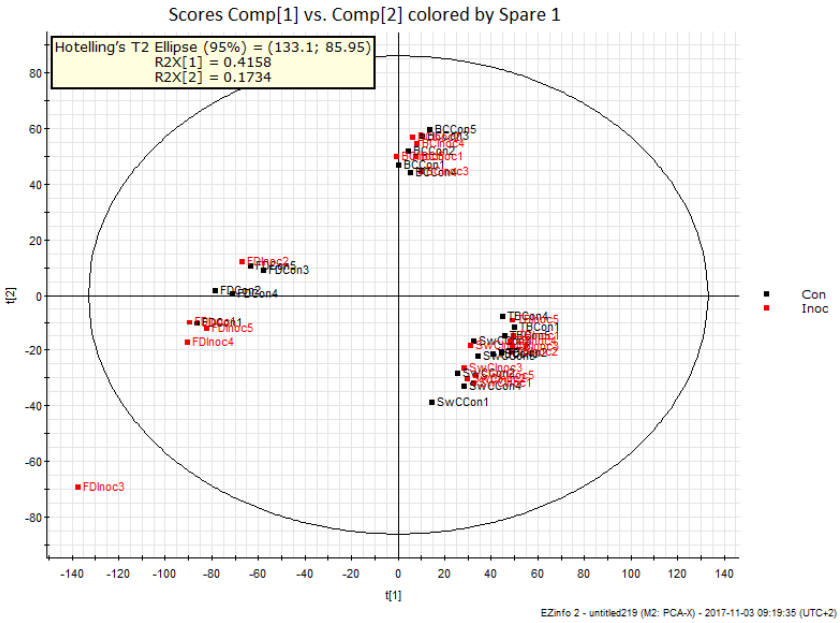



Figure 4.2b. Score plots of principal component analyses (PCA) for parameter adjustment based on LC/MS citrus metabolome **Season 1** Intensity threshold – 100 counts: (Corresponds with Table 4.2). No separation between inoculated (red) and control (black) samples.

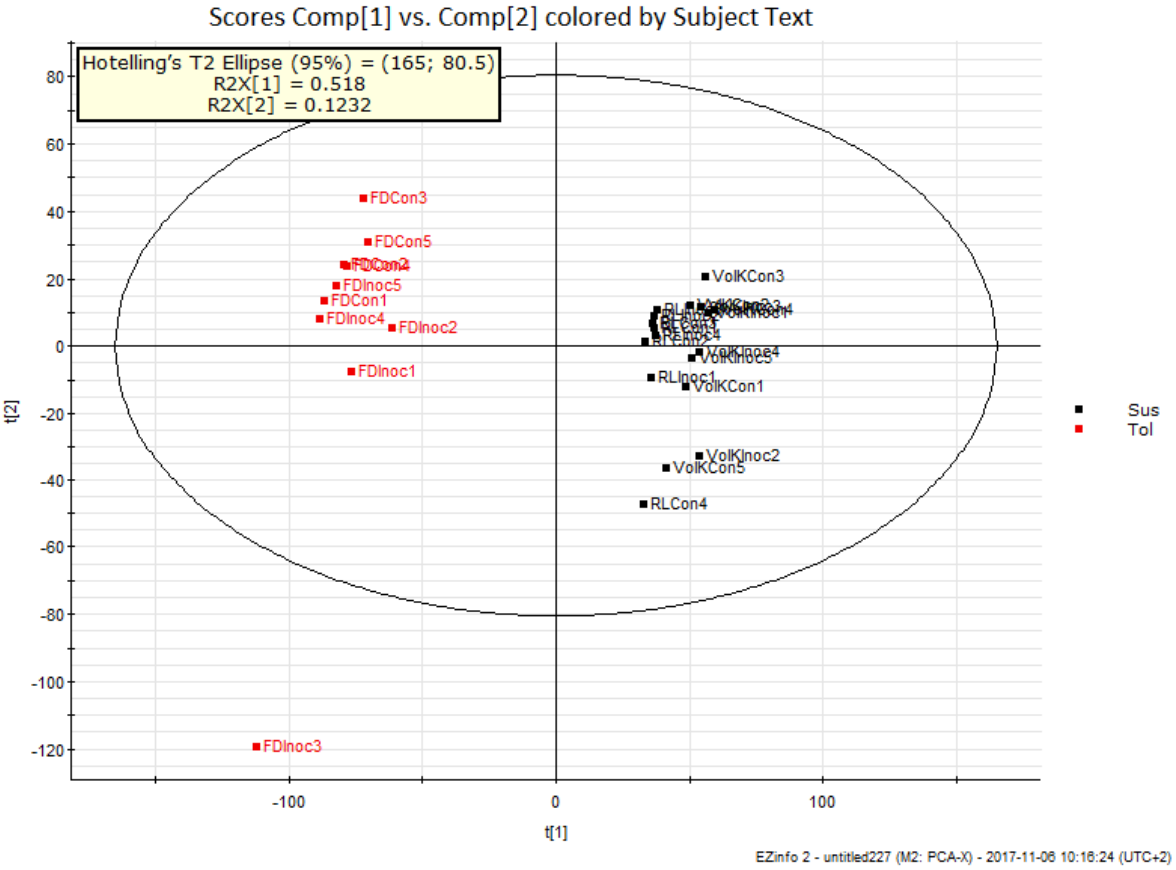



Figure 4.3a. Principle component analysis (PCA) score plot based on LC/MS citrus metabolome **Season 1** Tolerant (red) *versus* Susceptible (black) citrus rootstock species only. Clear separation between FD (tolerant) and RL & Volk (susceptible). Significantly high $R^2X$ 1 value= 0.518.

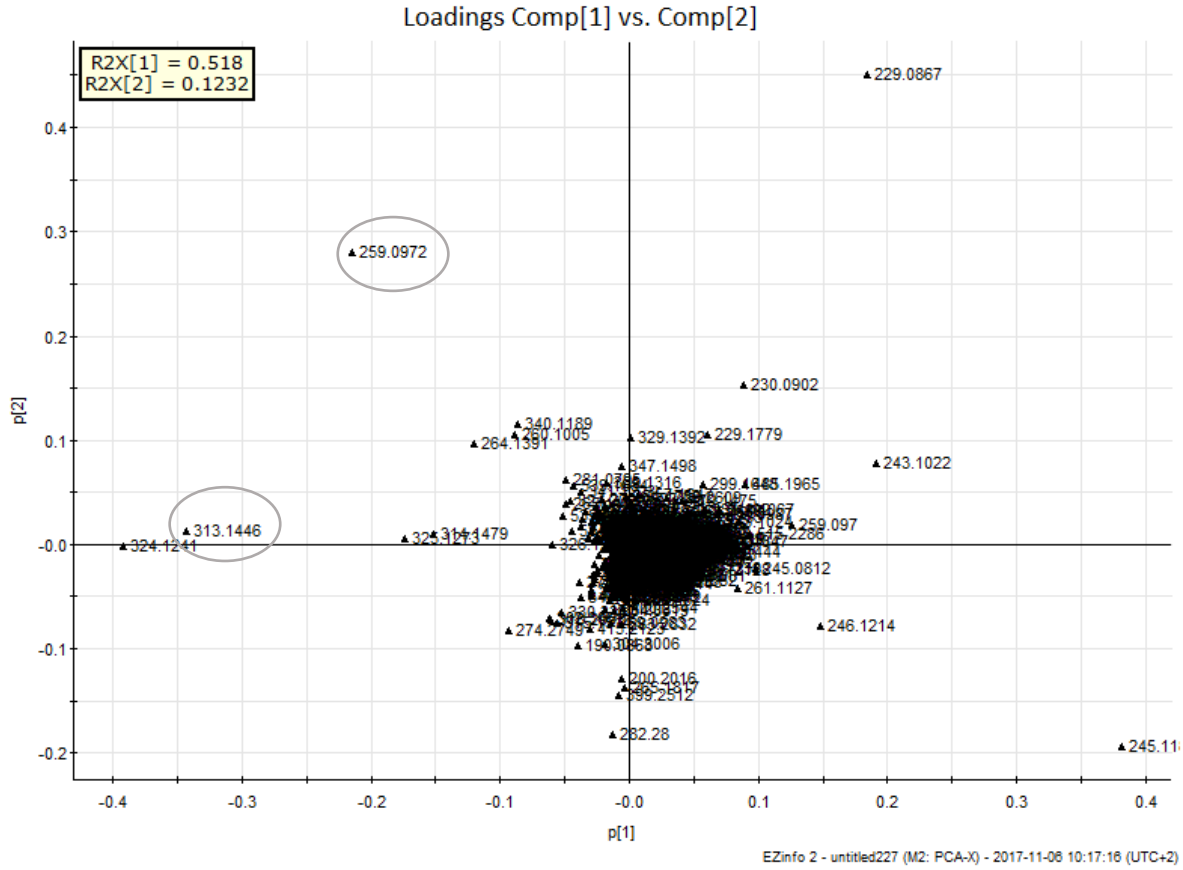



Figure 4.3a-1. Loadings plot for the PCA **Season 1** Tolerant *versus* Susceptible citrus rootstock species based on LC/MS citrus metabolome. Circles highlight features of importance to separate sample groups.

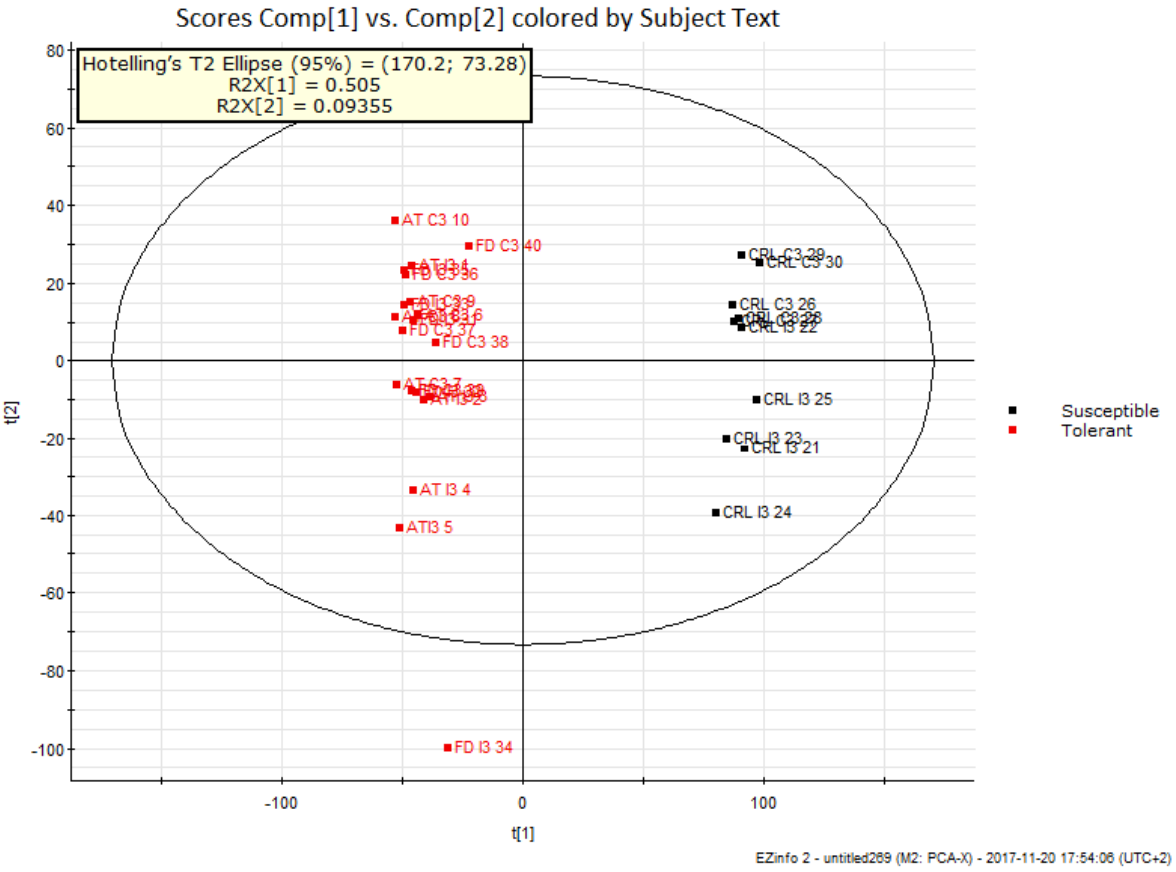



Figure 4.3b. Principle component analysis (PCA) score plot based on LC/MS citrus metabolome root **Season 2** root material extracts. Tolerant *versus* susceptible citrus rootstock species only. Clear separation between FD & AT- tolerant and CRL –susceptible rootstocks. Significantly high $R^2X$ 1 value =0.505.

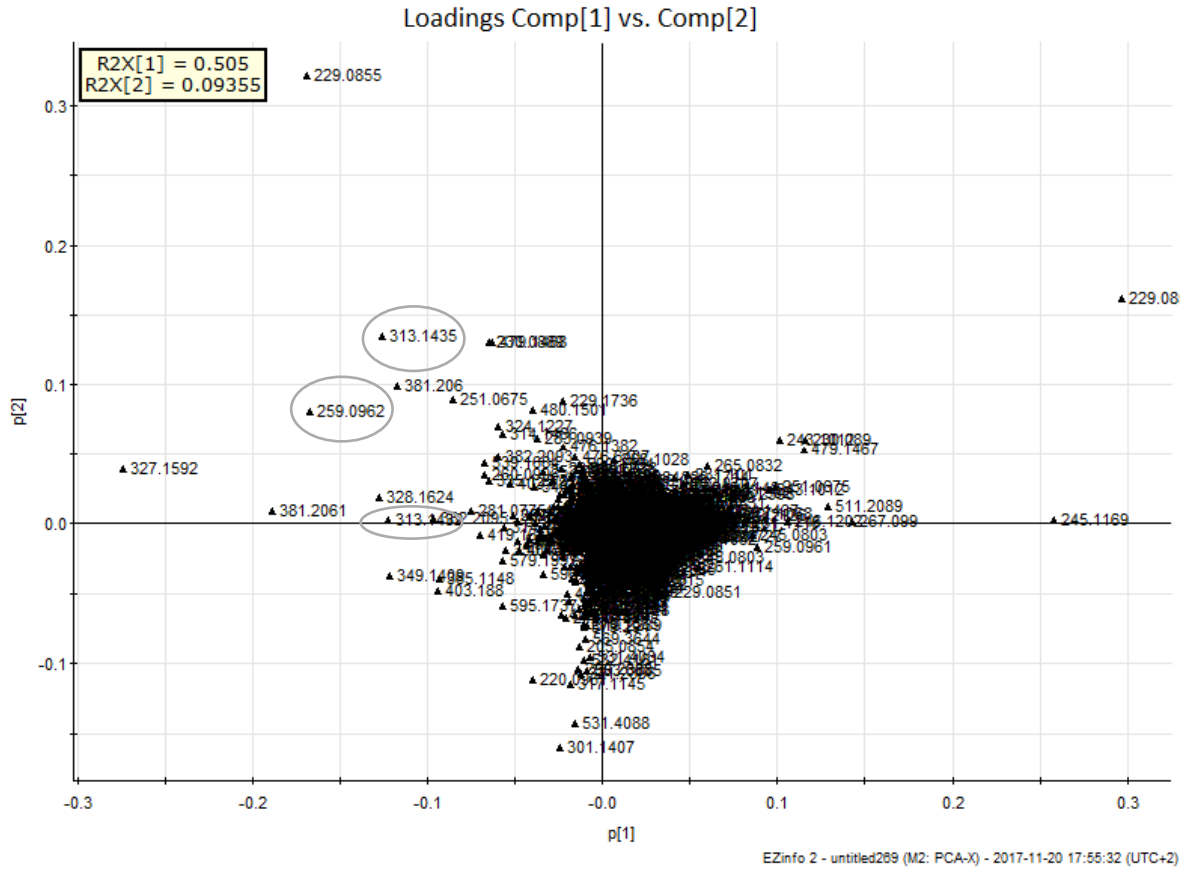



Figure 4.3b-1. Loadings plot for the PCA scores plot. Tolerant *versus* susceptible citrus rootstock species based on LC/MS citrus metabolome **Season 2**. Circled features recurrent from previous season as potential biomarkers for tolerance in citrus rootstocks.

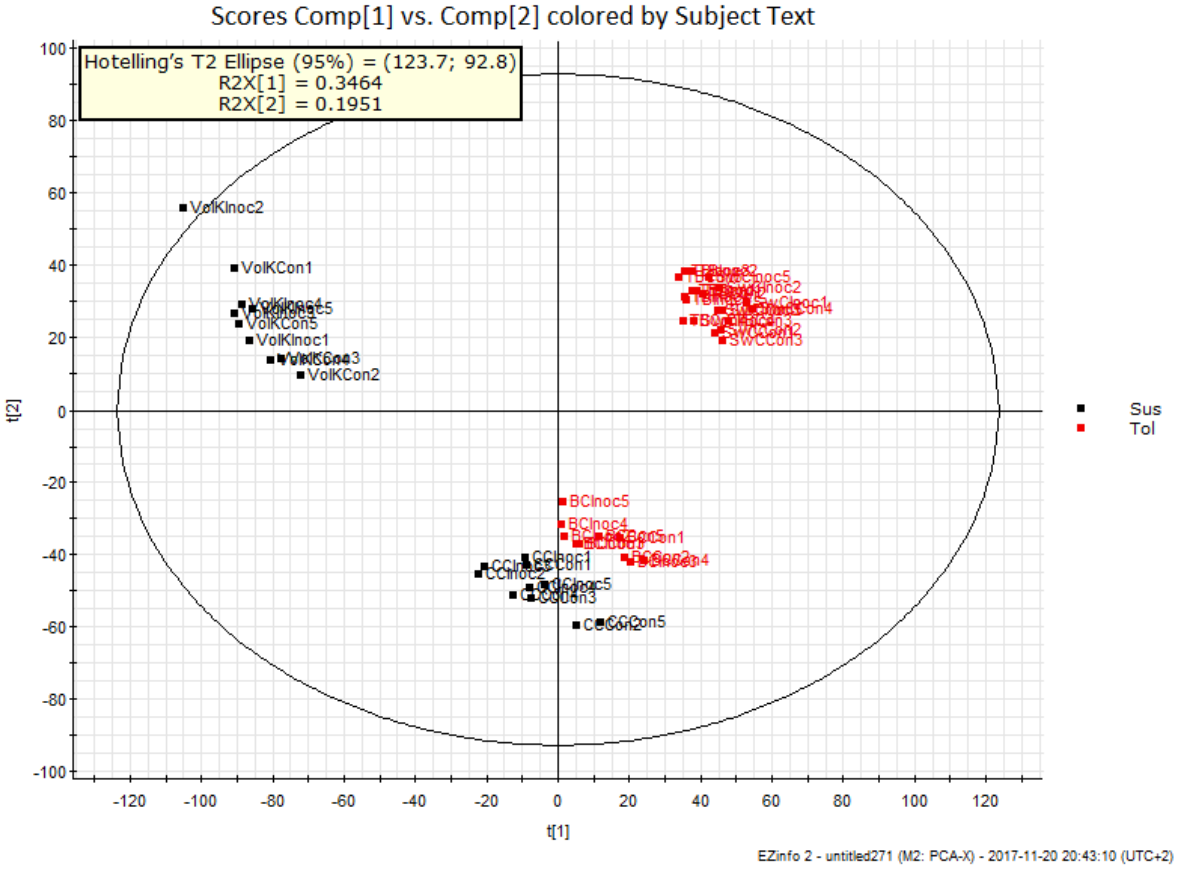



Figure 4.3c. Principle component analysis (PCA) score plot based on LC/MS citrus metabolome **Season 1** Tolerant (red) *versus* susceptible (black) citrus rootstocks including hybrids. Clear separation between TB, SWC and BC-tolerant and Volk and CC-susceptible citrus rootstocks. $R^2X$ 1 value= 0.3464.

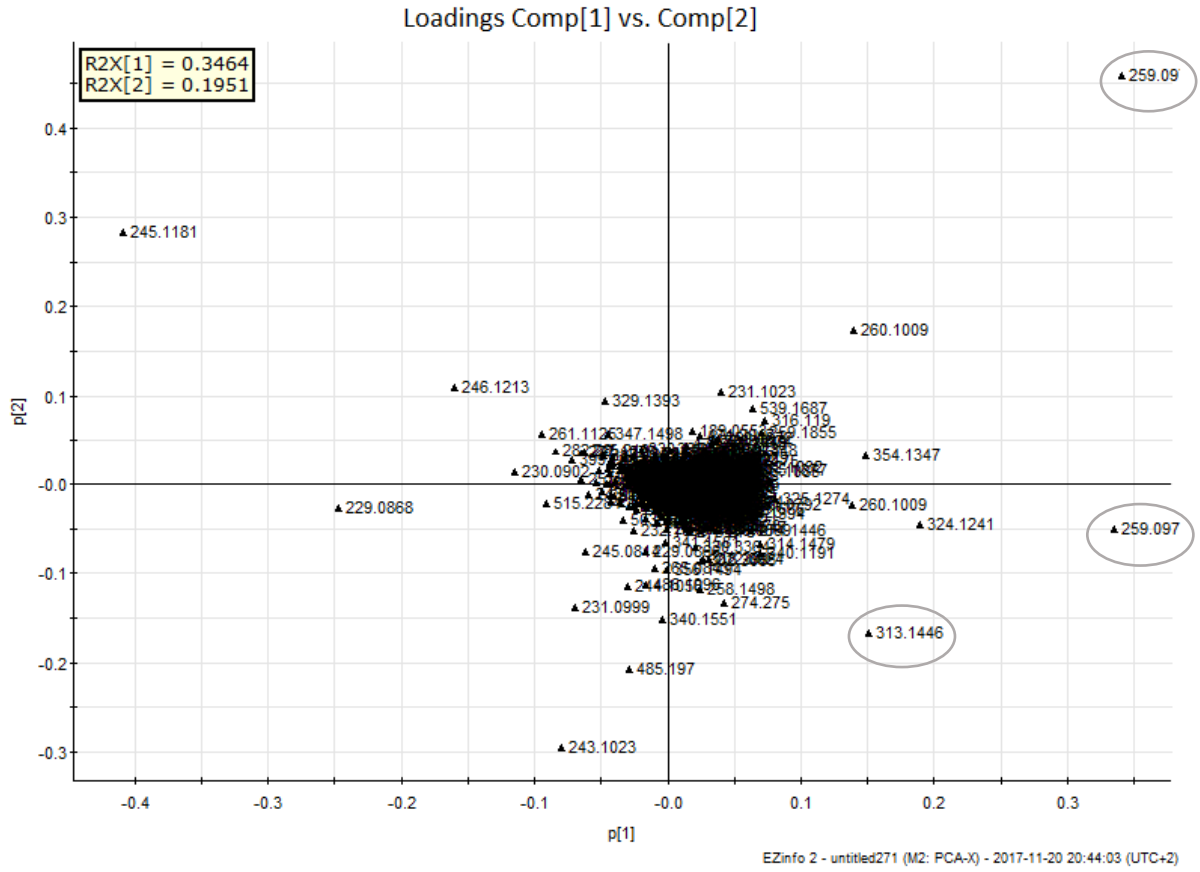



Figure 4.3c-1. Loadings plot for the PCA scores plot. Tolerant *versus* Susceptible citrus rootstock species based on LC/MS citrus metabolome **Season 1**. Circled features important for contrasting Tolerant rootstocks *versus* susceptible rootstock hybrids.

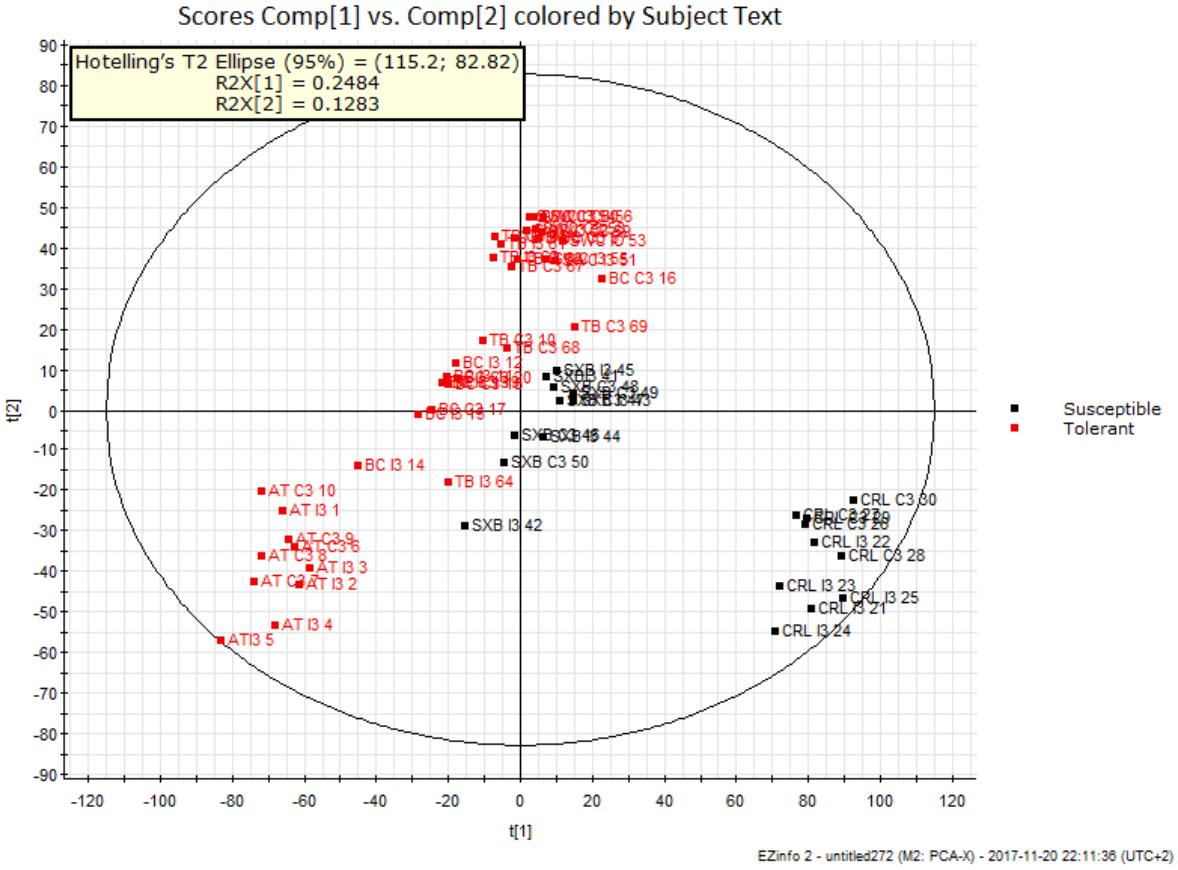



Figure 4.3d. Principle component analysis (PCA) score plot based on LC/MS citrus metabolome **Season 2** Tolerant (red) *versus* susceptible (black) citrus rootstocks including citrus rootstock hybrids. Clear separation between AT, BC TB, and SWC -tolerant and SXB and CRL-susceptible citrus rootstocks. $R^X2$ 1 value= 0.2438.

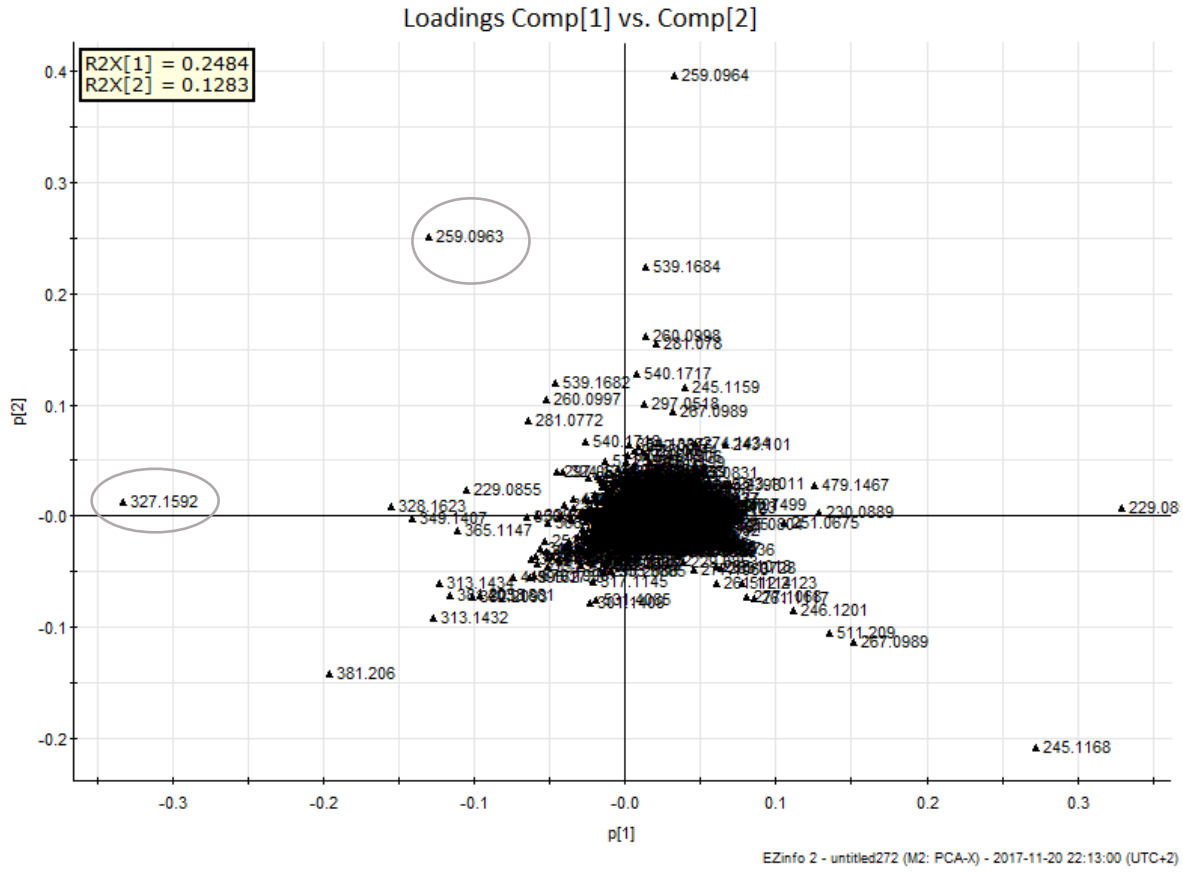



Figure 4.3d-1. Loadings plot for the PCA scores plot based on LC/MS citrus metabolome. Tolerant *versus* Susceptible citrus rootstock species **Season 2**. Circled features important for contrasting Tolerant rootstocks *versus* susceptible rootstock hybrids and recur over two season forming a pattern for potential tolerance biomarkers.

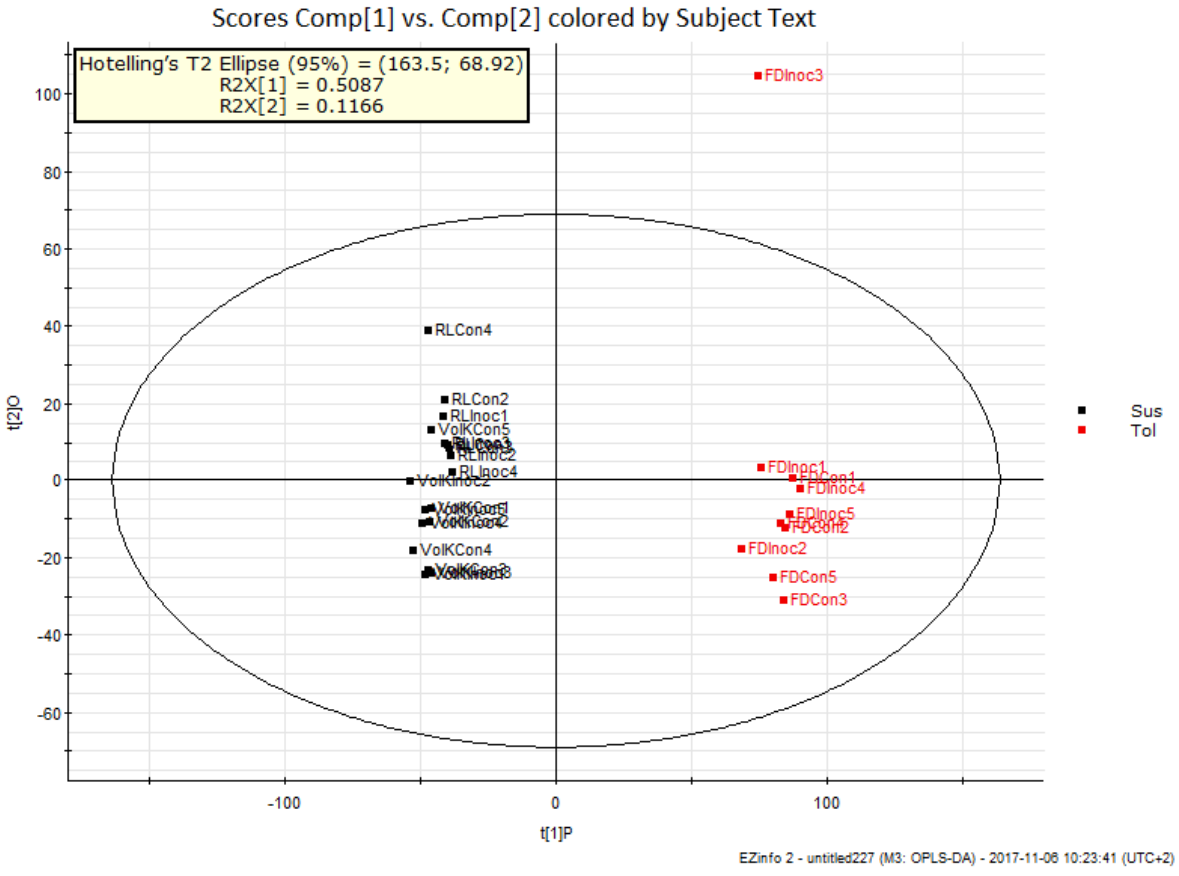



Figure 4.4a. Orthogonal partial least squared discriminant analysis (OPLS-DA) of citrus rootstock root LC/MS fingerprint **Season 1**. Citrus rootstock species only according to Tolerant (red) *versus* susceptible (black). Three rootstocks included FD (tolerant) and RL and Volk (susceptible) rootstock species.

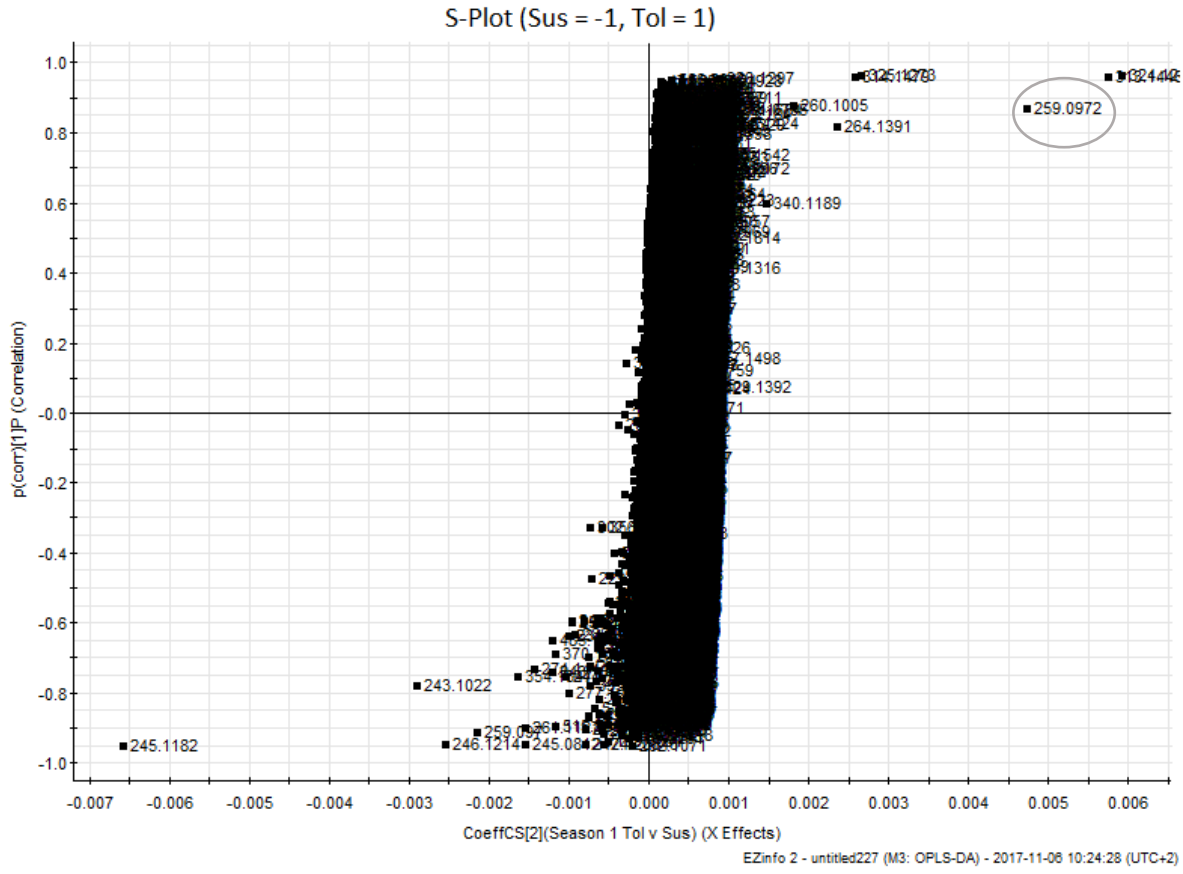



Figure 4.4a-1. S-Plot of the OPLS-DA Citrus rootstock species only **Season 1**. Variables situated far out in the S-plot are statistically relevant and represent potential discriminating features. The markers at the bottom left and top right of the curve, with p corr-[1] < -0.5 and > 0.5, occur predominantly in the tolerant (Tol = 1) and susceptible (Sus = -1) cultivars. Each feature is identified by accurate mass e.g. circled- 259.0972.

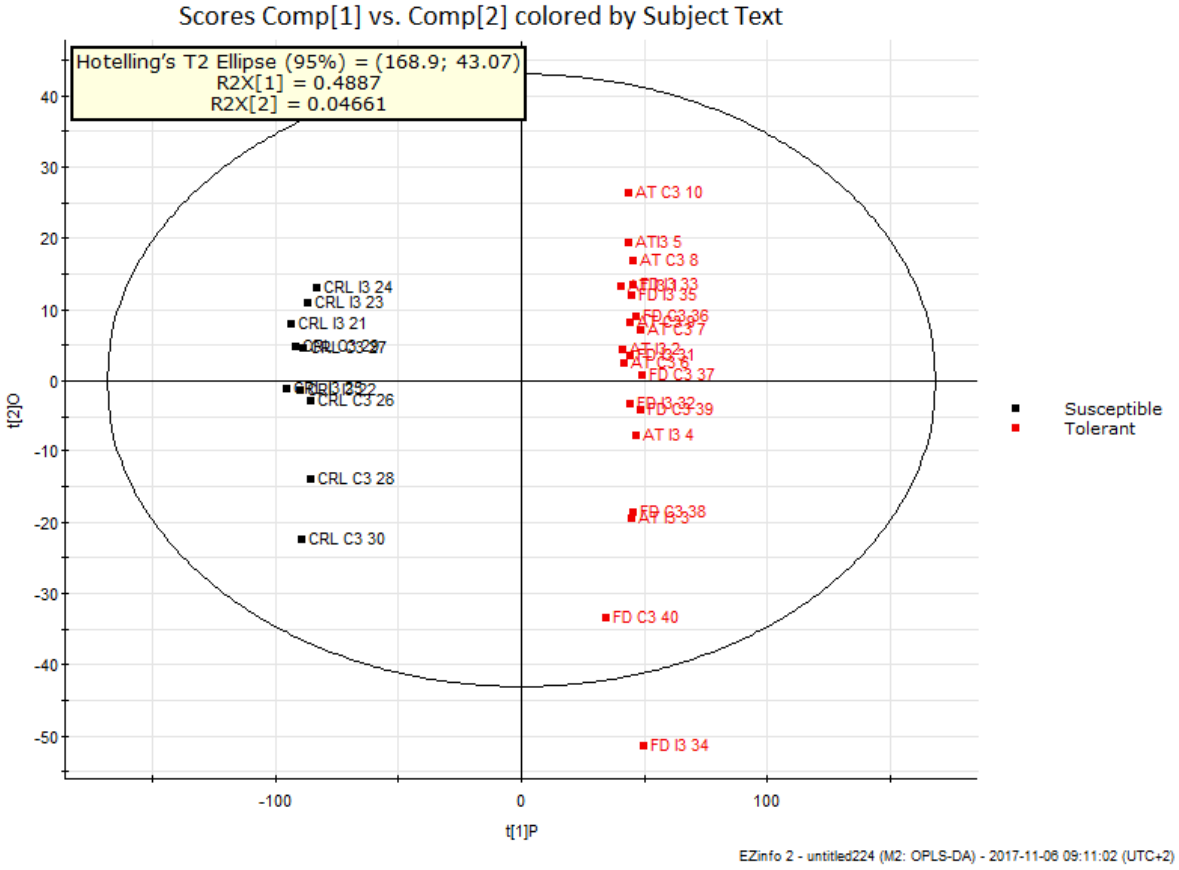



Figure 4.4b. Orthogonal partial least squared discriminant analysis (OPLS-DA) of citrus rootstock root LC/MS fingerprint **Season 2**. Citrus rootstock species only according to Tolerant (red) *versus* susceptible (black). Three rootstocks included FD and AT-tolerant and CRL -susceptible rootstock species.

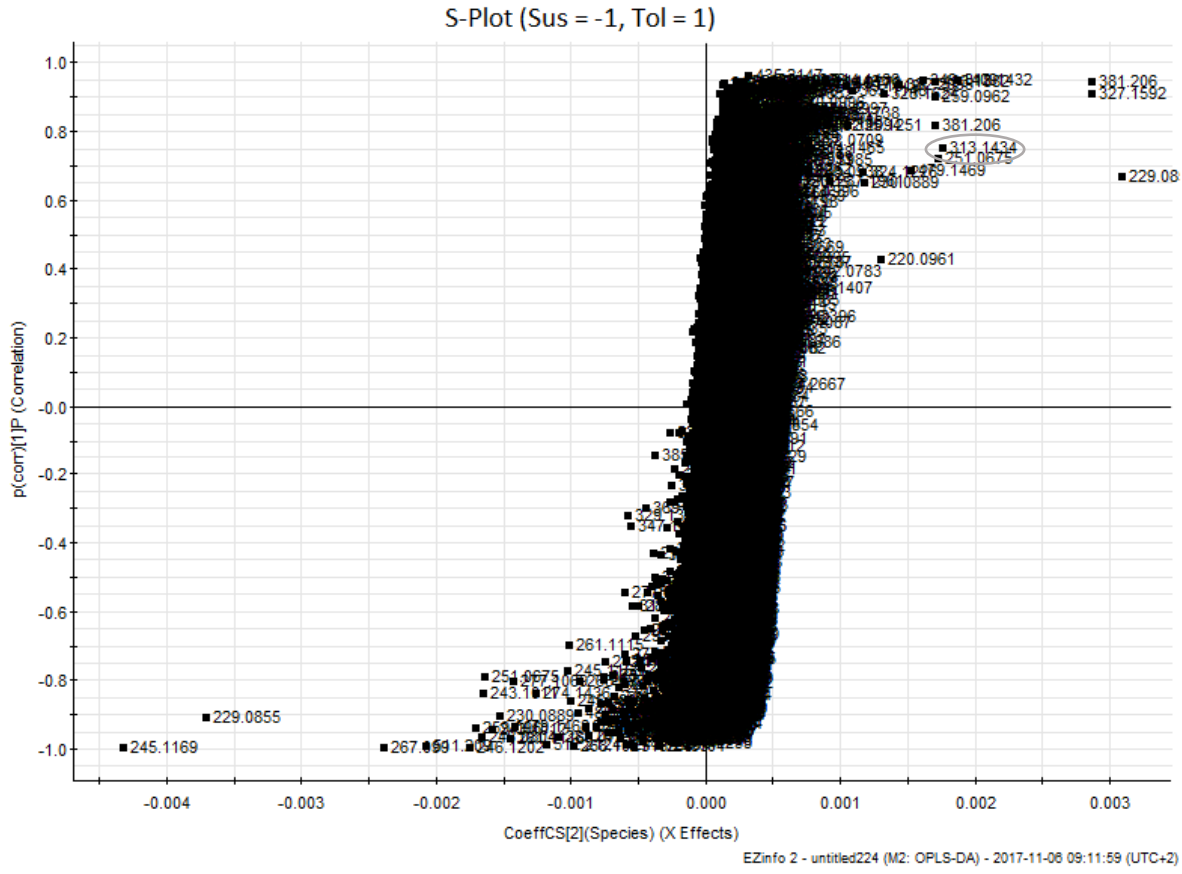



Figure 4.4b-1. S-Plot of the OPLS-DA Citrus rootstock species only **Season 2**. Variables situated far out in the S-plot are statistically relevant and represent potential discriminating features. The markers at the bottom left and top right of the curve, with p corr-[1] < -0.5 and > 0.5, occur predominantly in the tolerant (Tol = 1) and susceptible (Sus = -1) cultivars. Each feature is identified by accurate mass e.g. circled- 313.1434.

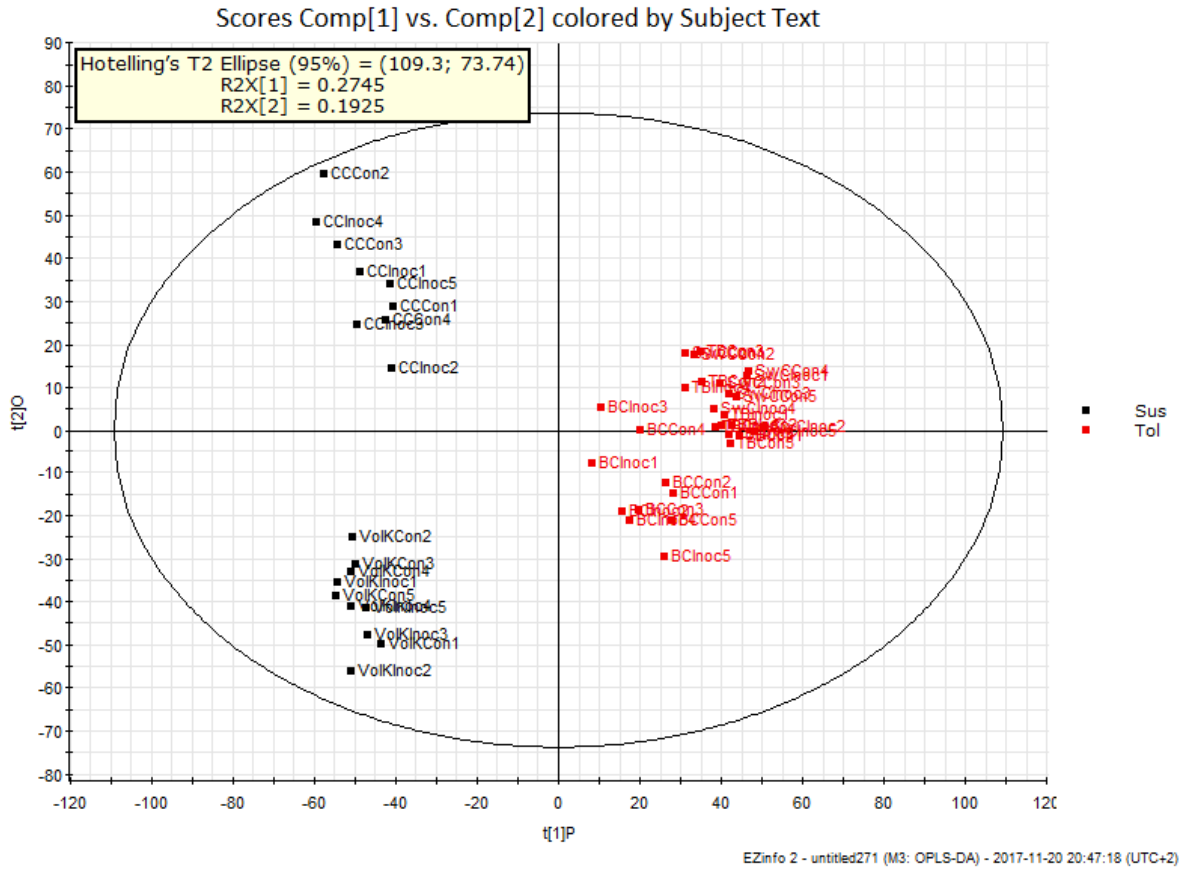



Figure 4.4c. Orthogonal partial least squared discriminant analysis (OPLS-DA) of citrus rootstock root LC/MS fingerprint **Season 1**. Citrus rootstocks according to Tolerant (red) *versus* Susceptible (black). Five rootstocks included: BC, SWC TB (tolerant) and CC and Volk (susceptible) rootstock. OPLS-DA model Citrus rootstock Hybrids Tolerant *versus* Susceptible.

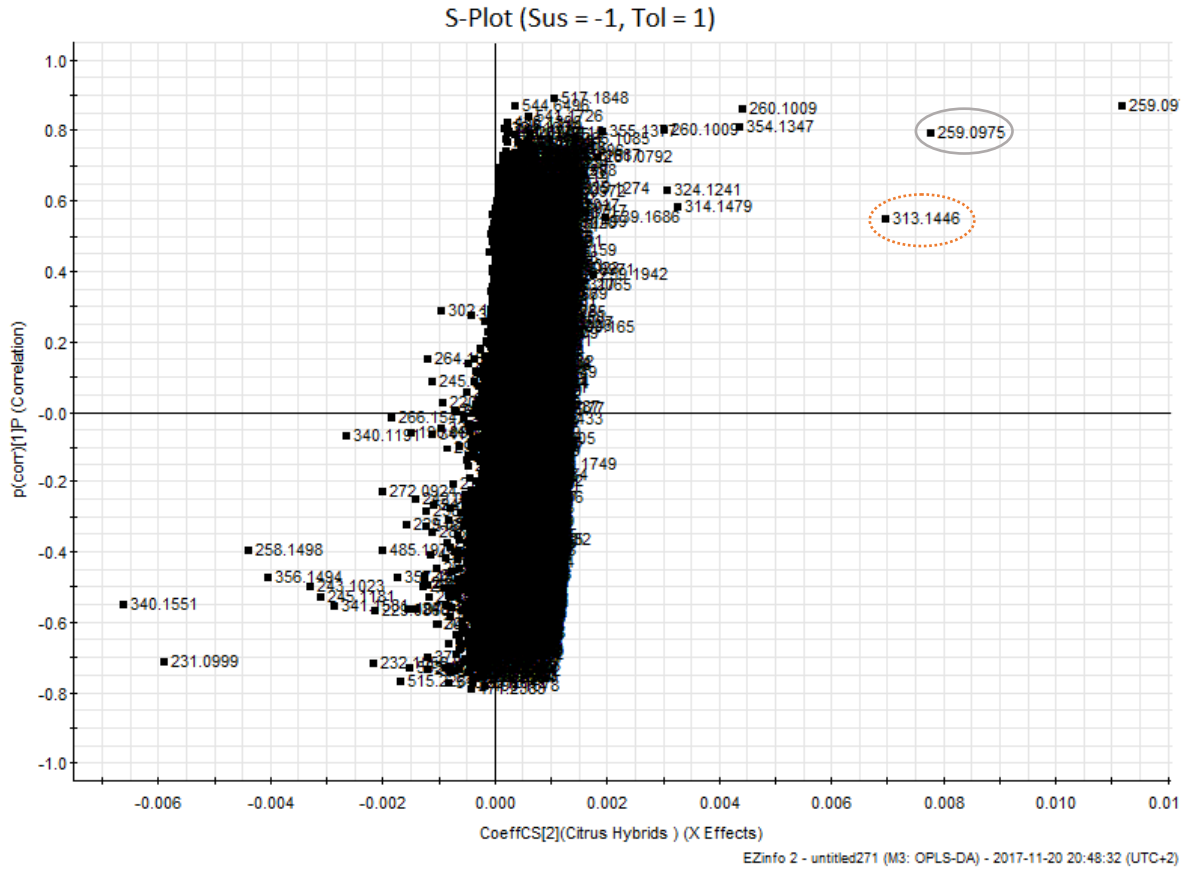



Figure 4.4c-1. S-Plot of the OPLS-DA Citrus rootstock species only **Season 1**. Variables situated far out in the S-plot are statistically relevant and represent potential discriminating features. The markers at the bottom left and top right of the curve, with p corr-[1] < -0.5 and > 0.5, occur predominantly in the tolerant (Tol = 1) and susceptible (us = -1) cultivars. Each feature is identified by accurate mass e.g. full circle- 259.0975. Dash circle (313.1446 *m/z*) corresponds with features of importance in Season 1 trend line plot 4 below (Fig. 4.5a).

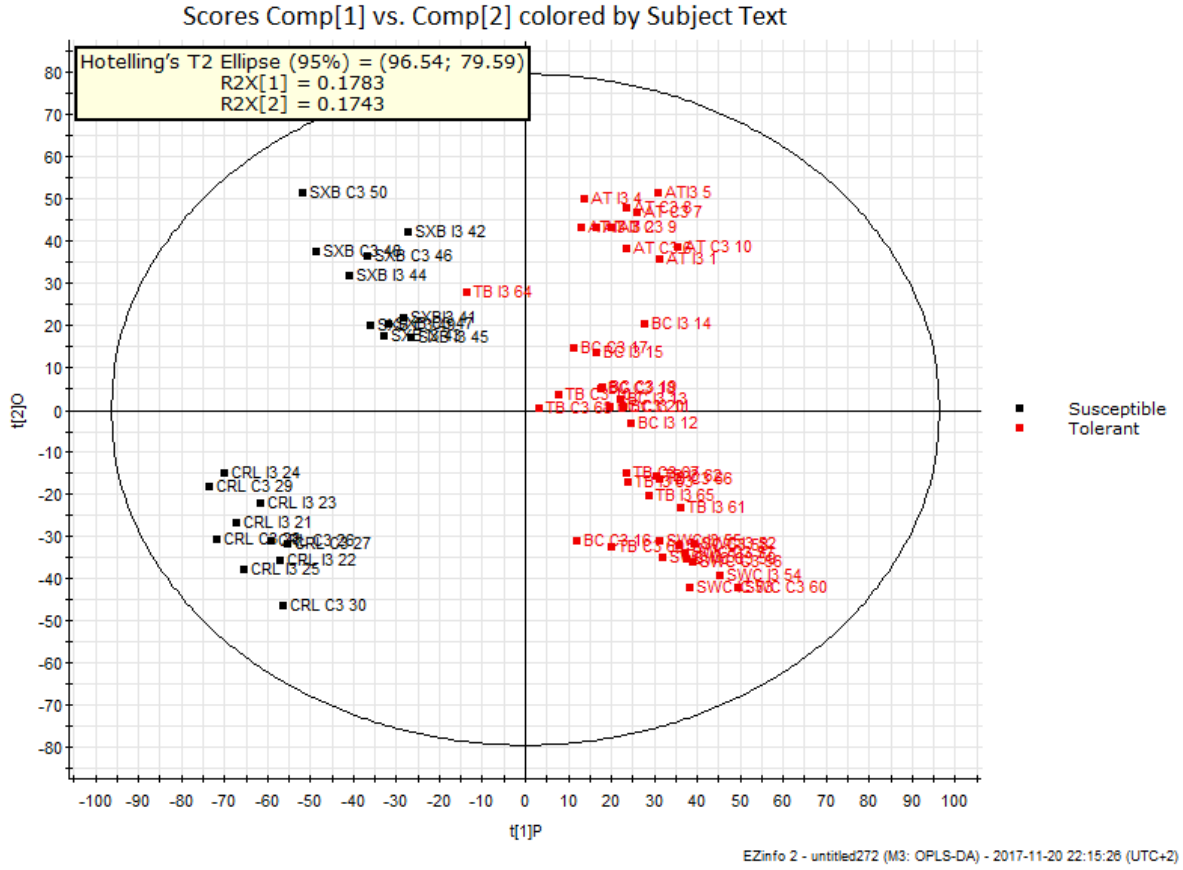



Figure 4.4d. Orthogonal partial least squared discriminant analysis (OPLS-DA) of citrus rootstock root LC/MS fingerprint **Season 1**. Citrus rootstock hybrids according to Tolerant (red) *versus* Susceptible (black). Six rootstocks included AT, BC, SWC and TB-tolerant and CRL and SXB-susceptible rootstock hybrids.

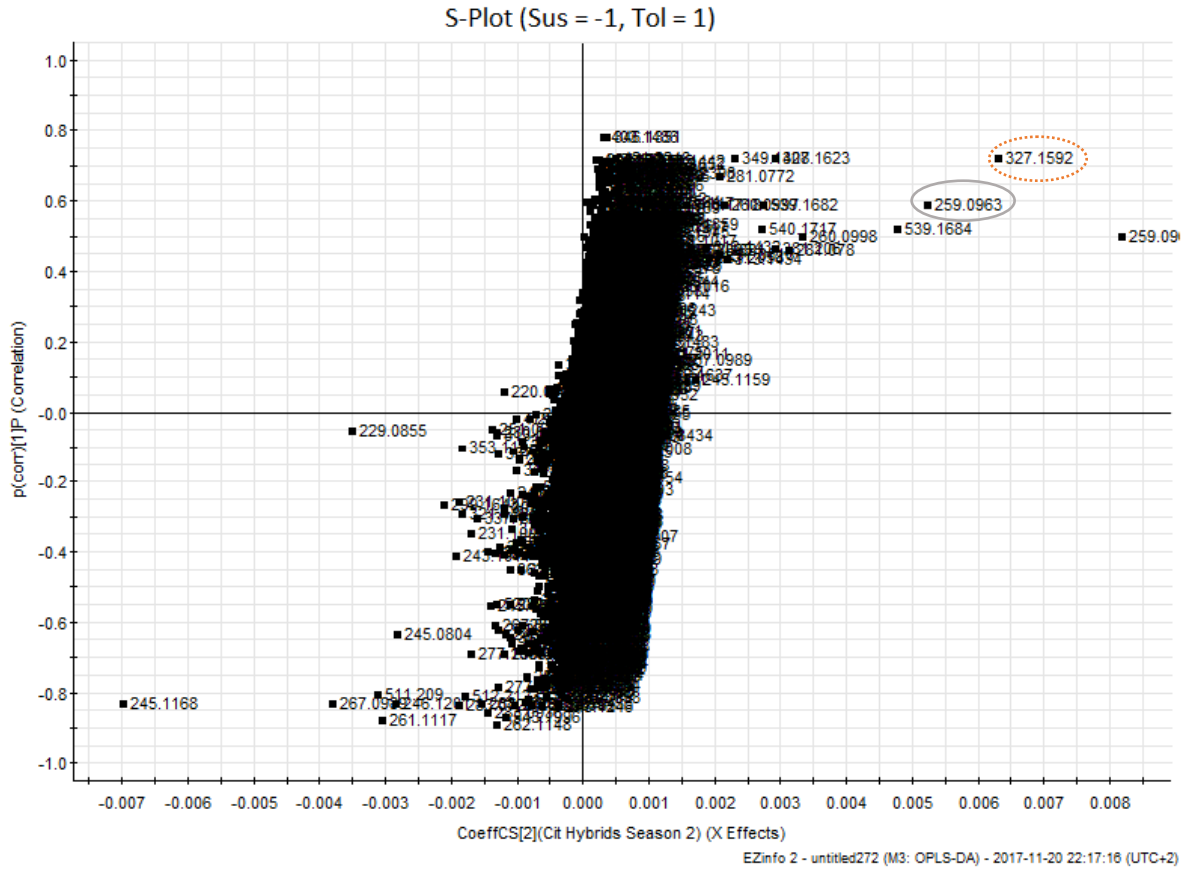
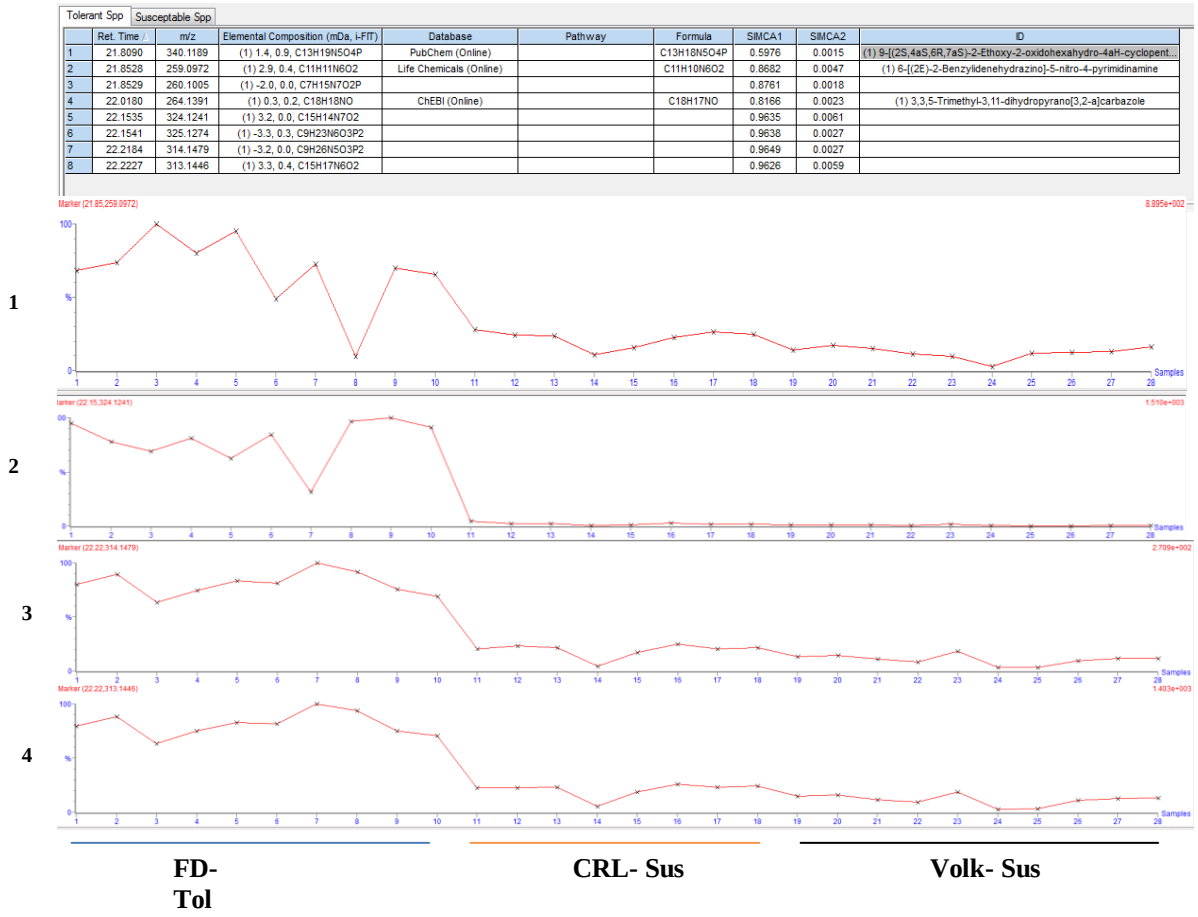



Figure 4.4d-1. S-Plot of the OPLS-DA Citrus rootstock hybrids **Season 2**. Variables situated far out in the S-plot are statistically relevant and represent potential discriminating features. The markers at the bottom left and top right of the curve, with p corr-[1] < -0.5 and > 0.5, occur predominantly in the tolerant (Tol = 1) and susceptible (Sus = -1) cultivars. Each feature is identified by accurate mass e.g. circled- 259.0963. Dash circle (327.1592 *m/z*) corresponds with features of importance in Season 2 citrus hybrids trend line plot 4 below (Fig. 4.6a).

Trend Line Plots

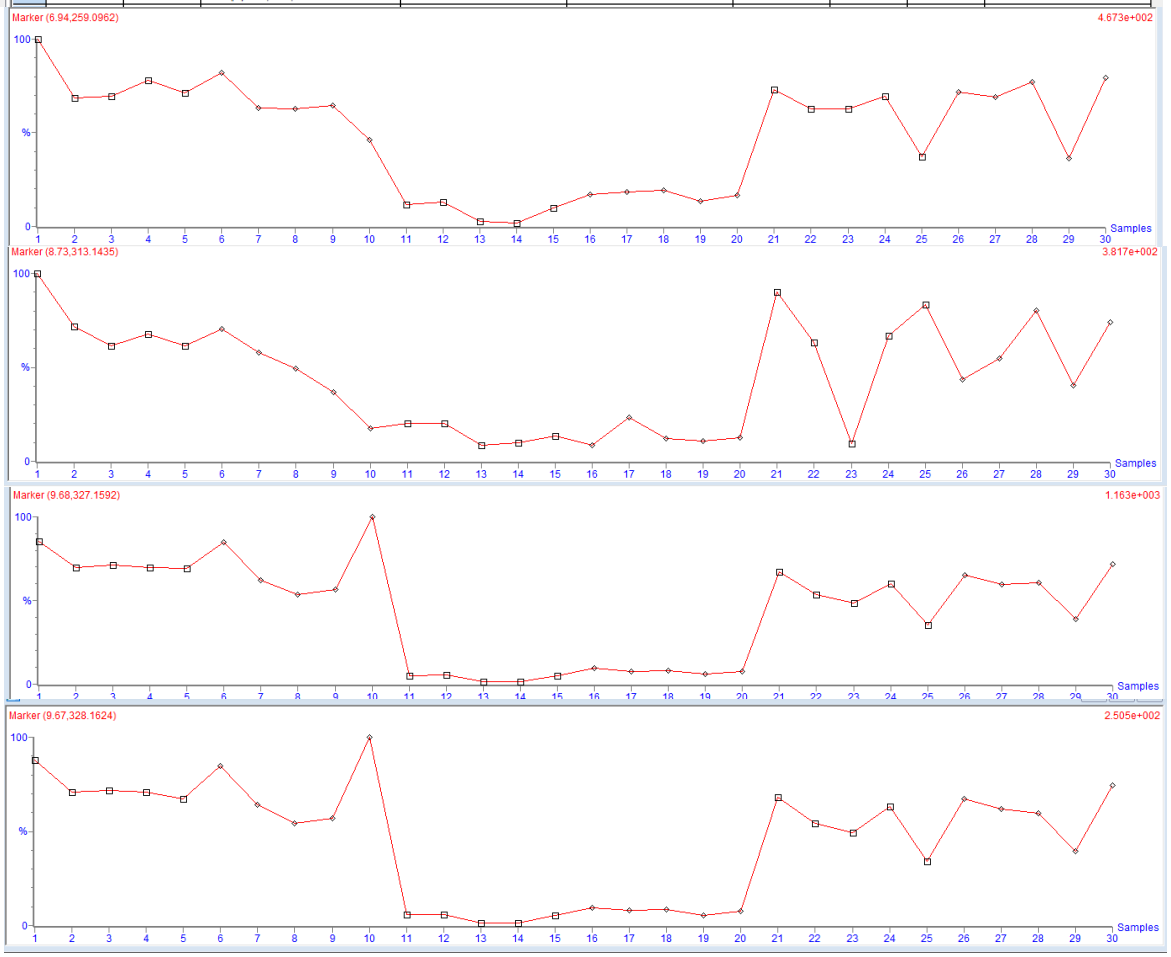



| | Ret. Time | m/z | Elemental Composition (mDa, i-FIT) | Database | Pathway | Formula | SIMCA1 | SIMCA2 | ID |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 21.8090 | 340.1189 | (1) 1.4, 0.9, C13H19N5O4P | PubChem (Online) | | C13H18N5O4P | 0.5976 | 0.0015 | (1) 9-[(2S,4aS,6R,7aS)-2-Ethoxy-2-oxidohexahydro-4aH-cyclopent... |
| 2 | 21.8528 | 259.0972 | (1) 2.9, 0.4, C11H11N6O2 | Life Chemicals (Online) | | C11H10N6O2 | 0.8682 | 0.0047 | (1) 6-[(2E)-2-Benzylidenehydrazino]-5-nitro-4-pyrimidinamine |
| 3 | 21.8529 | 260.1005 | (1) -2.0, 0.0, C7H15N7O2P | | | | 0.8761 | 0.0018 | |
| 4 | 22.0180 | 264.1391 | (1) 0.3, 0.2, C18H18NO | ChEBI (Online) | | C18H17NO | 0.8166 | 0.0023 | (1) 3,3,5-Trimethyl-3,11-dihydropyrano[3,2-a]carbazole |
| 5 | 22.1535 | 324.1241 | (1) 3.2, 0.0, C15H14N7O2 | | | | 0.9635 | 0.0061 | |
| 6 | 22.1541 | 325.1274 | (1) -3.3, 0.3, C9H23N6O3P2 | | | | 0.9638 | 0.0027 | |
| 7 | 22.2184 | 314.1479 | (1) -3.2, 0.0, C9H26N5O3P2 | | | | 0.9649 | 0.0027 | |
| 8 | 22.2227 | 313.1446 | (1) 3.3, 0.4, C15H17N6O2 | | | | 0.9626 | 0.0059 | |

Figure 4.5a. Trend line plot **Season 1**-Tolerant citrus rootstock species *versus* susceptible citrus rootstock species. Tolerant FD (blue line) (1-10) shows features with % intensity above 50. Susceptible CRL (orange line) and Volk (Black line) (11-28) intensity below 50%. 1, 2, 3 and 4 top markers detectible at greater intensity within FD then in CRL or Volk. Example 4: the feature (22.22_313.1446) can be visualised as circled with a dash line in S-plot Figure 4.4c-1 and clearly distinguishes between the tolerant citrus rootstock species and the two susceptible rootstock species.

Tol spp | sus spp | Untitled

| | Ret. Time | m/z | Elemental Composition (mDa, i-FIT) | Database | Pathway | Formula | SIMCA1 | SIMCA2 | ID |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.2358 | 287.1962 | (1) -0.9, 0.0, C14H27N2O4 | KEGG (Online) | | C14H26N2O4 | 0.6629 | 0.0010 | (1) (4bS,6R)-6-Methoxy-' |
| 2 | 5.0486 | 220.0961 | (1) -4.3, 0.6, C11H15N3P | | | | 0.4278 | 0.0013 | |
| 3 | 6.5272 | 229.0855 | (1) -1.0, 0.0, C14H13O3 | KEGG (Online) | | C14H12O3 | 0.6890 | 0.0032 | (1) (2-Hydroxy-4-methoxy... |
| 4 | 6.5277 | 230.0889 | (1) 2.5, 0.1, C10H18NOP2 | | | | 0.6728 | 0.0012 | |
| 5 | 6.5301 | 480.1501 | (1) -0.8, 0.7, C17H23N9O6P | | | | 0.6486 | 0.0010 | |
| 6 | 6.5303 | 479.1468 | (1) 1.4, 1.1, C25H23N2O8 | | | | 0.6445 | 0.0013 | |
| 7 | 6.5306 | 251.0675 | (1) -0.6, 0.4, C12H7N6O | | | | 0.7467 | 0.0018 | |
| 8 | 6.9396 | 259.0962 | (1) -0.8, 0.0, C15H15O4 | KEGG (Online) | | C15H14O4 | 0.9045 | 0.0017 | (1) 2-Isopropyl-3-methoxy... |
| 9 | 6.9493 | 281.0775 | (1) -1.2, 0.1, C13H9N6O2 | | | | 0.9425 | 0.0009 | |
| 10 | 8.4742 | 324.1227 | (1) -0.9, 0.4, C19H18NO4 | KEGG (Online) | | C19H17NO4 | 0.6865 | 0.0012 | (1) (12bS)-6,7,12b,13-Tetr... |
| 11 | 8.7319 | 313.1435 | (1) -0.5, 0.1, C19H21O4 | KEGG (Online) | | C19H20O4 | 0.7599 | 0.0018 | (1) Benzyl butyl phthalate |
| 12 | 8.7349 | 335.1250 | (1) -0.6, 0.3, C17H15N6O2 | | | | 0.8253 | 0.0011 | |
| 13 | 9.6720 | 328.1624 | (1) -4.3, 0.1, C10H28N5O3P2 | | | | 0.9119 | 0.0013 | |
| 14 | 9.6743 | 365.1148 | (1) 1.9, 0.3, C22H22OPS | | | | 0.9166 | 0.0011 | |
| 15 | 9.6765 | 327.1592 | (1) -0.4, 0.0, C20H23O4 | KEGG (Online) | | C20H22O4 | 0.9116 | 0.0028 | (1) 5,5'-Diallyl-3,3'-dimetho... |
| 16 | 9.6798 | 349.1409 | (1) -2.1, 0.2, C16H22N4O3P | | | | 0.9499 | 0.0016 | |
| 17 | 10.4175 | 382.2093 | (1) 1.3, 0.7, C11H29N9O4P | | | | 0.8224 | 0.0009 | |
| 18 | 10.4176 | 403.1879 | (1) 1.0, 0.2, C21H27N2O6 | | | | 0.8598 | 0.0009 | |
| 19 | 10.4195 | 381.2060 | (1) -3.6, 0.4, C23H30N2OP | | | | 0.8197 | 0.0017 | |
| 20 | 10.8737 | 314.1466 | (1) -1.5, 0.3, C10H25N3O6P | | | | 0.9471 | 0.0009 | |
| 21 | 10.8750 | 313.1432 | (1) 1.9, 0.6, C15H17N6O2 | | | | 0.9488 | 0.0019 | |

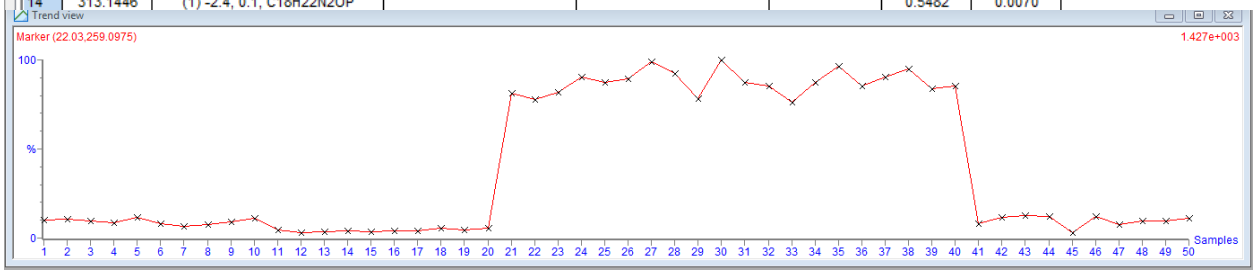



**AT-Tol** **CRL-Sus** **FD-Tol**

Figure 4.5b. Trend line plot **Season 2**-Tolerant citrus rootstock species *versus* susceptible citrus rootstock species. Tolerant AT (blue line) (1-10) and FD (red line) (21-30) shows features with % intensity above 50%. e.g. trend line 3 = 9.68_327.1592. This feature is circled with a dash line in S-plot Figure 4.4d-1. The feature has high potential as a metabolic marker. Susceptible rootstock CRL(orange line) (11-20) intensity below 50%.

Tol Hybrids | Sus Hybrids

| | m/z | Elemental Composition (mDa, i-FIT) | Database | Pathway | Formula | SIMCA1 | SIMCA2 | ID |
|---|---|---|---|---|---|---|---|---|
| 1 | 539.1686 | (1) 0.4, 0.4, C25H32O11P | | | | 0.5519 | 0.0020 | |
| 2 | 281.0792 | (1) 1.8, 0.6, C12H13N2O6 | eMolecules (Online) | | C12H12N2O6 | 0.7242 | 0.0018 | (1) 2,2'-[1,4-Phenylenebis(... |
| 3 | 259.1942 | (1) -1.4, 1.4, C12H27N4S | | | | 0.3929 | 0.0018 | |
| 4 | 260.1009 | (1) -1.6, 0.0, C7H15N7O2P | | | | 0.8619 | 0.0044 | |
| 5 | 259.0975 | (1) 3.2, 0.7, C11H11N6O2 | eMolecules (Online) | | C11H10N6O2 | 0.8695 | 0.0112 | (1) 5-[(E)-(3,5-Dimethyl-1,... |
| 6 | 260.1009 | (1) -1.6, 0.0, C7H15N7O2P | | | | 0.8003 | 0.0030 | |
| 7 | 259.0975 | (1) 3.2, 0.6, C11H11N6O2 | eMolecules (Online) | | C11H10N6O2 | 0.7949 | 0.0078 | (1) 5-[(E)-(3,5-Dimethyl-1,... |
| 8 | 286.1085 | (1) -4.1, 0.7, C13H22NO2P2 | | | | 0.7763 | 0.0014 | |
| 9 | 325.1274 | (1) 2.4, 0.1, C6H18N10O4P | | | | 0.6337 | 0.0014 | |
| 10 | 324.1241 | (1) -4.1, 0.5, C16H24NO2P2 | | | | 0.6303 | 0.0031 | |
| 11 | 355.1377 | (1) -4.9, 1.1, C11H21N10P2 | | | | 0.7956 | 0.0019 | |
| 12 | 354.1347 | (1) 3.2, 0.0, C16H16N7O3 | eMolecules (Online) | | C16H15N7O3 | 0.8113 | 0.0043 | (1) Ethyl {5-[(2-methylphen... |
| 13 | 314.1479 | (1) -3.2, 0.9, C9H26N5O3P2 | | | | 0.5853 | 0.0033 | |
| 14 | 313.1446 | (1) -2.4, 0.1, C18H22N2OP | | | | 0.5482 | 0.0070 | |

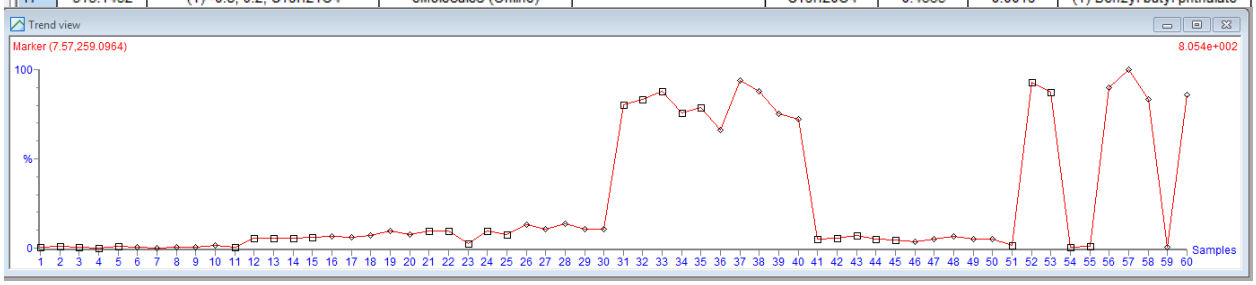



**BC- Tol** **CRL-Sus** **SWC- Tol** **TB- Tol** **Volk-Sus**

Figure 4.6a. Trend line plot **Season 1**-Tolerant citrus rootstock hybrids *versus* susceptible citrus rootstock hybrids. Tolerant (blue line) BC (1-10) does not in this case show high % intensity. SWC and TB (20-40) shows features with % intensity above 50%. Susceptible (orange line) CRL (11-20) and Volk (41-50) below 50%. The marker 22:03_259.0975 circled with a dash line in S-polt Figure 4.4c-1.

Tol Hybrids (S2) | Sus Hybrids (S2)

| | m/z | Elemental Composition (mDa, i-FIT) | Database | Pathway | Formula | SIMCA1 | SIMCA2 | ID |
|---|---|---|---|---|---|---|---|---|
| 1 | 259.0963 | (1) 2.0, 1.1, C11H11N6O2 | eMolecules (Online) | | C11H10N6O2 | 0.5896 | 0.0052 | (1) 5-[(E)-(3,5-Dimethyl-1,... |
| 2 | 540.1718 | (1) 2.1, 0.3, C12H32N9O11P2 | | | | 0.5882 | 0.0016 | |
| 3 | 260.0997 | (1) -3.8, 1.3, C13H14N3O3 | ChemShuttle (Online) | | C13H13N3O3 | 0.5863 | 0.0021 | (1) 3-(4-Amino-1-oxo-1,3-... |
| 4 | 539.1682 | (1) -2.7, 1.1, C27H24N8O3P | | | | 0.5898 | 0.0027 | |
| 5 | 281.0772 | (1) -1.5, 0.3, C13H9N6O2 | eMolecules (Online) | | C13H8N6O2 | 0.6683 | 0.0021 | (1) 8-Phenylimidazo[1,2-b]... |
| 6 | 540.1717 | (1) 2.0, 0.7, C12H32N9O11P2 | | | | 0.5184 | 0.0027 | |
| 7 | 260.0998 | (1) 4.5, 1.0, C13H15N3OP | | | | 0.4966 | 0.0033 | |
| 8 | 259.0964 | (1) -0.6, 0.0, C15H15O4 | eMolecules (Online) | | C15H14O4 | 0.4985 | 0.0082 | (1) 2-Isopropyl-3-methoxy... |
| 9 | 297.0518 | (1) 2.2, 0.2, C9H18N2O3PS2 | | | | 0.4484 | 0.0021 | |
| 10 | 539.1684 | (1) -2.5, 0.1, C27H24N8O3P | | | | 0.5211 | 0.0048 | |
| 11 | 281.0780 | (1) -0.7, 0.6, C13H9N6O2 | eMolecules (Online) | | C13H8N6O2 | 0.4577 | 0.0031 | (1) 8-Phenylimidazo[1,2-b]... |
| 12 | 313.1434 | (1) -0.6, 0.0, C19H21O4 | eMolecules (Online) | | C19H20O4 | 0.4326 | 0.0022 | (1) Benzyl butyl phthalate |
| 13 | 328.1623 | (1) -4.4, 0.1, C10H28N5O3P2 | | | | 0.7220 | 0.0029 | |
| 14 | 327.1592 | (1) -0.4, 0.0, C20H23O4 | eMolecules (Online) | | C20H22O4 | 0.7220 | 0.0063 | (1) 5,5'-Diallyl-3,3'-dimetho... |
| 15 | 349.1407 | (1) -2.3, 0.6, C16H22N4O3P | | | | 0.7221 | 0.0023 | |
| 16 | 381.2058 | (1) -0.8, 0.0, C24H29O4 | eMolecules (Online) | | C24H28O4 | 0.4399 | 0.0019 | (1) 9-(4-Methoxyphenyl)-3... |
| 17 | 313.1432 | (1) -0.8, 0.2, C19H21O4 | eMolecules (Online) | | C19H20O4 | 0.4665 | 0.0019 | (1) Benzyl butyl phthalate |



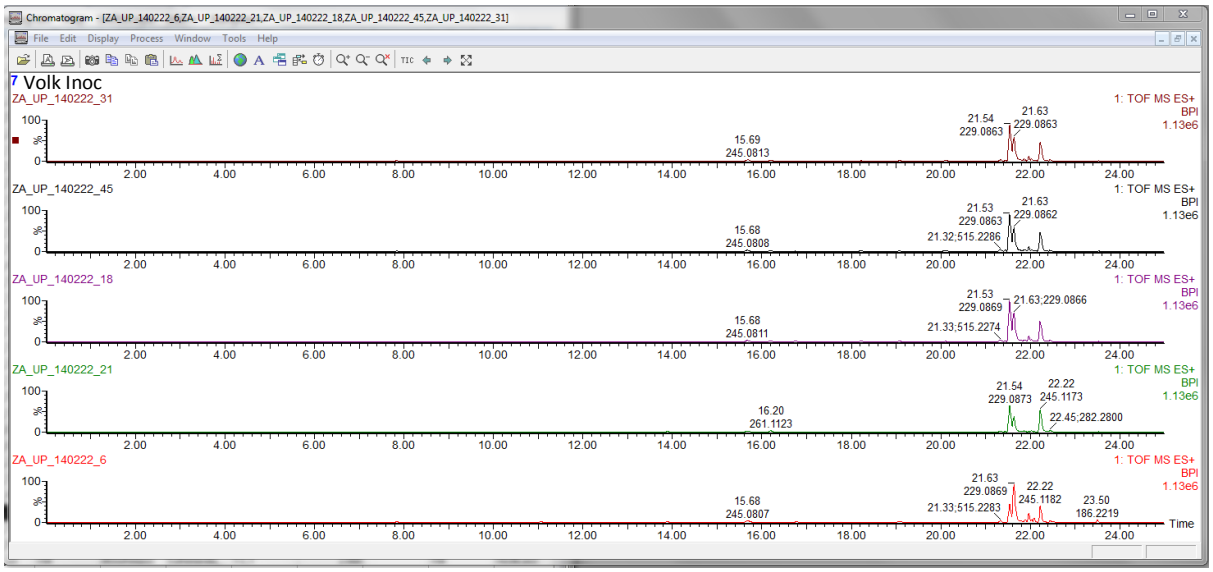

**AT & BC- Tol** **CRL- Sus** **SWC- Tol** **SXB- Sus** **TB- Tol**

Figure 4.6b. Trend line view **Season 2**- Tolerant citrus rootstock hybrids *versus* susceptible citrus rootstock hybrids. Tolerant (blue line) AT and BC (1-10) do not in this case show high % intensity. SWC (31-40) and TB (51-60) shows features with % intensity above 50%. Susceptible (orangeline) CRL (21-30) and SXB (41-50) below 50%. The marker 7.57_259.0964 circled with a dash line in S-polt Figure 4.4d-1.

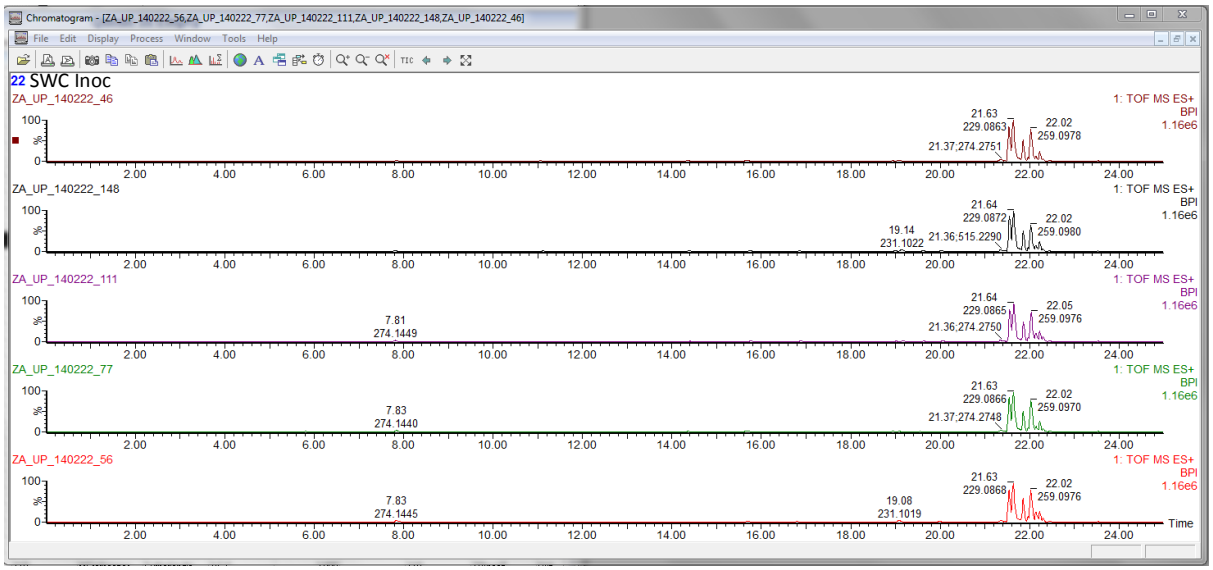
## APPENDIX 1: EXAMPLE CHROMATOGRAMS FROM SEASON 1 AND 2

The chromatograms below represent visual examples from MarkerLynx software highlighting examples from both Season 1 and Season 2

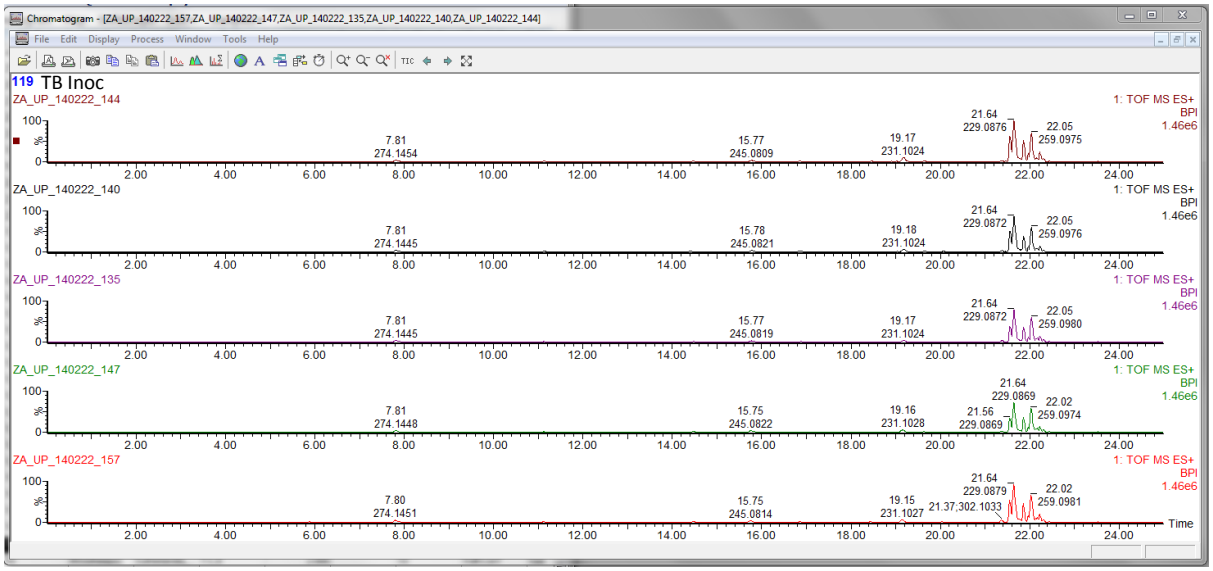
Season 1 chromatograms for Volkamer lemon: Top- extracts for control plants; Bottom- extracts for inoculated plants.

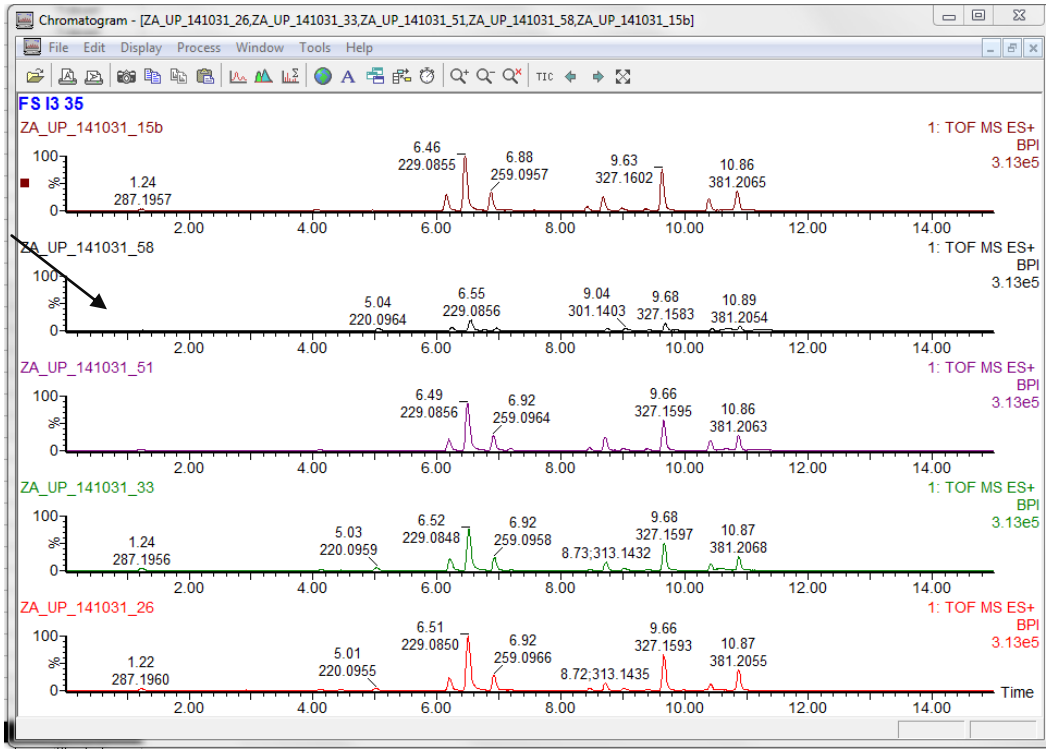
Season 1 chromatograms for Swingle citrumelo: Top- extracts for control plants; Bottom- extracts for inoculated plants.

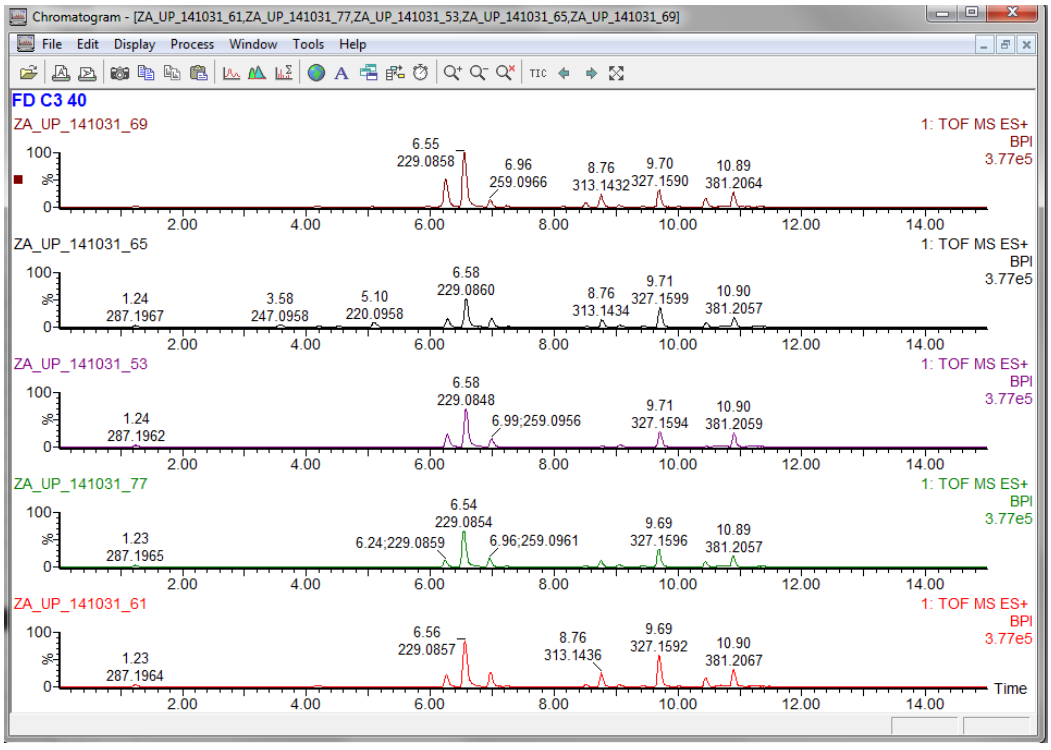



Season 1 chromatograms for Tera Bella citrumelo: Top- extracts for control plants; Bottom- extracts for inoculated plants.

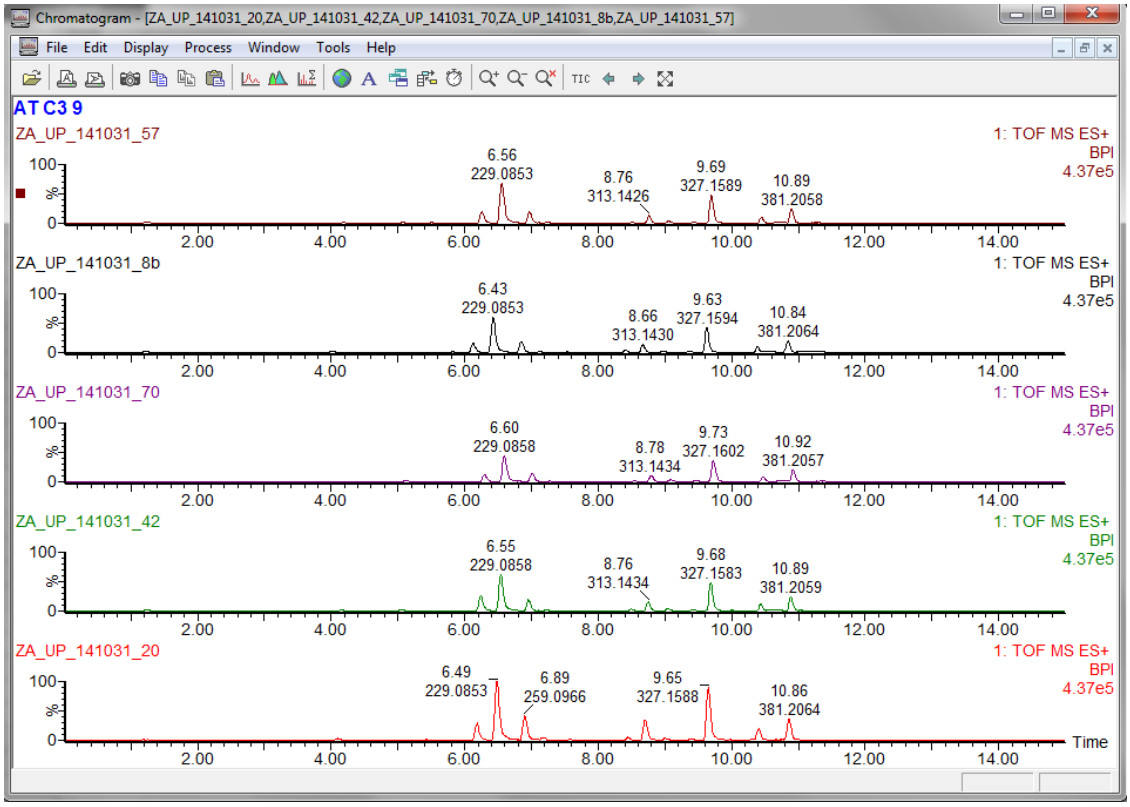
Season 2 chromatograms for Flying dragon: Top- extracts for inoculated plants; Bottom- extracts for control plants.

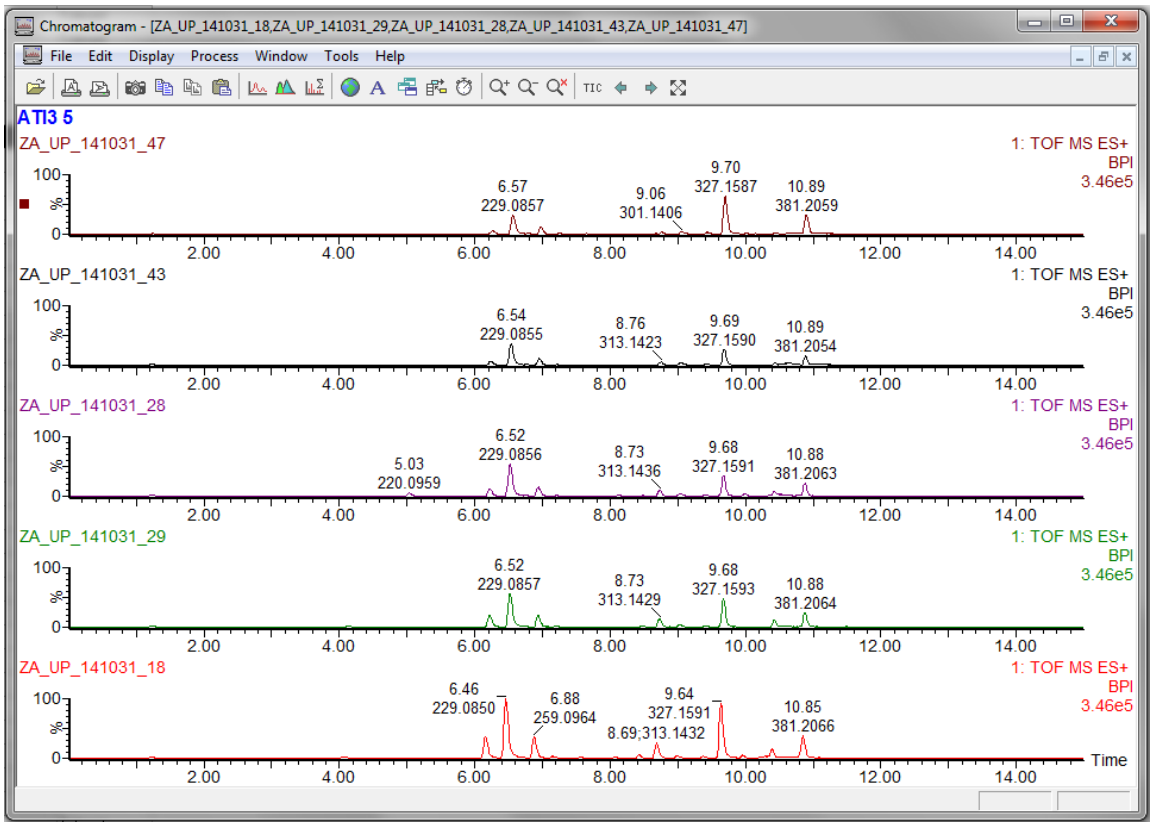


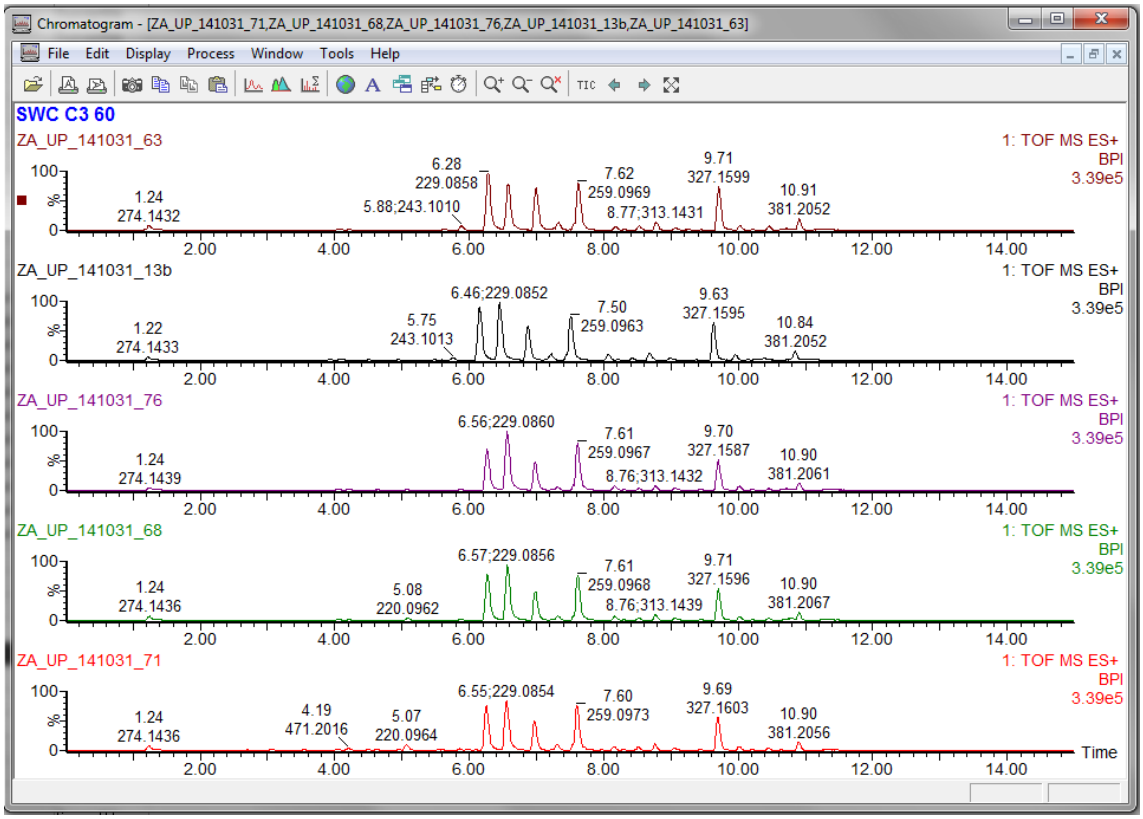

Arrow points to LC injection error resulting in poor chromatogram.

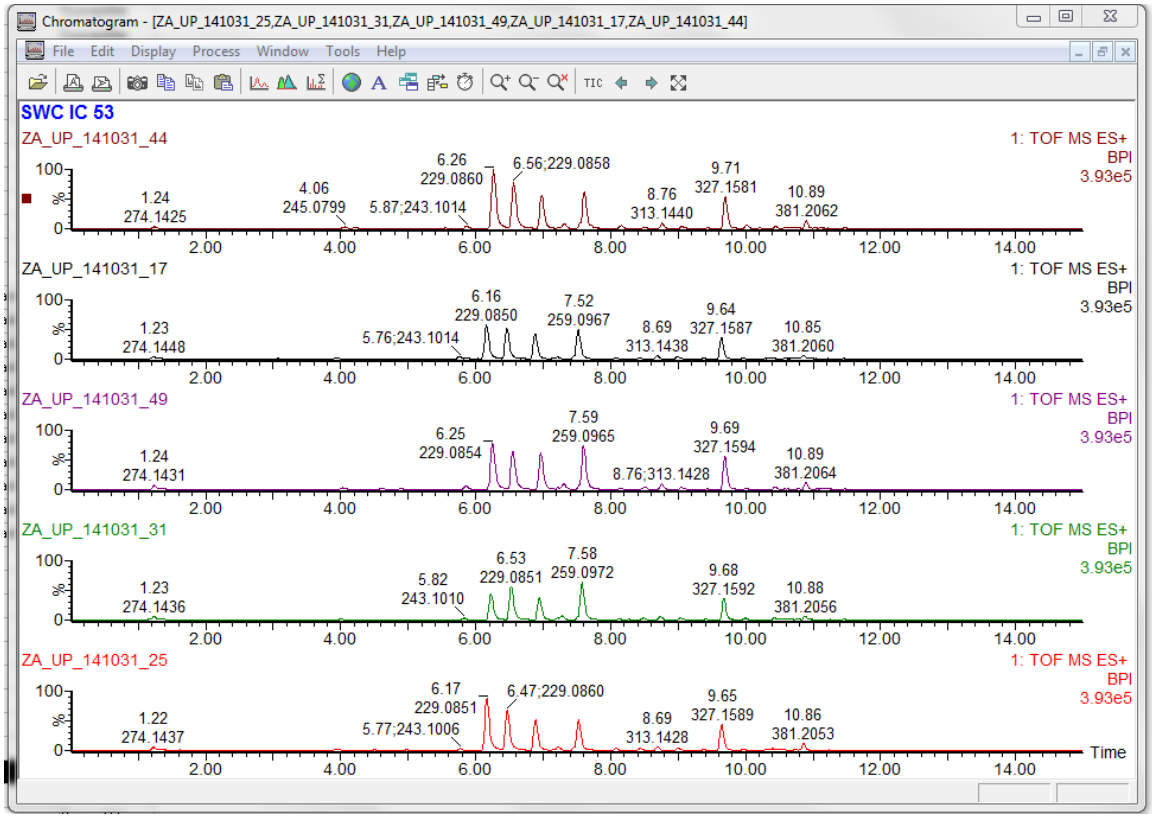
Season 2 chromatograms for Australian trifoliate: Top- extracts for control plants; Bottom- extracts for inoculated plants.



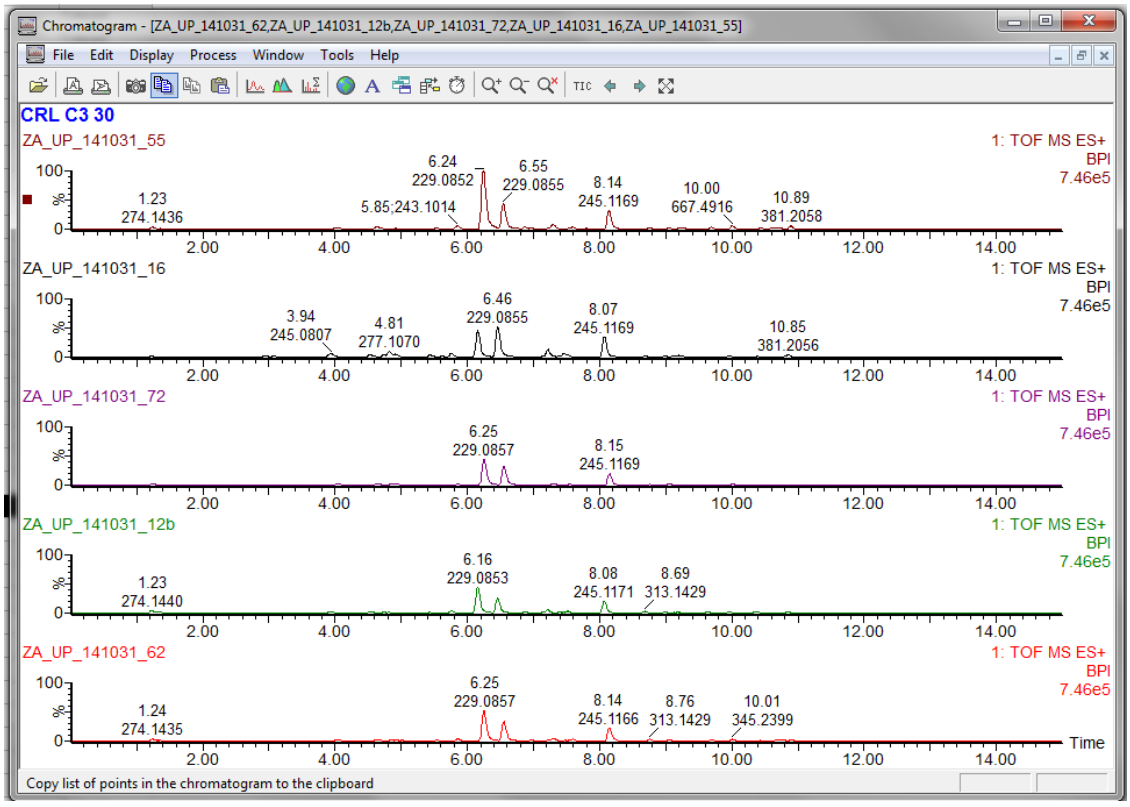



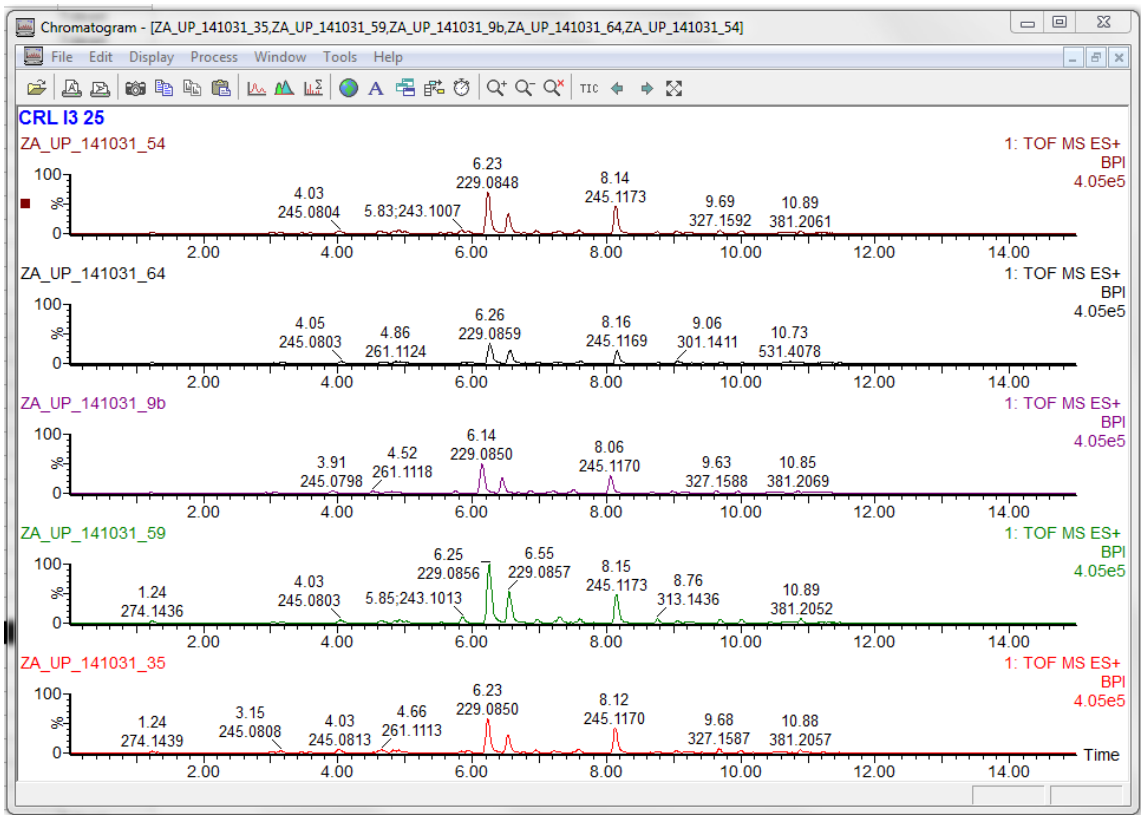
Season 2 chromatograms for Swingle citrumelo: Top- extracts for control plants; Bottom- extracts for inoculated plants



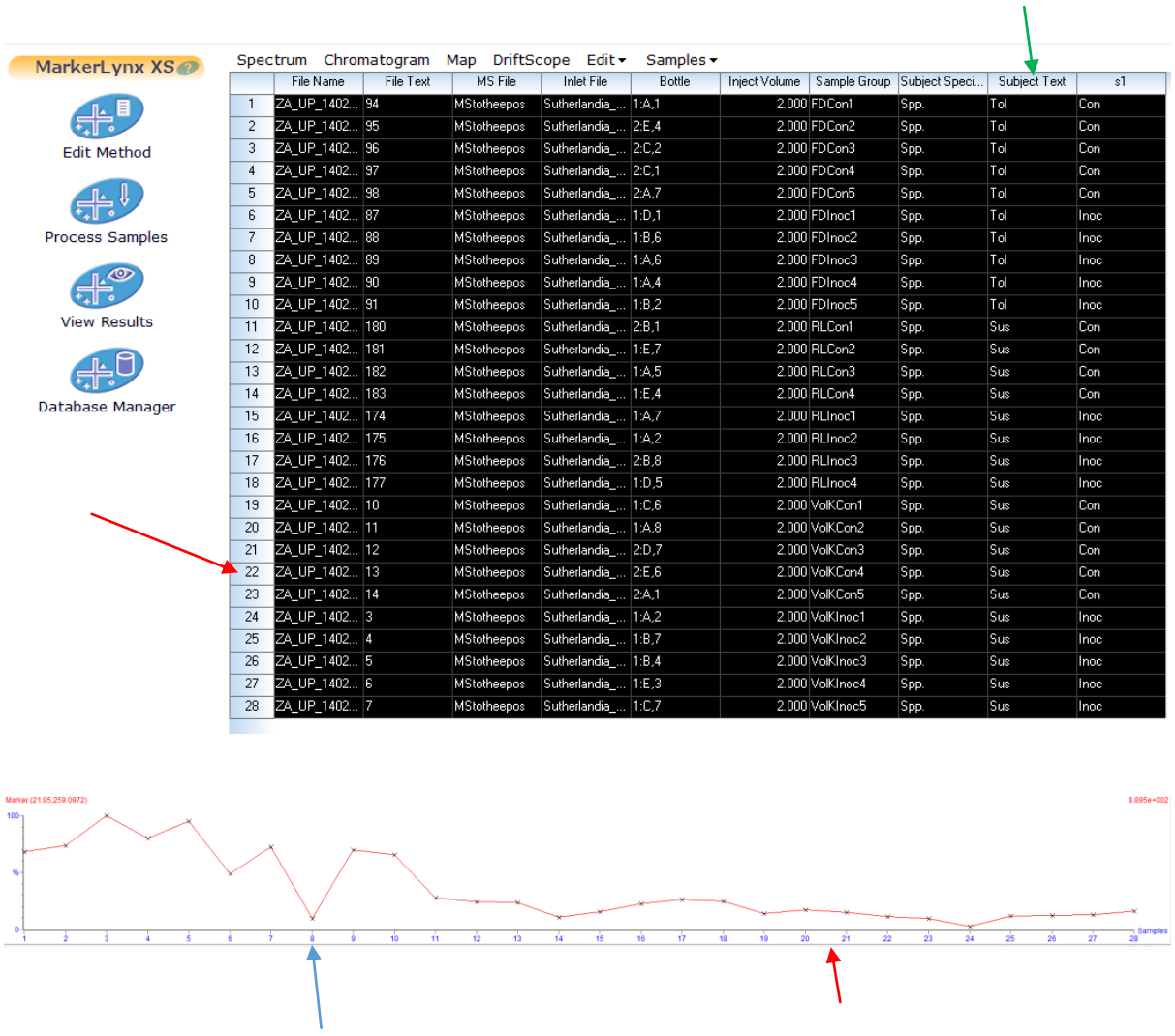
Season 2 chromatograms for Cairn rough lemon: Top- extracts for control plants; Bottom- extracts for inoculated plants.



## APPENDIX 2: TREND LINE PLOT INTERPRETATION

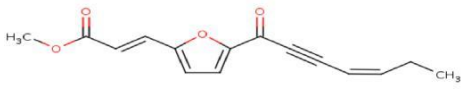
The best method for trend line plot interpretation is to view the plot in conjunction with the sample list prepared in MarkerLynx XS. The red arrows point to the number of sample (1 to 28) in the vertical column in the MarkerLynx sample list image and in the horizontal axis in the trend line below. The samples (1-28) are individual biological replicates separated with LC/MS. The green arrow points to the subject text, in this case whether the rootstock replicate was tolerant (Tol) or susceptible (Sus). In the example below the first 10 samples: 1-10 are FD tolerant rootstocks. From 11 to 28 the rootstock are 11-18 = RL and 19-28 = Volk susceptible rootstocks.



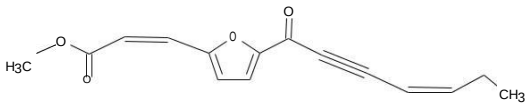

| | File Name | File Text | MS File | Inlet File | Bottle | Inject Volume | Sample Group | Subject Speci... | Subject Text | s1 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ZA_UP_1402... | 94 | MStotheepos | Sutherlandia_... | 1:A,1 | 2.000 | FDCon1 | Spp. | Tol | Con |
| 2 | ZA_UP_1402... | 95 | MStotheepos | Sutherlandia_... | 2:E,4 | 2.000 | FDCon2 | Spp. | Tol | Con |
| 3 | ZA_UP_1402... | 96 | MStotheepos | Sutherlandia_... | 2:C,2 | 2.000 | FDCon3 | Spp. | Tol | Con |
| 4 | ZA_UP_1402... | 97 | MStotheepos | Sutherlandia_... | 2:C,1 | 2.000 | FDCon4 | Spp. | Tol | Con |
| 5 | ZA_UP_1402... | 98 | MStotheepos | Sutherlandia_... | 2:A,7 | 2.000 | FDCon5 | Spp. | Tol | Con |
| 6 | ZA_UP_1402... | 87 | MStotheepos | Sutherlandia_... | 1:D,1 | 2.000 | FDInoc1 | Spp. | Tol | Inoc |
| 7 | ZA_UP_1402... | 88 | MStotheepos | Sutherlandia_... | 1:B,6 | 2.000 | FDInoc2 | Spp. | Tol | Inoc |
| 8 | ZA_UP_1402... | 89 | MStotheepos | Sutherlandia_... | 1:A,6 | 2.000 | FDInoc3 | Spp. | Tol | Inoc |
| 9 | ZA_UP_1402... | 90 | MStotheepos | Sutherlandia_... | 1:A,4 | 2.000 | FDInoc4 | Spp. | Tol | Inoc |
| 10 | ZA_UP_1402... | 91 | MStotheepos | Sutherlandia_... | 1:B,2 | 2.000 | FDInoc5 | Spp. | Tol | Inoc |
| 11 | ZA_UP_1402... | 180 | MStotheepos | Sutherlandia_... | 2:B,1 | 2.000 | RLCon1 | Spp. | Sus | Con |
| 12 | ZA_UP_1402... | 181 | MStotheepos | Sutherlandia_... | 1:E,7 | 2.000 | RLCon2 | Spp. | Sus | Con |
| 13 | ZA_UP_1402... | 182 | MStotheepos | Sutherlandia_... | 1:A,5 | 2.000 | RLCon3 | Spp. | Sus | Con |
| 14 | ZA_UP_1402... | 183 | MStotheepos | Sutherlandia_... | 1:E,4 | 2.000 | RLCon4 | Spp. | Sus | Con |
| 15 | ZA_UP_1402... | 174 | MStotheepos | Sutherlandia_... | 1:A,7 | 2.000 | RLInoc1 | Spp. | Sus | Inoc |
| 16 | ZA_UP_1402... | 175 | MStotheepos | Sutherlandia_... | 1:A,2 | 2.000 | RLInoc2 | Spp. | Sus | Inoc |
| 17 | ZA_UP_1402... | 176 | MStotheepos | Sutherlandia_... | 2:B,8 | 2.000 | RLInoc3 | Spp. | Sus | Inoc |
| 18 | ZA_UP_1402... | 177 | MStotheepos | Sutherlandia_... | 1:D,5 | 2.000 | RLInoc4 | Spp. | Sus | Inoc |
| 19 | ZA_UP_1402... | 10 | MStotheepos | Sutherlandia_... | 1:C,6 | 2.000 | VolKCon1 | Spp. | Sus | Con |
| 20 | ZA_UP_1402... | 11 | MStotheepos | Sutherlandia_... | 1:A,8 | 2.000 | VolKCon2 | Spp. | Sus | Con |
| 21 | ZA_UP_1402... | 12 | MStotheepos | Sutherlandia_... | 2:D,7 | 2.000 | VolKCon3 | Spp. | Sus | Con |
| 22 | ZA_UP_1402... | 13 | MStotheepos | Sutherlandia_... | 2:E,6 | 2.000 | VolKCon4 | Spp. | Sus | Con |
| 23 | ZA_UP_1402... | 14 | MStotheepos | Sutherlandia_... | 2:A,1 | 2.000 | VolKCon5 | Spp. | Sus | Con |
| 24 | ZA_UP_1402... | 3 | MStotheepos | Sutherlandia_... | 1:A,2 | 2.000 | VolKInoc1 | Spp. | Sus | Inoc |
| 25 | ZA_UP_1402... | 4 | MStotheepos | Sutherlandia_... | 1:B,7 | 2.000 | VolKInoc2 | Spp. | Sus | Inoc |
| 26 | ZA_UP_1402... | 5 | MStotheepos | Sutherlandia_... | 1:B,4 | 2.000 | VolKInoc3 | Spp. | Sus | Inoc |
| 27 | ZA_UP_1402... | 6 | MStotheepos | Sutherlandia_... | 1:E,3 | 2.000 | VolKInoc4 | Spp. | Sus | Inoc |
| 28 | ZA_UP_1402... | 7 | MStotheepos | Sutherlandia_... | 1:C,7 | 2.000 | VolKInoc5 | Spp. | Sus | Inoc |

The corresponding trend line plot below the sample list shows the prominence of the marker at 21.85_259.0972 (rt_*m/z*) in samples 1-10, all above 50% intensity. The percentage intensity is the vertical axis of the trend line plot. In the susceptible rootstocks RL and Volk the marker is lower in relative intensity (below 50%). This marker clearly distinguishes the two susceptible rootstocks from the tolerant rootstock. It is detected at great intensity.

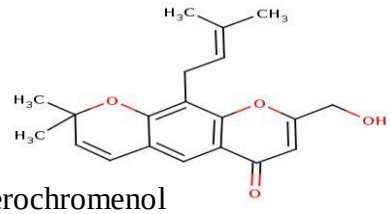
## APPENDIX 3: CHEMICAL STRUCTURES OF THE PUTATIVELY IDENTIFIED BIOMARKERS.



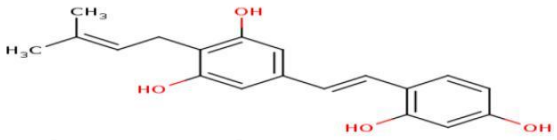
(E)-Wyerone



(Z)-Wyerone



Pulverochromenol



4’-prenyloxyresveratrol

Chong *et al.*, (2006) utilised a 1:1 ethyl acetate-ethanol organic solvent mixture to extract secondary metabolites from plants overnight. In this case the mixture yielded the secondary metabolites proposed as playing a role in root rot tolerance.

# CHAPTER 5

## Metabolite-marker based predictive model for *P. nicotianae* tolerance in citrus rootstocks

**Abstract**

In this study we report on a metabolomics approach for rapid assessment of citrus rootstocks for tolerance against root rot caused by *P. nicotianae* by identifying ion features that correspond with the phenotypic trait of tolerance. We were able to develop a model that can predict for this trait in citrus rootstocks. Root rot of citrus trees caused by *Phytophthora nicotianae* is responsible for severe economic losses in citriculture. Use of resistant rootstocks is an effective method of managing this problem, however, breeding and selection of new citrus rootstocks is a time consuming undertaking. The objective was to develop a method for rapid assessment of rootstocks for *P. nicotianae* tolerance, using a metabolomics approach to identify metabolic markers for the phenotypic trait of tolerance. Healthy citrus roots from four tolerant and four susceptible rootstock varieties were used for LC/MS based metabolite analysis with the objective of identifying potential biomarkers. Organic solvent extractions of the roots were prepared and analysed by mass spectrometry based liquid chromatography, which produced 367 ion features (retention time and m/z). Orthogonal partial least squares discriminant analysis of peak abundance using MarkerLynx software allowed for the identification of ion features that differentiate tolerant and susceptible rootstocks. Using descriptive and inferential statistics based on the ion features of uninoculated tolerant vs. susceptible rootstocks, applying logistic regression, 14 top markers were identified and two of them (22.03_259.0975 and 22.21_313.1445: retention time (rt):mass to charge ratio (m/z) were accepted as potential metabolic markers. A model that can potentially predict tolerance in citrus rootstocks with > 98% accuracy is presented. This model can potentially speed up the screening of citrus rootstocks for root rot tolerance if integrated into a rootstock breeding and selection program.

## 5.1 Introduction

*Phytophthora* species, particularly *P. citrophthora, P. nicotianae,* and *P. palmivora*, remain important soil and water borne pathogens affecting citrus production worldwide (Boava *et al*., 2011; Graham and Feichtenberger, 2015; Panabieres *et al*., 2016), impacting negatively on the profitability of citriculture (Matheron *et al*., 1998; Adaskaveg *et al*., 2014; Meitz-Hopkins *et al*., 2014). In South Africa *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) is the predominant causal agent of fibrous root rot and tree decline of citrus (Thompson *et al*., 1995; Meitz-Hopkins *et al*., 2014). No commercial citrus rootstocks are 100% immune to *Phytophthora* root rot (Castle, 1987; Siviero *et al*., 2006) resulting in rootstocks with varying tolerance to these pathogens (Boava *et al*., 2011). Citrus rootstock tolerance is defined by Graham, (1990) as the capacity of infected rootstocks to withstand infection. However, the innate mechanisms by which plants defend themselves against pathogen invasion is one that is yet to be fully elucidated (Talon and Gmitter, 2008; Pérez-Clemente *et al*., 2013; Matsukawa *et al*., 2017).

The use of citrus rootstocks with greater tolerance to *P. nicotianae* is considered the most effective and affordable long term method of managing citrus root rot (Castle, 2010; Adaskaveg *et al*., 2014). Citrus breeding programs aim to replace existing stocks with rootstocks of increased disease tolerance whilst maintaining favourable agronomic qualities (Grosser *et al*., 1995; Castle, 2010; Schinor *et al*., 2013; Adaskaveg *et al*., 2014). Pathogenicity screening is the common method used to determine tolerance/susceptibility of rootstocks. However, the breeding and selection of new citrus rootstocks is an arduous, decades long undertaking requiring new methods to accelerate trait identification (Castle, 2010; Curtolo *et al*., 2017). Fernie and Schuaer, (2008) illustrate that metabolomics-based

approaches can reduce the time for development of elite lines in crop improvement strategies. While metabolomics allows for greater insight into biological systems (Saito and Matsuda, 2010), it is important to remain aware of the challenges, limitations and bottlenecks associated with its application (Matsukawa *et al*., 2017).

This notwithstanding, it is essential to explore what possibilities these techniques and technologies can reveal for the citrus rootstock-*P. nicotianae* problem to better protect citrus plant production. Our objective was to develop a method for rapid assessment of rootstocks for *P. nicotianae* tolerance, using a metabolomics approach to identify metabolic markers for the phenotypic trait of tolerance based on metabolite abundance. Plant metabolomics tools have been used to demarcate citrus genotypes in phenotyping studies (Arbona *et al*., 2009) and in diagnostic studies, for example, to identify potential citrus Huanglongbing (HLB) tolerance biomarkers (Cevallos-Cevallos *et al*., 2009). Albrecht *et al*., (2016) used plant metabolomics applications to identify metabolic profiles associated with disease response and disease tolerance while investigating citrus HLB. The burgeoning prominence of applying metabolomics technology in systems biology enables greater capacity to develop powerful diagnostic and predictive tools for biomarker discovery (Schudoma *et al*., 2012; Fernandez *et al*., 2016) as investigated in this chapter.

The identification of resistance related metabolites as potential biomarkers for tolerance traits was investigated in barley (Bollina *et al*., 2011; Kumaraswamy *et al*., 2011) and in wheat (Hamzehzarghani *et al*., 2008; Paranidharan *et al*., 2008). Resistance related metabolites are small molecules or secondary metabolites, which are detected in higher abundance in uninoculated resistant or tolerant plants as opposed to susceptible plants (Hamzehzarghani *et*

*al*., 2008; Kumaraswamy *et al*., 2011). These metabolites, in particular from disease free plants are constitutive, and have potential uses as biomarkers for rapid screening of plant genotypes for disease tolerance (Kumaraswamy *et al*., 2011). Biomarkers are organic indicator compounds that can be used as tracers of a given biological trait (Simoneit, 2005; Schudoma *et al*., 2012; Menard *et al*., 2013). Metabolic markers are therefore sub-categories of biomarkers and can be diagnostic, prognostic, or predictive markers (Fernandez *et al*., 2016; Kumar *et al*., 2017).

In Chapter 3, citrus rootstocks were categorized as being either tolerant, moderately tolerant or susceptible to *P. nicotianae* root rot based on greenhouse assessments and all root material from the green-house experiments were stored as frozen samples. In this chapter organic solvent extracts from selected tolerant and susceptible rootstocks were analysed using Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS-MS) aiming to identify metabolites that best distinguish between the two groups, for use as potential metabolic markers. Computational statistics yielded potential biomarkers based on metabolite abundance. The predictive model was developed by selecting markers corresponding with tolerance, as identified in OPLS-DA and verified through descriptive and inferential statistics. We propose that the current study, on the development of such a model has the potential application towards the rapid identification of tolerant citrus rootstocks based on metabolite abundance. However, necessary follow-up is required to better satisfy the complexities faced when applying metabolomics approaches to trait discovery and predictive model formulation (Fernandez *et al*., 2016; Kumar *et al*., 2017; Matsukawa *et al*., 2017).

## 5.2 Materials and methods

### 5.2.1 Plants

Frozen root material of rootstocks used in Experiment 4 (Chapter 3, section 3.2.3) (season 2:2012- seedlings 11 months post sowing; *P. nicotianae* screening in soil/sand potting mixture) were used. Four uninoculated tolerant rootstocks (Benton citrange; Flying dragon; Swingle citrumelo and Terra Bella citrumelo) and four uninoculated susceptible rootstocks (Cairn rough lemon; Carrizo citrange; Volkamer lemon and X639-hybrid) were selected for analysis.

### 5.2.2 Metabolite extraction and chromatography

Using five replicates per treatment, for the above mentioned rootstocks, their roots were excised, individually frozen in liquid nitrogen and crushed to a fine powder using a mortar and pestle. One gram powdered root material was transferred to glass tubes and 3 ml cold ethyl acetate-ethanol (1:1) (Merck, HPLC grade) mixture was added (Chong *et al.*, 2006). Tubes were capped, vortexed for 30 seconds and allowed to settle for extraction overnight in the dark at room temperature. After 24 hours, extracts were recovered from the tubes and transferred to clean, labelled glass tubes before being evaporated to dryness in a fume hood. Resultant residues were suspended in 0.5 ml methanol (Merck® HPLC grade) to produce crude extracts. Aliquots of 50 µl for each rootstock extract were then transferred to Eppendorf tubes and stored at -20 °C until biochemical analyses. UPLC/MS-MS analyses of the 124 samples was performed by the Central Analytical Facility at the University Stellenbosch, South Africa as previously described (Chapter 4 section 4.2.1).

### 5.2.3 UPLC/MS-MS output processing

After parameter selection for peak alignment using MarkerLynx software (Waters, MA, USA) (Peters *et al*. 2009), advanced multivariate algorithms for orthogonal partial least squares discriminant analyses (OPLS-DA) were automatically generated. The multivariate analysis was performed using MarkerLynx XS from MassLynx software version 4.1 to identify markers that clearly distinguish between uninfected tolerant and susceptible rootstocks.

### 5.2.4 Data reduction and model development

To verify potential metabolic markers identified through OPLS-DA by signal strength, i.e greater abundance in tolerant as opposed to susceptible rootstocks, m/z data from MarkerLynx were exported to MS-Excel. Data were further analysed in SAS (SAS/STAT software v9.3. SAS Institute) for the calculation of descriptive and inferential statistics based on a test hypothesis of the ion features for tolerant vs. susceptible rootstocks using logistic regression. The markers to enter the model were selected using a three-step variable reduction method. The rules were: 1- if the number of missing values for the tolerant group is less than five in the 20 samples; 2- if the number of missing values for the susceptible group is less than five in the 19 samples; 3- if the average tolerant signal is 10% higher than that of the average susceptible signal (fold change approach (Kumar *et al*., 2017)). The ion features that met conditions 1 to 3 were accepted as top markers according to signal strength from the top 10% of the signal strength in rootstock extracts. Missing values (less than 5) were then imputed by the minimum value observed for particular ion features (variable), per group, i.e. tolerant/susceptible. The numbers of missing values per observation were counted, and a weight variable created so that observations that had fewer missing values carried more weight. A stepwise logistic regression procedure was performed on 39 samples and top

markers in order to select the best possible variables for a 1, 2, 3, or 4 variable model using Firth's penalised maximum likelihood estimation method in order to circumvent quasi separation of the data points. The probability modelled was GroupOriginal = "Control-Tolerant".

## 5.3 Results and Discussion

### 5.3.1 Multivariate data analysis

By means of MarkerLynx software 366 ion features were selected from the 124 citrus root extracts. The selected markers across the 124 sample injections were aligned by retention time and base peak *m/z* (Fig 5.1). The peak areas of an early, middle and late eluting peak were plotted against chromatogram sequence number to test the reproducibility of the peak area. No correction was made for peak area of the citrus samples, as this change, in the standards, was less than 20% for the late eluting peak and less than 10% for the middle and early eluting peaks, and the Coefficients of Variance (CV%) was low. The markers identified by MarkerLynx with OPLS-DA multivariate analysis are displayed in Figure 5.1. The markers at the bottom left and top right of the curve, with p corr[1] < -0.5 and > 0.5, occur predominantly in either the tolerant or susceptible cultivars respectively.

### 5.3.2 Data reduction and model development

Table 5.1 shows potential markers derived from the three rule data reduction procedure developed to accept features with specific characteristics regarding resistance/tolerance related constitutive metabolites. The three rule procedure was based on signal strength and selected markers that are unique for the two test groups (tolerant or susceptible). It was valuable to select features on this basis to exclude features that would provide conflicting

information relating to the overall purpose of the model (Fernandez *et al*., 2016). The markers were the same as those identified through OPLS-DA (Fig. 4.2). The two features selected were 22.03_259.0975 and 22.21_313.1445 (retention time and m/z) which appeared as predominant features for tolerant citrus rootstocks as indicated by multivariate OPLS-DA (Fig. 5.2). A two-variable model was decided upon, since the score $\chi^2$ value's increase flattened out after adding more variables (Table 5.2). The combination was decided upon after evaluating the three- and four-variable models, and observing the variables that appeared most frequently. Firth's penalized maximum likelihood procedure was then employed for fitting a two-variable, logistic regression model (Table 5.4). All p-values were less than 0.01 (Table 5.3 and 5.4) for separating the two classes and were therefore statistically significant.

This yielded the following model:

$$\frac{\qquad}{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}$$

The decision criterion was therefore:

$$\text{Decision} = \begin{cases} \text{Tolerant if} & p \geq 0.5 \\ \text{Susceptible if} & \text{otherwise.} \end{cases}$$

Rootstocks with $p > 0.5$ may thus be classified as *P. nicotianae* tolerant. Table 5.5 summarises the fit of the model indicating a 98% concordance with the predictive capacity of the model to select for tolerant citrus rootstocks. Biomarkers can be identified through

untargeted metabolite fingerprinting approaches to compare the patterns between the metabolome of tolerant genotypes *versus* susceptible genotypes (Kumaraswamy *et al*., 2011; Monteiro *et al*., 2013; Wolfender *et al*., 2013). Biomarkers are important for their uses in mapping quantitative trait loci in the form of metabolite markers or eQLT (Fernie and Schuaer, 2008) and have significant application as predictive tools in marker assisted plant breeding (Steinfath *et al*. 2010; Falke and Mahone 2013; Monteiro *et al*. 2013; Ernst *et al*. 2014; Fernandez *et al*. 2016). An advantage in using ion features as metabolic markers for phenotypic traits is that there is no requirement for annotation (Arbona *et al*. 2009; Cevallos-Cevallos *et al*. 2009). Upon further investigation and statistical work to compliment these initial findings, the metabolic markers and prediction model outlined here has the potential to be applied as the basis for citrus rootstock breeders to identify *P. nicotianae* tolerant rootstocks prior to pathogenicity screening, by including only those rootstocks that contain the proposed tolerance biomarkers. A further advantage is the constitutive nature of the potential markers. This has the potential to help plant breeders assess lists of citrus rootstocks for *P. nicotianae* tolerance more rapidly.

The metabolic markers can be used to predict the selected trait of *P. nicotianae* tolerance prior to time consuming greenhouse screening by including rootstocks found to constitutively contain these markers (Menard et al., 2013). The development of the prediction model outlined in this study represents an initial attempt to explore the possibilities plant metabolomics approaches can avail. Although the model is specific for *P. nicotianae* tolerance biomarkers, a similar approach may be used to develop models for other *Phytophthora* species.

## 5.4 Conclusion

In this chapter we investigated resistance related metabolites and satisfied our objectives towards developing a method for rapid assessment of citrus rootstocks for *P. nicotianae* tolerance using plant metabolomics approaches. This was achieved by identifying ion features that correspond with the phenotypic trait of tolerance and developing a model that can predict for this trait. This will help reduce the long periods required for evaluation and selection of new rootstock genotypes from breeding programs. It is important to offset the very real complexities or limitations involved in citrus plant breeding through investigation of new methods as proposed herein. In the previous chapter, we tentatively assigned putative identities to top markers identified as having potential use as tolerance/resistance related using MassLynx. A similar trend of top markers was found in the current chapter based on metabolite abundance calculated with logistic regression model in SAS (SAS/STAT software v9.3. SAS Institute).

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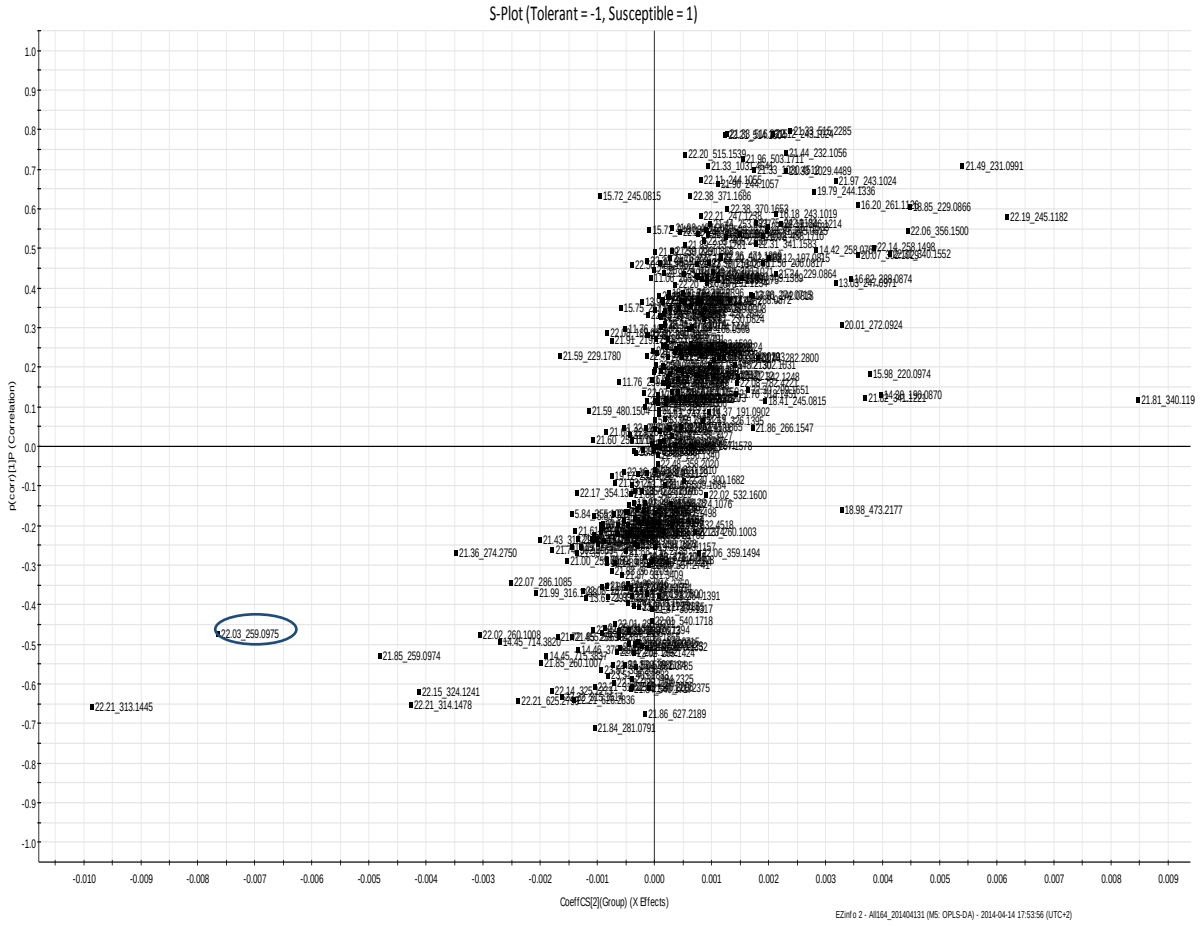


Figure 5.1. MarkerLynx S-plot for the markers that differentiate tolerant (lower left quadrant) and susceptible (top right quadrant) cultivars. The markers at the bottom left and top right of the curve, with p corr[1] < -0.5 and > 0.5, occur predominantly in the tolerant and susceptible cultivars respectively. Each feature is identified by retention time underscore accurate mass e.g. 22.03_259.0975 (blue circle).

**Table 5.1** Potential marker compounds highlighting signal strength of top 14 tolerance ion features for input in stepwise logistic procedure (Group Original = Control Tolerant).

| Retention Time/Mass *(Min_m/z*) | New Name | Average Signal Strength |
|---|---|---|
| 22.03_259.0975 | var2 | 964 |
| 22.15_324.1241 | var4 | 548 |
| 22.21_313.1445 | var24 | 659 |
| 22.21_314.1478 | var27 | 122 |
| 22.14_325.1273 | var29 | 102 |
| 22.02_260.1008 | var30 | 144 |
| 21.97_243.1024 | var40 | 237 |
| 21.85_259.0974 | var43 | 727 |
| 22.17_354.1347 | var49 | 141 |
| 21.85_260.1007 | var75 | 107 |
| 21.59_251.0686 | var76 | 110 |
| 21.59_229.0868 | var79 | 2783 |
| 21.59_230.0901 | var82 | 385 |
| 21.81_340.1190 | var125 | 113 |

**Table 5.2** Best models resultant from stepwise logistic regression

Regression Models Selected by Score Criterion

| Number of Variables | Score Chi-Square | Variables Included in Model |
|---|---|---|
| 1 | 12.3027 | var24 |
| 1 | 11.8962 | var27 |
| 1 | 10.0857 | var75 |
| 2 | 27.5811 | var24; var30 |
| 2 | 27.5281 | var2; var24 |
| 2 | 26.9825 | var27; var30 |
| 3 | 29.0697 | var2; var24; var27 |
| 3 | 29.0291 | var24; var27; var30 |
| 3 | 28.9949 | var24; var30; var125 |
| 4 | 30.9668 | var24; var30; var49; var125 |
| 4 | 30.8906 | var2; var24; var49; var125 |
| 4 | 30.4867 | var27; var30; var49; var125 |

**Table 5.3** Testing global Null hypothesis: BETA=0

| Test | Chi-Square | DF | Pr > ChiSq |
|---|---|---|---|
| Likelihood Ratio | 29.1262 | 2 | <.0001 |
| Score | 27.3772 | 2 | <.0001 |
| Wald | 11.5249 | 2 | 0.0031 |

**Table 5.4** Analysis of penalized maximum likelihood estimates

| Parameter | DF | Estimate | Standard Error | Wald Chi-Square | Pr > ChiSq |
|---|---|---|---|---|---|
| Intercept | 1 | -6.2214 | 1.6723 | 13.8412 | 0.0002 |
| var2 | 1 | 0.00266 | 0.00006 | 17.8785 | <.0001 |
| var24 | 1 | 0.0111 | 0.00324 | 11.7381 | 0.0006 |

**Table 5.5** Association of predicted probabilities and observed responses

| | | Test | |
|---|---|---|---|
| Percent Concordant | 98.4 | Somers'D | 0.968 |
| Percent Discordant | 1.6 | Gamma | 0.968 |
| Percent Tied | 0.0 | Tau-a | 0.497 |
| Pairs | 380 | C | 0.984 |

# CHAPTER 6

**Summary**

## 6.1 Summary

The following chapter briefly outlines the thesis according to its working chapters culminating in our conclusions and recommendation for further research. During greenhouse screening experiments covered in Chapter 3, citrus rootstocks were categorised as susceptible, intermediately tolerant and tolerant to the root rot pathogen *Phytophthora nicotianae*. The tolerant rootstocks were Australian trifoliate, Benton citrange, Flying Dragon, Swingle citrumelo, Terra Bella citrumelo and Yuma citrange. Of particular interest to citriculture is their use as replacement planting material for the management of the root rot problem.

Plant metabolomics strategies were applied to interrogate the citrus metabolome to compliment traditional pathogenicity screening of citrus rootstock tolerant to *P. nicotianae*, where organic solvent root extracts were analytically separated by means of UPLC/MS. In Chapter 4 MassLynx online data processing software was used for metabolite fingerprinting to assess the larger data sets produced from UPLC/MS. The software allowed for the detection and putative identification of discriminating metabolite characteristics between tolerant and susceptible citrus rootstocks. Of interest to this study were ion features which occurred in samples from two seasons as prominent markers in *P. nicotianae* tolerant rootstocks. Top markers associated with tolerant rootstocks included 259.0963, 313.1433 and 327.1592 (m/z) putatively identified as Wyerone, 4'-prenyloxyresveratrol and Pulverochromenol respectively. This allowed us to find evidence from literature linking the markers with plant self defense in other crops, thus supporting the conception that they are related to tolerance in citrus rootstocks. In Chapter 5 a predictive model for the rapid selection of tolerant citrus rootstocks was developed based on metabolite abundance

calculated with logistic regression model in SAS (SAS/STAT software v9.3. SAS Institute). The same fingerprint or trend of markers was identified as in chapter four using the SAS software. The model can potentially speed up the screening of citrus rootstocks for root rot tolerance if integrated into a rootstock breeding and selection program. This can be achieved by selecting new test rootstocks with the proposed markers during tolerance screening. The application of such a model does however require further development and refinement for a non-model crop such as citrus. To the best of our knowledge, this work represents a first scientific approach applying plant metabolomics strategies to interrogate the citrus rootstock metabolome for *P. nicotianae* defense related metabolites. These results highlight the benefits of plant metabolomics approaches as a complimentary component to traditional plant pathogen screening and for trait discovery. The identification of metabolites that are unknown and yet repeatedly detected between experiments is of great importance for developing new biological understanding of the plant-pathogen interactions (Hall and Hardy 2012). Further accurate mass determinations and analysis of multiple mass fragments would be necessary as part of the future process of metabolite annotation. We need to point out however that it is not always essential to identify or annotate biomarkers for plant breeding purposes (Arbona *et al*. 2009; Cevallos-Cevallos *et al*. 2009).

In this study, we associate the potential of reported top ion features for use as biomarkers owing to previous reports of disease mitigation from other crops based on putative identification. For the potential to be realised it will be important to fully annotate the markers and better determine functionality so as to elucidate the mechanisms for tolerance (Johnson *et al*., 2016). The complexities of phytochemicals leave plant biologists with daunting challenges. However in this case semi-quantification or repeated detection of specific ion features from season to season may be sufficient to add to growing understanding

of the citrus rootstock-*P. nicotianae* disease interaction (Hall and Hardy, 2012). Metabolomics is assisting greatly to bring the complexities of plant secondary metabolites into better view, and will continue to help plant biologists interpret plant stresses (Hall and Hardy, 2012). The potential of plant metabolomics is clearly demonstrated and good examples presented throughout this text along with the findings outlined. The onus remains to evaluate all components of the ever-evolving techniques and technological equipment developed to study the plant metabolome (Hall and Hardy, 2012). For stakeholders within the South African citrus industry, the deeper understanding gained into the notorious plant-pathogen system discussed in this thesis, enhances their position as a top producer and exporter of citrus fruits. These findings are revelatory and significant in relation to future marker assisted citrus rootstock breeding for *Phytophthora nicotianae* root rot tolerance.

## 6.2 References

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